Differences between the root and horn cells of the human medial meniscus from the osteoarthritic knee in cellular characteristics and responses to mechanical stress

Abstract

Background: Many histological, mechanical, and clinical studies have been performed on the medial meniscus posterior root attachment, as it often tears in patients with osteoarthritic knee. Medial meniscal root repair is recommended in clinical situations; however, to date, no studies have examined the differences between meniscus root and horn cells. The aim of this study was, therefore, to investigate the morphology, reaction to cyclic tensile strain, and gene expression levels of medial meniscal root and horn cells.

Methods: Meniscus samples were obtained from the medial knee compartments of 10 patients with osteoarthritis who underwent total knee arthroplasty. Root and horn cells were cultured in Dulbecco's modified Eagle's medium without enzymes. The morphology, distribution, and proliferation of medial meniscal root and horn cells, as well as the gene and protein expression levels of Sry-type HMG box 9 and type II collagen, were determined after cyclic tensile strain treatment.

Results: Horn cells had a triangular morphology, whereas root cells were fibroblast-like. The number of horn cells positive for Sry-type HMG box 9 and type II collagen was considerably higher than that of root cells. Although root and horn cells showed similar levels of proliferation after 48, 72, or 96 h of culture, more horn cells than root cells were lost following a 2-h treatment with 5 and 10% cyclic tensile. Sry-type HMG box 9 and α1(II) collagen mRNA expression levels were significantly enhanced in both cells after 2- and 4-h cyclic tensile strain (5%) treatment.

Conclusions: Medial meniscus root and horn cells have distinct morphologies, reactions to mechanical stress, and cellular phenotypes. Our results suggest that physiological tensile strain is important to activate extracellular matrix production in horn cells.

Introduction

The meniscus is a fibrocartilaginous tissue that plays an important role in controlling complex biomechanical responses of the knee to tension, compression, and shear stress [1]. In the adult human, the perimeniscal capillary plexus comprises the outer 10–25% of the meniscus, whereas the inner 75–90% meniscus is composed of avascular tissue [2]. The avascular inner meniscus has a more pronounced chondrocytic phenotype [3]. Hence, human cells derived from this inner region exhibit chondrocytic morphology and produce type II collagen (COL2), a cartilage-specific extracellular matrix (ECM) component [4–6]. Alternatively, outer meniscus cells have a fibroblastic morphology and primarily synthesize type I collagen (COL1), which resists circumferential tensile stress [4]. Further, the nuclear translocation of Sry-type HMG box (SOX) 9 is stimulated and α1(II) collagen (*COL2A1*) expression is enhanced by cyclic tensile strain (CTS) in inner meniscal cells [7]. Studies have also demonstrated that mechanical stimuli regulate the expression of growth factors, ECM proteins, and catabolic molecules in the menisci [3,7–10].

Many histological, mechanical, and clinical studies of meniscal attachments have been reported [11–15]. Meniscal attachments are ligamentous tissues anchoring the menisci to the underlying subchondral bone [16] that transition into the fibrocartilaginous structure of the meniscal body [13]. It has also been reported that the meniscal root might continue into the outer region of the meniscus, where it merges with the more fibrocartilage-like inner region of the tissue [13]. Moreover, it was demonstrated that the medial posterior attachment has a significantly greater elastic modulus and ultimate stress compared to corresponding parameters in the other three attachments, namely the medial meniscus (MM) anterior root, and lateral meniscus anterior and posterior roots [11].

MM root repair is recommended to prevent subsequent cartilage degeneration following MM posterior root tear (MMPRT) [12], as the loss of hoop stress secondary to meniscal insufficiency from root tears leads to medial compartment overload and osteoarthritis. Accordingly, favorable clinical outcomes have been reported after the

transtibial pullout repair of the MMPRT [17]. Although meniscal root and horn cells have not been defined previously [14,15], the meniscal root is not considered a fibrocartilaginous body but rather an insertional ligament [13], whereas the meniscal root and horn have been clearly distinguished [18]. However, currently no studies have characterized the differences between meniscus root and horn cells. We hypothesized that MM horn cells would be reduced more so than root cells after mechanical stretch and that chondrogenic gene expression is higher in horn cells than root cells. The aim of this study was therefore to investigate the morphology of medial meniscal root and horn cells, as well as gene expression levels in these cells.

Materials and Methods

Specimen preparation

This study was approved by our Institutional Review Board, and all patients provided written informed consent. Meniscus samples were obtained from the medial knee compartments of 10 patients with osteoarthritis who underwent total knee arthroplasty (Fig. 1A, B). Osteotomy of the tibial surface was performed using a System 6 sagittal saw (Stryker, Kalamazoo, MI) without damaging the supplemental fibers and tibial insertions of the MM posterior root (Fig. 1A). The meniscal root was defined as the insertional ligament-like region from the attachment to the tibial surface except for the fibrocartilaginous body (Fig. 1C). Among the study participants, there were three men and seven women, with a mean age of 70.5 (range, 59–85) years. Relatively less-damaged medial menisci were included based on macroscopic observations after severely damaged tissues were excluded from the study.

