Original Research

Effects of Conditioned Media From Human Umbilical Cord-Derived Mesenchymal Stem Cells on Tenocytes From Degenerative Rotator Cuff Tears in an Interleukin 1β–Induced Tendinopathic Condition

Min Ji Lee,*† PhD, Kyungtaek Park,‡ PhD, Seung Yeon Lee,* MS, Kwi-Hoon Jang,* PhD, Sungho Won,‡§‖ PhD, and Chris Hyunchul Jo,*†¶ MD

Investigation performed at SMG-SNU Boramae Medical Center, Seoul National University College of Medicine, Seoul, Republic of Korea

Background: Evidence suggests that mesenchymal stem cells (MSCs) are safe for treating different tendinopathies. Synovial fluid is a pooled environment of biomarkers from the inflammatory and degenerative joint cavity. Understanding the effects of synovial fluid on MSCs is important, as it is the first microenvironment that administered MSCs encounter. Several studies have reported that exposure to osteoarthritic synovial fluid–activated MSCs increased the release of soluble factors; however, the paracrine effects of shoulder synovial fluid–stimulated umbilical cord-derived MSCs (SF-UC-MSCs) on tendinopathy have yet to be investigated.

Purpose: To assess the effects of the conditioned media from SF-UC-MSCs on tenocytes from degenerative rotator cuff tears in an interleukin-1β (IL-1β)–induced tendinopathic condition.

Study Design: Controlled laboratory study.

Methods: UC-MSCs were isolated and cultured from healthy, full-term deliveries by cesarean section. Tenocytes were isolated and cultured from patients with degenerative rotator cuff tears. Conditioned media were obtained from UC-MSCs stimulated with synovial fluid. To evaluate the gene expression of proinflammatory and anti-inflammatory cytokines, enzymes and their inhibitors, matrix molecules, and growth factors, the tenocytes were cultured with IL-1β and 50% of the conditioned media from the SF-UC-MSCs; quantitative, real-time, reverse transcriptase polymerase chain reaction was also performed. A prostaglandin E2 (PGE2) assay was performed to investigate the PGE2 level secreted by the tenocytes. Western blotting was performed to examine protein synthesis of collagen type I and III. Cell viability, senescence, and apoptosis assays were also performed.

Results: The conditioned media from the SF-UC-MSCs interfered with the inflammatory gene expression on tenocytes induced by IL-1β, but it increased the gene expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-3. Meanwhile, the conditioned media decreased the PGE2 level on cells induced by IL-1β. It did increase the type I/III ratio of gene expression and protein synthesis, mainly through the induction of type I collagen. Conditioned media of SF-UC-MSCs reversed senescence and apoptosis induced by IL-1β.

Conclusion: Study findings indicated that the conditioned media from SF-UC-MSCs had anti-inflammatory effects and cytoprotective effects on IL-1β–treated tenocytes from degenerative rotator cuff tears.

Clinical Relevance: UC-MSCs have useful potential for the treatment of tendinopathy in practice.

Keywords: conditioned media; degeneration; interleukin 1β; mesenchymal stem cells; tendinopathy; tenocyte; umbilical cord

Tendinopathy is a complex multifaceted tendon disorder, often referred to as a nonrupture injury in the tendon or paratenon in response to overuse.[2,32,52] The condition is characterized by pain and swelling in a tendon with a decline in function and reduced exercise tolerance, which

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often leads to tendon rupture.[44] To date, there has been a lack of effective treatments for tendon regeneration, with the primary approach to management relying on nonoperative methods such as physical therapy, exercise, nonsteroidal anti-inflammatory drugs (NSAIDs), and periodic local corticosteroid injections.[7] NSAIDs and corticosteroids provide short-term pain relief (7-14 days) but lose effectiveness in the long term.[2,13] Surgery remains the last option because of its invasiveness and inconsistent outcomes.[7] The prominent histopathologic and molecular features of tendinopathy include degeneration and disorganization of extracellular matrix (ECM), loss of mechanical properties, tenocyte apoptosis, increased immune cells, and inflammatory mediators—such as cytokines, nitric oxide, prostaglandins, and lipoxins—in both animal and human tendinopathies.[17,43] The inflammatory cytokines and chemokines are produced by various immune cells and activated tenocytes; these molecules play central roles in ECM remodeling, and adverse ECM remodeling dysregulates the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which could detrimentally change the synthesis and turnover of ECM components. To the best of our knowledge, current treatments do not completely repair or regenerate the diseased tendon to its native composition, structure, and mechanical function.[38]

Mesenchymal stem cells (MSCs) hold promising results for treating tendon disorders, and several clinical studies suggest they are safe for treating different tendinopathies.[45] Animal research investigating the effects of MSCs on tendon injury has demonstrated that it improves the histological and biomechanical properties of the tendon, therefore increasing the rate of tendon healing and maturation.[20] The principal consequences of MSCs on regeneration are the immunomodulatory and anti-inflammatory effects through paracrine mechanisms, in that the secretion of cytokines, growth factors, and extracellular vesicles contribute to communication between cells.[57] The immunomodulatory ability of MSCs is not innate but could be induced by inflammatory cytokines present in the injured microenvironment.[21] Because of the invasive harvesting procedures, low collection efficiency, decreased quality with age, and the reported risk of ectopic bone formation associated specifically with bone marrow-derived MSCs, fetal MSCs are preferred for their noninvasiveness and superior proliferation efficiency.[38,63] Umbilical cord-derived MSCs (UC-MSCs), one of the fetal MSCs, meet the requirements for clinical use in terms of proliferation, differentiation potential, immunomodulatory and anti-inflammatory effects, and ethical concerns.[36]

Synovial fluid is the natural collection of biochemical markers and catabolic molecules from the inflammatory and degenerative joint microenvironment.[54] Several studies have demonstrated that stimulating MSCs with osteoarthritis synovial fluids promotes the MSCs' release of immunomodulatory factors, which induce macrophage polarization of M0 into M2-like phenotype, inhibit T cell proliferation, and promote regulatory T cell expansion.[12,26] Conditioned medium from synovial fluid-exposed MSCs has shown significantly higher mRNA expression of indoleamine-pyrrole 2,3-dioxygenase (IDO), and interleukin (IL)-6 than the control medium.[37] However, no report evaluated the paracrine effects of shoulder synovial fluid-stimulated UC-MSCs (SF-UC-MSCs) on tendinopathy.

This study aimed to assess the effects of shoulder SF-UC-MSCs conditioned media on tenocytes from degenerative rotator cuff tears with IL-1β–induced tendinopathic conditions. We hypothesized that conditioned media from UC-MSCs stimulated by synovial fluid would have anti-inflammatory, promatrix synthesis, and cytoprotective effects on tenocytes from degenerative rotator cuff tears in IL-1β–induced tendinopathic conditions.

METHODS

The overall study protocol and tissue-collection procedures were approved by the institutional review board at our institution. An outline of the study procedure is shown in Figure 1. Abbreviations used within the text are listed in Appendix Table A1.

Isolation and Culture of Tenocytes from Degenerative Human Rotator Cuff Tendons

Degenerative tenocytes from patients undergoing arthroscopic rotator cuff repair (n = 3; mean age, 57.3 ± 7.6 years) were harvested, isolated, and cultured. Tendon tissue was minced into 1-2 mm fragments, then treated with 0.3% collagenase II (Worthington, Lakewood, NJ, USA) in HG-DMEM (Hyclone, Logan, USA) containing antibiotics for 2 hours with gentle agitation. After adding DPBS, undigested tissue was removed using a 100 μm cell strainer (SPL Life Sciences), and cells were collected, washed, and resuspended in a culture medium consisting of high-glucose DMEM (Hyclone)

¶Address correspondence to Chris Hyunchul Jo, MD, Department of Orthopedic Surgery, SMG-SNU Boramae Medical Center, Seoul National University College of Medicine, 20 Boramae-ro 5-gil, Dongjak-gu, Seoul 07061, Republic of Korea (email: chrisjo@snu.ac.kr).
*Department of Orthopedic Surgery, SMG-SNU Boramae Medical Center, Seoul National University College of Medicine, Seoul, Republic of Korea.
†Department of Translational Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea.
‡Institute of Health and Environment, Seoul National University, Seoul, Republic of Korea.
§Interdisciplinary Program of Bioinformatics, College of Natural Sciences, Seoul National University, Seoul, Republic of Korea.
‖Department of Public Health Sciences, Graduate School of Public Health, Seoul National University, Seoul, Republic of Korea.
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Ethical approval for this study was obtained from Seoul Metropolitan Governance, Seoul National University Boramae Medical Center (reference No. 20120405/06-2012-78/118).

