The effects of hyaluronic acid on fibronectin fragment mediated cartilage chondrolysis in skeletally mature rabbits

J. M. Williams*†‡, J. Zhang†, H. Kang†, V. Ummadi† and G. A. Homandberg†
Departments of *Anatomy, †Biochemistry and ‡Internal Medicine (Section of Rheumatology), Rush Medical College at Rush Presbyterian St Luke’s Medical Center, 1653 West Congress Parkway, Chicago, IL, 60612-3864, U.S.A.

Summary

Objective: Intraarticular Na-Hyaluronate (HA) exerts a beneficial effect on adolescent rabbits after fibronectin fragment (Fn-f) mediated cartilage injury. We extended our studies to a population of rabbits which have reached full skeletal maturity.

Design: Adult male NZW rabbits received an injury with Fn-f and no further treatment; an injection of HA followed by Fn-f injury, or Fn-f injury followed by a single or weekly intraarticular injection of HA. All animals were sacrificed 38 days after receiving the Fn-f injury. After sacrifice, proteoglycan (PG) content was determined from articular cartilage from the medial femoral condyles and tibial plateaus. The patellae were processed for histology.

Results: Cartilage PG contents were significantly reduced after Fn-f injection (P=0.0167) and were only slightly improved with HA pre-treatment. However, post-treatment with HA resulted in significant improvements in cartilage PG content when compared to Fn-f only (single HA, P=0.01; weekly HA, P=0.01). Loss of Safranin-O staining, cell loss, osteophyte formation and inflammation were present in the patellae following Fn-f injection. Pre-treatment with HA reduced these changes. More significant protection of cartilage and restoration of Fn-f injury were noted in animals receiving post-treatment with HA.

Conclusions: These results suggest that 38 days after Fn-f injury the lost PG content induced by Fn-f injection is substantially restored by post-treatment with HA. Otherwise normal, intact articular cartilage26. In contrast, spontaneous restoration of lost matrix PGs following intraarticular HA in rabbits that have reached full skeletal maturity. © 2003 Published by Elsevier Science Ltd on behalf of OsteoArthritis Research Society International.

Key words: Articular cartilage repair, Hyaluronic acid, Experimental arthritis, Therapeutic interventions.

Introduction

We have demonstrated that administration of a commercially available form of hyaluronic acid (HA) reduces fibronectin fragment (Fn-f) mediated bovine cartilage chondrolysis1 and human knee cartilage chondrolysis2 in vitro. We have also reported that HA has some reparative potential and exerts a beneficial effect on adolescent rabbits days after an Fn-f mediated cartilage injury3. These results suggested that HA has a prophylactic effect in this model of Fn-f-induced articular cartilage injury by promoting the restoration of proteoglycans (PGs) that had been lost from the tissue. However, spontaneous restoration of lost matrix PGs following intraarticular Fn-fs can occur in younger animals4. Subsequently, we have shown that restoration of lost PGs does not occur to any significant degree during the 4–5 weeks after the induction of the Fn-f-mediated injury in adult rabbits that have reached skeletal maturity5. This finding is consistent with the view that with aging, articular cartilage has a diminished capacity to restore lost matrix macromolecules.

HA is a biologic material that has received a great deal of interest as a potential agent of therapeutic intervention in arthritis. HA forms the backbone to which cartilage PG is attached via an HA binding region6 thus forming the macromolecule aggrecan. HA is also a major component of synovial fluid7 and plays a central role in the formation of the synovial joint8. High molecular weight HA has been used in the treatment of human9–15 and animal osteoarthritis (OA)16–23. An ameliorative effect of HA has also been demonstrated in a papain-induced model of articular cartilage injury in guinea pigs24 and rabbits25. Intraarticular injections of HA effectively coat the articular surface of otherwise normal, intact articular cartilage26. In contrast, treatment with HA of cartilage where the surface has been disrupted results in deeper penetration of HA into the crevices formed by the surface fibrillation and deeper penetration into the cartilage matrix. The biologic significance of this observation is not known. The observation that HA can cover the articular surface might suggest that HA acts as a prophylactic barrier against agents (e.g. cytokines), which induce matrix degradation. However, the major beneficial effect of HA may not be simply as a physical barrier but as a mediator of enhanced anabolic processes. For example, it has been reported that in cultured human explants, HA can restore PG content...
in damaged cartilage and this is associated with enhanced PG synthesis. We have utilized an animal model to test the efficacy of HA. Injection of Fn-f into rabbit knee joints has been shown to rapidly enhance loss of PG within a few days. We have recently reported that the injection enhances levels of MMPs and temporarily suppresses synthesis of PG in the injected joint. This model was used by us to demonstrate that HA decreased Fn-f mediated cartilage matrix PG depletion in adolescent animals. We have now extended our studies to a population of rabbits that have reached full skeletal maturity. This study has particular relevance to the use of HA/800 in humans since the vast majority of patients receiving HA/800 therapy have reached skeletal maturity. The design of this study was to (1) determine if HA/800 can block Fn-f mediated damage in the knee joints of skeletally mature rabbits and (2) determine if HA/800 administered following the Fn-f-mediated injury can enhance the restoration of matrix PGs lost from the articular matrix.