Cells and cell culture

Meniscal samples (n = 5) derived from the root and horn were minced separately using a scalpel. Attached cells (passage 0) were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) without enzymes. They were

then incubated at 37 °C in 95% air with 5% CO_2 and subcultured at a density of 2500 cells/cm^2 on non-coated polystyrene tissue culture dishes (BD Biosciences, Bedford, MA) as previously described [19]. The medium was changed every 3 days. Cells at passage 1 were used for RNA extraction, whereas those at passage 2 were used for immunofluorescence staining and cell proliferation assays. Further, cells at passage 3 were used for CTS treatment.

Cellular morphology and collagen synthesis

Cultured cells derived from the meniscal root and horn (passage 1, day 1) were observed using a phase-contrast microscope (Olympus, Tokyo, Japan). Next, the area, perimeter, and transformation index (TI) were measured using ImageJ (version 1.47) to reveal the morphological characteristics as previously described [20]. TI was determined as described by Fujita et al. [21] using the following formula: [perimeter (μm)]^2 / 4π [cell area (μm^2)]. This index is suitable for comparisons of cell shape as it is dependent on cell shape yet independent of cell size. Therefore, circular cells are assigned an index of 1, whereas a cell with long processes and a small soma would have a larger index [21]. Relative values were normalized based on the values in root cells for each sample. Immunofluorescence staining was performed as previously described [7]. The cells were fixed with methanol for 10 min and with acetone for 1 min (Sigma). The slides were incubated with a rabbit anti-SOX9 polyclonal antibody (1:500 for 1 h, Abcam, Cambridge, UK) and a mouse anti-COL2 monoclonal antibody (1:100 for 1 h, Kyowa Pharma Chemical, Toyama, Japan). Bovine serum albumin solution without the primary antibody was used as a negative control. Alexa Fluor 488-conjugated anti-rabbit antibody for SOX9 and anti-mouse antibody for COL2 (1:200 for 30 min, Invitrogen, Carlsbad, CA), Alexa Fluor 568-conjugated phalloidin (1:40 for 20 min, Molecular Probes, Eugene, OR), and Hoechst 33342 (1:1000 for 5 min, ICN Biomedicals, Aurora, OH) were used to detect specific markers, and the cells were examined under a fluorescence microscope (Olympus). SOX9-positive and COL2-positive cell percentages were measured as the ratios of cells positively stained with corresponding antibodies to the total cell count over an area of 670 × 670 μm. Meniscus root and horn cell cultures were analyzed five

times per replicate (total of three replicates), and the mean value was calculated.

Histological analyses

Five samples were fixed in a 95% ethanol solution and then decalcified in a 20% EDTA solution (Fig. 1B). Coronal sections (6-μm thickness) were sequentially assessed by safranin-O staining as previously described [22] to examine the cell morphology and distribution. The meniscal root was defined as the insertional ligament region from the attachment to the tibial surface, excluding the fibrocartilaginous body (Fig. 1).

Cell proliferation assay

After cell count and density adjustments, root and horn cells were seeded in microplates at a density of 10^4 cells/well with culture medium (500 μL) and incubated for 24, 48, 72, and 96 h prior to the addition of Accutase (Innovative Technologies, San Diego, CA). After collection, cell counts were performed for each treatment, and the data were used for analysis. Cell counts were performed manually with Toluidine blue using a microscope (Olympus) in a blinded manner five times per replicate (total of three replicates), and the mean value was calculated.

Cyclic tensile strain

Polydimethylsiloxane stretch chambers (STREX, Osaka, Japan) were coated with 100 mg/mL of rat tail COL1 (BD Biosciences) as described previously [23]. Root and horn cells were seeded onto stretch chambers (culture surface of 4 cm^2) at a concentration of 15,000 cells/cm^2. The cells were incubated on the COL1-coated chambers for 24 h under the same conditions as those mentioned in the "Cells and cell culture" subsection before the stretching experiments.

Cell proliferation assay after CTS

Uni-axial CTS (0.5 Hz, 5% or 10% stretch) was applied using a STB-140 system (STREX) for 2 h [22]. Root and horn cells in the stretch chambers were incubated for 24 h prior to cell

counts. Non-stretched meniscus cells cultured on stretch chambers were used as 0% stretch controls. Each experiment was performed using four chambers per replicate (total of three replicates), and the mean value was calculated.

