High molecular weight hyaluronan promotes repair of IL-1β-damaged cartilage explants from both young and old bovines
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Summary
Objective: The addition of exogenous high molecular weight hyaluronic acid (HA) reverses cartilage damage caused by fibronectin fragments (Fn-fs) added to explant cultures of bovine and human cartilage and by Fn-fs in an experimental in vivo model of rabbit knee joint damage. Our objective was to test whether HA was also effective in an IL-1 damage model and whether this repair was stable and occurred in older bovine cartilage.

Design: Bovine cartilage explants from 18-month-old or 6-year-old bovines in 10% serum/Dulbecco’s modified Eagle’s medium were exposed to Fn-f or to IL-1 and the ability of 1 mg/ml HA of 800 kDa to block damage or promote restoration of proteoglycan (PG) after the damage was measured. The damage phase as well as the exposure to HA were varied.

Results: Exposure of exogenous HA decreased Fn-f-mediated damage, but did not decrease IL-1β-induced cartilage damage. If explants from 18-month-old bovines were damaged by a 7-day exposure to Fn-f or IL-1 and then exposed for 7 days to HA, PG was restored. This reparative activity persisted up to 4 weeks after the removal of HA from the culture medium. The restoration of PG did not occur in 0.1% serum-free cultures, was less when the exposure to the Fn-f was doubled and failed when exposure to IL-1β was doubled. In explants from 6-year-old bovines damaged with IL-1β for 7 days, HA fully restored PG content to normal levels.

Conclusions: The reparative activities of HA occur not only in a Fn-f damage model, but also in an IL-1 damage model and occur with older bovine cartilage.

Key words: Hyaluronic acid, Hyaluronan, Fibronectin fragment, Cartilage, Cartilage damage, Osteoarthritis, Proteoglycan.

Abbreviations: DMB, dimethylmethylene blue, DMEM, Dulbecco’s modified Eagle’s medium, Fn-f, fibronectin fragment, HA, hyaluronic acid or hyaluronan, MMP, matrix metalloproteinase, MMP-3, matrix metalloproteinase-3: stromelysin-1, OA, osteoarthritis, PG, proteoglycan, SD, standard deviation.

Introduction
Hyaluronic acid or hyaluronan (HA), has been tested as a therapeutic intervention in the treatment of osteoarthritis (OA)1. Intra-articular HA treatment of the knee of patients with OA has been shown to reduce painful symptoms and improve joint mobility2–6. One report suggested that repeated intra-articular injections of HA weakly affect the structural progression of OA in clinical patients7; however, that conclusion will need to be strengthened with further studies. In contrast to the reports of efficacy2–6, a recent placebo-controlled trial failed to show positive results8. Further, the results of a study that showed that HA reduced one parameter of pain when compared with saline controls9 or with low molecular weight HA forms10, have been challenged11. The complexities of interpretation of these clinical trials and discussion of limitations of some of these studies have been reviewed12.

Investigations of the effects of HA in animal models have been less controversial. It has been shown in a Pond–Nuki canine model, which involves severance and resection of the anterior cruciate ligament, that HA causes significant reduction of cartilaginous lesions and delays progression of cartilage degradation13. In another anterior cruciate ligament animal model in rabbits, HA decreased damage to femoral condyles as measured by gross inspection, cartilage thickness, presence of collagen cross-links and hexosamine content14. In a bilateral canine osteoarthritis rabbit model, HA was effective up to 6 months after injection and prevented progression of OA15. HA also reduced histopathology in a partial meniscectomy model of OA in rabbit knee16. In another partial meniscectomy rabbit model, HA enhanced meniscal regeneration and inhibited cartilage degeneration as long as 6 months post-surgery, suggesting a long-term effect. In this study, there was a trend toward increased proteoglycan (PG) content and less morphological degradation in HA-treated medial tibial articular
cartilage. In one study of a rabbit anterior cruciate ligament transection model, the effect of HA on matrix metalloproteinases (MMPs) and cytokines was investigated. It was found that HA reduced the grade of cartilage damage, but did not block MMP production in cartilage, although it did so in synovium.