Methods

ANIMALS AND EXPERIMENTAL GROUPS

Adult skeletally mature male New Zealand White rabbits (10–11 months of age) were housed individually in caged provided in the Comparative Research Center at The Rush Presbyterian St Luke’s Medical Center (IACUC #95074). Animals were fed Purina Rabbit Chow and food intake monitored daily. Forty-six animals were randomly assigned to five groups (Table I). Determination of the experimental groups and the number of animals per group were determined in consultation with a biostatistician at the investigators' institution. An original group size of 10 animals per group was planned. However, animal deaths not related to the study resulted in fewer animals for some groups as noted in Table I. Animals were anesthetized after which they received a single injection of 1 μM Fn-f into the left knee. Intraarticular injections of HA (0.3 ml of a 10 mg/ml solution: molecular weight 800 000 Da; herein referred to as HA/800) were carried out in a similar fashion. The HA/800 was extracted from chicken combs and was supplied by the Seikagaku Corporation (Tokyo, Japan).

The right non-injected knee served as a control. An additional 10 animals served as untreated controls. As noted more completely in Table I injected animals received a single intraarticular injection of Fn-f and no further treatment, an intraarticular injection of HA/800 followed 3 days later by an Fn-f injection, an intraarticular injection of Fn-f followed 3 days later by a single injection of HA/800, or an intraarticular injection of Fn-f followed 3 days later by a 5 weekly intraarticular injections of HA/800. Animals were sacrificed 38 days after receiving the Fn-f injection. After sacrifice the patellae were processed for histology and stained with Safranin-O to demonstrate matrix PGs and counterstained with fast green or with hematoxylin and eosin. Histologic parameters were used to determine a Composite Histology Score for comparing changes in the patellae. Cartilage PG content was determined from full thickness shavings of articular cartilage from the medial femoral condyles and medial tibial plateaus.

Table I

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated injury</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-injury HA/800</td>
<td>8</td>
<td>0</td>
<td>f</td>
</tr>
<tr>
<td>Post-injury HA/800</td>
<td>9</td>
<td>f</td>
<td></td>
</tr>
<tr>
<td>Post-injury HA/800</td>
<td>10</td>
<td>f</td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sacrifice

F—0.3 ml fibronectin fragment at 1 μM 29/50 kDa.

—0.3 ml of a 10 mg/ml HA (about 800 kDa) solution in sterile saline.

INJECTION PROCEDURE

Animals were anesthetized by an intra-muscular injection of a mixture of ketamine (80 mg/cc; Abbot Laboratories, North Chicago, IL) and acepromazine (2.0 mg/cc; Phoenix Pharmaceuticals, St Josephs, MO) at a dose of 1.0 cc/1.5 kg body weight after which the left knee was washed with 70% isopropyl alcohol. Intraarticular injections were performed under aseptic conditions by passing a 26-gauge needle attached to a tuberculin syringe through the joint capsule lateral to the patellar tendon. Injected joints were moved through a few extension/flexion movements to ensure even distribution of the injected material throughout the joint cavity. Animals were then placed in their pens and permitted to recover from the anesthetic. All animals tolerated the injection procedure well with no adverse reactions.

