의 학 박 사 학 위 논 문

Therapeutic Mechanisms of Human Adipose-Derived Mesenchymal Stem Cells in a Rat Tendon Injury Model

힘줄 손상 백서 모델에서의 인간 지방유래 중간엽 줄기세포의 치료 기전

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서울대학교 대학원

의학과 재활의학 전공

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이 논문을 의학박사 학위논문으로 제출함

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Therapeutic Mechanisms of Human Adipose-Derived Mesenchymal Stem Cells in a Rat Tendon Injury Model

by

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A thesis submitted to the Department of Rehabilitation Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Rehabilitation Medicine at the Seoul National University College of Medicine

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Approved by thesis committee:

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Abstract

Therapeutic Mechanisms of Human Adipose-Derived Mesenchymal Stem Cells in a Rat Tendon Injury Model

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Introduction: Mesenchymal stem cell (MSC) in treating tendon injury is well investigated by experimental animal models and several clinical trials have also reported safety and efficacy of the MSC therapy. However, therapeutic mechanisms of MSC for tendon injury have not fully understood and there is no *in vivo* study that MSCs can function as differentiated cells after transplantation. We aimed to investigate whether MSCs can differentiate into the tenogenic lineage and secrete their own proteins using a xenogeneic MSC transplantation model.

Methods: Bilateral Achilles tendons of 57 SD rats were given full-thickness rectangular injuries at the tendon insertion site to the midsubstance. They were randomly assigned to 3 groups after the modeling: 1) human adipose-derived mesenchymal stem cells (hASC)

implantation with fibrin glue (10^6 cells in 60 μL) (Cell group), 2) fibrin glue injection with cell media by the same volume (Fibrin group), and 3) identical surgical procedure without any treatment (Sham group). After 2 and 4 weeks after modeling, all groups were evaluated by morphological, biomechanical, and histopathological (using modified Bonar score) analyses. Viability of tagged hASC was observed by immunofluorescence staining and protein expressions (collagen type I/III and tenascin-C) were evaluated by immunohistochemistry and Western blot analyses.

Results: Rupture rate of Cell group (11.1%) was lower than the rates of Sham (25.0%) and Fibrin (22.2%) groups. Cross sectional areas of tendons in Cell group were decreased ($P = 0.008$) while those in Sham group were increased ($P = 0.005$) from 2 to 4 weeks. At 2 weeks, ultimate tensile strength and stiffness of Cell group (49.4 ± 17.4 N and 10.1 ± 3.9 N/mm) were significantly higher than those of Sham group (31.2 ± 7.5 N, $P = 0.037$ and 4.7 ± 1.4 N/mm, $P = 0.010$, respectively). Stiffnesses of Cell group at 2 and 4 weeks were also significantly higher than those of Fibrin group ($P = 0.037$ in both). However, there were no significant differences of total modified Bonar score among three groups at both 2 and 4 weeks. From the immunofluorescent scanning at 1, 2, and 4 weeks after cell implantations, tagged hASCs were all observed. Cell group showed higher optical densities in immunohistochemistry than those of Sham and Fibrin groups in human-specific collagen type I at both 2 and 4 weeks and in human-specific tenascin-C at 2 weeks. Western blot analysis also revealed human-specific collagen type I expression was higher than that of Sham group.

Conclusions: Implanted hASCs to rat tendon injury model survived for 4 weeks and secreted human-specific collagen type I and tenascin-C. Human stem cells biomechanically enhanced rat tendon healing superior to sham and active control groups. To the best of the author's knowledge, this is the first *in vivo* report which proves the cell-originated protein synthesis by the implanted stem cells.

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Keywords: Mesenchymal stem cell transplantation, Heterologous transplantation, Tendon injuries, Achilles tendon, Collagen type I

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MSC = mesenchymal stem cell

hASC = human adipose-derived mesenchymal stem cells

CM-Dil = chloromethyl-1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

DAPI = 4′,6-diamidino-2-phenylindole

CSA = cross sectional area

H & E = hematoxylin and eosin

COL1 = collagen type I

COL3 = collagen type III

TnC = tenascin-C

IHC = immunohistochemistry

OD = optical density

MMP = matrix metalloproteinase

RT-PCR = real-time polymerase chain reaction

Contents

Introduction

Mesenchymal stem cell (MSC) is drawing a big attention as a novel regenerative therapy in the musculoskeletal field. MSC applications in treating tendon injury or tendinopathy have been well investigated by several experimental models [1-4] and a few clinical trials [5, 6]. From the 52 weeks' follow up, MSC injection seems to be safe and efficacious in even cases with chronic and refractory tendinopathy [6]. There are also another active-recruiting clinical trials about MSC treatments on rotator cuff tear (NCT02298023), Achilles tendinopathy (NCT02064062), and lateral epicondylosis (NCT02131077). Nonetheless, therapeutic mechanisms of MSC for tendon injury have not fully understood.

Generally, MSC-based cell therapy can induce the tissue healing by three mechanisms [7]. First, MSCs can modulate the immune response from their immuno-modulatory functions by direct cell-to-cell contact and release of soluble immunosuppressive factors [8]. Second, MSCs can secrete a broad variety of cytokines, chemokines, and growth factors which may prevent the adjacent cells from apoptosis and promote their proliferation [9]. This paracrine theory is also supported by which administration of conditioned medium of MSCs is able to reproduce the beneficial effects of MSCs for tissue repair [10]. Lastly, MSCs have capacity to differentiate into various cell lineages including bone, cartilage, tendon, fat, bone marrow stroma, and muscle [11]. Among the three mechanisms, the last one is the most intuitive process of MSC applications because it can substitute the wounded tissue and make the tissue-replacement not by merely humeral or immunologic reactions on recipient tissues.

There are several *in vitro* studies which proposed that MSC differentiated into the tenocytes. Morita *et al.* reported two studies which suggested that stem cell-to-tenocyte differentiation by *in vitro* experiments [12, 13]. One also reported that rhesus MSCs differentiated into tenocytes after BMP 12 gene transfection to MSCs, which was identified by morphological observation and molecular biological assay such as immunocytochemistry [14]. However, to our knowledge, there is no *in vivo* study that MSCs can differentiate into the tenogenic lineage and secrete the cell-originated proteins.

In the present study, I aimed to investigate whether MSCs can differentiate into the tenogenic lineage and secrete their own proteins. To prove the cellular function of MSCs, a xenogeneic cell transplantation model was planned: human adipose-derived mesenchymal stem cells (hASC) into rat tendon. This method can support to identify the MSC differentiation and discriminate whether the secreted protein is originated from donor cells (human) or recipient tendon tissues (rat) by using the species-specific monoclonal antibody. I hypothesized that hASCs can differentiate into the human tenogenic lineage and the cells can also secrete human-specific proteins.

Materials and Methods

1. Study design

Fifty-seven 13-week-old male Sprague-Dawley rats (body weight 390-410 g) were used in this study. All rats had the adaptation period of one week in the laboratory. Temperatures were maintained as 22°C and brightness was controlled in 12-hour cycle. Food and water was supplied *ad libitum*. 54 S-D rats were randomly divided into 3 groups; 1) hASC implantation with fibrin glue (Cell group), 2) fibrin glue injection with cell media by the same volume (Fibrin group), and 3) identical surgical procedure without any treatment (Sham group). Outcome parameters (rupture rate, cross sectional area, biomechanical, histopathological, immunohistochemistry, and Western blot) were assessed after 2 and 4 weeks from each modeling. Another 3 rats were used to verify the hASCs viability for 1, 2, and 4 weeks after cell transplantation by immunofluorescent staining. The Institutional Animal Care and Use Committee of Seoul National University Hospital approved all the procedures described below (No. 14-0242-S1A0).

2. Stem cell preparation

hASCs were isolated from lipoaspirates of human subcutaneous fat tissue obtained from healthy donors who provided informed consent. Donor suitability assessment was performed in accordance with the Guideline on the Requirements for Quality Dossier of Biological Products in Clinical Trials of the Korean Ministry of Food and Drug Safety. The lipoaspirates were washed with phosphate buffered saline and digested in an equal volume of phosphate buffered saline containing 1% bovine

serum albumin and 0.025% collagenase type I (Invitrogen, Gaithersburg, MD) for 80 minutes at 37°C with intermittent shaking. The isolated stromal vascular fraction was cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) that was supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1 ng/mL human basic-fibroblast growth factor to obtain a sufficient number of cells for injection. After harvesting cells by trypsinization, the cells were suspended in Dulbecco's Modified Eagle's Medium and packaged into single-use vials. hASCs were manufactured using an ASC bank which was established with subcutaneous adipose tissues harvested from a healthy donor. As the batch size of the hASC bank from one donor is at least more than 50, the ASCs at passage 3 to 4 obtained from one healthy donor were used for this clinical trial. The manufacturing procedure was performed according to the Good Manufacturing Practices authorized by the Korean Ministry of Food and Drug Safety. For lot-release testing, hASCs were assessed for cell appearance, viability, identification, purity, content, and potency. The potency was assessed as a viable cell counting. hASCs are known to have diverse biological functions including self-differentiation potential, anti-inflammatory effect, and various growth factor-releasing effect resulting in promoting wound healing and tissue regeneration not by the one of specific mechanisms. In this regard, total amount of viable cells that could exert their biological function was determined by a reasonable potency testing item. In the process of ASC bank, MSC characteristics such as self-renewal, cell morphology, doubling time, karyotype, cell surface markers, and biological function which includes growth factor releasing and immune suppressive activity were thoroughly tested. Only hASCs met the all testing requirements including test items mentioned above are banked after culturing. All these procedure follows the 'Cell Bank Process' which is a standard operating procedure that outlines how to establish cell banking system. The minimum criteria for release were 80% cell viability and less

than 1% of CD45-positive cells (a measure of purity). In addition, hASCs were screened for contamination with adventitious agents, mycoplasma, bacteria, fungi, and viruses 3 days before packaging to comply with the recommendation of 'Guidance on specifications and test methods for cell therapy products' from Korean Food and Drug Administration. Sterility test was performed once more with the sampled final product after packaging [6].