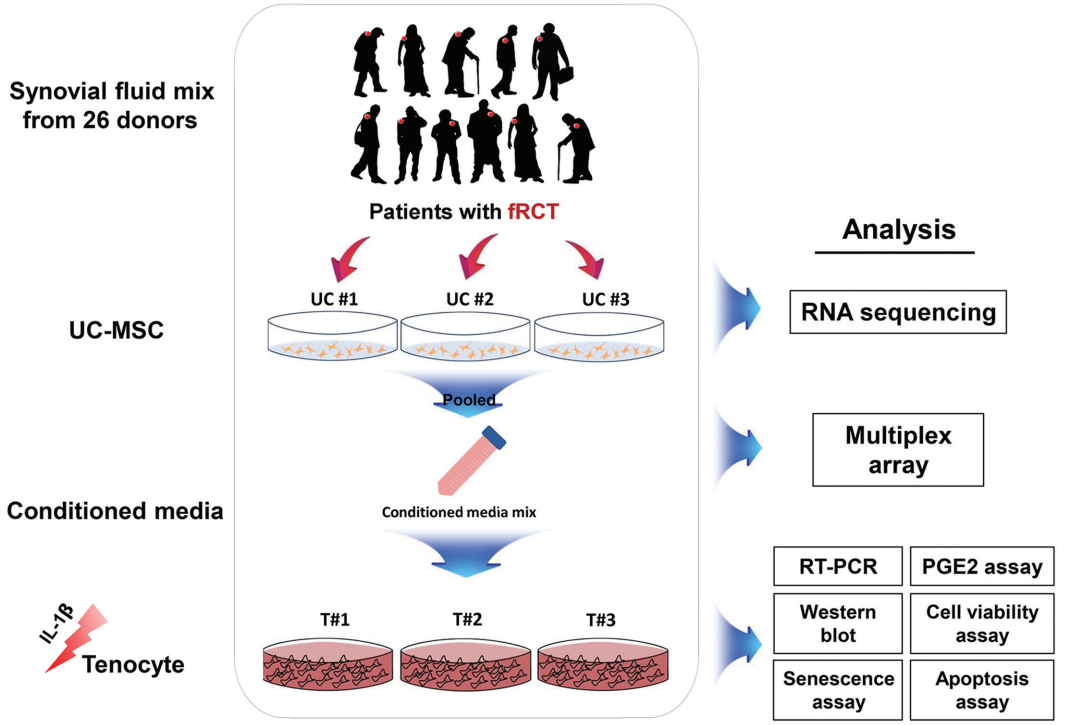

Figure 1. Schematics of the study procedure. Synovial fluid from 26 patients with full-thickness rotator cuff tears (fRCTs) was used to treat umbilical cord (UC)-derived mesenchymal stem cells (UC-MSCs). UC-MSCs and synovial fluid-treated UC-MSCs were analyzed using RNA sequencing, and their conditioned media were analyzed using a protein array. The effects on IL-1β–treated tenocytes of synovial fluid-treated UC conditioned media versus UC conditioned media were observed using RT-PCR, PGE2 assay, Western blot, cell viability assay, senescence assay, and apoptosis assay. See Appendix Table A1 for abbreviation expansions not mentioned here.

supplemented with 10% FBS (Hyclone) and antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Welgene). Cells were plated at 2-5 × 10^4 cells/cm^2 at 37°C in a humidified 5% CO_2 atmosphere, with medium changes every 2-3 days. At 80% confluence, cells were detached with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Welgene) and replated at a 1:3 ratio. Tenocytes from passages 2-5 were used.

Isolation and Culture of UC-MSCs

Human UCs were obtained from healthy, full-term deliveries by cesarean section after receiving informed consent. The UCs were washed 2 to 3 times with Dulbecco PBS (Welgene) to remove blood products, measured for length and weight, and then cut into minimal cube explants with 2 to 4 mm sizes by surgical scissors. The cube explants (1 g) were aligned at regular intervals in 15-cm culture dishes and were allowed to firmly attach to the bottom of the dish for 60 minutes in a 5% CO_2 incubator with humidified air at 37°C. Then, a culture medium consisting of low-glucose DMEM (Hyclone) supplemented with 10% FBS (Hyclone) and antibiotic-antimycotic solution was gently added into the dishes. The medium was replaced twice a week. Nonadherent cells were removed by medium changes. When cells reached 60% to 80% confluence, they were detached by incubation for 3 minutes with trypsin-EDTA. The tissues were removed through a 100-μm cell strainer, and the cells were centrifuged at 500g for 5 minutes at 20°C and then replated at a density of 3 × 10^3 cells/cm^2.

Acquisition of Synovial Fluids and Preparation of Conditioned Media

Synovial fluid samples were obtained from 26 patients with full-thickness rotator cuff tears who were undergoing arthroscopic rotator cuff repair. The samples were centrifuged at 4°C and 1000g for 15 minutes to remove cell debris, and the supernatants were stored at –80°C until required. The synovial fluid samples from the donors were mixed in equal volumes. After being allowed to attach for 24 hours, the UC-MSCs (n = 3) were stimulated with a mixture of synovial fluids (10% vol/vol) for 6 hours. To eliminate contamination by the synovial fluid, the culture medium was replaced with serum-free DMEM, and cells were cultured for 24 hours. UC-MSCs in passage 4 were used for the preparation of the conditioned media.

Characteristics of the Synovial fluid-Treated UC-MSCs and Their Conditioned Media

Three SF-UC-MSCs and 3 untreated UC-MSCs were used for RNA sequencing. Total RNA was extracted using

TRIzol RNA Isolation Reagents (Life Technologies). The quantity and quality of the total RNA were evaluated using the Agilent 2100 Bioanalyzer RNA Kit (Agilent). The isolated total RNA was processed for preparing the mRNA sequencing library using the Illumina TruSeq-Stranded mRNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. The quality and size of the libraries were assessed using the Agilent 2100 Bioanalyzer DNA Kit (Agilent). All libraries were quantified by quantitative PCR using CFX96 Real-Time System (Bio-Rad) and sequenced on the NextSeq500 sequencers (Illumina) with a paired-end 75bp plus single 8bp index read run. The pair-ended reads were trimmed of their adaptor sequences using Trimmomatic (v 0.39)[6] followed by mapping to the Hg38 reference genome and ensemble genes using STAR (Version 2.7.8a).[19] The mapped reads were counted using HTSeq (Version 0.12.4).[1]

Next, differentially expressed genes (DEGs) between the SF-UC-MSC and UC-MSC groups were extracted by DESeq2 (Version 1.26.0),[41] with false discovery rates <0.05 and log2 fold changes >0.5. Enrichr[9] was then used to test whether the upregulated DEGs by synovial fluid were associated with the Gene Ontology (GO) Biological Process aspect (2021). The supernatants were centrifuged at room temperature, 700*g* for 8 minutes, and stored at –80°C until required. Human Antibody Array L-507 Membrane Kit (RayBiotech) was used to detect 507 cytokines, chemokines, growth factors, soluble receptors, and other bioactive factors in the conditioned media according to the manufacturer's instructions. Membrane signals were visualized by a LAS-4000 device (GE Healthcare), and the signals on the membrane were observed using the LAS-4000 device; the spot intensities were determined using the Protein Array Analyzer macro in Image J (http://rsb.info.nih.gov/ij/macros/toolsets/ProteinArrayAnalyzer.txt). To ensure consistency, the spot intensities were normalized using positive controls and analyzed by RayBiotech analysis tool software, which was provided by the supplier. Based on the ratio of signal intensity between stimulated UC-MSCs and untreated UC-MSCs, the top 480 proteins that showed at least a 2-fold increase were selected. Furthermore, we extracted proteins belonging to the GO terms "response to growth factor" (GO:0070848) and "negative regulation of immune response" (GO:0050777), according to the Amigo 2 database.[4] With these selected proteins, pathway analyses were performed using BioPlanet 2019 as well as GO Biological Process 2021, implemented in Enrichr.