ANALYTICAL PROCEDURES

Both knees were opened immediately after sacrifice and examined grossly. Full thickness shavings of articular cartilage from the central, habitually loaded region of the
medial femoral condyle and medial tibial plateau were obtained for determination of PG content. Cartilage samples were weighed and then digested in 1.0 ml of 50 mM phosphate buffer, pH 6.0, containing 10 mM EDTA, 10 mM cysteine and 27 μg/ml papain for 8 h at 60°C. The digests were then subjected to the DMB assay as described in modified form29. Thus, cartilage PG content was expressed as μg PG/mg cartilage wet weight. In order to rule out possible systemic effects of Fn-f injury, all contralateral noninjected knee PG contents were compared for significant differences. The mean PG content value for the right contralateral knees of all groups studied was 34.3±8.5 and there were no differences among these groups. Cartilage PG contents were then expressed as a % of the contralateral control knee and examined for differences among the groups. The PG content data were analysed by a one-way analysis of variance followed by post-hoc Bonferroni corrected t-tests to determine which specific differences were significant. For histology, samples of the patellae, including the parapatellar synovium, were fixed for one week in 10% neutral buffered formalin containing 5% cetyl pyridinium chloride, decalcified in formic acid: sodium citrate (44% formic acid: 20% sodium citrate) for two weeks and embedded in paraffin. Microscopic sections (8 μm) of the samples were stained with Safranin-O to demonstrate matrix PGs and counterstained with fast green30 or with hematoxylin and eosin. Sections from the left (injected) knees and the untreated control right knees of each experimental animal were stained concurrently to control for variations in uptake of the stain. A previously published scoring method31 was used to quantify histologic change using 6 parameters (articular surface, Safranin-O staining, cell loss, cell clusters, osteophytes, synovial membrane). Scoring was made for sections while blinded to the reader and later summarized for comparisons. The patellar histology scores were analysed by the Kruskal–Wallis test followed by Mann–Whitney tests to determine which specific differences were significant.

Results

All animals were maintained in good general health, ate and drank a normal amount and had no noticeable differences in physical activity when compared with similar animals involved in other studies but maintained in the same room. Animals ranged in ages from 10.0 to 11.8 months and weighed 4.2±0.4 kg. Animals exhibited a normal weight gain during the study. All joints that received intraarticular Fn-f were slightly swollen but were normal 3 days after the injection. On day 38, knees from animals injected with Fn-f were grossly normal. All contralateral non-injected right knees and knees from untreated control animals were normal in every respect throughout the entire experiment.

CARTILAGE PG CONTENT

Cartilage PG content levels were significantly reduced (71%±12 of control knee; mean±S.D.; P=0.001) 38 days after Fn-f injection in animals not receiving HA/800 treatment (Fig. 1). Cartilage PG content levels remained similarly reduced 38 days after Fn-f injection in animals pretreated with HA/800 (84%±11), and in animals that received a single HA/800 post-treatment injection following Fn-f injury (86%±10). In contrast, weekly HA/800 post-treatment injections following Fn-f injury resulted in significantly restored cartilage PGs (90%±12; P=0.017).

There was a significant group effect for the PG data (P<0.001 in the analysis of variance). Figure 1 shows means and standard deviations of the five groups. The untreated control PG mean was significantly higher than the means for the injury only (no HA/800 treatment) group (P=0.001), the pre-injury single HA/800 treatment group (P=0.032) and the post-injury single HA/800 treatment group (P=0.037), but was not different from the post-injury weekly HA/800 treatment group (P=0.194). For all groups of animals injected with Fn-f, only the post-injury weekly HA/800 treatment group had a higher mean than the untreated Fn-f injury group (P=0.017) and was not statistically different from the untreated control group.

HISTOLOGY OF PATELLA

No histologic changes were noted in the right contralateral non-injected knees of any animals in the groups.
studied. Loss of Safranin-O, indicating loss of cartilage PGs, ranged from none to marked in all groups receiving Fn-f injection (Table II). In general, Fn-f injected groups receiving no HA/800 treatment had a higher number of animals with more pronounced PG loss. Animals receiving pre-treatment with HA/800 had less PG staining loss suggesting partial restoration of lost cartilage PGs. Restoration of PGs was greatest in animals receiving HA/800 treatment after Fn-f injection. Only three of 10 animals 38 days after Fn-f injection and no treatment exhibited significant cell loss. Cell loss was not noted in any other animals. Surface fibrillation was not noted in any animal. Osteophytes, which consisted of a small bud of cartilage at the joint margin, were noted in four of 10 animals 38 days after Fn-f injection and no HA/800 treatment. Osteophytes were seen in two of eight animals pre-treated with HA/800, no animals receiving a single post-treatment of HA/800 and only one of 10 animals receiving weekly post-treatment HA/800. Inflammation of the synovial membrane was noted in three of 10 animals 38 days after Fn-f injection and in no animals receiving HA/800 treatment. Composite patella scores were highest (indicating more damage) in animals 38 days after Fn-f injection and no HA/800 treatment. Composite histology scores of the patellae expressed as total composite score± S.D. Letters refer to statistical measures as follows: (a) different from the intact untreated control group \( P=0.001 \); (b) different from the post-injury HA (single injection) group \( P=0.0040 \); (c) different from the post-injury HA (weekly injection) group \( P=0.0042 \); (d) different from the intact untreated control group \( P=0.01 \).