Reverse transcription-polymerase chain reaction (PCR) and quantitative real-time PCR analysis after CTS

RNA samples were obtained from cultured meniscus cells immediately after CTS. Uni-axial CTS (0.5 Hz, 5% stretch) was applied using a STB-140 system (STREX) for 2 or 4 h [22]. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). RNA samples (1,000 ng) were reverse-transcribed using the ReverTra Ace kit (Toyobo, Osaka, Japan). The obtained cDNA was then subjected to PCR amplification in the presence of specific primers using exTaq DNA polymerase (TaKaRa, Ohtsu, Japan). For all RT-PCR fragments, the reaction was allowed to proceed for 30–35 cycles. The following specific primer sets were used [24]: 5′-CTG AAC GAG AGC GAG AAG-3′, 5′-TTC TTC ACC GAC TTC CTC C-3′ for *SOX9*; 5′-AAT TCC TGG AGC CAA AGG AT-3′, 5′-AGG ACC AGT TGC ACC TTG AG-3′ for *COL2A1*; 5′-ATC CAG CTG ACC TTC CTG CG-3′, 5′- GGG AGG TCT TGG TGG TTT TG-3′ for α1(I) collagen (*COL1A1)*; 5′-CAT CAA GAA GGT GGT GAA GCA G-3′, 5′-CGT CAA AGG TGG AGG AGT GG-3′ for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). Quantitative real-time PCR analyses were performed using a FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Basel, Switzerland) as described previously [25]. The cycle number crossing the signal threshold was selected from the linear part of the amplification curve. *G3PDH* amplification data were used for normalization.

Statistical analysis

All experiments were repeated at least three times independently, and similar results were obtained from multiple replicates. Data are expressed as the mean ± standard deviation.

Statistical analyses were performed using EZR software (Saitama Medical Center Jichi Medical University, Tochigi, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing). Differences among groups were compared using the Mann–Whitney U test or a one-way ANOVA. Post-hoc comparisons were performed using the Tukey test. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$).

Results

Morphology and immunohistochemistry

Distinct cell morphologies were observed based on phase-contrast microscopy analysis. Specifically, the root cells showed a spindle-shaped fibroblastic morphology, whereas the horn cells were triangular in shape (Fig. 2A, B). Furthermore, the relative area of the horn cells was significantly higher than that of the root cells, and the relative TI of root cells was significantly higher than that of the horn cells, whereas the relative perimeter of both cell types was similar (Fig. 2C–E). Immunostaining revealed SOX9 and COL2 production in both root and horn cells. Image analysis demonstrated that relative SOX9 and COL2 densities were 25-fold and 5-fold higher, respectively, in horn cells than in root cells (Figs. 3, 4).

Response to CTS

The number of both root and horn cells increased significantly ($P < 0.05$) by approximately 2.0-fold at 96 h of culture, and the proliferation rates of both cell types were similar (Fig. 5A). The density of only horn cells was significantly reduced after a 2-h CTS (10%) treatment compared to that after 0% treatment (controls) ($P < 0.05$). More horn cells than root cells were lost after 5 and 10% CTS (2 h; Fig. 5B).

CTS enhances SOX9 and COL2A1 mRNA expression in meniscal horn cells

RT-PCR analyses revealed that the expression levels of the chondrocytic genes *SOX9* and *COL2A1* were barely detectable, even after CTS treatment, in root cells, whereas the mRNA levels of both markers were enhanced in horn cells after 2 and 4 h of CTS treatment (5%) (Fig. 6A). Quantitative real-time PCR analyses revealed that *SOX9* and *COL2A1* gene expression levels increased in both meniscal root and horn cells after CTS compared to those under CTS-free conditions ($P < 0.05$), and significantly higher gene expression levels were observed in horn cells than in root cells at all conditions (5% CTS; $P < 0.05$; Fig. 6B, C), whereas *COL1A1* gene expression levels were similar in both cells under all conditions (after CTS; Fig. 6D).

Discussion

The most important finding of this study is that MM root and horn cells have distinct morphological characteristics and show different cellular phenotypes. Cellular responses to mechanical stress underlie many critical functions such as development, morphogenesis, and wound healing [26]. It was previously reported that inner meniscus cells maintain a more pronounced chondrogenic phenotype than outer meniscus cells [4] and exhibit chondrocytic morphology and chondrogenic gene expression after CTS [7]. In the present study, meniscal horn cells showed characteristics similar to those of inner meniscus cells. This result was consistent with a previous report demonstrating that the root might continue into the outer portion of the meniscus, where it merges with more fibrocartilage-like inner portions of the tissue [13].