Treatment of Tenocytes with IL-1β and Conditioned Media for the Evaluation of Gene Expression, Protein Synthesis, PGE2 level, Viability, Senescence, and Apoptosis

After allowing them to attach for 24 hours, cells were treated with 0.1 ng/mL of IL-1β (recombinant human IL-1β/IL-1F2 protein; CF, 201-LB/CF; R&D Systems) and conditioned media from SF-UC-MSCs (50% vol/vol) for 24 hours in DMEM supplemented with 2% FBS and antibiotic solution. Cells were treated exceptionally with 10 ng/mL IL-1β to evaluate prostaglandin E2 (PGE2) and apoptosis. Nontreated cells were used as a control.

Real-Time Reverse Transcription PCR

Reverse transcription and amplification were performed with genes related to the following: (1) proinflammatory cytokines: IL-1β, TNF-α, IL-6, and COX-2; (2) degradative enzymes and their inhibitors: MMP-1, MMP-9, and MMP-13, TIMP-1 and TIMP-3, ADAMTS-4 and ADAMTS-5; (3) anti-inflammatory cytokines: IL-4, IL-10, and IL-1 receptor antagonist (IL-1ra); (4) matrix molecules: type I and III collagen, decorin (DCN), and tenascin-C (TNC); (5) growth factors: TGF-β1, TGF-β2, and TGF-β3; IGF-1, and bFGF. Gene expressions were normalized versus GAPDH as follows: The cycle number at which the transcript of each gene was detectable (threshold cycle; Ct) was normalized against the Ct of GAPDH (referred to as ΔCt). Gene expressions relative to GAPDH are expressed as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{GAPDH}$. All experiments were performed in triplicate.

PGE2 Assay

After seeded at a density of $2 \times 10^4/cm^2$ in the 24-well plates and allowed to attach for 24 hours, tenocytes were treated with 10 ng/mL IL-1β, and the conditioned media from SF-UC-MSCs in DMEM supplemented with 2% FBS and antibiotic solution for 1 day. PGE2 was estimated in the culture supernatants using a Parameter Prostaglandin E2 Assay Kit (R&D Systems) according to the manufacturer's instructions. All experiments were performed in triplicate. To determine the baseline level of PGE2, the supernatant from tenocytes treated without IL-1β was examined as a control.

Western Blot

Cell protein extracts were prepared using PRO-PREP Protein Extraction Solution (iNtRON, Sungnam). Equal amounts of protein extracts for each group were electrophoresed into 10% SDS-PAGE gels. The electrophoresed proteins were blotted onto a polyvinylidene difluoride membrane with a 0.45-μm pore size. The membranes were blocked with TBS-T buffer containing 5% skim milk for 1 hour at room temperature and incubated with a primary antibody overnight at 4°C. Primary antibodies were as follows: collagen type I antibody (#LS-C343921, LSBio), collagen type III antibody (#sc271249; Santacruz Biotechnology), and β-actin antibody (#sc47778; Santacruz Biotechnology). The membranes were washed with TBS-T, and those were incubated with an HRP-conjugated secondary antibody diluted 1 to 4000 for 45 minutes. The membranes were washed with 1× TBS-T and scanned using ImageQuant LAS4000 mini (GE Healthcare Life Sciences). Densitometric quantification was analyzed with

TABLE 1
The Top 10 Signature Genes Upregulated Uniquely in UC-MSCs or SF-UC-MSCs[a]

Gene	Description	Log2lFCl	*P*	FDR
IL1R1	Interleukin 1 receptor type 1	2.006	2.48E-25	1.15E-21
PTGES	Prostaglandin E synthase	3.481	1.95E-22	5.42E-19
ABCA7	ATP binding cassette subfamily A member 7	1.328	6.25E-16	7.91E-13
H1-2	H1.2 linker histone, cluster member	1.43	1.30E-13	1.34E-10
MATN2	Matrilin 2	1.216	1.06E-12	9.26E-10
DPYSL3	Dihydropyrimidinase like 3	0.829	1.42E-12	1.20E-09
RBP1	Retinol binding protein 1	1.472	3.36E-12	2.53E-09
SNCAIP	Synuclein alpha interacting protein	1.93	4.23E-12	3.02E-09
PLIN2	Perilipin 2	1.323	7.30E-12	4.84E-09
METTL7A	Methyltransferase like 7A	1.913	1.45E-11	8.61E-09

[a]FC, fold change; FDR, false discovery rate; SF-UC-MSCs, synovial fluid–stimulated umbilical cord-derived mesenchymal stem cells; UC-MSCs, umbilical cord-derived mesenchymal stem cells.

ImageQuant LAS4000 mini. The protein synthesis levels were normalized to those of β-actin from the same samples.

Cell Viability Assay

After seeded at a density of 5×10^2 cells/cm^2 in the 96-well plates and allowed to attach for 24 hours, cells were treated with IL-1β and conditioned media from the SF-UC-MSCs in DMEM supplemented with 2% FBS and antibiotic solution. Cell viability was determined using the EZ-CyTox Cell Viability Assay Kit (Daeil Lab Service) according to the manufacturer's instructions. Briefly, 10 μL of assay reagent was added to the 96-well plate on days 1, 2, and 5. Cells were incubated for 3 hours at 37°C in a humidified atmosphere of 5% CO_2. Plates were read on a SpectraMax plus384 (Molecular Devices) at 450 nm. All experiments were performed in triplicate.

Senescence Assay

Tenocytes were seeded at a density of 3×10^4 cells/cm^2 in the 12-well plates, allowed to attach for 24 hours, treated with conditioned media from the SF-UC-MSCs with 0.1 ng/mL IL-1β, and cultured for 5 days. Senescent cells were identified by the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology Inc) according to the manufacturer's instructions. The medium was removed, and cells were washed in PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes at room temperature. Cells were washed twice with PBS and then incubated at room temperature overnight with a staining solution (30 mM citric acid/sodium phosphate [pH 6], 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] solution). Cells were rinsed 1 time with PBS and stained with DAPI at room temperature for 5 minutes. The cells were photographed at 200× magnification using a fluorescence inverted microscope (LEICA DMI4000B) in 5 random plate areas. The percentage of senescent cells was calculated by dividing the number of β-galactosidase–positive cells by the number of DAPI-positive cells. All experiments were performed in triplicate.

Apoptosis Assay

Tenocytes were seeded at a density of 2×10^4 cells/cm^2 in 6-well plates, allowed to attach for 24 hours, and treated with conditioned media from the SF-UC-MSCs with 10 ng/mL IL-1β for 5 days. TUNEL was performed to detect apoptotic cells using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Briefly, the cell suspension was smeared onto a silanized microscope slide gently and fixed with 4% paraformaldehyde for 25 minutes at 4°C. Slides were immersed twice in PBS for 5 minutes each time. Cells were permeabilized by 0.2% Triton X-100 in PBS for 5 minutes. Slides were immersed twice in PBS for 5 minutes each time. An equilibration buffer of 100 μL was added to the cells at room temperature for 5 minutes. TdT reaction mix was added to the cells on the slides, and the slides were covered with plastic coverslips. Slides were incubated for 60 minutes at 37°C in a humidified chamber. Slides were immersed in 2× standard saline citrate for 15 minutes and washed 3 times in PBS for 5 minutes each time. The cells were stained with DAPI at room temperature for 5 minutes, and were then photographed at 400× magnification using a confocal laser scanning microscope (LEICA SP8 X) in 10 random plate areas. The percentage of apoptotic cells was calculated by dividing the number of TUNEL-positive cells by the number of DAPI-positive cells. All experiments were performed in duplicate.

Statistical Analysis

All data values are shown as mean ± standard deviation. Data were compared between groups using 1-way analysis of variance with post hoc analysis of Bonferroni multiple comparison test. The analysis was performed using SPSS (Version 13.0; IBM). Differences with $P < .05$ were considered to be statistically significant.

RESULTS

Effects of Synovial Fluids on the Gene Profile of UC-MSCs and the Protein Profiles of the Conditioned Media From SF-UC-MSCs

A total of 305 genes were significantly upregulated in the SF-UC-MSCs compared with the untreated UC-MSCs. The most significant genes were IL1R1 and PTGES (Table 1). The analysis of DEGs revealed that upregulated genes in the SF-UC-MSCs were associated with cell cycle processes, including kinetochore and microtubule cytoskeleton organization (Figure 2A). We then compared the proteins secreted by the SF-UC-MSCs and untreated UC-MSCs (Figure 2, B-E). A total of 480 of 507 bioactive factors were increased in SF-UC-MSCs secretion by >2-fold compared with the control. Growth factors such as Epo, chemokines, such as CTACK, and cytokines, such as IL-1ra, were found to be highly secreted from SF-UC-MSCs (Figure 2C). Notably, the proteins with the highest expression levels in SF-UC-MSCs were associated with cell signal transduction, inflammatory response, and cell proliferation as well as growth factor, cytokine, and chemokine activity (Figure 2D).

