The long-term effects of hyaluronan during development of osteoarthritis following partial meniscectomy in a rabbit model

K. Kobayashi*, M. Amiel*, F. L. Harwood*, R. M. Healey*, M. Sonoda†, H. Moriya† and D. Amiel*  
*Department of Orthopaedics, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0630, U.S.A.  
†Department of Orthopaedic Surgery, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-0856, Japan

Summary

Objective: The long-term effect of hyaluronan (HA) on meniscus remodeling and articular cartilage preservation was assessed during the development of osteoarthritis following partial meniscectomy in a rabbit model.

Design: Approximately 60% of the region of each medial meniscus of 20 rabbit knees was excised bilaterally. The left knee joint was treated with five weekly intraarticular injections of 0.3 ml of HA, beginning 1 week after surgery. The right control knee was injected with PBS on the same schedule. Six months after surgery, animals were killed and the medial menisci and tibial articular cartilage were evaluated morphologically, histologically and biochemically.

Results: Meniscal regeneration was observed as newly synthesized translucent tissue, and image analysis revealed that the amount of this tissue was significantly greater in the HA-treated menisci than in the vehicle-treated menisci. Safranin-O staining and image analysis revealed the increased presence of glycosaminoglycans in the HA-treated menisci relative to vehicle-treated menisci while vascularity and biochemical parameters (hydration, total GAGs and reducible collagen crosslinks) were statistically similar in HA- and vehicle-treated menisci. Gross morphologic grading with India ink revealed a trend for less deterioration of tibial articular cartilage in the HA group (P=0.06) while Mankin’s score of the HA-treated tibial articular cartilage was marginally lower than that of the vehicle group (P=0.06). Biochemical assessments showed a trend for higher total GAGs concentration in the HA-treated tibial articular cartilage when compared to the vehicle treatment group (P=0.06).

Conclusion: The present study has demonstrated that following partial meniscectomy, treatment with hyaluronan can enhance meniscal regeneration and may inhibit articular cartilage degeneration as long as six months post surgery. © 2000 OsteoArthritis Research Society International

Key words: Osteoarthritis, Hyaluronan, Partial meniscectomy, Meniscal remodeling.

Introduction

Osteoarthritic changes in the tibial articular cartilage following total or partial meniscectomy have been reported in both human and animal studies.1–7 The clinical long-term data have also shown that articular cartilage degeneration cannot be avoided even if partial meniscectomy is performed arthroscopically.5–10 Several human clinical11–17 and animal model7,18,19 studies have reported a meniscal synthetic response to total or partial meniscectomy. Such response has been referred to as meniscal regeneration and meniscal remodeling corresponding to total and partial meniscectomies, respectively.20 Other studies have reported that meniscal regeneration has a chondroprotective effect on articular cartilage following meniscectomy.7,11,18

Intraarticular injection of hyaluronan (HA) has been utilized in many countries as a pain relief procedure for knee osteoarthritis (OA). A protective effect of HA on articular cartilage during the early stage of OA following total meniscectomy has been reported.1,4-5 We postulate that non-surgical clinical treatments such as injection of HA are important for the long-term preservation of the articular cartilage and enhancement of meniscus matrix remodeling following meniscal injury and/or partial meniscectomy. The rationale for this treatment for OA patients is based on the known biological effects of HA. For example, HA has been demonstrated to stimulate synovial cells isolated from OA joints to synthesize HA in vitro,21 to inhibit the release of glycosaminoglycans (GAGs) from papain-treated articular cartilage matrix,22 and to stimulate tissue inhibitor of metalloproteinase-1 (TIMP-1) in articular chondrocytes.23 As for soft tissue healing, HA increases angiogenesis and decreases inflammatory response in ligament healing24 and stimulates the process of wound healing in the skin.25 In a rabbit model, our laboratory has recently demonstrated active collagen remodeling and increased expression of type I procollagen mRNA in the HA-treated menisci up to 3 months following partial meniscectomy.26 Although, when using an animal model, HA has been shown to clear from the joint cavity within 20 h following intraarticular injection,27 several long-term improvements in joint pain and
function have been observed clinically after HA treatment in OA patients. It was also demonstrated that patients with OA of the knee who received several intraarticular injections of HA experienced decreased progression of the disease 1 year later, as judged by arthroscopy, than controls who received no HA injections. Although the basis for this long-term effect on symptoms in some patients after intraarticular HA injection is unknown, these reports led us to hypothesize that HA might have a long-term chondroprotective effect during the development of OA. Thus far, there have been no reports assessing such long-term effects of HA on articular cartilage during the development of OA following partial meniscectomy.