In the posterior third of the medial menisci of human cadaveric knees, average compressive strains of 2.2% and 6.3% were observed in the medial-lateral and superior-inferior directions, as well as an average tensile strain of 3.8% in the anterior–posterior direction, based on computed tomography imaging [27]. Studies have simulated physiological force using various mechanical stimuli. Herein, a lower number of horn cells was observed following CTS compared to that of root cells. Considering that cell stretch

would induce mechanical extension of cytoplasmic macromolecules, the activation of ion channels, and the phosphorylation of mechanotransducers [26], we postulate that horn cells might become fragile under continuous mechanical stress, as was previously demonstrated for meniscus inner cells [5]. However, since cellular behavior is dynamic under stretching conditions *in vivo*, further examination is required to comprehensively describe the observed weakness of horn cells.

In the present study, 5% CTS significantly enhanced the mRNA expression levels of *SOX9* and *COL2A1* in both meniscal root and horn cells. However, with regard to the RT-PCR results, an increase in *SOX9* and *COL2A1* gene expression levels might have been observed in root cells after CTS treatment because of the extremely low expression demonstrated in CTS-free conditions. Although horn cells likely have low healing potential, similar to that of the inner meniscus cells derived from the avascular region [2], physiological tensile strain might be important to activate ECM production in meniscal horn cells. This supports previous conclusions that transtibial pullout repair of the MMPRT decreases the proton density-weighted imaging signal intensity of the MM posterior segment in postoperative magnetic resonance images [28]. Accordingly, this phenomenon might indicate that MM posterior root repair induces a compositional change in the MM posterior segment. Meniscus degeneration following MMPRT might also be suppressed by pullout repair, which restores meniscal hoop tension. Furthermore, the results of the present study might be similar for healthy and injured menisci in which horn cells are more chondrogenic; however, further studies are necessary to confirm this. Therefore, meniscus repair for injury in younger patients after trauma, like anterior cruciate ligament injury, would also be recommended to recover the chondrogenic potential and prevent the degeneration of the meniscus or femorotibial cartilage.

Several limitations have been noted in this study. Migrated cells, rather than tissues, were used throughout the study, and although the gene expression level of transcription factors necessary for chondrogenesis, such as *SOX9*, was reported to remain

236 unchanged, cultured meniscus cells might undergo dedifferentiation during monolayer
237 culture [29,30]. Changes in the intracellular signals caused by CTS treatment or the actual
238 amount of proteins following CTS treatment were not examined. Although we observed that
239 *SOX9* and *COL2A1* gene expression levels in horn cells were significantly enhanced by CTS,
240 we did not examine the mechanisms underlying these phenomena, which should be
241 addressed in future studies. In addition, PCR analyses were performed only at 0, 2, or 4 h
242 after 5% or 10% uniaxial CTS. It was difficult to perform CTS treatment for a long period
243 because most cells detach over time. More pronounced differences might have been obtained
244 if these data were acquired 30 min, 1 h, or > 24 h after 2.5 or 7.5 % CTS treatment.
245 Furthermore, the study samples were from older adults who were affected by osteoarthritis.
246 Further investigations using healthy menisci or animal models will be required to understand
247 the properties of meniscus cells at the surface of injured menisci and other differences
248 between meniscus root and horn cells. In conclusion, MM root and horn cells have distinct
249 morphologies and reactions to mechanical stress and show different cellular phenotypes. Our
250 results suggest that physiological tensile strain is important for the activation of ECM
251 production in horn cells.

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Figure legends

Fig. 1. Meniscal sample. (A) Gross appearance. (B) Isolated and fixed medial meniscus (MM). (C) Safranin-O-stained MM. (D) Cells from MM posterior root (PR). (E) Cells from MM posterior horn (PH). LM: lateral meniscus. Dotted line: slice surface; yellow/red arrowheads: horn/root cells.

Fig. 2. Morphology of cell types observed under a phase contrast microscope and quantification of each cell type. (A) Root cells. (B) Horn cells. (C) Relative area. (D) Relative perimeter. (E) Relative transformation index. **P* < 0.01. Bar = 100 μm.

Fig. 3. Immunofluorescence staining for SOX9 and F-actin. (A) Respective images. (B) Ratio of SOX9-positive cells. **P* < 0.05. Bar = 100 μm.

Fig. 4. Immunofluorescence staining for COL2 and Hoechst staining. (A) Respective images. (B) Ratio of COL2-positive cells. **P* < 0.05. Bar = 100 μm.

Fig. 5. Results of cell proliferation assay using both root and horn cells. (A) The results of simple manual cell counts. (B) The results of manual cell counts after a 2-h cyclic tensile strain (CTS) treatment. **P* < 0.05.

Fig. 6. Effect of cyclic tensile strain on *SOX9*, *COL2A1*, and *COL1A1* expression. (A) Results of reverse transcription PCR analyses. (B–D) Results of quantitative real-time PCR analyses. **P* < 0.05.