Among the highly expressed proteins, we focused on proteins included in the GO terms "response to growth factor" and "negative regulation of immune response." The proteins related to the former were associated with TGF-β and BMP signaling pathways (Supplemental Tables S1 and S2, available separately), and the proteins related to the latter were associated with JAK-STAT, TSLP, and Th1/Th2 differentiation pathways (Supplemental Tables S3 and S4). In particular, highly secreted proteins from SF-UC-MSCs that were involved in anti-inflammatory effects were IL-1ra (14,051.23-fold), TGF-β1 (48.47-fold), HGF (44.16-fold), IL-10 (33.39-fold), and GRN (8.345-fold). These results suggest that exposure of UC-MSCs to synovial fluids promotes the cell cycle of UC-MSCs and their secretion of cytokines and growth factors, which are related to signal transduction, cell proliferation, and immune response.

Effects of the Conditioned Media From SF-UC-MSCs on the Gene Expression of Proinflammatory Cytokines, Degenerative Enzymes, and Their Inhibitors, Anti-inflammatory Cytokines, Growth Factors, and PGE2 Secretion

For proinflammatory cytokines, IL-1β significantly upregulated the gene expression of IL-1β, TNF-α, IL-6, and COX-2 by 619.8-fold, 4.2-fold, 2668.2-fold, and 75.3-fold, respectively (Figure 3A). Additional treatment with conditioned media from SF-UC-MSCs downregulated their gene expression by 0.25-fold, 0.71-fold, 0.80-fold, and 0.29-fold, respectively.

With respect to anti-inflammatory cytokines, IL-1β significantly downregulated the gene expressions of IL-4 by 0.48-fold; however, IL-1β upregulated those of IL-10 and IL-1ra by 1.4-fold and 211.1-fold (Figure 3D), respectively. The conditioned media further increased the upregulated IL-4 expression by 1.6-fold and significantly decreased the IL-10 and IL-1ra expression by 0.68-fold and 0.45-fold, respectively.

The secretion of PGE2, an important marker of tendinopathy, was investigated using a PGE2 assay. PGE2 was not detectable in the negative control group, but IL-1β significantly induced the secretion of PGE2 in tenocytes, reaching a level of 5.28 ng/mL. However, when treated with the conditioned media, the PGE2 level decreased to 4.57 ng/mL (Figure 3B).

For matrix degradative enzymes and their inhibitors, IL-1β significantly upregulated the gene expression of MMP-1, MMP-9, and MMP-13 by 90.6-fold, 4.7-fold, and 64.4-fold, respectively (Figure 3C). Subsequent treatment with the conditioned media from SF-UC-MSCs significantly downregulated MMP-1 expression by 0.63-fold, whereas no significant change was observed in MMP-9 and MMP-13 expression. IL-1β upregulated the gene expression of ADAMTS-4 and ADAMTS-5 by 5.2 and 2.2 folds, respectively. Subsequent treatment of conditioned media did not change their expression. The expression of TIMP-1 was significantly increased with IL-1β treatment by 1.6-fold, and conditioned media further increased its expression by 1.5-fold. The expression of TIMP-3 was significantly decreased with IL-1β treatment by 0.47-fold, whereas additional treatment of conditioned media upregulated its expression by 1.2-fold.

IL-1β significantly downregulated the gene expression of TGF-β1, TGF-β2, and TGF-β3, which are growth factors related to tendon healing, by 0.82-fold, 0.49-fold, and 0.41-fold compared with the control group, respectively. However, treatment of the conditioned media rescued their gene expression by 1.2-fold, 1.3-fold, and 1.2-fold, respectively (Figure 3E). In contrast, the gene expression of IGF-1 and bFGF was significantly upregulated by 24.9-fold and 3.4-fold with IL-1β, respectively, and further increased to 1.3-fold and 1.3-fold, respectively, with the conditioned media treatment.

Effects of the Conditioned Media From SF-UC-MSCs on the Gene Expression of Matrix Molecules and Protein Synthesis of Matrix Molecules

For type I and III collagen, which are the primary components of the ECM in tendons, IL-1β significantly downregulated the gene expression of type I collagen by 0.60-fold compared with the control group. Additional treatment of the conditioned media significantly increased its expression by 1.4-fold. Type III collagen expression, on the other hand, remained unchanged by both IL-1β and the conditioned media (Figure 4A). In terms of the expression ratio of type I/III collagen, IL-1β significantly decreased the ratio by 0.51-fold, whereas the conditioned media significantly increased the ratio by 1.5-fold. The gene expression of important ECM components in tendons, including sceleraxis (SCX), DCN, and TNC, was investigated. IL-1β significantly upregulated the gene expression of DCN by 1.7-fold and downregulated the gene expression