There have been many studies on the effects of HA in simpler systems, for example in cartilage explant or chondrocyte culture models. HA has also been shown to block PG release caused by addition of catabolic cytokines to cultured cartilage; however, the mechanism is unknown. One possible mechanism may be that HA acts as an antioxidant and blocks reactive oxygen species involved in cytokine pathways. In fact, HA was shown to inhibit IL-1-induced superoxide anion in bovine chondrocytes. HA was also shown to increase PG synthesis in bovine articular cartilage in the presence of IL-1. In our investigation of the mechanisms by which HA suppresses cartilage damage, we have utilized a model of cartilage damage involving the ability of fibronectin fragments (Fn-f) to damage cartilage explants, through elevation of catabolic cytokines and also induces aggregan-like cleavage. This model has been reviewed. A particular useful feature of this model is that, while many of the reported studies of HA utilize cytokine models, this model is upstream of cytokine pathways and allows us to examine the effects of HA in a more physiologic pathway of a more complex nature.

In application of this model, our studies showed that HA decreased the loss of cartilage PG in Fn-f-damaged cartilage in vitro, in both bovine and human cartilage. Further, in a counterpart Fn-f in vivo model in which injection of Fn-f into rabbit knee joints causes marked PG loss, HA was also effective. However, HA was also found to restore PG lost from bovine and human cartilage explants previously damaged by Fn-f. These reports were the first to show that HA had reparative activities in vitro. Since the damaging agents were removed to test for repair, there are likely mechanisms other than blocking penetration of the mediator that would be required to explain repair. This HA-mediated restoration of PG was associated in human cartilage with enhanced PG synthesis. Whether or not the enhanced PG synthesis is due to suppression of cytokine activities through anti-oxidant activities or enhancement of anabolic pathways through other mechanisms, is not yet clear.

In order to address the question as to whether HA acts to block cytokine activities or affects other novel pathways, we have investigated as to whether the effects of repair observed in Fn-f-damaged cartilage apply to IL-1 damaged cartilage as well. We have also investigated whether this restoration of PG is persistent and, thus, of a useful nature, and whether it occurs in older bovine cartilage.

Materials and methods

All common chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). HA (800 kDa) and 5-aminofluorescein-labeled HA (800 kDa) were provided by Seikagaku Corporation (Tokyo, Japan). Recombinant human IL-1β (cat. number: 201-LB) was from R & D Systems, Inc. (Minneapolis, MN). Recombinant human IGF-1 was from UBI (Lake Placid, NY).

ISOLATION OF FN-F

Fn-fs were generated from human Fn as described. The Fn-f tested was a well-characterized amino-terminal 29-kDa Fn-f that has been found to be the most potent and is referred to as the Fn-f.

CARTILAGE EXPLANT CULTURES

Cartilages from 18-month-old as well as cartilage from older 6-year-old bovines were studied (Research 87, Marlboro, MA). Only macroscopic-appearing normal cartilage was used in all the cases. Culturing of slices of bovine metacarpophalangeal cartilage was performed as described in Dulbecco’s modified Eagle’s medium (DMEM) containing 50 U/ml penicillin-streptomycin in 10% fetal bovine serum/DMEM or in DMEM alone, and with 50–80 mg cartilage per 2 ml/well. The medium was changed every other day. Each datum was based on assays of three culture wells treated identically.

PG-CONTENT OF CARTILAGE AND ASSAYS OF PG DEPLETION

The total amount of PG/mg wet weight cartilage was determined by assays with dimethylmethylen blue (DBM) reagent after treatment of 50–80 mg of cartilage in 1.0 ml of 50 mM phosphate buffer, pH 6, containing 10 mM EDTA and 10 mM cysteine, with 50 µg/ml papain for 16 h at 65°C as originally described. To assay PG, a volume of 0.8 ml DBM reagent, prepared as described, was mixed with 190 µl of 1.37 M GuHCl and 10 µl of test sample. After 2 min, a spectrophotometer was used to determine absorbances at 525 and 595 nm, using water as a blank. The ratios of 525 nm absorbance over 595 nm absorbance were used to establish a standard curve with purified PG and a standard curve of 0.5–3 µg/ml purified bovine PG was used to estimate PG contents in the papain digests. The data are reported as µg PG/mg wet weight cartilage based on a mean and standard deviation (SD) of at least three cartilage samples. The data were compared by unpaired t test analysis. A P value <0.05 was considered significant.