3. Fluorescent cell labeling and immunofluorescence staining

To distinguish between implanted hASC and host-derived proliferated rat cells, the hASCs were labeled using the chloromethyl-1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (CM-Dil) (C7001, CellTracker; Invitrogen Life Technologies, Carlsbad, CA) according to a modification of the manufacturer's protocol. Briefly, from a 1 mg CM-Dil/ml stock solution in culture-grade DMSO, 8 μM solutions were made in 500 μL HBSS, vortexed, and then combined with 2 x 10^6 hASCs in 500 μL HBSS, to give 10^6 cells/ml in 4 μM CM-Dil labeling solution. CM-Dil cell suspensions were incubated for 30 min at 37 °C and then for 15 min at 4 °C. After labeling, cells were washed three times with PBS at 400×g for 10 min at 25 °C and resuspended in fresh medium [15]. After harvesting each tissue, specimens were fixed in 4% PFA for 24 hours, then immerged in 10%, 20%, and 30% sucrose solution for 24 hours each, sequentially. After sucrose was removed, tissues were embedded in OCT compound, then stored in liquid nitrogen tank at -70°C. Blocks were cut into 5 μm-thick cryostat sections and mounted on superfrost slides. The 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used for nuclear counterstain. Specific fluorescence was analyzed using laser scanning microscopy (BX-UCB; Olympus Co., Tokyo, Japan).

4. Surgical procedure and treatment

Anesthesia was induced and maintained with 5% and 2% of isoflurane dissolved in 40%/60% and 25%/75% oxygen/nitrogen applied via chamber and nose cone, respectively. Anesthesia depth was adjusted to the level abolishing abdominal contractions to tail pinch. After anesthesia with isoflurane inhalation in the prone position, the surgical procedures were performed under sterile conditions after shaving and disinfection of both hindlimb. With use of aseptic technique, an approximately 15-mm horizontal skin incision was performed on the medial side over the Achilles tendon by No. 10 surgical blade. Great care was taken not to harm the tendon itself in the skin incision. The Achilles tendon was freed from surrounding tissue. After completely exposed, two No. 11 surgical blades assembled with a plastic rack printed on a 3D-printer (Figure 1A) were used to make a standardized parallel incision in the tendon 0.8 mm apart and 5 mm long, spanning from the tendon-bone insertion at the calcaneus to the mid-tendon. To make rectangular defects, micro-scissors were used to remove the scored sections, such that a full-thickness defect was created in the midsubstance [16] (Figure 1B). The plantaris tendon was resected about 5 mm-length around the rectangular defects of Achilles tendon to prevent an internal splint effect [17]. Then, a 60 μL volume of mixtures with 10^6 hASCs and fibrin glue was inoculated into the intratendinous defect in Cell group. A dual-syringe injection system (Greenplast kit, Green Cross, Seoul, Korea) with 26-gauge needles was loaded with 30 μL thrombin mixed with 10^6 hASCs in the first syringe and 30 μL fibrinogen in the other syringe (Figure 1C and 1D). In Fibrin group, the defect was filled with the same volume (60 μL) of fibrin glue with cell media. The identical surgical procedure without any treatment was conducted in Sham group. The skin was

closed in an intracutaneous fashion with non-absorbable 4-0 silk suture in all three groups. No immobilization device was applied and weight-bearing was not restricted. All rats were received subcutaneous injection with 1 mg/kg meloxicam and 20 mg/kg cefazolin before operation and the next day of operation.

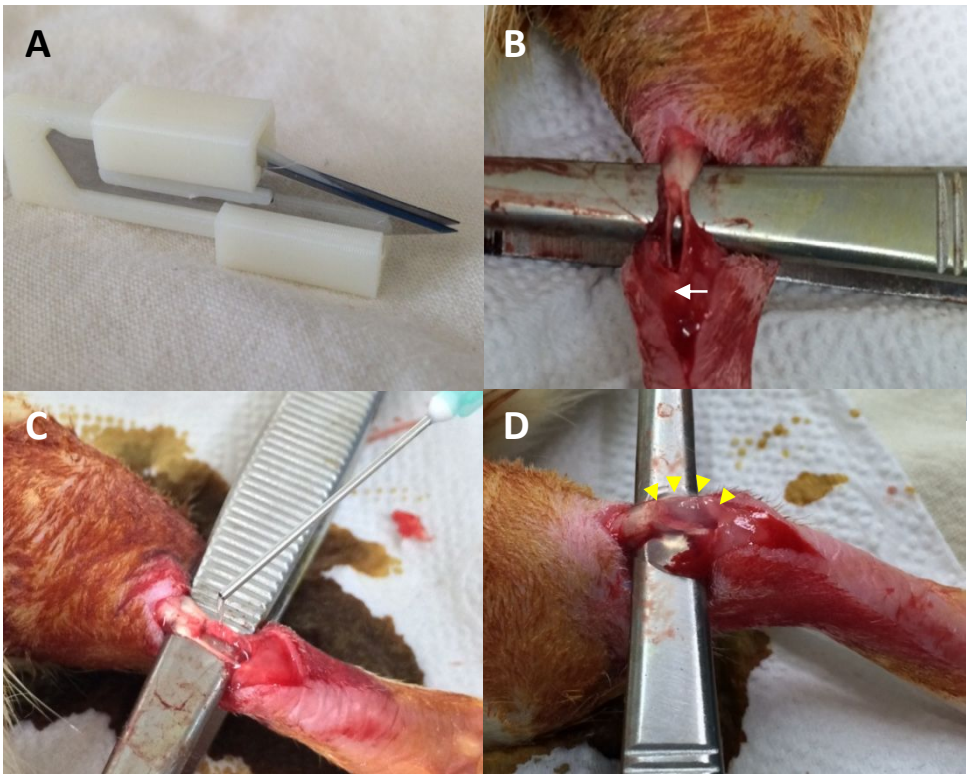

Figure 1. Intratendinous rectangular tendon defect and cell implantation

Surgical blades assembled with plastic racks (A) and a full-thickness rectangular intratendinous defect (0.8mm x 5mm) of the Achilles tendon (B) spanning from the tendon-bone insertion at the calcaneus (arrow) to the mid-tendon. Inoculation of human adipose-derived mesenchymal stem cells with fibrin glue into the intratendinous defect (C) and the coagulated injectates (yellow arrowheads) before the skin closure (D).

5. Rupture rate and cross sectional area calculation

After being euthanized by means of carbon dioxide inhalation, tendons were harvested at 2 and 4 weeks from each modeling. Tendon rupture was defined as ≥ 1 mm-sized visible gapping between tendon end and calcaneal bone with partial or complete loss of continuity [1]. After assuming the cross section of tendon is an ellipse, tendon cross sectional area (CSA) at the mid-portion of the surgical defect was calculated by two perpendicular thickness (major axis = a and minor axis = b) as below: $CSA = \pi \times a \times b/4$. Each thickness was measured by a digital vernier caliper.

6. Biomechanical test

All tendons for the biomechanical test (6 tendons in each group) were harvested still attached to the foot, and the triceps surae muscle was transected through the muscle belly, well proximal to the tendon repair. All the harvested specimen were stored in -80°C to prevent tissue damage. Before the test, tissues were thawed at room temperature for more than 4 hours. Each thawed Achilles tendon was moistened with soaked gauzes by phosphate-buffered saline solution to prevent drying out. The distal end around metatarsal bone was securely press-fixed with metallic clamp while the proximal end (triceps surae muscle) was frozen with liquid nitrogen and fixed in a customized frame produced by a 3D-printer (Figure 2). To make sure that the liquid nitrogen did not affect the healed tendon itself, the surface temperature of tendons was measured before and after applying liquid nitrogen. The temperature difference was less than 2˚C in all specimens. The tendons were then mounted onto a biomechanical testing machine (JSV-H100; JISC, Tokyo, Japan) with an 100 N load cell. The construct was initially set to a

basic axial stress of 0.1 N preload for straightening and adjustment. After that, the length between calcaneal bone and myotendinous junction was measured as an initial length. Each tendon was then axially pulled at a constant speed of 10 mm/min until maximal load to failure. Ultimate tensile strength (N), stiffness (N/mm), and Young's modulus (MPa or N/mm^2) were measured from each tendon's stress-strain curve [18]. All tests were conducted with being blind to groups and weeks of specimens.