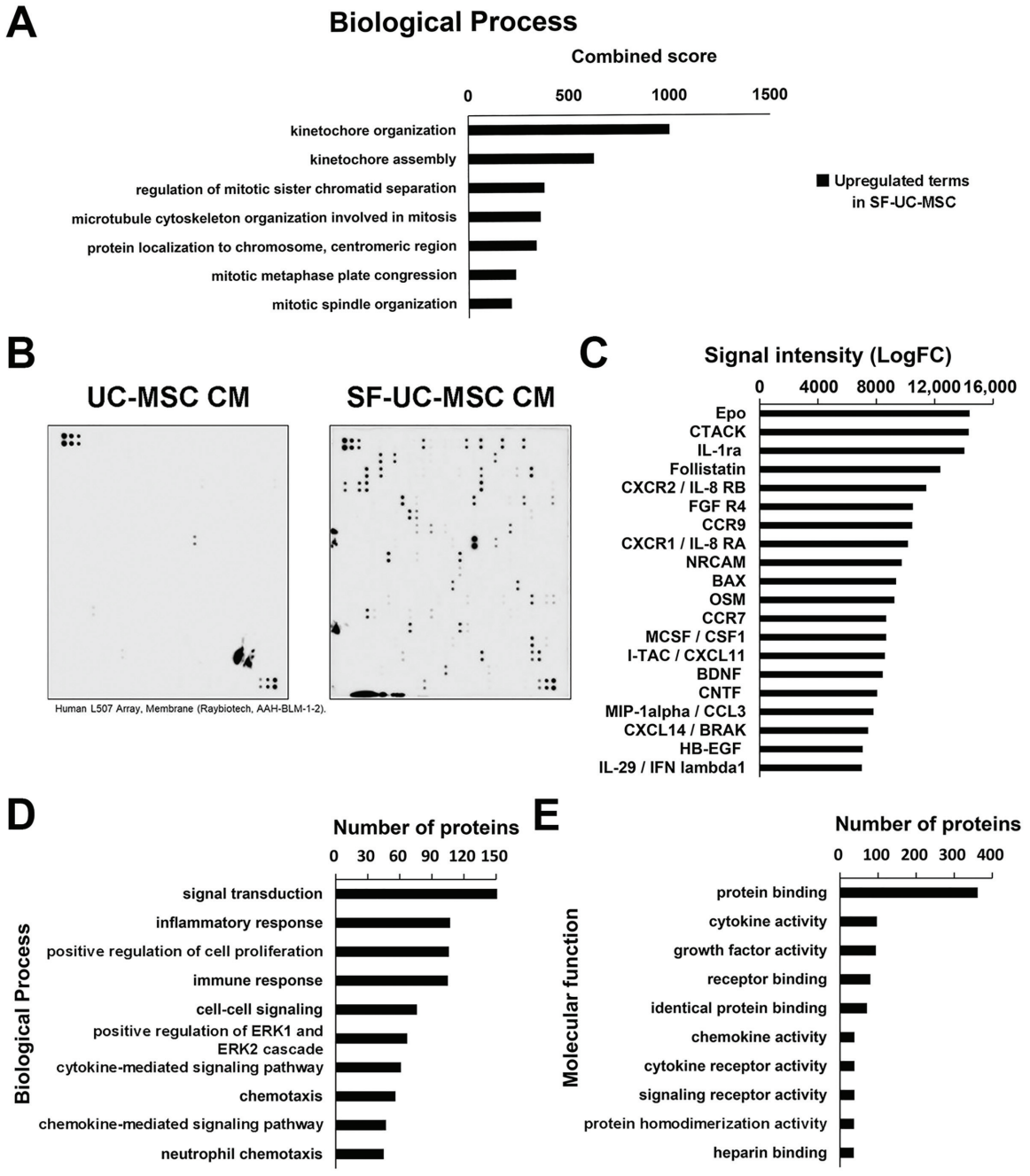

Figure 2. (A and B) Analysis of the RNA sequences in untreated UC-MSC and SF-UC-MSC conditioned media (CM). Top signature genes upregulated uniquely in SF-UC-MSC compared with untreated UC-MSC. Adjusted $P < .01$, false discovery rate <0.05, and Log2|FC| > 0.5. (A) The combined score of differentially expressed genes in SF-UC-MSC compared with untreated UC-MSC. (B-E) The secretome of UC-MSC and SF-UC-MSC were analyzed using a human L507 array. (C) The top 20 highly upregulated secreted bioactive factors in SF-UC-MSC conditioned media compared with UC-MSC conditioned media. GO enrichment analysis of upregulated differentially expressed genes in SF-UC-MSC conditioned media: (D) biological process and (E) molecular function. FC, fold change. See Appendix Table A1 for abbreviation expansions not mentioned here.

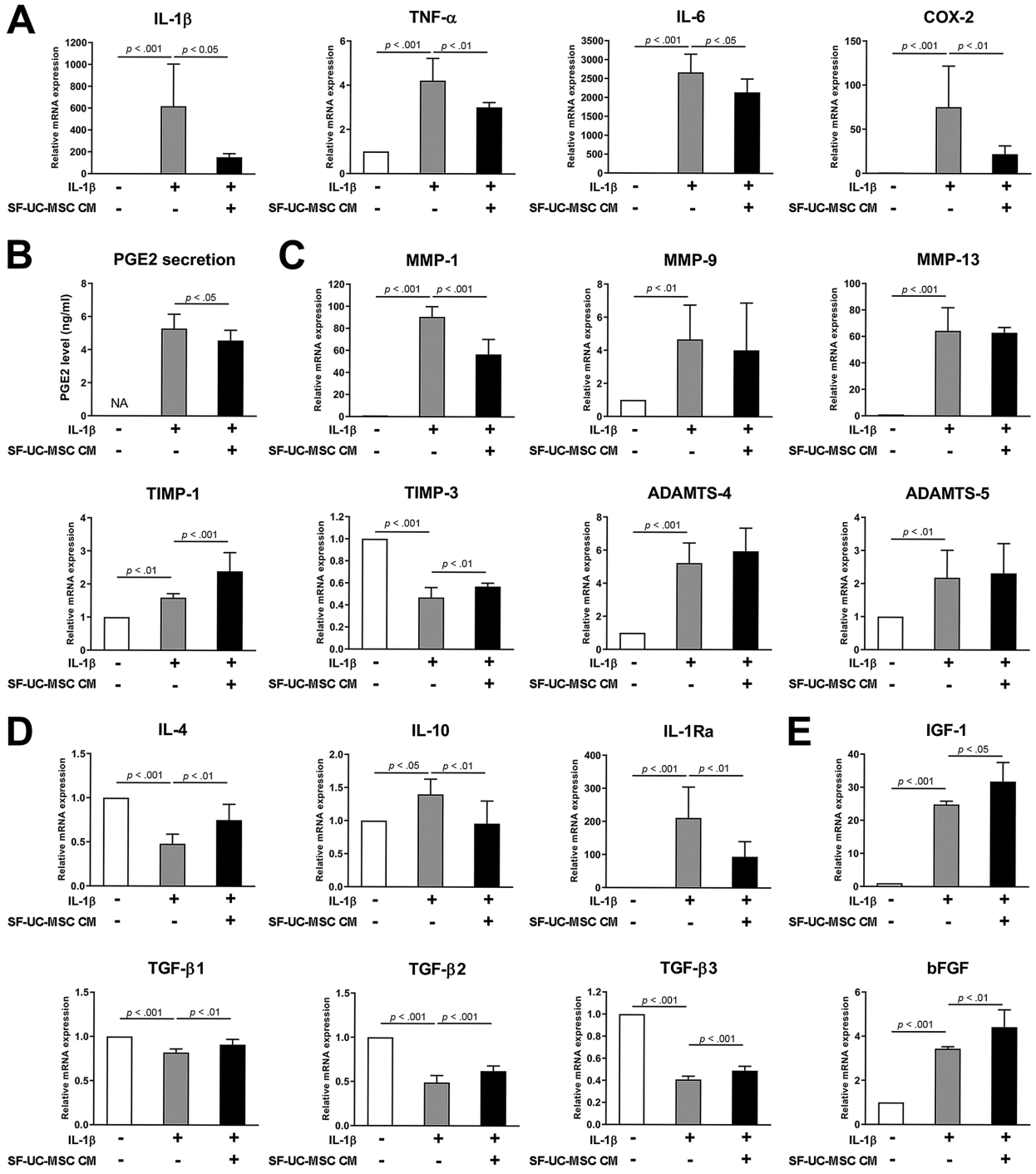

Figure 3. Effects of the conditioned media from SF-UC-MSCs on the gene expression (left to right). (A) Proinflammatory cytokines, (B) PGE2 secretion, (C) degradative enzymes and their inhibitors, (D) anti-inflammatory cytokines, and (E) growth factors. Tenocytes were treated with 0.1 ng/mL recombinant human IL-1β and the conditioned media (50% vol/vol) for 24 hours. Non-treated cells were used as a negative control. The quantitative results are means ± SD of 3 independent experiments. Statistics were determined by analysis of variance with Bonferroni post hoc analysis, except for the PGE2 assay (paired *t* test, IL-1β group vs conditioned media group). See Appendix Table A1 for abbreviation expansions.