TESTS OF EFFECTS OF HA ON BLOCKING FN-F OR IL-1β-MEDIATED DAMAGE, ON RESTORING PG AND ON STABILITY OF REPAIR

To test for blocking activity, to 10% serum/DMEM cultures, HA was typically added to a final concentration of 1 mg/ml. After 4 h, the Fn-f was added to a final 0.1 µM concentration or IL-1β was added to a final 10 ng/ml concentration. Every 7 days during the 28 to 42-day culture period, cartilage was removed, the dry weight was determined and the cartilage was subjected to assays of PG content. To test for restoration of PG, cartilage was cultured with 0.1 µM Fn-f or 10 ng/ml IL-1β for 7 or 14 days in 10% serum/DMEM in order to remove about half of the total cartilage matrix PG. At 7 or 14 days, HA was added to a final 1 mg/ml concentration. Every 7 days, for up to 42 days, cartilage was removed for assays of total PG content. To test for stability of repair, cartilage was cultured with Fn-fs or IL-1β for 7 days as described before to deplete about half of the cartilage PG and then cultured with 1 mg/ml HA in 10% serum/DMEM for an additional period. The HA was then removed at various times, and culturing continued in 10% serum/DMEM alone. Slices were removed for PG content analysis each of the 7 days.
BINDING OF HA TO CARTILAGE

Fl-HA (provided by Seikagaku Corp, Tokyo, Japan) was added at a final concentration of 1 mg/ml to cultured 18-month-old bovine cartilage in 10% serum/DMEM for 7 days. Cartilage was recovered, washed exhaustively with DMEM and subjected to cryosectioning (10 µm). The sections were then mounted in glycerol on slides and visualized with an Olympus BH-2 microscope equipped with epifluorescence using appropriate filters for fluorescein. A ×10 objective and ×2 ocular were used to photograph with 400 ASA Kodak Tri-X film pushed to 1600 ASA with 4 min per exposure.

Results

RESTORATION OF PG BY HA DID NOT REVERSE AFTER REMOVAL OF HA FROM FN-F-DAMAGED CARTILAGE

We had shown earlier that 1 mg/ml HA blocked the ability of the Fn-f to deplete PG content in cultures in 10% serum/DMEM and that HA also partially restored PG to normal or above levels by day 21 in cartilage first treated with Fn-f32,33. In order to investigate the stability of HA-mediated restoration of PG, cartilage was first treated with Fn-fs for 7 days in 10% serum/DMEM to deplete PG, and was then treated with 1 mg/ml HA for periods of 7, 14, 21 or 35 days during a 42-day culture period. As shown in Fig. 1(A), 7 days of HA treatment (F [d0–7]; HA [d7–14] curve) was sufficient for a full restoration of PG, and the restoration was stable up to day 42 or up to 4 weeks after the HA was removed. At days 14, 21, 28, 35 and 42, the PG content for the F [d0–7]; HA (d7–14) curve were significantly different than Fn-f treatment at \( P \) values of 0.045, 0.01, 0.003, 0.002 and 0.001, respectively. The curves for HA (d7–21) and HA (d7–42) were very similar to the HA (d7–14) curve with similar levels of significant difference when each was compared with Fn-f treatment. PG content in the repair curves appeared to be greater than control levels, but did not reach significant difference.

In another set of experiments, the response of cartilage exposed to Fn-f for twice as long, for 14 days, was investigated. Figure 1(B) shows that restoration of PG to control levels occurred after an exposure to HA for as short a period as 7 days (F [d0–7]; HA (d7–14) curve) \( P = 0.18 \) for 7 days when experimental compared with control). A decreased effect is suggested by the decrease in PG content at day 35 for the days 14–28 HA treatment \( P < 0.005 \) for experimental vs control. This decreased effect was also observed at day 42.

When cartilage was damaged with Fn-f in the presence of 0.1% serum present throughout the experiment, a greater degree of PG depletion occurred, but HA, added at day 7, did not promote restoration of PG (data not shown). Therefore, either the greater degree of damage or the lower concentration of serum components decreased the potential for repair.