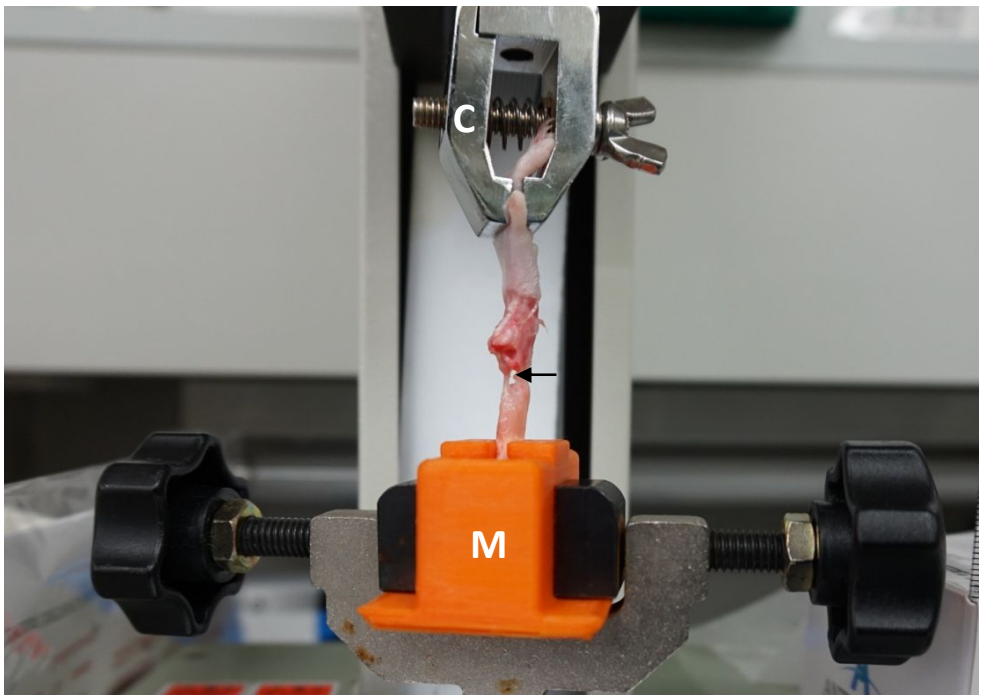

Figure 2. Two clamps for biomechanical test and a ruptured tendon during the test.

The distal end (metatarsal bone) was securely press-fixed with metallic clamp (C) while the proximal end (triceps surae muscles) was frozen in a customized mold (M). A black arrow shows the torn area of the osteotendinous junction during biomechanical test.

7. Tissue preparation and histological analysis

Achilles tendons for histological analysis (6 tendons in each group) were harvest between the calcaneus and the musculotendionus junction. After 10% formalin fix for a day, specimens were embedded in paraffin and cut into 3 μm sections by coronal direction, and stained with either hematoxylin and eosin (H & E) or Alcian blue. From the stained tissue, histopathological analysis of the Achilles tendon were analyzed using the semi-quantitative histopathological scale (modified Bonar score) [19]. Briefly, this scale consists of five features, each graded as 0, 1, 2, or 3: (1) cell morphology, (2) collagen arrangement by polarizing microscope observation, (3) cellularity, (4) vascularity, and (5) ground substance by Alcian blue staining. As total score, a tendon with no observable pathology will score 0 while the worst pathology will score 15. Each slide was examined by one pathologist who was blind to groups and weeks.

8. Immunohistochemistry

The distribution of collagen type I (COL1), type III (COL3), and tenascin-C (TnC) was assessed by means of immunohistochemical (IHC) staining. . To evaluate whether COL1 is synthesized by the differentiated hASC (donor) or already existed rat cells (recipient), two antibodies were used: human-specific anti-COL1 monoclonal antibody (ab138492; Abcam, Cambridge, MA) or general anti-COL1 polyclonal antibody (ab84956; Abcam) which is reactive on both humans and rats. General anti-COL3 polyclonal antibody (ab23746; Abcam) was also used. TnC is a protein which are secreted by tenocytes or fibroblasts and also found in the process of tendon healing [20, 21]. Because anti-TnC monoclonal antibody (ab58954; Abcam) is also human-specific, it can help to discriminate whether the distributed

protein is expressed by human cells or not.

Tissue sections were 3 μm sized cut and placed on slides. Using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems Inc., Tucson, AZ), slides were stained as follow procedure. Sections were deparaffinized using EZ Prep solution. CC1 standard (pH 8.4 buffer contained Tris/Borate/EDTA) was used for antigen retrieval. Inhibitor D (3% H_2O_2, Endogenous peroxidase) was blocked for 4 min at 37°C temperature. Slides were incubated with each primary antibody (human-specific anti-COL1, general anti-COL1, general anti-COL3, and human-specific anti-TnC) at dilutions of 1:50 [22], 1:400 [23], 1:400 [24], and 1:20 [25] for 32 min at 37°C and a secondary antibody of Omnimap anti Mouse HRP for 20 min at 37°C. Slides were incubated in DAB+ H_2O_2 substrate for 8 min at 37°C followed by Hematoxylin and Bluing reagent counterstain at 37°C. Reaction buffer (pH 7.6 Tris buffer) was used as washing solution. The absence of staining was identical to that obtained when a non-specific primary antibody was used [26]. The glass slides were then examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan). The photographs of the specimens under 400× magnifications were taken in 10 different fields using Leica Application Suite EZ (Leica, Wetzlar, Germany). All the photographs were taken in the same scale. Human skin was used as a positive control tissue in human-specific protein analyses.

After IHC staining, sections stained with each antibody were quantitatively analyzed in a random order using ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/) by color deconvolution with 10 high power fields (400×) as reported previously [27]. Briefly, IHC images stained with DAB and hematoxylin leads to the production of three images, namely, DAB, hematoxylin,

and a complimentary image. Using a IHC profiler [28], which is currently compatible for use with Microsoft Windows operating system, the pixel intensity values for any color range from 0 to 255, wherein, 0 represents the darkest shade of the color and 255 represent the lightest shade of the color as standard. To determine the correct optical density (OD) vectors for the RGB channel of Hematoxylin and DAB, the protocol as previously described by Ruifrok *et al* was followed [29]. Since the optical density is proportional to the concentration of the stain, the amount of stain present will be a factor determining the OD which is defined as log (maximal intensity/mean intensity), where maximal intensity = 255 for 8-bit images. IHC staining intensity was assessed by mean OD of 10 different fields in each specimen.

9. Western blot analysis

From each tendon (6 tendons in each group), 30-40mg proteins were extracted by homogenization with the PRO-PREPTM protein extraction solution (iNtRON Biotechnology Inc., Seongnam-si, Korea). Homogenates were centrifuged at 12,000 rpm at 4°C for 30min. The protein concentrations in homogenates were measured using the BCA protein assay reagent (Pierce, Rockford, IL). The protein samples were loaded an equal amount per well, and then gel electrophoresis about 100 min (with 80V for 20 min and 120V for 80 min). After gel electrophoresis, proteins were transferred NC membranes with 400mA for 180 min on ice. NC membranes were blocked with 5% skim milk-PBS/T at room temperature for 1 hour. Used primary antibodies were as follows; human-specific anti-COL1 (ab138492; Abcam), diluted 1:750; general anti-COL1 (ab6308; Abcam), diluted 1:750; general anti-COL3 (MAB3580; Anova, Taipei, Taiwan), diluted 1:750; anti-matrix metalloproteinase (MMP)-2 (ab86607; Abcam), diluted 1:750; anti-MMP-9

(ab76003; Abcam), diluted 1:750; and monoclonal anti-α tubulin produced in mouse (T9026 sigma; Sigma-aldrich, St. Louis, MO), diluted 1:10000. Samples were incubated at room temperature for 1 hour, washed with PBS/T buffer for 10 min, repeated 5 times. The secondary antibodies were applied and incubated for 1 hour at room temperature, then washed with the PBS/T buffer for 10 min, repeated 5 times. Signal bands were detected using chemiluminescence kit (Pierce, Rockford, IL) and were quantified with ImageJ. The ratios of each protein to those of the α-tubulin were calculated.

10. Statistical analysis

Differences in rupture rates between two groups were analyzed by the Chi-square tests. Comparing the cross sectional area of tendon among 3 groups was conducted by analysis of variance and Fisher least significant difference post hoc test. Because the sample size of each outcome variable was not enough, Kruskal-Wallis test was used to compare the results of biomechanical, histological, and ODs from IHC and WB among 3 groups. Mann-Whitney *U* test was also used for *post hoc* comparisons between two groups. All statistical analyses were performed using PASW Statistics 18.0 for Windows (SPSS Inc., Chicago, IL). P values < 0.05 were considered to be statistically significant.

Results

1. Rupture rate and cross sectional area of tendon

Rupture rate of Cell group (11.1%) tended to be lower than the rates of Sham (25.0%) and Fibrin (22.2%) groups although the differences were not statistically significant ($P = 0.126$ and $P = 0.206$, respectively) (Table 1). Among 3 groups, CSAs were different at both 2 ($P = 0.030$) and 4 ($P = 0.001$) weeks. From 2 to 4 weeks after modeling, CSA of Sham group was increased (8.3 ± 2.3 to 10.8 ± 2.8 mm^2; $P = 0.005$) while that of Cell group was decreased (10.7 ± 3.0 to 8.4 ± 3.8 mm^2; $P = 0.008$). CSA of Cell group was higher than that of Sham group at 2 week ($P = 0.006$). However at 4 weeks, CSA of Cell group was lower than that of Sham group ($P = 0.007$). (Figure 3).

Table 1. Rupture rates in each group

Groups	Sham	Fibrin	*P*-value*	Cells	*P*-value*	*P*-value**
2weeks	**16.7% (3/18)**	**22.2% (4/18)**	0.674	**5.6% (1/18)**	0.289	0.148
4 weeks	**33.3% (6/18)**	**22.2% (4/18)**	0.457	**16.7% (3/18)**	0.248	0.674
Total	**25.0% (9/36)**	**22.2% (8/36)**	0.781	**11.1% (4/36)**	0.126	0.206

Compared with Sham* and Fibrin group** at the same period (by Chi-square test).