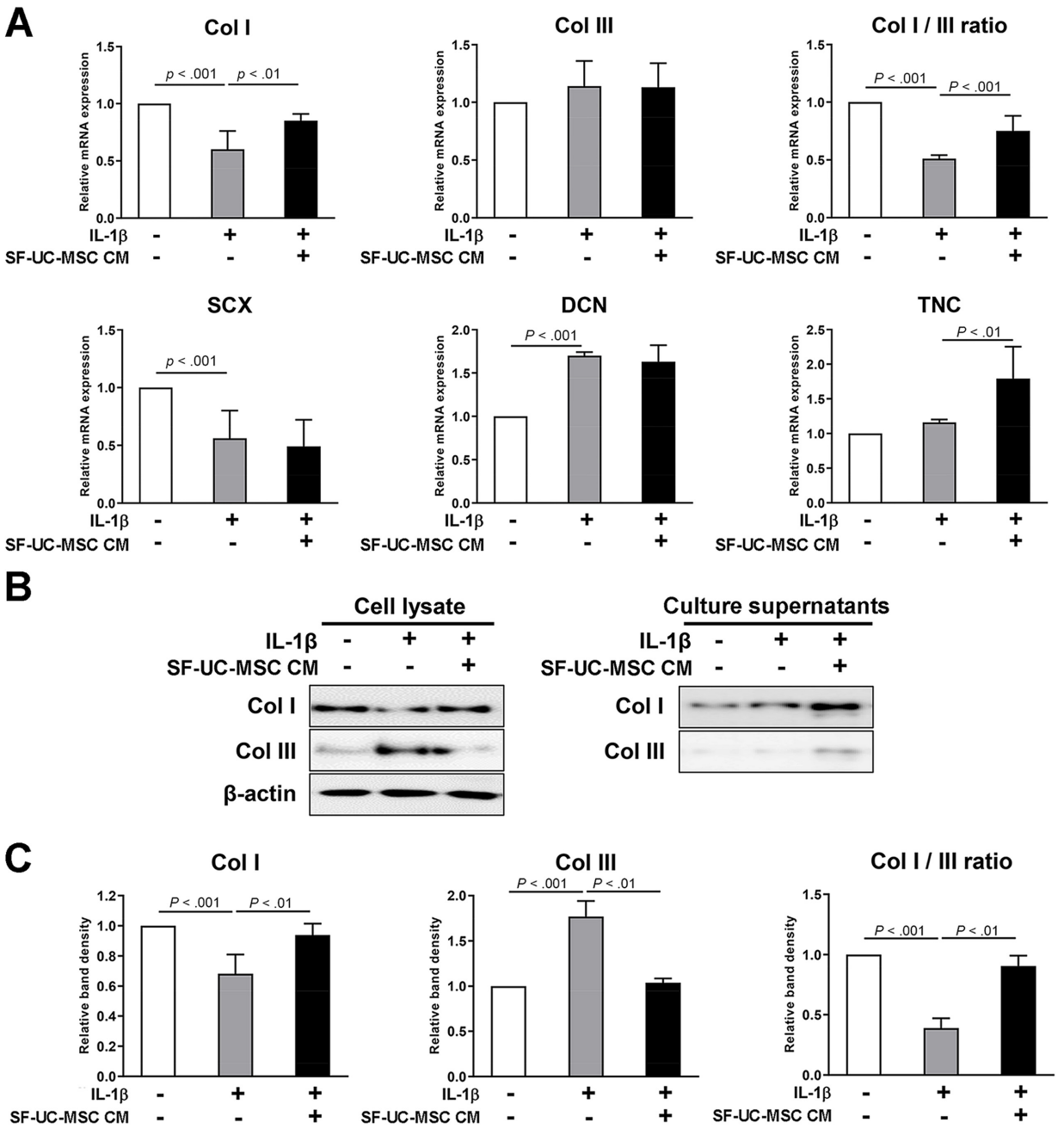

Figure 4. Effects of the conditioned media from SF-UC-MSCs on (A) the gene expression of matrix molecules, (B) protein synthesis of matrix molecules in cell lysate and culture supernatants using Western blot analysis, and (C) the quantification of type I and II collagen, as well as their ratio, were analyzed. Tenocytes were treated with 0.1 ng/mL recombinant human IL-1β, and the conditioned media (50% vol/vol) for 24 hours. The protein levels in cell lysates were standardized using β-actin, and an equal volume of culture supernatant was loaded into each lane. Nontreated cells were used as a negative control. The quantitative results are means ± standard deviation of 3 independent experiments. Statistical analysis was conducted using analysis of variance with Bonferroni post hoc analysis. See Appendix Table A1 for abbreviation expansions.

of SCX; subsequent treatment with conditioned media did not result in a significant change. However, IL-1β did not change the gene expression of TNC, while the conditioning media significantly upregulated its expression by 1.6-fold.

The protein synthesis of type I and type III collagen was investigated (Figure 4B). IL-1β significantly inhibited the protein synthesis of type I collagen by 0.64-fold. However, additional treatment of the conditioned media increased its

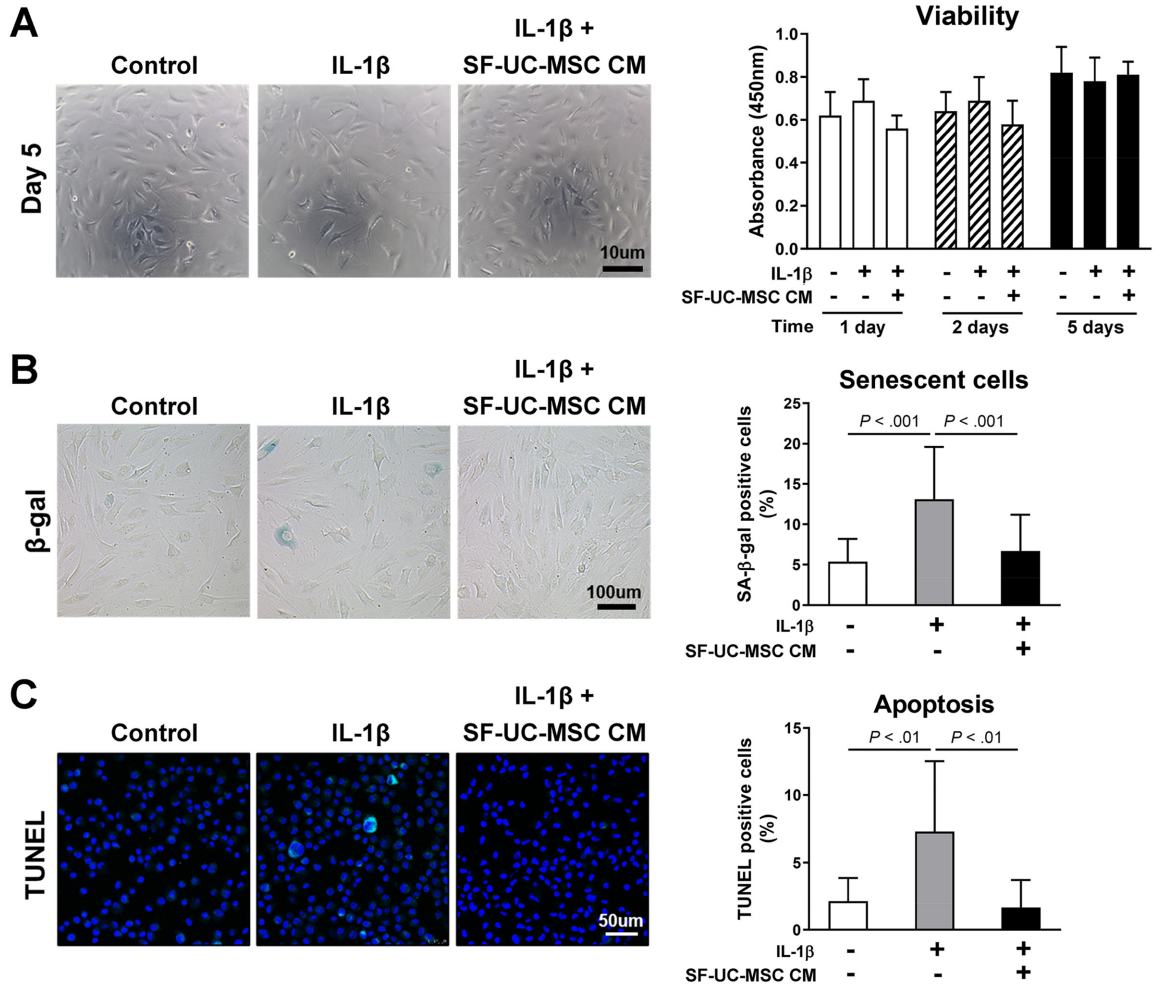

Figure 5. Effects of conditioned media from SF-UC-MSCs on tenocyte viability, senescence, and apoptosis. Tenocytes were treated with 0.1 ng/mL recombinant human IL-1β, and the conditioned media (50% vol/vol) for 24 hours. Nontreated cells were used as a negative control. (A) Representative images and quantification of cell viability showed no significant differences between the treatment of IL-1β and conditioned media (at 100 × magnification). (B) Representative image and the percentage of tenocytes staining positive for senescence-associated (SA) β-galactosidase (β-gal) after treatment with IL-1β or conditioned media (at 200 × magnification). The percentage of SA-β-gal–stained cells was calculated as the number of stained cells in total cells. (C) Representative image and the percentage of tenocytes staining positive for TUNEL (400 × magnification). The percentage of TUNEL-stained cells was calculated as the number of stained cells in total cells. The quantitative results are means ± standard deviation of 3 independent experiments. Statistical analysis was conducted using analysis of variance with Bonferroni post hoc analysis. See Appendix Table A1 for abbreviation expansions not mentioned here.

synthesis by 1.4-fold (Figure 4C). On the other hand, the protein expression of type III collagen was upregulated by 1.6-fold with IL-1β compared with the control group, and treatment with the conditioned media inhibited its synthesis by 0.88-fold. In terms of the synthesis ratio of collagen type I/III, IL-1β significantly decreased this ratio by 0.39-fold, whereas the conditioned media increased this ratio by 2.3-fold. These results suggest that the conditioned media increased the gene expression levels of growth factors related to tendon healing compared with the IL-1β group. Moreover, the conditioned media promoted the synthesis of type I collagen and inhibited the synthesis of type III collagen, which may facilitate tendon healing.