In other experiments, when cartilage was first damaged by Fn-f and HA was substituted with 1 mg/ml methylcellulose, with viscosity similar to that of HA, restoration of PG did not occur (data not shown). Further, methylcellulose did not block Fn-f-mediated damage.

In order to determine as to how long cartilage treated with HA retained the HA and where HA bound, Fl-HA was added to the 10% serum/DMEM media of cultured cartilage between day 0 and 7, and the excess Fl-HA was removed at day 7. Sections were analyzed for fluorescence at various time points. Figure 2 shows that in controls not treated with HA (–HA) there was no detectable fluorescence. Ph shows a typical phase contrast of the control tissue. C7, C14 and C28 show cartilage after treatment with Fl-HA between day 0 and 7, and the excess Fl-HA was removed at day 7, 14 and 28. Similar cultures were also treated with Fn-f between day 0 and 7 and as well as Fl-HA, and the media Fl-HA was removed as with the control cultures. F7, F14 and F28 show the respective Fn-f-treated sections. Note that fluorescence is still detected up to 21 days after removal of HA.
from the culture media in either the HA control or Fn-f-treated cartilage and that Fn-f treatment does not appear to either enhance the amount of Fl-HA bound between day 0 and 7 or decrease the amount bound at a later time point. In all the tissues, fluorescence was observed throughout the full thickness.

HA DID NOT DECREASE DAMAGE BY IL-1β BUT DID REVERSE DAMAGE CAUSED BY IL-1β IN THE ABSENCE OF HA

In order to determine whether the blocking and reparative effects of HA were unique to Fn-f-mediated damage, another catabolic mediator, IL-1β, was used as a test.
agent. Figure 3(A) shows that preincubation of cartilage with 1 mg/ml HA, followed by addition of IL-1β, resulted in a major loss of PG by day 7 (P<0.0002 for experimental vs control), a loss similar to that of IL-1β alone (P<0.02 for experimental vs control). However, HA did facilitate restoration of PG in cartilage first exposed to IL-1β for 7 days as shown in Fig. 3(B). Restoration occurred to levels that were significantly greater than IL-1β-treated levels (P<0.03 for IL-1β vs IL-1β plus HA). The restored levels at days 28 and 35 were also significantly greater than the control untreated levels (P<0.03 and 0.02, respectively).

RESTORATION OF PG BY HA DID NOT OCCUR AFTER REMOVAL OF HA FROM IL-1β-DAMAGED CARTILAGE IF THE DAMAGE PERIOD WAS PROLONGED

The stability of restoration of PG in IL-1β-damaged cartilage was investigated (Fig. 4). Panel A shows that restoration of PG occurred after only a 14-day treatment with HA (IL[d0–7]; HA[d7–28]). This degree of restoration was stable through day 42 (P=0.003 for IL-1β vs IL-1β; HA at day 42). Restoration of PG was more complete after HA treatment for 28 days (IL[d0–7]; HA[d7–28] curve) and was at control levels at day 28 (P=0.8 when experimental compared with untreated control) and above control levels at days 35 and 42 (P=0.028, 0.0124, respectively). Similar results were observed with half the concentration of IL-1β, 7.5 ng/ml (data not shown). As with the earlier data, HA-treated cartilage, whether first damaged or not, showed PG contents later in culture that were greater than non-HA treated controls (P=0.03, 0.015, respectively, for days 35 and 42).

The effect of a longer damage period which would also delay introduction of HA by an additional period was also tested. As shown in Fig. 4(B), a longer treatment for 14 days with IL-1β prevented HA from causing a stable restoration of PG. Addition of HA for 14 days, followed by its removal, did not allow a regain of PG up through day 21. At day 28, there was an increase in PG content with HA-treated cartilage to a level indistinguishable from control.
levels ($P=0.07$ for both damage/HA curves). However, by day 35, the attempted repair had failed and PG contents were indistinguishable from IL-1β treatment ($P>0.36$ for both compared with control). The repair curves remained at damaged levels at day 42.