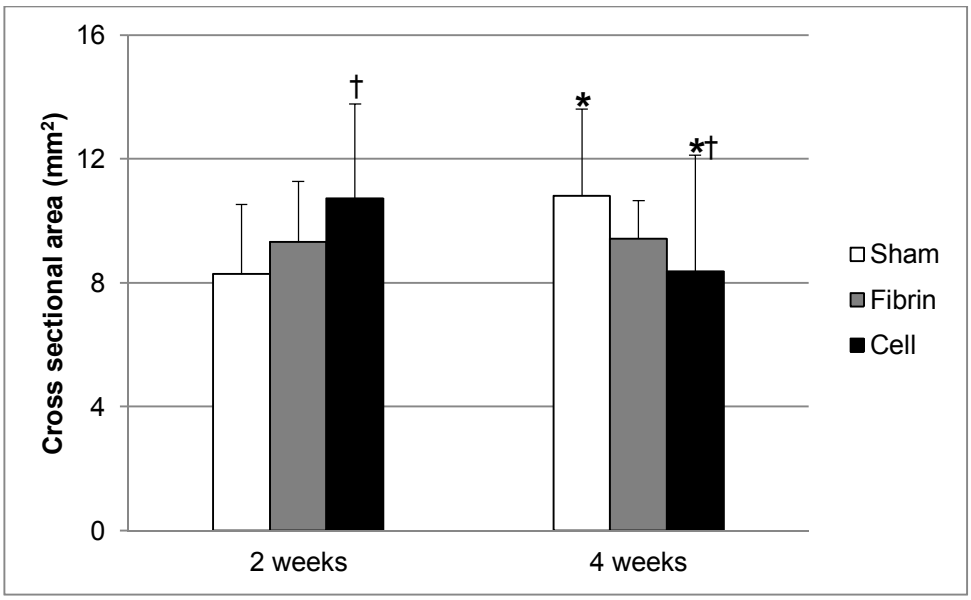

Figure 3. Cross sectional areas of rat tendons in each group

$P < 0.05$ by post-hoc test using Fisher least significant difference after analysis of variance compared with the same group* at 2 weeks and the sham group† at the same period.

2. Biomechanical test

During the biomechanical test, 6 specimens (2 in Sham group, 3 in Fibrin group, and 1 in Cell group) were slipped out of the plastic frame. Therefore, 30 specimens were finally analyzed for the biomechanical test. Stiffness was the only different variable at 2 weeks among 3 groups ($P = 0.012$) and there were no different biomechanical outcomes at 4 weeks. Ultimate tensile strength and stiffness of Cell group were significantly higher than those of Sham group at 2 weeks (49.4 ± 17.4 N *vs*. 31.2 ± 7.5 N, $P = 0.037$ and 10.1 ± 3.9 N/mm *vs*. 4.7 ± 1.4 N/mm, $P = 0.010$, respectively). Although ultimate tensile strength of Cell group at 4 weeks tended to be higher than that of Sham group, it was not significant ($P = 0.201$). From 2 to 4 weeks from modeling, a significant temporal change was only observed at the stiffness of Fibrin group (6.1 ± 1.3 N/mm to 10.0 ± 7.6 N/mm, $P = 0.010$). Stiffnesses of Cell group at 2 and 4 weeks were also significantly higher than those of Fibrin group ($P = 0.037$ in both). While Young's modulus of Cell group tended to be higher than the other groups at both 2 and 4 weeks, the differences were not statistically significant (Figure 4).

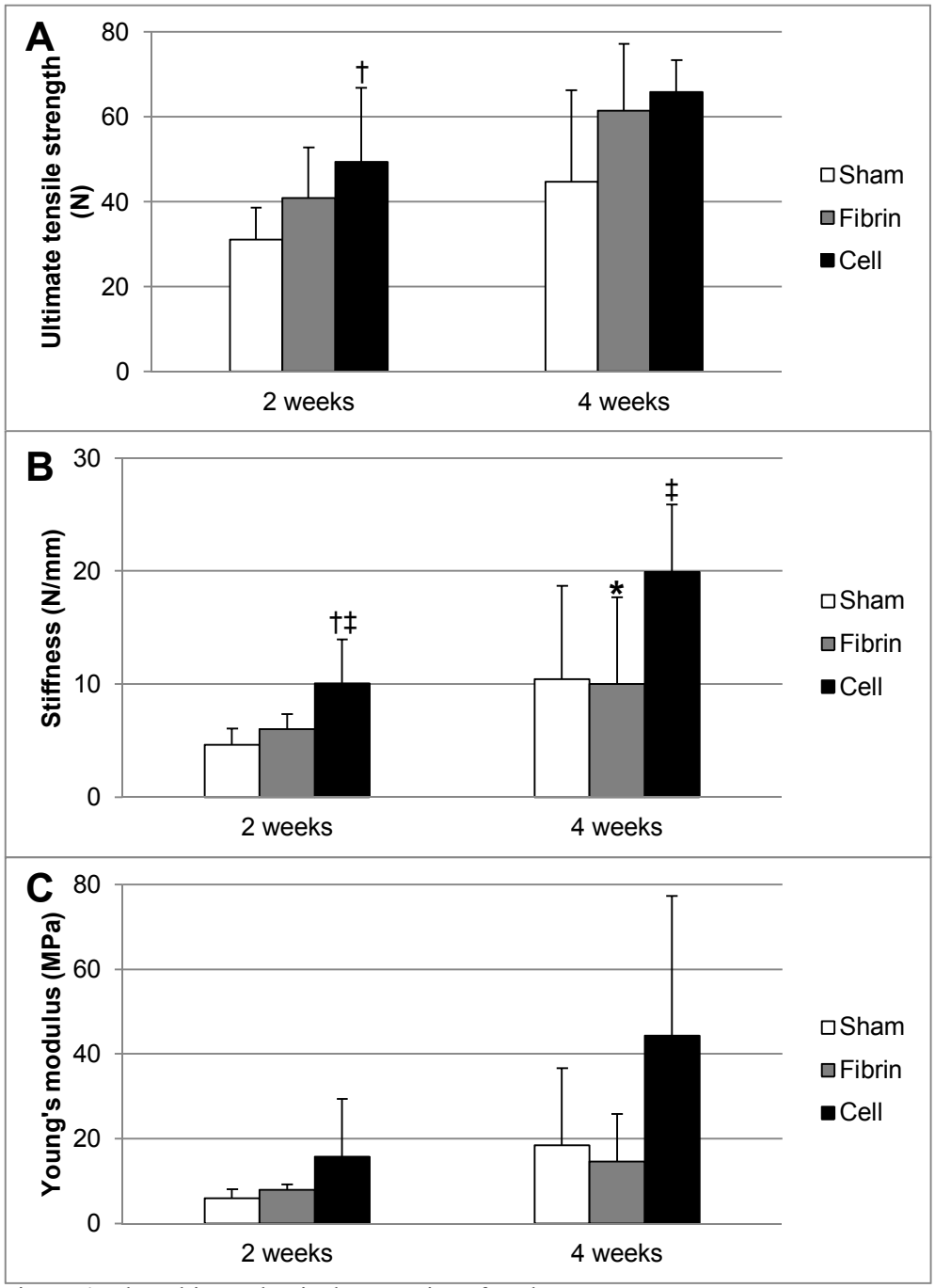

Figure 4. Three biomechanical properties of each group.

(A) Ultimate tensile strength, (B) Stiffness, and (C) Young's modulus. $P < 0.05$ by Mann-Whitney's U test compared with the same group* at 2 weeks; the Sham† and Fibrin‡ groups at the same period.

3. Histological analysis (modified Bonar score)

The longitudinal defect and recovered tissue can be histologically observed by both H & E and Alcian blue staining (Figure 5). A total modified Bonar score was statistically different among 3 groups at 2 weeks ($P = 0.045$), but not at 4 weeks ($P = 0.355$). At 2 weeks, Sham group showed significantly lower total score than Fibrin group (6.8 ± 1.0 *vs*. 8.5 ± 1.4, $P = 0.022$) (Figure 6).

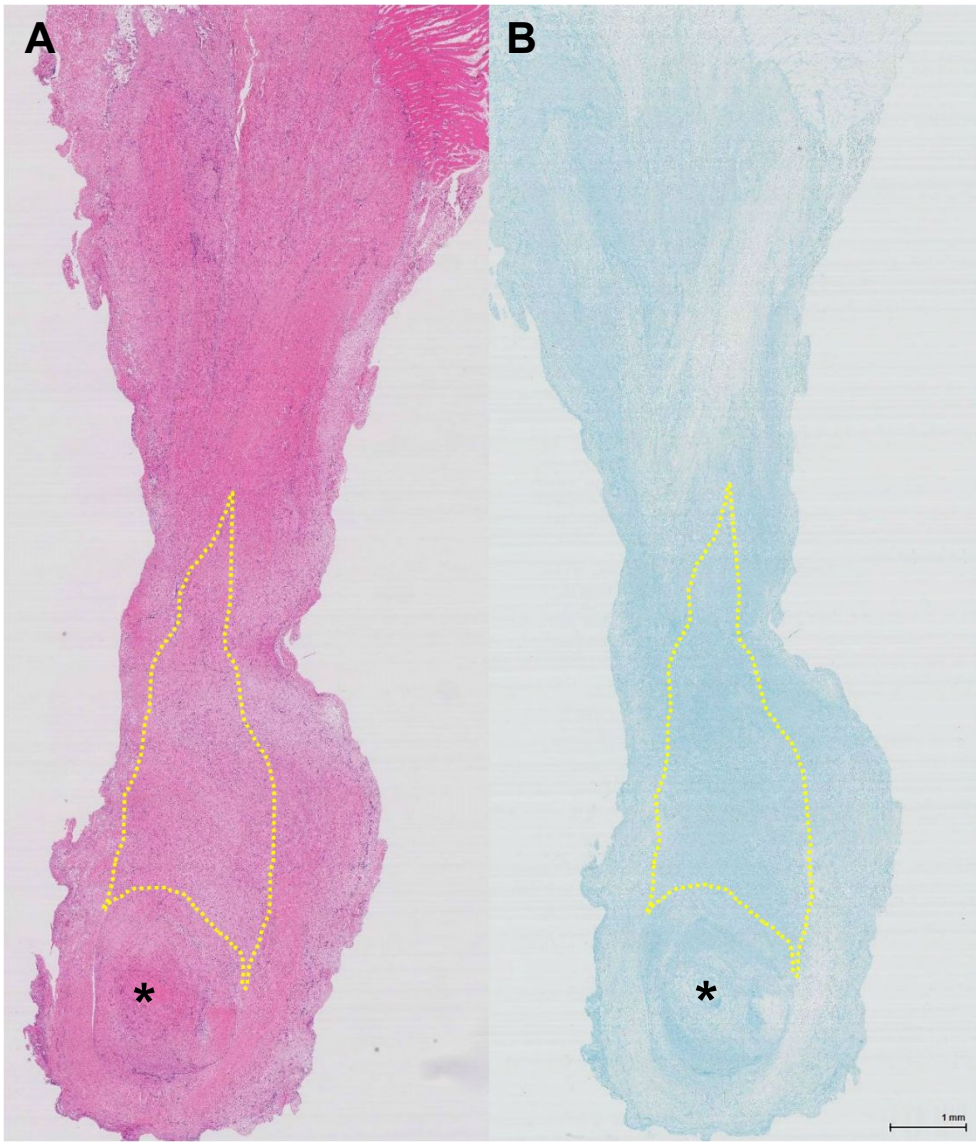

Figure 5. Histological analysis of one harvested tendon

Representative sections in Sham group after 2 weeks from modeling (mid-coronal section, magnification 40×). A: H & E staining and B: Alcian blue staining. Dotted polygons and asterisks represent the hypothetical intratendinous defect which was recovering histologically and the core of calcaneal bone, respectively.