Employing a rabbit model, the present study reports the effects of HA treatment on the medial meniscus and on articular cartilage 6 months after partial meniscectomy of the medial meniscus. Evaluation of the tissues includes gross morphology, histology and quantitative biochemical analysis.

Materials and methods

SURGICAL MODEL AND POST-SURGICAL TREATMENT

Twenty skeletally mature New Zealand white rabbits 7–8 months old and weighing 3.5–4.0 kg were used in this study. Each animal was anesthetized with an intramuscular injection of ketamine (80–100 mg/kg) and xylazine (7–10 mg/kg). Both hind limbs were shaved and disinfected with Betadine solution and the medial menisci were partially removed from both knees as follows. A medial parapatellar incision and arthroscopy were performed. The patella was dislocated laterally and the knee placed in full flexion. In the region medial to the incision, the joint capsule was separated from the synovial tissues and the medial collateral ligament (MCL) was exposed. A longitudinal incision was made just anterior to the MCL to separate the synovial tissues in front of the medial meniscus and the femur. The synovial tissues were cut transversely, turned over and grasped to pull out the medial meniscus. This surgical procedure allowed the medial meniscus to be exposed without release of the MCL. Partial medial meniscectomy was then performed using a No. 11 surgical blade. The inner area, approximately 60% of each medial meniscus, was excised except in the region of the anterior and posterior horn ligament. No bleeding was observed on the meniscectomized surface. Great care was taken not to injure the articular cartilage surface or the cruciate ligaments.

Following partial meniscectomy, full range of motion of the joint was re-established and the joint was washed with sterile saline. The capsule was closed with 4-0 monofilament polypropylene sutures, and the skin was closed with 3-0 nylon sutures. After surgery, the rabbits were returned to cage activity (60 × 60 × 40 cm). The rabbits were given intramuscular injection of analgesics (buprenorphine HCl 0.01–0.02 mg/kg) for 3 days and antibiotics (enrofloxacin 1.0–1.3 mg/kg) for 7 days after surgery.

Beginning 1 week after surgery, the left knee joints received intraarticular injections of 0.3 ml of hyaluronate (MW=8×10^5 Daltons; Seikagaku Corp., Tokyo, Japan) once a week for 5 weeks, in a protocol similar to that used clinically. The right knees were injected with a vehicle (carrier of HA: phosphate buffered saline). During intraarticular injection the animals were anesthetized intramuscularly with small doses of ketamine and xylazine. All rabbits were killed at 6 months post-surgery with an intra-cardiac injection of a mixed solution of pentobarbital sodium, phenytoin sodium, ethyl alcohol and propylene glycol. Immediately after death the medial menisci and tibial articular cartilage from HA and vehicle treated knees were dissected and randomly designated for the following assessments.

QUANTITATIVE MORPHOLOGIC ASSESSMENT

Quantitative morphologic analysis of the medial menisci (N=6) and tibial articular cartilage (N=8) was evaluated as described previously. Photographs of the menisci were scanned and total meniscal area and area of new growth were calculated by image analysis using NIH Image 1.60 software, National Institutes of Health, USA. To evaluate articular cartilage damage the medial tibial plateaus were brushed with India ink and examined for signs of erosion and/or fibrillation. The examination was performed blindly by two observers and averaged to minimize the effects of observer bias. Changes in the articular cartilage were graded as follows: Grade 1 (intact surface)—surface normal in appearance and did not retain ink; grade 2 (minimal fibrillation)—site appeared normal before staining, but the India ink showed fibrillation; grade 3 (overt erosion)—areas were apparent before staining and retained ink as intense black patches; grade 4 (ulceration)—loss of cartilage exposing the underlying bone.

HISTOLOGICAL ASSESSMENT

Menisci (N=4) and articular cartilage (N=6) were fixed in 10% buffered formalin with 1% cetylpyridinium chloride (CPC) and embedded in paraffin. The articular cartilage together with bone tissue was decalcified in ethylene-diamine tetraacetic acid (EDTA) before embedding. Six micrometre-thick sagittal sections were cut and stained with hematoxylin and eosin (H&E) for cellular detail and safranin-O/fast green for the presence of GAGs. The specimens were evaluated by light microscopy. Mean density of safranin-O stained area in menisci was determined using NIH image analysis (1.60) software while histological changes in the articular cartilage of the medial tibial plateau were evaluated according to the Mankin’s grading system: histological sections stained with safranin-O/fast green, were analysed for abnormalities in structure, cell population, safranin-O stain (GAGs) distribution, and tidemark integrity. Scores were given a histologic-histochemical grade.

