Comparison of Posterior Root Remnant Cells and Horn Cells of the Medial Meniscus

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Running title: Characteristics of root remnant cells

Contributions of authors

Takayuki Furumatsu designed the study and prepared the manuscript. Takayuki Furumatsu and Shinichi Miyazawa contributed to the data collection. Ximing Zhang, Yuki Okazaki, and Takaaki Hiranaka contributed to the analysis and interpretation of data. All authors have critically reviewed the manuscript, approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Abstract

Purpose/Aim of the study: Previous studies of meniscal attachment have noted distinctions between medial meniscus posterior root and horn cells. However, the characteristics of root remnant cells have not been explored in detail. The purpose of this study was to evaluate the gene expression levels, proliferation, and resistance to mechanical stress of remnant and horn cells.

Materials and Methods: Medial meniscus tissue samples were obtained from patients who underwent total or uni-compartmental knee arthroplasty. Cellular morphology, sry-type HMG box 9, type II collagen, and chondromodulin-I gene expression levels were analyzed. Collagen synthesis was assessed by immunofluorescence staining. Proliferation analysis after 4 h-cyclic tensile strain was performed.

Results: Horn cells displayed triangular morphology, whereas root remnant cells appeared fibroblast-like. *Sry-type HMG box 9* mRNA expression levels were similar in both cells, but *type II collagen* and *chondromodulin-I* mRNA expressions were observed only in horn cells. The ratio of type II collagen-positive cells in horn cells was 12-fold higher than that in root remnant cells, whereas the ratio of sry-type HMG box 9-positive cells was similar. A significant increase in proliferation was observed in root remnant cells compared to that in horn cells. Further, under cyclic tensile strain, the survival rate was higher in root remnant cells than in horn cells.

Conclusions: Medial meniscus root remnant cells showed higher proliferation and resistant properties to cyclic tensile strain than horn cells and showed no chondromodulin-I expression. Preserving the medial meniscus posterior root remnant during pullout repair surgery might maintain mechanical stress-resistant tissue and support healing.

Keywords: medial meniscus; posterior root remnant cells; posterior horn cells; collagen synthesis; anti-angiogenic gene

Introduction

The meniscus comprises crescent-shaped cartilage on the medial and lateral articular surfaces of the tibial plateau. The meniscus is mainly composed of 70% water and 30% organic matter, and primarily collagen[1]. In the meniscus of humans, 10–25% of the outside area is rich in blood supply, whereas 75–90% of the interior area is composed of avascular tissue[2]. The cells from the vascularized area are fibroblast-like, whereas the cells in the avascular area are chondrocyte-like in shape[3, 4].

Meniscal root tears are radial and/or oblique tears within 1 cm of the meniscus insertion, which lead to failure to convert axial loads into transverse hoop stresses[5]. The medial meniscus (MM), especially the posterior root, bears greater knee pressure and is easily damaged in the axial and radial stress of the knee joint[6, 7]. The proportion of MM posterior root tears (PRTs) might represent approximately 20−30% of all medial meniscus tears[8]. After suffering from MMPRT, the ability of the meniscus to transmit hoop tension is disrupted and the meniscus will extrude to the side of the joint capsule[9]. Further, the peak contact stress of the medial tibiofemoral joint increases by 25%, which is similar to the peak stress after meniscectomy[10, 11].

There are currently several treatment options for MMPRT, including transtibial pullout repair[12]. Some surgeons will confirm the complete MMPRT and then shave and refresh the remnant of the posterior root. Although it has been reported that preserving ACL remnants during ACL reconstruction leads to improved stability and synovial coverage[13], there are no studies about whether remnants of the posterior root should be preserved. The purpose of this research was to evaluate the gene expression levels, proliferation, and resistance to mechanical stress of MM posterior root remnant and horn cells. We hypothesized that MM

posterior root remnant cells would exhibit a higher survival rate and proliferation after mechanical stress, while showing lower collagen and anti-angiogenic gene expression levels compared to those in horn cells.