HA DID NOT BLOCK IL-1β-MEDIATED DAMAGE IN 6-YEAR-OLD BOVINE CARTILAGE, BUT DID PROMOTE REPAIR TO THE SAME LEVEL AS DID IGF-1

To test whether HA blocked IL-1β-mediated PG depletion, cartilage was obtained from 6-year-old bovines and cultured separately. Only normal looking cartilage, as ascertained by gross macroscopic analysis, was used. The cartilage did not have apparent fibrillation. Separate culturing was performed because of the greater potential for variability of older cartilage. As shown in Fig. 5, the control cartilages to which nothing was added, except for specimen D, showed a significant decrease in PG content by day 7 when treated with IL-1β. The $P$ values for day 7 data for specimens A–D, respectively, when control data were compared with IL-1 (d0–7)-treated samples were 0.0045, 0.0001, 0.0002 and 0.096. HA was not effective in blocking IL-1β-mediated PG depletion in any of the specimens, as also shown earlier for the 18-month-old cartilage. The $P$ values for day 28 data for specimens A–D, respectively, when IL-1 (d0–7) and IL-1β, HA (d0–28) data were compared were 0.127, 0.20, 1.0 and $>1.0$, respectively.

However, when IL-1β was added for 7 days to deplete PG and then removed and HA was added, there was significant restoration of PG in the four different cartilage specimens within 7 days of HA treatment. The $P$ values for day 14 data for specimens A–D, respectively, when IL-1 (d0–7) data were compared with IL-1 (d0–7); HA (d7–28) treatment were 0.045, 0.0068, 0.03 and 0.042, respectively. By day 21, restoration was almost to control levels of PG. The $P$ values for day 21 when control and HA-treated data were compared were $<0.17$ for specimens A, B and C and 0.08 for specimen D. By day 28, PG restoration was more complete.

Two additional 6-year-old bovine sources showed similar results (data not shown). In other experiments, treatment with IL-1β for 14 days before HA treatment did not allow significant restoration of PG (data not shown), as also demonstrated for the cartilage explants from 18-month-old bovines.

Fig. 5. Effect of HA on decreasing IL-1β-mediated damage in 6-year-old bovine cartilage and promoting repair—To test blocking activity, cartilage from 6-year-old bovines was cultured in 10% serum/DMEM alone (Control) or with 10 ng/ml IL-1β in 10% serum/DMEM from day 0 to 7 (IL [d0–7]) or with both IL-1β and 1 mg/ml HA from day 0 to 28 (IL, HA [d0–28]). To test for repair, cartilage was cultured with 10 ng/ml IL-1β from day 0 to 7, to remove PG and then incubated with 1 mg/ml HA in 10% serum/DMEM from day 7 to 28 (IL [d0–7]; HA [d7–28]). Controls for continuous IL-1β for days 0–28 are not shown, but were similar to day 0–7 treatment. PG content was measured every 7 days.
Similar trends were observed for specimen B. Greater than control (day 32, each treatment enhanced PG content to levels control cartilage had decreased compared with day 24. At combination vs HA or IGF-1). At day 32, the PG content in not significantly better than HA or IGF-1 alone ($P < 0.2$ for experimental vs control). The combination was also tested the stability of this repair, which has not yet been reported. We also investigated another parameter, the role of serum and found that the restoration of PG did not occur if the serum was lowered from 10 to 0.1%. Thus, serum appears to be important in providing greater vitality for repair. This blocking activity or ability of HA to facilitate restoration of PG in 10% serum cultures was not due to a viscosity effect, as shown by our experiments with methylcellulose. The activity was not due to interaction between HA and Fn-f, since we reported earlier that HA and the Fn-f do not interact as shown by gel filtration chromatography. The reparative activity also appeared to be stable in that the removal of HA after only a 7-day exposure to the damaged cartilage still allowed a full restoration of PG in Fn-f-damaged cartilage. It should also be noted that the concentration of HA we have used throughout these studies was 1 mg/ml. Since the synovial fluid concentration of HA is 2–4 mg/ml, as reviewed, we are not studying effects due to the use of abnormally high concentrations of HA.