VASCULAR ASSESSMENT OF MEDIAL MENISCI

Animals (N=4) were anesthetized with intramuscular ketamine and xylazine. Each abdominal descending aorta was exposed and cannulated with a polyethylene catheter. One hundred and fifty milliliters of filtered India ink was perfused into the aorta with continuous manual pressure and the animals killed. Modified Spalteholz technique was used for tissue clearing. The menisci were fixed in 10% buffered formalin with 1% CPC for 3 days and washed in running water for 8 h. After dehydration by passing through changes of 70% to 100% ethanol, specimens were immersed in methyl salicylate until tissue clearing was advanced. Sagittal sections with 1–2 mm thickness were investigated under light microscopy.
BIOCHEMICAL ASSESSMENT

Biochemical characterization of whole medial menisci \((N=6)\) included quantitative measurements of water content, total GAGs, and the reducible collagen cross-link, dihydroxylysinoonorleucine (DHLNL). Articular cartilage \((N=6)\) was dissected as completely as possible from the tibial plateau for determination of water content and total GAGs.

**Hydration:** Hydration levels in menisci and cartilage were determined by measuring wet and dry weights in these tissues and calculating percentage water content.

**Total glycosaminoglycans:** Total GAGs concentration was determined in menisci and tibial plateau cartilage by measuring the concentrations of hexosamine contained in the tissues. Two to four milligrams of dry tissue were hydrolysed in 6 N HCl at 100°C for 5 h. The amount of hexosamine was then calculated as previously described. Results are expressed as micrograms hexosamine per mg of dry tissue.

**Reducible collagen cross-links:** The reducible collagen cross-link DHLNL was quantified in medial menisci in order to assess collagen remodeling in this tissue. Samples of lyophilized tissue were reduced with tritium-labeled sodium borohydride and then hydrolysed in 6 N HCl. Isolation of the cross-link was accomplished by cation-exchange HPLC and quantitation was achieved with in-line liquid scintillation spectrometry. Results are expressed as counts per minute (cpm) per picomole of total collagen.

STATISTICAL ANALYSIS

Quantitative data were presented in the text as mean±standard deviation of the mean. For comparisons of quantitative measures between the vehicle- and the HA-group, the values were subjected to statistical analysis using paired Student's \(t\)-test. A modified arcsine transformation was applied to proportional data as a variance stabilizing measure. For the semi-quantitative scales of morphologic and histologic changes, the scores were ranked and between-group differences were tested using the Mann–Whitney \(U\) test.

**Results**

**MENISCUS**

Gross assessment of the medial menisci at 6 months following partial meniscectomy revealed the presence of newly deposited translucent tissue in both vehicle-treated and HA-treated specimens [Fig. 1(a)]. However, image analysis revealed that the mean of the percentage new tissue area [translucent tissue area/total area of medial meniscus] in the HA-treated medial menisci was significantly \((P<0.05)\) higher than in the vehicle-treated menisci: 19.2±4.0% vs 13.9±3.8%, respectively; Fig. 1(b)].

Histological evaluation (safranin-O staining) revealed localized staining of GAGs in the region of new tissue growth [Fig. 2(a)]. Image analysis demonstrated that such localized staining was present at an increased \((P<0.05)\)
level in the HA-menisci relative to the vehicle-treated menisci [Fig. 2(b)]. India ink injection studies demonstrated a similar vascular network in HA-treated and vehicle-treated menisci. Quantitative biochemical analysis of menisci from vehicle- and HA-treated knees demonstrated no statistically significant differences in water content, total GAGs or DHLNL.

ARTICULAR CARTILAGE

Figure 3 shows representative specimens of tibial plateau from vehicle-treated and HA-treated joints following partial meniscectomy. Morphological grading with India ink revealed that two vehicle-injected knees and no HA-injected knees exhibited grade 3 pittings into the cartilage surface on the posteromedial aspect of the tibial plateau. Minor fibrillation of the medial tibial articular cartilage (grade 2) was observed in five vehicle-injected rabbits and four HA-injected rabbits (Table I). A trend for an overall lower grading (P=0.09) was seen in the HA group than in the vehicle group. Gross examination revealed no detectable osteoarthritic changes in the femoral condyles in either group 6 months after partial meniscectomy. Histological
examination (safranin-O staining) revealed variably fibrillated cartilage surfaces and vertical clefts reaching into the radial zone in two of four vehicle-treated tibial plateau specimens and no HA-treated specimens (Fig. 4). Minor changes in the intensity of safranin-O staining of HA-treated cartilage were observed, but the tide marks were preserved with normal appearance in all samples from the HA-treatment group. Mankin's grading system showed that the average value in the vehicle-treated tibial cartilage had a trend for a higher score compared to the HA-treated cartilage \((P=0.06)\).