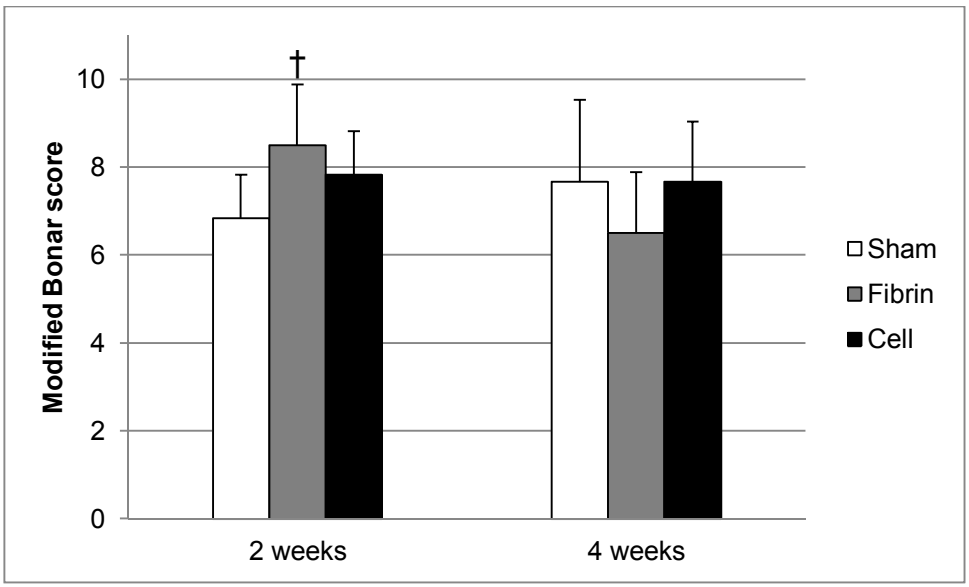

Figure 6. Histopathological outcomes by modified Bonar score.

$P < 0.05$ by Mann-Whitney's U test compared with the Sham† group at the same period.

4. Immunohistochemistry and immunofluorescent staining

ODs of general COL1 were not statistically different among 3 groups at both 2 (P = 0.103) and 4 (P = 0.472) weeks. Cell group tended to have higher ODs of general COL1 than Sham and Fibrin groups at both 2 and 4 weeks while the findings were not statistically significant (Figure 7A). ODs of general COL3 were not also statistically different among 3 groups at both 2 (P = 0.372) and 4 (P = 0.076) weeks. As to general COL3, all groups showed a declining tendency from 2 to 4 weeks and the only Cell group had a significant OD reduction (254.9 ± 45.2 to 187.7 ± 49.4, P = 0.037) (Figure 7B).

ODs of human-specific COL1 were statistically different among 3 groups at both 2 (P = 0.004) and 4 (P = 0.020) weeks. All groups showed positively stained IHC results with human-specific COL1 in a more or less degree. However, Cell group had significantly higher ODs at both 2 (229.5 ± 115.3) and 4 (137.1 ± 26.3) weeks than those of Sham (89.8 ± 20.3, P = 0.004 and 68.7 ± 44.7, P = 0.033, respectively) and Fibrin groups (95.2 ± 22.2, P = 0.006 and 44.5 ± 13.9, P = 0.011, respectively) (Figure 7C and 8). As to human-specific TnC, ODs were statistically different among 3 groups at only 2 weeks (P = 0.012) and that of Cell group (170.1 ± 39.1) were higher than those of Sham and Fibrin groups (70.2 ± 38.9, P = 0.009 and 90.9 ± 35.6, P = 0.028, respectively) (Figure 7D and 9). From the immunofluorescent scanning, CM-Dil labeled hASCs were observed at 1, 2, and 4 weeks after cell implantations (Figure 10).

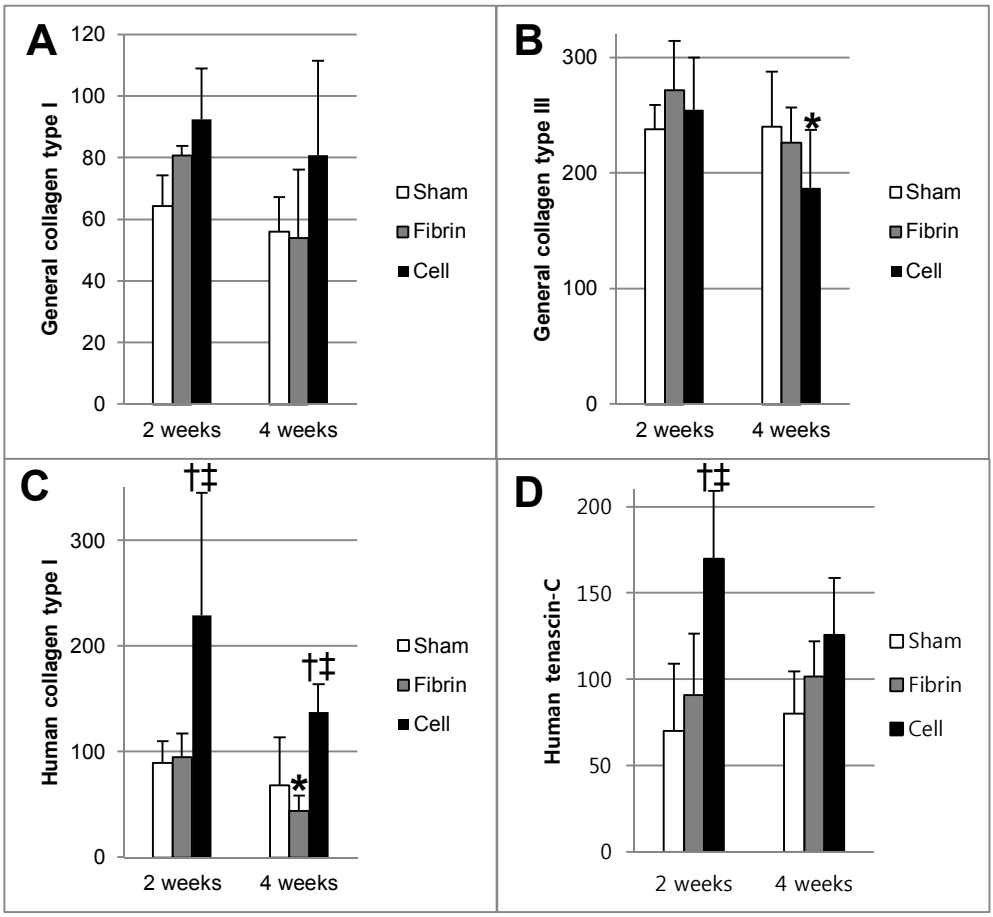

Figure 7. Quantification of four proteins by immunohistochemistry

(A) general collagen type I, (B) general collagen type III, (C) human-specific collagen type I, and (D) human-specific tenascin-C antibodies. $P < 0.05$ by Mann-Whitney's U test compared with the same group* at 2 weeks; the Sham† and Fibrin‡ groups at the same period.