Effects of the Conditioned Media From SF-UC-MSCs on Viability, Senescence, and Apoptosis

Treatment with IL-1β or conditioned media did not affect the viability of tenocytes on days 1, 2, and 5 (Figure 5A). IL-1β significantly increased the number of β-galactosidase positive cells by 2.4-fold. In contrast, the conditioned media significantly reduced the number of β-galactosidase–positive cells by 0.51-fold (Figure 5B). The number of TUNEL-positive cells was increased by 3.4-fold in the IL-1β group, and the conditioned media significantly reduced the number of apoptotic cells by 0.23-fold (Figure 5C). These results suggest that the conditioned media

have cytoprotective effects on inflammatory tenocytes by inhibiting cell senescence and apoptosis.

DISCUSSION

The most important findings of this study are as follows: First, exposure of UC-MSCs to synovial fluids from patients with rotator cuff disease increased both their proliferation and the secretion of immunomodulatory factors. Second, treatment with conditioned media from SF-UC-MSCs suppressed the gene expression of proinflammatory cytokines, matrix-degradative enzymes, and PGE2 secretion in an IL-1β–induced in vitro tendinopathic model. Third, SF-UC-MSC conditioned media modulated the ECM by decreasing MMP-1 expression by 0.63-fold and increasing type I collagen by 1.4-fold. Last, senescence and apoptosis induced by IL-1β were reversed by SF-UC-MSC conditioned media by 0.51-fold and 0.23-fold, respectively. Taken together, these findings support the hypothesis that conditioned media from SF-UC-MSCs would have anti-inflammatory effects as well as cytoprotective effects against IL-1β–induced tendinopathic conditions.

One of the challenges in cell-based therapies is the inadequate retention and survival of transplanted cells at the target site. After local administration, several factors contribute to insufficient retention, including cell death caused by the hostile and inflammatory microenvironment at the disease site and poor integration into tissue.[39,65] Synovial fluid is the natural microenvironment with numerous biochemical markers and catabolic molecules during inflammatory and degenerative joint processes.[54] During inflammation, synovial cells secrete a large amount of synovial fluid containing IL-1β, TNF-α, MMPs, and PGE2, which indicate the initiators of the tendinopathic pathway. These proinflammatory mediators induce apoptosis, the elaboration of pain mediators, and MMPs, which degrade collagens and proteoglycans.[24,48] When MSCs are injected into the tendon injury site, the synovial fluid is the first microenvironment they encounter. However, the effects of MSCs on synovial fluid from patients with rotator cuff disease have not been studied yet, as research has primarily focused on synovial fluid from knee arthritis.[12,15,26,37] In knee osteoarthritis, inflammatory cytokines—such as IL-1, IL-6, IL-8, IL-17, and TNF-α—are detected in synovial fluid from patients and correlated with the severity of symptoms. Recent studies have reported that inflammatory microenvironments, such as those in knee osteoarthritic or rheumatoid arthritis conditions, can stimulate the immunomodulatory activity of MSCs, helping restore abnormal immune responses.[12,26] Factors such as IFN-γ play a role in the immunomodulatory function of MSCs, while cytokines such as IL-1, IL-6, and TNF-α can impair the function of MSCs. The impact of an inflammatory microenvironment on MSCs can vary depending on the conditions (acute vs chronic, mild vs severe). Acute inflammation may benefit the immunomodulatory activity of MSCs, while chronic inflammation can impair these cells. This helps explain the inconsistent findings regarding the influence of an inflammatory microenvironment on MSCs reported in previous studies.[23] Interestingly, Fan et al[23] reported that synovial fluid from patients with osteoarthritis blunted the chondroprotective effects of human UC-MSCs and significantly worsened knee osteoarthritis progression in a rat model. Because of these concerns, it is important to verify the effectiveness of MSC in the presence of synovial fluids from disease patients. Our study is the first to explore the paracrine effects of SF-MSCs from patients with severe rotator cuff disease, compare the mRNA levels and secreted proteins of UC with UC stimulated by synovial fluid from rotator cuff disease, and validate their anti-inflammatory effects on inflamed tenocytes.

The present study showed that synovial fluid-stimulated cell cycles and proliferation, as supported by the upregulated RNA levels of IL1R1 and PTGES, result in an upregulated cell cycle process. IL1R1 was found to be associated with the MSC proliferation induced by IL-1β through the ERK1/2 pathway.[56] Regarding PTGES, its role in MSCs exhibits both proliferative and anti-inflammatory functions, while in primary cells, it promotes inflammation by stimulating the production of proinflammatory cytokines.[40] The anti-inflammatory function of PTGES is frequently observed in MSCs, where PGE2 produced by MSCs serves to suppress the activity of immune cells, thereby mitigating inflammation during tissue injury.[61] The synovial fluid treatment further increased the secretion of proteins associated with signal transduction, inflammatory response, positive regulation of cell proliferation, and immune response. Among the highly secreted proteins, CTACK/CCL27 (a chemokine), IL-1ra (an anti-inflammatory cytokine), and growth factors—including erythropoietin, BDNF, CNTF, HB-EGF, HGF, and follistatin—were identified. CTACK/CCL27 is an inflammatory chemokine that chemoattracts T cells and enhances stem cell homing to the wound site,[30,31,58] and IL-1ra is an interleukin-1 receptor antagonist that prevents the binding of IL-1, thereby reducing inflammation and promoting healing.[3] The growth factors erythropoietin, BDNF, CNTF, HB-EGF, and HGF play a significant role in stimulating cellular processes involved in MSC or tendon healing—such as cell proliferation, migration, and tissue regeneration. Moreover, follistatin acts as a modulator of these growth factors, exerting both inhibitory and stimulatory effects on cellular processes and contributing to the intricate regulation of MSC or tendon healing.[33,42,47,59,62] This enhancement of MSC immunomodulation aligns with previous research findings. It was observed that the immunomodulatory effect was more pronounced in osteoarthritis SF-MSC conditioned media compared with MSC conditioned media, as indicated by elevated levels of IDO, IL-6, CXCL8, and CCL2.[26,37] Similarly, conditioned media stimulated by proinflammatory cytokines—such as IFN-γ or TNF-α—also exhibited increased immunomodulatory effects, characterized by elevated levels of IDO, factor H5, PGE2, TGF-β, and HGF.[21,51] In contrast, the research conducted by Zayed et al[64] did not demonstrate an increase in the viability and proliferation of bone marrow-derived MSCs when exposed to normal allogeneic synovial fluid. However,

they did observe an increase in the concentration of IDO in the conditioned media of bone marrow-derived MSCs and synovial fluid-derived MSCs when treated with IFN-γ, indicating that inflammatory cytokines have a more significant effect on inducing immunomodulation in MSCs compared with exposure to normal synovial fluid. Therefore, it can be considered that exposure to an inflammatory microenvironment—including inflammatory synovial fluids—has positive effects on both the proliferation of MSCs and their secretion of immunomodulatory factors.

IL-1β, TNF-α, and IL-6 are 3 crucial mediators in acute tendon injury, orchestrating a range of internal and external cellular mechanisms during the inflammatory phase, leading to characteristic clinical symptoms such as heat, swelling, and pain.[22] Our data revealed that SF-UC-MSC conditioned media effectively reduced IL-1β, TNF-α, and IL-6 levels while simultaneously increasing IL-4, an anti-inflammatory cytokine, in IL-1β–treated tenocytes. Previous studies have reported that IL-4 suppresses PGE2 production by inhibiting COX-2 in A549 and RH2 NSCLC cell lines, and in human monocytes and peritoneal macrophages.[14,16,28] While the specific interaction between IL-4 and PGE2 in tenocytes has not been documented previously, our findings showed that SF-UC-MSC conditioned media downregulates COX-2 gene expression and reduces PGE2 secretion while upregulating IL-4 gene expression in inflamed tenocytes. This balance between IL-4 and PGE2 is pivotal in inflammation regulation. PGE2, a significant contributor to tendinopathy development, sustains inflammation and pain in humans.[43] Studies on synovial fluids from arthritis patients and animals have reported an upregulation in PGE2 levels, ranging from 7.35 to 1200 pg/mL.[18,25,27,29,34] In a study by Bergqvist et al,[5] tendon-derived stromal cells from diseased supraspinatus secreted 8800 pg/mL of PGE2 when exposed to 10 ng/mL of IL-1β, while negligible concentrations were detected without IL-1β. Similarly, Koshima et al[34] found that IL-1β production in tissue culture of torn tendons in rabbits led to increased COX-2 and PGE2 levels, with PGE2 reaching a maximum concentration of about 1200 pg/mL at 7 days after the tear. PGE2 levels are commonly investigated to assess anti-inflammatory effects, particularly in studies involving osteoarthritis chondrocytes. Several studies on human osteoarthritis chondrocytes have demonstrated that the use of chemicals such as chrysin and tormentic acid can reduce PGE2 levels from approximately 250 to 1100 pg/mL to 150 to 500 pg/mL. Furthermore, the PGE2 level in the synovial fluid of rheumatoid arthritis patients was successfully reduced from 330 pg/mL to 236 pg/mL through intra-articular injection of hyaluronate, resulting in improved joint pain.[27] Notably, our study revealed that under IL-1β conditions, PGE2 secretion significantly increased to 5.28 ng/mL. However, when treated with SF-UC-MSC conditioned media, it decreased by 0.87-fold, concurrently with a reduction in COX-2 expression. SF-UC-MSC conditioned media demonstrates significant effects on key inflammatory mediators, effectively reducing IL-1β, TNF-α, and IL-6 levels while increasing IL-4 and, notably, downregulating COX-2 gene expression and reducing PGE2 secretion in inflamed tenocytes. This balance between proinflammatory and anti-inflammatory factors underscores its potential as a promising approach for inflammation regulation in tendon healing.