Materials and Methods

Material collection

This study was approved by the Institutional Review Board (Okayama University NO.1608-019). Meniscal tissue samples were acquired from nine patients with osteoarthritis who were diagnosed with MMPRT and subjected to total or sub-compartmental knee arthroplasty. Six women and three men were included with a mean age of 67 years (range, 53–75 years). An osteotomy was performed by using a System 8 sagittal saw (model 8208; Stryker Instruments, Kalamazoo, MI), and the posterior segment of the meniscus was preserved as much as possible (Fig 1(a)). The serial numbers and patient information were recorded. The root remnant was defined as scar-like tissue located at the terminal MM posterior torn root from the attachment of the tibia surface (Fig 1(a)). Root remnant and horn tissues were carefully cut to show the section. (Fig 1(b)).

Histological analyses and cell culture

The selected meniscal tissues (n=4) were fixed in a 95% ethanol solution. Then, the tissues were decalcified in a 20% EDTA solution. Coronal sections (6 μm thickness) of the samples were prepared using safranin O staining as previously described[14] (Fig 2(a-c)).

The other meniscus samples (n=5) were divided into the remnant root and horn (Fig 1 and 2) and were used for other experiments. Each tissue was cut into small pieces area and

incubated on small dishes in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan), which including 1% penicillin/streptomycin (Sigma) and 10% fetal bovine serum (HyClone, South Logan, UT). When the cells were attached to the dish, they were recorded as the passage 0 cells. Cells were stored at atmospheric pressure at 37 °C with 5% CO_2 and 95% air. The culture medium was changed every 2 days. Cultured cells derived from the meniscus root remnant and horn cells were observed using a phase-contrast microscope (Olympus, Tokyo, Japan). Passage 1 meniscus cells (day 2) were then used (Fig 2(d-e)).

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

RNA samples were acquired from cultured remnant root and horn cells. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). Reverse transcription of RNA samples (1000 ng) was performed using Revertra Ace (Toyobo, Osaka, Japan). The acquired cDNA was then subjected to PCR amplification with specific primers using exTaq DNA polymerase (TaKaRa, Ohtsu, Japan). RT-PCR was carried out for 30–38 cycles. Quantitative real-time PCR analyses were accomplished using a FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Basel, Switzerland). The cycle number crossing the signal threshold was selected from the linear part of the amplification curve. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) amplification data were used for normalization. The following specific primers were used: a1(II) collagen (*COL2A1*), Sry-type HMG box 9 (*SOX9*) (5′-CTG AAC GAG AGC GAG AAG-3′, 5′-TTC TTC ACC GAC TTC CTC C-3′); Chondromodulin-I (*ChM-I*) (5′-GAA GGC TCG TAT TCC TGA GG-3′ and 5′-GGC ATG ATC TTG CCT TCC AG-3′), and *G3PDH* [2, 15-18].

Immunofluorescence staining

The root remnant and horn cells were fixed on type I collagen-coated chambers loaded with 1% paraformaldehyde solutions (Sigma). The expression of SOX9 was evaluated with a rabbit anti-SOX9 polyclonal antibody (1:500 for 1 h, Sigma-Aldrich, St. Louis, USA). The content of human meniscal type II collagen (COL2) was evaluated using a mouse anti-COL2 monoclonal antibody (working dilution of 1:500 for 1 h, Kyowa Pharma Chemical, Toyama, Japan) as described[18]. The negative control was bovine serum albumin solution without the primary antibody. The OUMS-27 chondrosarcoma cells (Okayama University, Okayama, Japan) were used as a positive control because of their high expression of SOX9 and COL2. An Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA) was used for SOX9 and COL2 detection (1:200 for 30 min) under a fluorescence microscope (Olympus). Moreover, Alexa Fluor 568 phalloidin (1:40 for 30 min, Molecular Probes, Eugene, OR) was used for F-actin staining with Hoechst 33,342 (1:1000 for 5 min, ICN Biomedicals, Aurora, OH)[15, 18]. The percentage of positive cells and total cells stained by corresponding antibodies for SOX9 and COL2 were measured in a 670×670 μm region. Root remnant and horn cells were analyzed repeatedly five times (a total of three replicates), and the average value was recorded.