In order to provide an explanation for the stability of the repair, we tested whether a brief 7-day exposure was sufficient for binding of fluorescent HA and whether the HA remained bound once the excess HA in the culture media was removed. Fluorescence microscopy showed that the HA that bound within a 7-day exposure period remained bound to cartilage for up to 21 days, whether the cartilage was exposed to Fn-f or not. Since a concentration of 1 mg/ml of fluorescent HA was used and the synovial fluid concentration of HA is 2–4 mg/ml, this binding was physiologic and not driven by an artificially high concentration of HA. Further, the fluorescence appeared to penetrate throughout the full thickness section of the cartilage and the HA appeared to surround the cells. We had reported earlier that most of the HA remained on the superficial cartilage surface; however, more sensitive detection does show that a very small fraction does become internalized. We have no explanation for how HA of 800 000 can penetrate and diffuse throughout the section. It is very possible that the very small fraction of HA that does penetrate is degraded and that this is the active fraction of HA. This will be difficult.

Discussion

We have reported that high molecular weight HA can suppress decreases in cartilage matrix PG content from both bovine metacarpophalangeal cartilage and human knee cartilage in the presence of Fn-fs, which we have shown to cause chondrocytic chondrolysis. We have also found that HA can promote restoration of PG in Fn-f-damaged bovine or human cartilage.

Our objectives were to determine whether blocking or restoration of PG were unique to Fn-f-mediated damage or would also occur in an IL-$1\beta$ damage system. We have reported that Fn-fs upregulate cytokines, such as IL-$1\beta$ and, thus, one might expect that HA-mediated repair of Fn-f-damaged cartilage should also be observed in an IL-$1\beta$ damage model. However, Fn-fs upregulate TNF-$\alpha$ and IL-$1\beta$ in a short-term pulse-like pattern, which leads to a relatively short pulse of MMP activity. Other catabolic cytokines such as IL-$1\alpha$ and IL-6 are also upregulated. However, the enhanced MMP release does not occur continuously. In contrast, in a typical IL-$1\beta$ damage model, IL-$1\beta$ is added continuously, but little is known as to whether MMP upregulation remains elevated during the culture period. Thus, because IL-$1\beta$ is often used for a cartilage explant damage model, it was important for us to determine whether HA would also be effective in an IL-$1\beta$ model.

We first confirmed that HA was effective in suppressing PG depletion in Fn-f-cultured 18-month-old bovine cartilage for up to 28 days and also in enhancing repair as we reported previously. Additionally, we also tested the stability of this repair, which has not yet been reported. We also investigated another parameter, the role of serum and found that the restoration of PG did not occur if the serum was lowered from 10 to 0.1%. Thus, serum appears to be important in providing greater vitality for repair. This blocking activity or ability of HA to facilitate restoration of PG in 10% serum cultures was not due to a viscosity effect, as shown by our experiments with methylcellulose. The activity was not due to interaction between HA and Fn-f, since we reported earlier that HA and the Fn-f do not interact as shown by gel filtration chromatography. The reparative activity also appeared to be stable in that the removal of HA after only a 7-day exposure to the damaged cartilage still allowed a full restoration of PG in Fn-f-damaged cartilage. It should also be noted that the concentration of HA we have used throughout these studies was 1 mg/ml. Since the synovial fluid concentration of HA is 2–4 mg/ml, as reviewed, we are not studying effects due to the use of abnormally high concentrations of HA.

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to demonstrate because of the relative difficulty in separat-
ing this small amount of HA from the surface-bound HA and
determining its mass. However, future experiments will
include work in that direction.

This observation of retention of HA within the cartilage
matrix for weeks after the initial treatment is also consistent
with our earlier report that a 1-day treatment with 1 mg/ml
HA decreased the effect of the Fn-f for up to 28 days32.
These collective results suggest that the effects of HA
persist after the excess is removed from the media and that
the reparative activity is not due to the solution phase HA in
the media, but rather to the bound HA. Thus, the efficacy of
HA in clinical studies should not be strictly dependent on
the synovial fluid concentration.