MMP-1, along with COX-2 and PGE2, can accelerate the degradation of the tendon ECM, subsequently influencing the mechanical properties of the tendon.[55] Among various MMPs, MMP-1 can degrade fibrillar collagen, particularly type collagen, which is the main component of tendons. TIMPs serve as natural inhibitors of various MMPs. A disruption in the delicate balance between MMPs and TIMPs can contribute to detrimental alterations in the microstructure and composition of a tendon, ultimately weakening its material properties. Consequently, regulating the homeostasis of MMPs/TIMPs is crucial for effective ECM remodeling.[43] In tendinopathic disorders, tendons often experience a shift in collagen composition, with a decrease in type I collagen, which provides mechanical strength, and an increase in type III collagen, leading to a more randomly oriented collagen network.[46] Our study observed an elevated expression of MMPs and ADAMTSs under inflammatory conditions. Interestingly, SF-UC-MSC conditioned media had a significant impact by reducing MMP-1 expression and increasing TIMP-1 and TIMP-3 levels. Furthermore, SF-UC-MSC conditioned media played a role in rebalancing the gene and protein synthesis of collagen under inflammatory conditions, promoting the upregulation of type I collagen and the reduction of type III collagen. This contrasts with a previous study on adipose-derived mesenchymal stromal cells and tenocytes that reported increased DCN and TNC expression but no shift in the type I to III collagen ratio.[60] Oshita et al[46] demonstrated that administration of adipose-derived stem cells initially increased the type III collagen to type I collagen ratio, followed by a decrease, suggesting that adipose-derived stem cell administration accelerated wound healing from the acute stage and normalized the tendon structure in tendinopathy at the chronic stage. In our previous studies using UC-MSCs in a rat model with a full-thickness rotator cuff tendon defect, UC-MSC treatment improved various parameters such as tendon thickness, inflammation, defect size, tissue connection, and slidability compared with saline. UC-MSCs also enhanced tendon matrix formation, collagen organization, fibroblast aspect ratio, and glycosaminoglycan levels compared with saline, resulting in increased ultimate failure load and ultimate stress at both 2 and 4 weeks.[63] Taken together, SF-UC-MSC conditioned media may offer a promising approach to tendon healing, particularly in countering the fibrotic healing tendency characterized by excessive and disorganized ECM deposition by regulating MMP and collagen synthesis.[15]

The progressive accumulation of matrix damage and inflammatory mediators can lead to reduced cell repair and increased cell apoptosis in tendinopathy.[43] In our study, we found that IL-1β increased the number of β-galactosidase–positive cells and TUNEL-positive cells, indicating enhanced senescence and apoptosis. Notably, the conditioned media reversed IL-1β induced senescence and apoptosis by approximately 0.51-fold and 0.23-fold, respectively. However, we did not observe significant changes in tenocyte proliferation, either by IL-1β or SF-UC-MSC conditioned media. Several studies have demonstrated that using MSC-conditioned media or direct co-culture with MSCs can enhance tenocyte proliferation in

the absence of IL-1β. Bone marrow MSC conditioned media has been shown to enhance rat tenocyte proliferation via the ERK1/2 signaling pathway, leading to changes in cell cycle distribution.[10] Co-culturing adipose-derived MSCs with tenocytes at a 3 to 1 ratio and employing adipose-derived MSC-conditioned media have been shown to enhance human tenocyte proliferation.[35] Similarly, bone marrow-derived MSCs have demonstrated increased proliferation, migration, and cell adhesion in rat tendon fibroblasts, as observed by Shimode et al.[53] However, our study first revealed that in the presence of IL-1β, the effects of MSC-conditioned media on tenocyte proliferation were negated, resulting in no significant enhancement. This aligns with the findings of Platas et al,[49] which showed that the conditioned media did not induce significant changes in osteoarthritic chondrocyte proliferation under 10 ng/mL IL-1β. The variations in our findings could be attributed to the characteristics of tenocytes derived from patients with degenerative rotator cuff disease, as well as the inflammatory state induced by IL-1β. MSC-conditioned media has been reported to have other functions on cells in tendinopathy, including enhancing the migration of tenocytes in a dose-dependent manner, inhibiting proliferation of peripheral blood mononuclear cell, and inducing macrophage to M2 polarization.[8,11,50] Our consistent data on the anti-inflammatory and cytoprotective effects of SF-UC-MSC conditioned media on inflammatory tenocytes suggests that exposing UC-MSCs to degenerative synovial fluids during injection could support tendon healing.

Limitations

This study has several limitations. First, we did not conduct assays for protein synthesis on all the genes we tested. Second, we exposed UC-MSCs to synovial fluid for 24 hours, representing the early response of UC-MSCs when encountering the synovial fluid microenvironment. Therefore, the study did not provide information on the long-term effects of synovial fluid on patients with inflammation of MSCs. Third, this study did not compare the effects of MSC-conditioned media and SF-UC-MSC conditioned media on inflamed tenocytes; thus, the differences between their respective impacts were not assessed. Fourth, the effects of SF-UC-MSC conditioned media on inflamed tenocytes were observed at 24 hours in the study. However, multiple time points may be needed, as during the tendon remodeling process, type III collagen reacts in the initial response, and type I collagen then replaces type III collagen over time.

CONCLUSION

The findings of this study showed that conditioned media from SF-UC-MSCs had anti-inflammatory effects and cytoprotective effects on IL-1β–treated tenocytes from degenerative rotator cuff tears, thus suggesting that the UC-MSCs could be used for the treatment of tendinopathy in practice.

ORCID iD

Chris Hyunchul Jo https://orcid.org/0000-0002-6161-5442

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APPENDIX

TABLE A1
Abbreviations Used

Term	Description
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
BAX	BCL2 associated X, apoptosis regulator
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BRAK	Breast and kidney-expressed chemokine
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CNTF	Ciliary neurotrophic factor
Col	Collagen
COX	Cyclooxygenase
CSF	Colony-stimulating factor
CTACK	Cutaneous T cell-attracting chemokine
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAPI	4′,6-diamidino-2-phenylindole
DCN	Decorin
DEG	Differentially expressed gene
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
Epo	Erythropoietin
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDR	False discovery rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRN	Progranulin
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
I-TAC/CXCL11	Interferon-inducible T-cell alpha chemoattractant
JAK-STAT	Janus kinase–signal transduction and transcription activation
MCSF/CSF1	Macrophage colony-stimulating factor
MIP-1α/CCL3	Macrophage inflammatory protein–1 alpha
MMP	Matrix metalloproteinase
NRCAM	Neuronal cell adhesion molecule
OSM	Oncostatin M
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PTGES	Prostaglandin E synthase
SCX	Sceleraxis
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF-UC-MSC	Synovial fluid–stimulated umbilical cord-derived mesenchymal stem cells
TBS-T	Tris-buffered saline containing 0.1% Tween 20
TdT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
Th1	T-helper cell type 1
TIMP	Tissue inhibitor of metalloproteinase
TNC	Tenascin
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UC-MSC	Umbilical cord-derived mesenchymal stem cell