Cell proliferation assay and cyclic tensile strain (CTS)

Proliferation assay and CTS experiments were performed as described[19]. Root remnant and horn cells were seeded into microplates (tissue culture grade, 96 wells, flat bottom). The cells were incubated at 37 °C with 95% air and 5% CO_2 with 100 μL/well of culture

medium for 0 h, 24 h, 48 h, 72 h, and 96 h prior to the addition of the cell proliferation reagent, water-soluble tetrazolium-1 (WST-1; Roche Diagnostics, Basel, Switzerland). Absorbance was measured using a microplate reader at wavelengths of 450 and 630 nm. Uni-axial CTS (0.5 Hz, 5% or 7% stretch) examination was performed using a STB-140 system (STREX, Osaka, Japan) for 4 h[16]. Root remnant and horn cells were incubated for 24 h in the stretching chambers before adding WST-1. The control group was unstretched meniscus cells, which were cultured on a stretching chamber. Each experiment used three chambers for 4 h, representing 0%, 5%, and 7%. Each experiment was replicated three times, and the average was recorded.

Statistical analysis

All experiments were performed using SPSS Statistics Version 25.0 (IBM Corp., Armonk, NY, USA) and all experiments were repeated at least three times. Data were expressed as the mean ± standard deviation. Differences among groups were compared by using the one-way ANOVA or Mann-Whitney U test. Statistical differences were set at $P < 0.05$.

Results

Root remnant cells were not stained red by Safranin O and showed a fibroblastic, slender morphology, whereas horn cells were stained red with Safranin O and showed a triangular morphology (Fig 2). Further, the root remnant cell counts were significantly greater than horn cell counts (Fig 2(b-c)). In the RT-PCR analyses, the expression of *SOX9* was similar in both cells, whereas the expressions of the chondrocytic gene *COL2A1* and anti-angiogenic gene *ChM-I* were barely detectable in root remnant cells (Fig 3(a)). Further,

quantitative real-time PCR analyses revealed significantly higher gene expression levels of *COL2A1* and *ChM-I* in horn cells than in root remnant cells (approximately 46- and 10-times higher, respectively), whereas *SOX9* gene expression levels were similar in both cells (Fig. 3(b-d)). Immunofluorescence staining revealed SOX9 and COL2 production in both root remnant and horn cells. The ratio of SOX9-positive cells was similar in both cells (Fig 4), whereas the ratio of COL2-positive cells was about 10-fold higher in horn cells than in root remnant cells (Fig 5, $P<0.01$).

The proliferation rate at 48, 72, and 96 h in root remnant cells was much greater than that of horn cells (Fig 6 (a), $P<0.01$). A significant decrease in the proliferation of horn cells after 4 h CTS (7%) was observed compared to that at 0% control and 5% CTS (Fig 6 (b), $P<0.05$), whereas cell proliferation in root remnant cells was similar even after 4 h CTS stimulation (5%, 7%) compared to that in controls (Fig 6 (b)). Further, root remnant cells demonstrated a higher survival rate than horn cells after uni-axial mechanical stretches.

Discussion

Medial meniscus posterior root remnant cells showed higher proliferation and survival rates after mechanical stress, as well as lower anti-angiogenic gene expression levels compared to those in horn cells, which confirmed our hypothesis. Because of this, preserving MM root remnants during pullout repair surgery might maintain proliferation potential. Meniscus cells in different locations have different characteristics[20, 21]. The inner meniscus cells maintain their chondrogenic phenotype, whereas the outer meniscus cells show a fibroblastic morphology and phenotype[2, 19]. Compared with the outer meniscus cells, the inner meniscus cells expressed more *SOX9* and *COL2A1*, maintaining higher

chondrogenic potential[2, 22]. Further, it was reported that *COL2A1* is expressed more in meniscus horn cells than in meniscus root cells, which results in main articular cartilage fibrillar collagen made from the extracellular matrix[18, 23, 24]. In this study, the results in horn cells were consistent with those in previous studies. The results of root remnant cells were somewhat different from those of previous studies on outer or root cells, in that expression of the transcription factor *SOX9* was similar to that in horn cells but expression of the chondrogenic gene *COL2A1* was barely detectable in root remnant cells.

COL2A1 is regulated differently in root remnant and horn cells, resulting in different expression levels[25, 26]. The SOX9 protein specifically binds the sequence in the first intron of human *COL2A1*, and the expression of *COL2A1* is directly regulated by SOX9 protein in vivo[27, 28]. The regulation of *SOX9* occurs at the transcriptional and post-transcriptional levels[29-31]. Furthermore, chondrocyte differentiation is modulated by various epigenetic factors, such as transforming growth factor (TGF)-β, TGF-β-regulated Smad3/4, and transcription factors/coactivators such as Scleraxis/E47 and p300[31-33]. We consider that the results of this study were caused by the epigenetic status, including histone modification and chromatin structure, directly influencing SOX9-regulated chondrocyte (*COL2A1*) differentiation[29]. This result is consistent with our previous reports, which demonstrated that the activation of cell clusters and their products in torn menisci appears to contribute to the regulation of cartilage expression[34]. The difference was mainly due to the variety of extracellular matrix and signaling pathways in root remnant and horn cells.