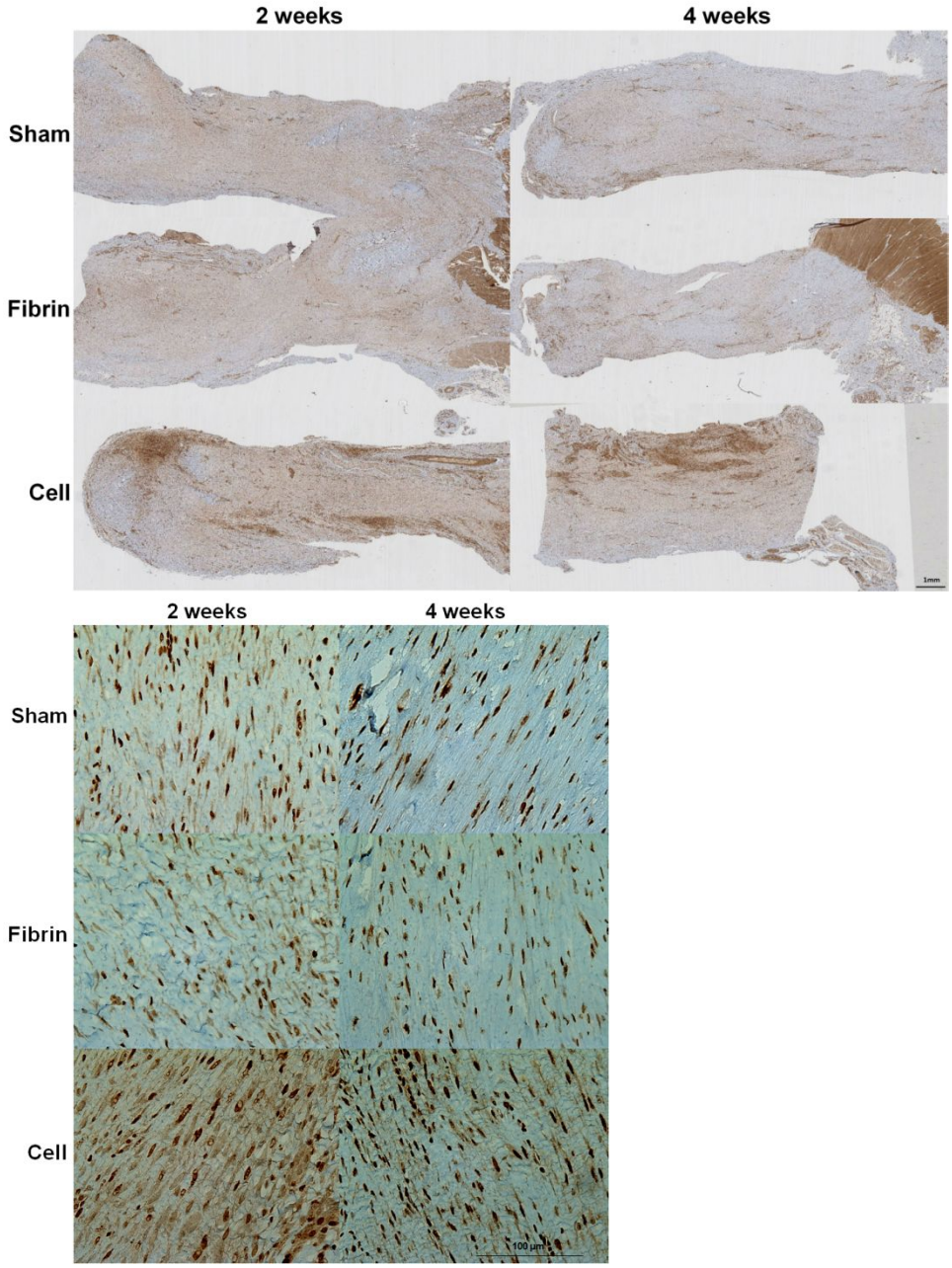

Figure 8. Immunostaining for human-specific collagen type I

Immunohistochemical staining images with human-specific collagen type I antibody of three groups at both 2 and 4 weeks after modeling (magnification: upper, 40× and lower, 400×).

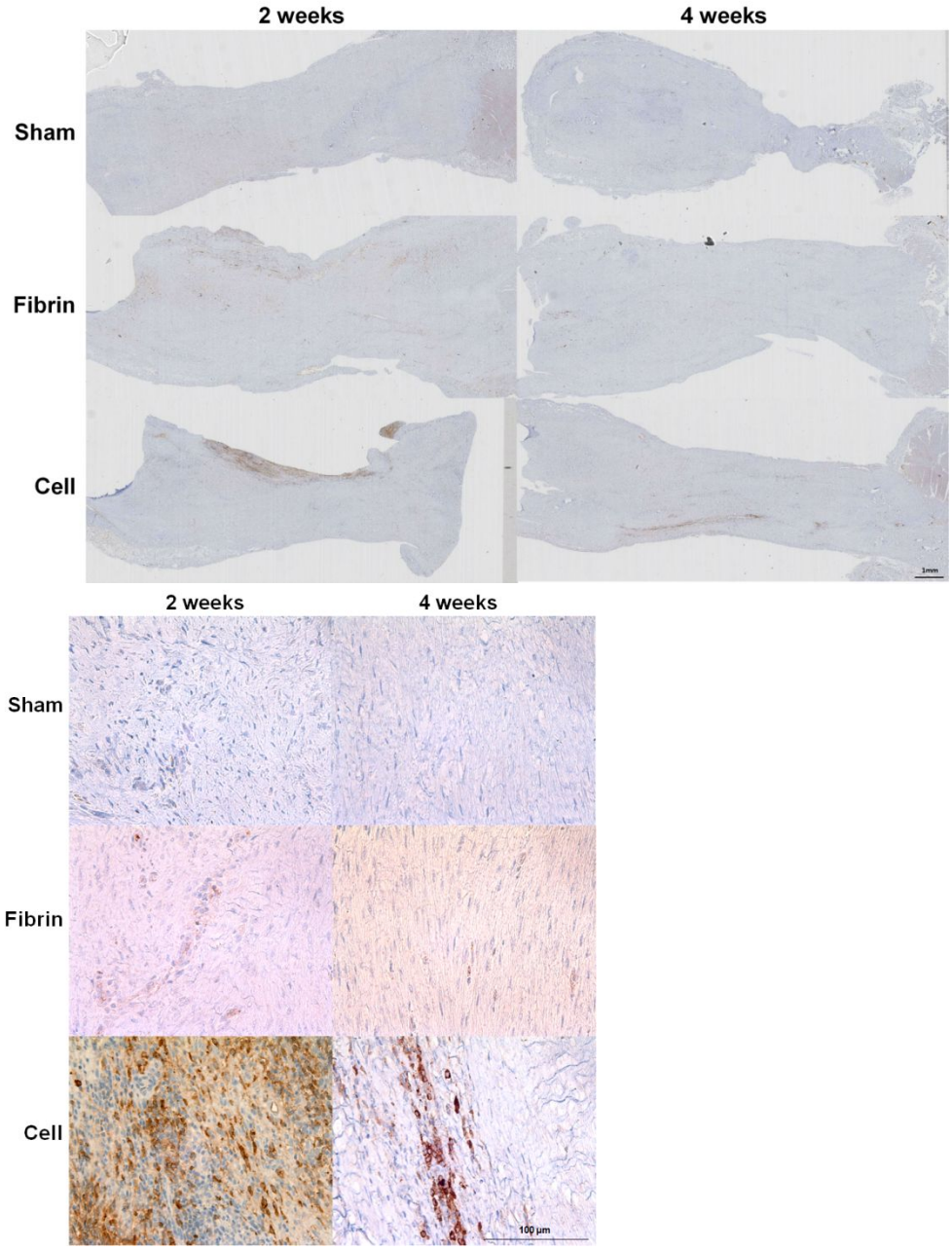

Figure 9. Immunostaining for human-specific tenascin-C

Immunohistochemical staining images with human-specific tenascin-C antibody of three groups at both 2 and 4 weeks after modeling (magnification: upper, 40× and lower, 400×).

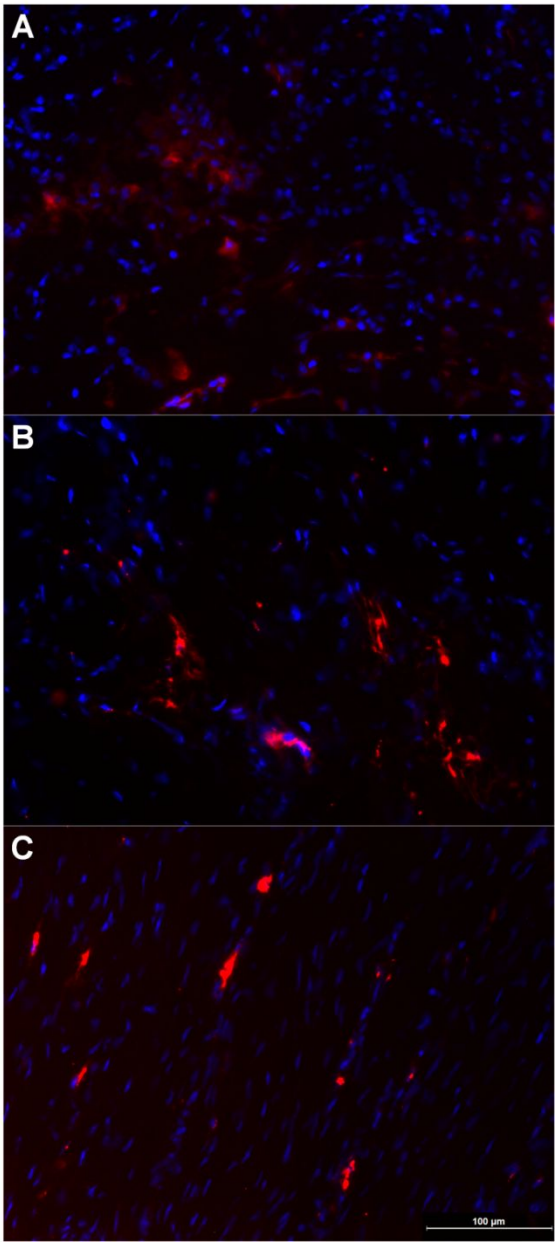

Figure 10. Fluorescently labeled human adipose derived mesenchymal stem cells

Immunofluorescent images (magnification 200×) of CM-Dil (red) tagged human adipose derived mesenchymal stem cells at 1 (A), 2 (B), and 4 (C) weeks from cell implantation. All images were merged with nuclear counterstained images with DAPI (blue).