<table>
<thead>
<tr>
<th>Group</th>
<th>Safranin-O loss</th>
<th>Cell loss</th>
<th>Fibrillation</th>
<th>Osteophytes</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury only</td>
<td>2/10 none</td>
<td>7/10 (&lt;25%)</td>
<td>None</td>
<td>4/10</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>3/10 slight</td>
<td>3/10 (25–50%)</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2/10 mild</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2/10 moderate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/10 marked</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-injury HA/800</td>
<td>1/8 none</td>
<td>8/8 (&lt;25%)</td>
<td>None</td>
<td>2/8</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2/8 slight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/8 mild</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/8 moderate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/8 marked</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-injury HA/800</td>
<td>5/9 none</td>
<td>9/9 (&lt;25%)</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>(single)</td>
<td>1/9 slight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/9 mild</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/9 moderate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/9 marked</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-injury HA/800</td>
<td>3/10 none</td>
<td>9/9 (&lt;25%)</td>
<td>None</td>
<td>1/10</td>
<td>None</td>
</tr>
<tr>
<td>(weekly)</td>
<td>3/10 mild</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/10 moderate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/10 marked</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>9/9 none</td>
<td></td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Table II Histologic analyses of the patellae

Fig. 2. Composite histologic scores of patellae expressed as total composite score± S.D. Letters refer to statistical measures as follows: (a) different from the intact untreated control group \( P=0.001 \); (b) different from the post-injury HA (single injection) group \( P=0.0040 \); (c) different from the post-injury HA (weekly injection) group \( P=0.0042 \); (d) different from the intact untreated control group \( P=0.01 \).
with no treatment was significantly higher than the untreated control group ($P=0.001$), the post-injury HA (single injection) treatment group ($P=0.004$) and the post-injury HA (weekly injection) treatment group ($P=0.0042$). The mean composite patella histology score for the pre-injury treatment group was not different from the group receiving Fn-f injury with no treatment, but was different from the intact untreated control group ($P=0.01$). Thus, both the post-injury single HA/800 and weekly HA/800 treatments reduced cartilage changes induced by Fn-f injury.

**Discussion**

We have reported that with adolescent rabbits, injected HA/800 can block the catabolic insult caused by Fn-f 2, a class of mediator which has been shown to up-regulate MMPs and greatly enhance degradation and loss of PG from bovine or human explants. Further, it was shown that Fn-fs up-regulate MMP-3 and temporarily suppress PG synthesis in the injected knee in a dose-dependent fashion in this model. This study was designed to determine if HA/800 can block Fn-f mediated damage in the knee joints of skeletally mature rabbits and to determine if HA/800 administered following the Fn-f-mediated injury can enhance the restoration of matrix PGs lost from the articular matrix. Pre-treatment of cartilage slices in vitro with HA has been shown to block Fn-f-induced PG loss. Similarly, pre-treatment of adolescent rabbits with intraarticular HA reduced Fn-f-mediated PG loss. In the present study, pretreatment of older, skeletally mature rabbits had no effect on Fn-f-mediated cartilage injury in the femoral condyles or tibial plateaus suggesting that HA may work in this model of older animals by enhancing PG synthesis of damaged cartilage. MMP-3 is up-regulated in the injected knee joint cartilage of mature rabbits but not in control knees and PG synthesis is suppressed to 50% of control levels by day 2 (data not shown). This PG depletion and MMP-production peaks early as a damage pulse, followed by an attempted rebound. These catabolic events are maximal by day 2. This would correspond to 1 day prior to administration of HA in the post-treatment groups in this study.

While many of the in vivo animal model tests and in vitro testing with cartilage explants of the chondroprotective activities of HA suggest that HA has beneficial effects, clinical studies have not yet clearly demonstrated efficacy in terms of structural alteration, although pain alleviation in the human subjects is clear. Since the cartilage substrate used for in vivo and in vitro testing is typically derived from younger animals, while the typical patient population used in tests of clinical HA is of older patients, a possible explanation for differences in beneficial effects may be that HA loses effectiveness as cartilage ages. This, in turn, could be due to a number of factors, including age related decreases in cell vitality or anabolic responses or increases in responses of cartilage to catabolic insults, such as increased catabolic cytokine or MMP levels. Aged related HA loses effectiveness as cartilage ages. This, in turn, can explain differences in beneficial effects may be that HA is ineffective on blocking Fn-f mediated damage to cartilage from 6-year-old bovines (Homandberg et al., personal communication). Thus, it is unlikely that cartilage from older animals is incapable of responding to HA treatment. Based on these results and in extrapolation to human trials, it is imperative to question why injection of HA into human patients does not result in structural modification as reported here for animal tissue.

**References**