Interestingly, HA did not block IL-1β-mediated damage as
it did in Fn-f-mediated damage. This may be because the
29-kDa Fn-f used in this study can bind matrix components
such as PG, while the IL-1β would not be expected to have
specific binding affinity for matrix components. We have
shown that treatment of cartilage with HA decreases pen-
etration of Fn-f into cartilage tissue and that this cannot be
due to interaction between HA and Fn-f since complexes
were not observed by gel filtration chromatography32. We
did not test whether or not HA blocked penetration of IL-1β
since others have shown that HA does not alter effects of
IL-1 on cartilage matrix synthesis, and this may suggest
that HA does not block penetration of IL-120,21.

Most importantly, this work does show that HA can be
effective in a cytokine model in terms of restoration of PG
and, thus, this work supports the notion of a general effect
of HA on cartilage repair. Since HA did not block IL-1-
mediated damage, but promoted repair, this may suggest that
the potential physical effect of HA on decreasing
penetration of Fn-f, as we have reported32,33, probably plays
only a small role in the overall chondroprotective
effect of HA. While it can be argued that HA does block
penetration of Fn-f and decreases damage, the most
important activity of HA may be more general, and may be
due to the enhancement of reparative processes.

Nonetheless, since HA helped restore PG in both Fn-f
and IL-1β-damaged cartilage, it is likely that the reparative
mechanism does not involve unique synergistic interaction
with elements of a Fn-f damage or repair pathway, unless
these elements would be similar in an IL-1β system. It is
more likely that HA has direct effects on catabolic and/or
anabolic pathways. It was interesting to note that a short
7-day exposure to HA was sufficient to initiate these effects
in either the Fn-f or IL-1 models. Thus, HA triggers anabolic
pathways or suppresses catabolic pathways that continue
after removal of the bulk of the HA, consistent with our
visualization of Fl-HA in cartilage weeks after removal of
HA from the media.

When cartilage was cultured with IL-1β for 14 days, the
restoration of PG was not stable and certainly not as stable
as with prolonged Fn-f treatment. While we cannot interpret
whether this relative failure to restore PG stably is due to
prolonged deleterious effects of IL-1β treatment or due to
the delay of introduction of HA, it does point out that there
may be differences in our Fn-f model vs cytokine models. In
our Fn-f models, a cytokine pulse is caused, which may be
milder than the effects of continuous incubation with cyto-
kines. Since restoration of PG was more complete with
Fn-f-damaged cartilage, also with a delayed introduction
of HA, it is likely that the lesser restoration of PG with
prolonged IL-1β treatment is not due to the timing of
introduction of HA, but rather due to the severity of the
treatment. It should be noted that we have shown that
continuous treatment with Fn-f does not decrease PG
content38–40, and that the chondrocytes respond to Fn-f
treatment initially with reduced PG and general protein
synthesis, but later show elevated rates of synthesis, which
by 28 days in culture, return to control values38–40. There-
fore, it is unlikely that the Fn-f have caused reduced cell
vitality during the more prolonged treatment. However, we
did not test the effect of prolonged treatment with IL-1β on
cell vitality.

Another interesting observation was that HA appeared to
enhance PG content to above normal levels. Such an effect
is not without precedent. We have observed this effect with
other agents as well, including antioxidants37,41,42 or syn-
thetic peptides that mimic the cell-binding domain of Fn43
or the growth factor, OP-144. Further, addition of lower
concentrations of Fn-f to cartilage explants, which cause
enhanced PG synthesis, also enhances PG content to above
normal levels38–40.

The observation that HA was effective on IL-1β-treated
6-year-old cartilage explants, though perhaps to a lesser
degree, was encouraging since older cartilage is thought to
be less capable of repair. In fact, it has been shown that
bovine cartilages of this age are much less responsive to
growth factors such as IGF-145. Nonetheless, IGF-1 as well
as HA, potently enhanced restoration of PG.

In summary, HA has been shown to be effective not only
in a Fn-f model, but also in a cytokine model of cartilage
damage. It has also been found to be effective long after
the excess solution form of HA is removed from the
cultures, and is also effective on older cartilage. Collect-
ively, these data add to a growing literature on the efficacy
of HA in in vitro models of cartilage damage. However,
caution should be exercised in extrapolating our results to
expectations of efficacy in clinical trials since the joint
presents a much more complex milieu than our culture
conditions.

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