Expression of the *ChM-I* gene is regulated by a variety of factors such as oxygen content, growth factors, DNA methylation, and histone acetylation[14, 35]. The *ChM-I* concentration in the MM cells is higher than that in the outer meniscus cells[14, 36]. *ChM-I* derived from the

inner meniscus can inhibit endothelial cell proliferation to preserve the avascularity of the meniscus[2, 37, 38]. Furthermore, ChM-I is a cartilage-specific 25-kDa glycoprotein that stimulates DNA synthesis and colony formation in cultured chondrocytes in the presence of fibroblast growth factor-2 (FGF2) [39, 40]. In this study, the *ChM-I* content in horn cells was significantly higher than that in root remnant cells, which demonstrates that horn cells maintain avascularity and more cartilage properties than root remnant cells.

In the posterior 1/3 of the MM of the human cadaveric knee, the average compressive strain in the medial-lateral and in the superior-inferior directions were determined to be 2.2% and 6.3%, respectively, and the average tensile strain in the anterior and posterior direction was 3.8%.[41] In previous studies, 5% CTS significantly enhanced the mRNA expression of *SOX9* and *COL2A1* in the posterior root cells and horn cells[15]. Although the root cells and horn cells showed similar levels of proliferation after 48, 72, or 96 h of culture, the proliferation rate of horn cells decreased significantly compared to that of root cells after 5% and 10% CTS for 2 h, which was similar to our results.

This study had several limitations. First, the samples were from elderly patients with osteoarthritis. Patients with MMPRT are generally younger than those who undergo knee arthroplasty. Second, we used cultured meniscus cells at low passage (≦2) in order to obtain enough meniscus cells. Next, intracellular signaling changes, which caused the inhibition of *SOX9* expression in root remnant cells, were not detected clearly. Moreover, this needs to be addressed further in future studies. Finally, the sample size should be expanded, and normal meniscus cell controls or animal meniscus controls should be included.

Conclusions

This study showed that *COL2A1* and *ChM-I* mRNA expression levels were observed only in horn cells and that MM root remnant cells showed higher proliferation and resistance properties to CTS than horn cells. Preserving MM root remnants during pullout repair surgery might be useful to maintain mechanical stress-resistant tissue and to support healing in terms of angiogenic activity.

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Declaration of Interests

The authors report no conflicts of interest.

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Figure legends

Figure 1. Meniscal tissue sample. (a) Gross appearance of medial meniscus. (b) Section of medial meniscus. Bars = 1cm.

Figure 2. Distribution and morphology of root remnant and horn cells of medial meniscus (MM). (a) Safranin-O-stained MM. (b) Root remnant cells were indicated by red arrowheads. (c) Horn cells were indicated by green arrowheads. (d) Root remnant cells showed fibroblastic morphology. (e) Horn cells showed triangular morphology. Bars = 100 μm.

Figure 3. Gene expressions in root remnant and horn cells. (a) Higher gene expression levels of *COL2A1* and *ChM-I* were detected in horn cells than in root remnant cells. (b) *SOX9* expression levels were similar. (c) *COL2A1* gene expression levels were 46-fold higher in the horn cells. (d) *ChM-I* gene expression levels were 10-fold higher in the horn cells. *P<0.05.

Figure 4. Immunofluorescence staining for SOX9 and F-actin. (a) Respective images. (b) The ratio of SOX9-positive cells was similar in both cells. Bars = 50 μm.

Figure 5. Immunofluorescence staining for COL2 and Hoechst. (a) Respective images. (b) The ratio of COL2-positive cells was about 10-fold higher in horn cells. Bars = 50 μm. *P<0.01.

Figure 6. (a) Proliferating activity of root remnant cells was significantly higher than that of horn cell after 48h. (b) Horn cells was decreased significantly after 4-h cyclic tensile strain (5 and 7%) stimulation, whereas root remnant cells were similar. *P<0.05, **P<0.01.