Biochemical analysis of the articular cartilage from the tibial plateau demonstrated no statistically significant differences in water content between vehicle-treated and HA-treated knees. However, the HA-treated tibial cartilage showed a marginally higher amount of total GAGs than vehicle-treated cartilage \((P=0.06; \text{Fig. 5}).\)

Discussion

The results of the present study support the hypothesis that intraarticular injections of HA enhance meniscal remodeling and provides some long-term (6 months) chondroprotection during the development of OA following partial meniscectomy. In a previous study, using the same model, newly synthesized translucent tissue in medial menisci was observed 3 months post-surgery, but we were unable to detect a statistical difference for the amount of this tissue between vehicle- and HA-injected knees. However, active collagen remodeling at 3 months was demonstrated biochemically, i.e. increased DHLNL concentration and increased expression of type I procollagen mRNA, in the HA-treated menisci when compared to the vehicle-treated menisci. These observations indicated that HA had the ability to enhance meniscal remodeling. In the current study, examination at 6 months post-surgery showed no difference for DHLNL concentration in the medial menisci between vehicle- and HA-treatments. Thus, it was speculated that the effect of HA mentioned above, had reached a steady state by 6 months following partial meniscectomy. As a consequence of increased collagen remodeling at 3 months, a significantly larger area of regenerated tissue in the HA-treated medial menisci, compared to vehicle-treated menisci, was seen at 6 months. Although we were unable to detect a significant difference biochemically for the amount of GAGs in the whole menisci between vehicle- and HA-treated knees, a stronger local safranin-O staining of GAGs in the regenerated tissue of HA-treated meniscus was observed. HA-enhanced meniscal remodeling might provide one of the positive effects of HA for the preservation of articular cartilage following partial meniscectomy, since meniscal regeneration was reported to have a protective role on articular cartilage after meniscectomy.\(^7,11,18\)

In a previous study we demonstrated no significant differences between the vehicle- and HA-treated cartilage morphologically or biochemically in a 3 month short-term study.\(^26\) However, in the present 6 month study we have shown a trend toward higher amounts of total GAGs \((P=0.06)\) and less morphological degradation \((P=0.09)\) in the HA-treated medial tibial articular cartilage than in

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**Table I**

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<th>OA grade</th>
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**Fig. 4.** Histological assessment (safranin-O/fast green) of a grade 3 vehicle-treated specimen of tibial articular cartilage at 6 months after partial meniscectomy and a grade 2 HA-treated specimen. Note the extensive fibrillation of articular surface and vertical clefts extending into the radial zone (arrow) in the vehicle treated specimen; the HA-treated cartilage, by contrast, contained a more intact surface and exhibited a more normal tide mark region (arrow).
vehicle-treated cartilage. These data suggest that a chondroprotective effect of HA was maintained between three and six months post surgery, even though HA was long cleared from the joint cavity by that time.

As for the basis of the long-term effects of HA, we postulate that intraarticularly injected HA diffuses in the synovial fluid and is incorporated into the synovial tissues. Other studies have shown that during the initial stages after injection of exogenous HA, HA in the synovial fluid decreases in molecular weight, and in later stages, the molecular weight of HA in the synovial fluid increases to a value greater than the original before the injection.39 This phenomenon has been explained by the theory that exogenous HA stimulates the production of endogenous HA by positive feedback.40 In a positive feedback mechanism, exogenous HA may induce synovial cells (type B) to synthesize endogenous HA of higher molecular weight.21 It has been shown that intrinsic HA is important for cell movement24,25 and the regulation of extracellular matrix aggregation and synthesis.22,41 These previous findings and our present results, collectively, suggest that a similar mechanism may underlie the long-term chondroprotective effects of HA after partial meniscectomy; there is the possibility that extrinsic HA incorporated into synovial tissues may activate the production of the intrinsic HA, which may have been up-regulated in long periods by the positive feedback mechanism. An increase in cartilage GAG content would result in a decrease in the hydraulic permeability of the cartilage and an increase in its stiffness on compression.42 In conclusion, the present study has supported one of the rationales for HA treatment following partial meniscectomy although the exact mechanism of long-term effects of HA during development of OA remains to be determined.

References