5. Western blot analysis

Western blot analysis revealed that protein expressions of general COL1 in Cell group tended to be higher than those of other groups although those were not statistically significant at both 2 and 4 weeks ($P = 0.055$ and $P = 0.196$, respectively) (Figure 11A). Human-specific COL1 expressions were different among 3 groups at 4 weeks ($P = 0.023$) and the ODs of Cell (0.363 ± 0.239) and Fibrin (0.274 ± 0.102) groups were higher than that of Sham group (0.065 ± 0.036, $P = 0.021$ in both) (Figure 11B). MMP-2 expressions of both Fibrin and Cell groups were higher than Sham group at 2 weeks ($P = 0.006$ and $P = 0.009$, respectively) and those were significantly decreased at 4 weeks ($P = 0.004$ and $P = 0.014$, respectively). At 4 weeks, MMP-2 expression of Cell group (0.579 ± 0.233) was also higher than those of Sham and Fibrin groups (0.197 ± 0.176 and 0.235 ± 0.201, $P = 0.033$ in both) (Figure 11C). With regards to MMP-9 expression, only Cell group showed a significant reduction from 2 to 4 weeks (0.474 ± 0.250 to 0.150 ± 0.052, $P = 0.034$) (Figure 11D).

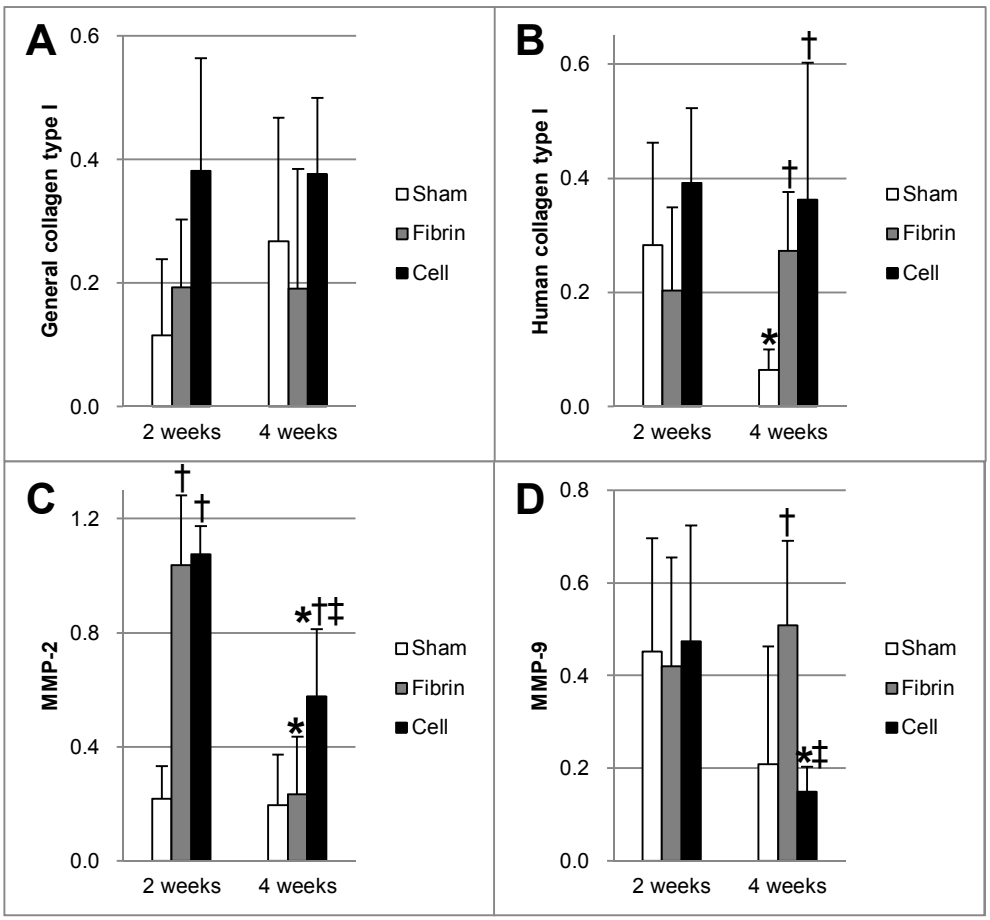

Figure 11. Quantification of four proteins by Western blot

(A) general collagen type I, (B) human-specific collagen type I, (C) MMP-2, and (D) MMP-9 (normalized by the value of α-tubulin). $P < 0.05$ by Mann-Whitney's U test compared with the same group* at 2 weeks; the Sham† and Fibrin‡ groups at the same period.

Discussion

After human MSCs implantation to rat tendon injury model, the cells were viable for 4 weeks inside the rat tendon. Tendons treated with MSC showed better biomechanical recovery than those of sham and active control (treated with only fibrin glue) groups. Especially, tendon stiffness of cell-treated group was higher than active control group at both 2 and 4 weeks. However, there was no definite histological recovery in cell group measured by modified Bonar scale. Although there were no differences of general collagen type I and III expressions among 3 groups, cell group had significantly higher human-specific collagen type I expression than other groups. From this *in vivo* xenogeneic cell transplantation model, it was proved that human MSC could help the recovery of injured tendon and the donor cell-originated protein secretion is one of the healing mechanism about MSC application.

MSCs on tendinopathy and tendon injury model

Tendinopathy is the most common musculoskeletal disease although many patients with tendinopathy spontaneously recovered [30]. However, several conservative managements produce unsatisfactory outcomes in some cases [31]. In these refractory cases, several biochemical agents such as dextrose solutions [32], autologous whole blood injection [33], and platelet-rich plasma [34, 35] have been used to promote tendon healing. However, there are limited evidences about the efficacy of these treatment [36], and a recent systematic review reported strong evidence against the effectiveness of platelet-rich plasma injection for treating

tendinopathy [37]. In such situations, MSC has recently emerged as the rising star in the musculoskeletal regenerative medicine. However, mechanisms and functions of the implanted MSC on the recipient tendon healing have not fully investigated yet.

Several experimental studies about MSC implantations on tendon injury have been used 'transection and suture' methods of animal Achilles tendon [1, 3, 4]. However, the surgical technique showed a high rupture rate (up to 26.2 %) even after applying long leg cast [1]. Furthermore, it is hard to observe the fresh healing tissue after cell injection because there is no tissue cleft after surgical suture. For these reasons, a new injury model which is full-thickness and longitudinal without fully rupturing the tendon [16] was borrowed in the current study. After using this method, a space at which implanted cells can settle down was secured and healing process can be also authentically observed by histopathological analysis. Additionally, the surgical lesions are around the osteotendinous junction which is one of the most frequently occurred area of tendinopathy [38]. Therefore, it might be pathophysiologically suitable model of tendon injury rather than 'transection and suture' model.

Process of tendon healing and the effect of fibrin glue

Clinically, tendon healing takes three steps during several weeks: inflammatory, proliferative, and remodeling phases. In the initial inflammatory phase, tenocytes gradually migrate to the wound and collagen type III synthesis is initiated [39]. It peaks during the proliferative phase and lasts for a few weeks. In the remodeling phase, the repair tissue changes from celluar to fibrous and a higher proportion of

collagen type I is synthesized [40]. The types of the collagen fibers in a repair tendon are different from those of a normal tendon because the interdigitation of collagen fibers are reduced and the portion of collagen type III is increased to 20-30% [41]. The qualities of the scar tissue improve with maturation which results from better interdigitation of the collagen fibers and increase in type I to type III collagen ratio [42]. In this study, cross sectional areas of injured tendons in Cell group were significantly decreased from 2 to 4 week while those in Sham group were increased during the same period. The identical tendencies were also observed in general collagen type III (by immunohistochemistry) and MMP-2 and -9 (by Western blot) expressions of Cell group. Therefore, it can be postulated that inflammatory phase of Cell group might be ended earlier than the other groups and consequently three steps of tendon healing were also proceeded more quickly owing to the MSC's tissue healing mechanism. As a result, tendon tissue edema (measured by cross sectional area) was improved earlier and biomechanical properties were also advanced only in the Cell group.

MSCs have been usually applied with several scaffolds to adhere to the recipient tissue. In this study, the fibrin glue was used as a scaffold. However, there have been several clinical reports to suggest that fibrin glue itself is also useful to repair tendon injury [43-45]. Hohendorff *et al.* studied 31 patients who had undergone Achilles tendon repair with fibrin glue and a half of patients (n = 15) also surgically augmented with the plantaris longus tendon [46]. After long term follow-up (average of 11.5 years), they concluded that surgical augmentation is not necessary and fibrin glue is enough to repair the ruptured Achilles tendon. However, in the current study, there were no benefits of biomechanical outcomes in Fibrin group superior to Sham group. Histopathological score of Fibrin group was even

worse than that of Sham group at 2 weeks. One also suggested there was no mechanically beneficial effects of fibrin glue although it might be helpful to attenuate adhesion formation at the early healing stage from a rabbit flexor tendon injury model [47]. Fibrin glue is able to act as a physical bridge between two injured tissues actually. However, if the tendon is not totally ruptured as the experimental model in this study, there might be no additional profit in tendon healing. Therefore, it is hard to expect any further chemical or biological healing mechanism of fibrin glue itself beyond the physical bridging role.

Xenotransplantation of mesenchymal stem cells

There have been several experimental studies about xenogeneic MSC transplantation. Jang *et al.* reported human MSCs with composite scaffolds effectively repaired osteochondral defect in a rabbit model [48]. They also suggest differentiated human MSCs showed superior healing of chondral lesion than undifferentiated cells. There was another study about human MSC application to rats which had damaged salivary glands by irradiation [49]. After MSC intravenous injection, human-specific DNA sequence can be detected in rat salivary gland by fluorescent in situ hybridisation technique. Furthermore, mucin and amylase which are secreted in gland can be found in the damaged salivary gland. Machova *et al.* also reported human MSC can support rat tendon healing after collagenase induced injury and they also observed the human cell survival at the site of the lesion for 6 weeks [50]. Although these MSC xenotransplantation (human cells to animals) studies showed positive effects on several kinds of tissue healing, no one has ever figured out whether the implanted xenogeneic cells can make donor cell-specific protein synthesis.

In this study, human-specific monoclonal antibodies which are reactive on not rat but human tissues were used. Higher concentrated antibodies (1:50) than the conventional staining (1:1000) were used to increase the detecting sensitivity in the immunohistochemistry because human-specific collagen type I and tenascin-C were secreted in the cell level, not in the tissue level. Under this staining process, tendon tissues in Sham and Fibrin groups which had no human cells were also weakly stained by human-specific antibodies owing to the interspecies cross-reactivity. However, optical densities of human-specific collagen type I and tenascin-C in Cell group were preponderantly higher than those in the other groups and these differences were also confirmed by the quantitative protein analysis using Western blot technique. Therefore, I could suggest that implanted human stem cells can secrete human-specific proteins in rat tendon.

These human-specific antibodies staining with higher concentration are a novel technique. Although several studies about xenogeneic MSC implantations have used donor cell-specific antibodies, they did not quantitatively observe any specific proteins from donor cells but simply traced the survival or existence of donor cells. One implanted human MSC into intervertebral discs in minipigs and detected collagen type I and II, and aggrecan [51]. However, these proteins were not human-specific. Allard *et al.* also reported xenotransplanted human stem cells in rodents model [52]. The authors analyzed human-specific biomarkers (Ku80, human mitochondria, and Alu) expression using immunohistochemistry by human-specific antibodies. However, these biomarkers are cell surface or cytoplasmic molecules which were used to identify the existence of human cells, not the secretion from human cells. In other words, they have just been tracking the human stem cells and

any functions of survived cells were not verified. Therefore, the current study is the first *in vivo* report which ascertained the cellular function of the implanted stem cells using the novel species-specific staining methods.

Tenascin-C and MMP proteins

TnC is an extracelluar matrix protein which is present in developing and mature tendons [53]. Higher TnC expression at the musculo-tendinous junction of a rat Achilles tendon was observed after increased physical loading [54]. It plays a role in proper alignment and orientation of collagen fibrils within the tendon [55]. The expression of TnC increased rapidly during the early period of recovery following tendon injuries and the increase in the expression of this protein before producing matrix proteins and related enzymes may be a necessary process in the remodeling of tendon [56]. In the current study, TnC was used as an identification marker of tenocyte. Because the anti-TnC antibody was specifically human-reactive, it can be speculated that significantly higher expression of human-specific TnC resulted from the cellular function of the tenogenically differentiated human stem cells. This phenomenon can also support the hASCs' function and differentiation in company with human-specific collagen type I expression.

Matrix metalloproteinase (MMP), which is a family of proteolytic enzymes [57], is an important regulators of extracellular matrix network remodeling and its level has been known to be altered during tendon healing [58]. Among the MMP family, MMP-2, MMP-3, and MMP-14 participate both in collagen degradation and in collagen remodeling, whereas MMP-9 and MMP-13 participate only in collagen degradation [59, 60]. Therefore, increased or maintained MMP-2 with decreased

MMP-9 could be an indicator of tendon healing while decreased MMP-2 with increased or maintained MMP-9 represent the status of collagen fiber degradation [61]. From Western blot analysis, both MMP-2 and MMP-9 in Cell group were decreased from 2 to 4 weeks. However, MMP-2 level in Cell group was significantly higher than those of the other groups and the temporal reduction of MMP-9 was only observed in Cell group. From these phenomena, it can be verified that resilient power of the injured tendon in Cell group was stronger than those in the other groups.

Limitations of the study

There were several limitations in this study. First, sample size was too small. Only 6 tendons in a group per each outcome test were examined. Furthermore, because of technical problem, only 4 specimens in both Sham and Fibrin groups at 4 weeks were analyzed for biomechanical test. In addition, a long-term effect of MSC beyond 4 weeks did not examined since merely two time points (2 and 4 weeks after modeling) were observed. Whether the cells can survive and the secreted human-specific proteins will not cause any immunological rejection beyond 4 weeks should be investigated to confirm the efficacy and safety of MSC. Therefore, studies with a larger sample and a longer follow-up periods are needed to draw a general conclusion. Second, any histopathological improvement was not shown from modified Bonar score measurement while there were definite advantages of morphological and biomechanical outcomes in Cell group. The Bonar score was originally designed for the assessment of patellar tendinopathy and the score is representative of the worst degree of tendon degeneration [19]. Therefore, it may be a suitable tool to assess any chronic or overuse conditions of tendinopathy [62, 63]. In acute conditions or experimentally injured defects as the current study, this

score might be fundamentally hard to give a true histopathological information although it is one of the most universal tools to evaluate the tendon pathology in several animal models with acute injury [2]. Third, there was one inevitable limitation of biomechanical test in the current study. Metatarsal bones were fixed as the distal portion of specimen using metallic clamp. In this technique, foot intrinsic muscles and ligaments could disturb the biomechanical properties of tendon. Although press-fixing the calcaneal bone is more ideal to observe the biomechanical property of pure Achilles tendon, tendon tissue could be partially jammed with calcaneal bone in the clamp and it was more easily slipped out of the clamp than metatarsal bone fixation. Therefore, there was no choice but to use the current fixing method at the distal portion of a specimen. Lastly, gene expression levels of collagen and tenascin-C were not studied. Real-time polymerase chain reaction (RT-PCR) which can measure the specific mRNA expression is helpful to verify the differentiation of MSC to the tenogenic lineage [12]. Zhang *et al*. examined not only IHC and RT-PCR but also cell morphology and cell doubling time to observe the *in vitro* differentiation of stem cell to tenocytes [64]. Although protein synthesis which was mainly investigated in the current study is the last step of the central dogma, the other genetic and cellular studies can reinforce the results.

Conclusion

Implanted human mesenchymal stem cells to rat tendon injury model survived for 4 weeks and secreted human-specific collagen type I and tenascin-C. Human mesenchymal stem cells biomechanically enhanced rat tendon healing superior to sham and active control groups. To the best of the author's knowledge, this is the first *in vivo* report which proves the cell-specific protein synthesis by the implanted stem cells.

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국문초록

서론: 중간엽 줄기세포를 이용한 힘줄손상의 치료효과는 많은 동물실험을 통해 확인되었고 현재 여러 임상시험을 통해 안전성과 유효성이 확인되고 있다. 그러나 힘줄손상에서 줄기세포의 치료 기전은 아직 명확히 밝혀지지 않았고 이식된 줄기세포가 생체 내에서 분화한 후 세포의 기능을 하는지 대한 증거도 부족한 상태이다. 본 연구에서는 인간 지방유래 중간엽 줄기세포를 손상된 백서의 힘줄에 주입한 후 줄기세포가 tenocyte 계열의 세포로 분화하고 인간 특이 단백질을 분비하는지에 대해 알아보고자 하였다.

방법: 57마리 SD 백서의 양쪽 아킬레스 힘줄에 직사각형 모양의 전층 손상을 가한 후 아래 3개의 그룹으로 무작위 배정되었다: 1) 수술 부위에 인간 지방유래 중간엽 줄기세포(10^6 개/60 μL)와 피브린 글루를 주사한 그룹(세포군), 2) 피브린 글루와 세포 배양액을 동량 주사한 그룹(피브린군), 3) 수술 후 아무 치료를 시행하지 않은 그룹(모의수술군). 수술 2주와 4주에 형태학, 생역학, 조직병리학(수정 보나르 지수 이용) 기법으로 각 그룹에서 아킬레스 힘줄의 회복 정도를 비교하였다. 또한 형광표지된 줄기세포의 생존 여부를 면역형광염색으로 확인하고 면역조직화학염색 및 웨스턴블롯 분석으로 콜라겐 I, III형 및 테나신 C 단백질의 발현도를 비교하였다.

결과: 수술 후 힘줄의 파열율은 세포군(11.1%)이 모의수술군(25.0%) 및 피브린군(22.2%)에 비해 낮음을 확인하였다. 세포군 힘줄의 단면적은 2주에 비해 4주차에 감소하나(P = 0.008) 모의수술군의 단면적은 같은 기간 동안 증가하였다(P = 0.005). 생역학 검사에서

2주차 세포군의 ultimate tensile strength와 stiffness가 모두 모의수술군에 비해 높았고(P= 0.037, P = 0.010), 2주 및 4주차에서 세포군의 stiffness는 피브린군에 비해 모두 높음을 확인하였다(모두 P = 0.037). 수정 보나르 지수상 3개 그룹간의 조직병리학적 차이는 확인되지 않았으나 면역형광염색 결과 수술 후 4주까지 인간 줄기세포가 백서 힘줄 조직 내에 생존함을 확인하였다. 면역조직화학염색상 세포군에서 피브린군 및 모의수술군에 비해 인간 특이 콜라겐 I형 단백질은 수술 후 2주(P = 0.004, P = 0.006) 및 4주차(P = 0.033, P = 0.011)에 인간 특이 테나신 C 단백질은 2주차(P = 0.028, P = 0.009)에 높게 발현되었다. 웨스턴블롯 분석에서도 인간 특이 콜라겐 I형 단백질이 수술 후 4주차에 모의수술군에 비해 높게 발현되었다(P = 0.021).

결론: 힘줄손상 백서 모델에 주입된 인간 중간엽 줄기세포는 백서 조직 내에 4주간 생존하였고 인간 특이 콜라겐 I형 및 테나신 C 단백질을 분비하였으며 손상된 백서 힘줄의 생역학적 회복에 도움이 되었다. 저자가 아는 바에 의하면 이번 연구는 이종 줄기세포 이식을 통해 생체 내에 주입된 줄기세포의 분화를 통한 세포 특이 단백질 분비를 확인한 첫번째 연구로 그 의미가 있겠다.

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주요어: 중간엽 줄기세포 이식, 이종 이식, 힘줄 손상, 아킬레스 힘줄, 콜라겐 I형 단백질

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