Assessment of the catabolic effects of interleukin-1β on proteoglycan metabolism in equine cartilage cocultured with synoviocytes

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I n the development of osteoarthritis, there is a disruption in extracellular matrix homeostasis with an overall balance toward cartilage catabolism. The enzymes of the MMP family deplete the extracellular matrix through decreased synthesis and increased degradation of essential matrix proteins including aggrecan and type II collagen. In a synovial joint, MMPs are synthesized by chondrocytes and synoviocytes. The activity of MMPs is tightly regulated at several levels including transcription, activation of precursor enzymes (zymogens), interaction with extracellular matrix components (collagen type II and aggrecan), and inhibition by tissue inhibitors of metalloproteinas. One of the most potent activators of MMPs in cartilage and synovium is the proinflammatory cytokine IL-1β.

To study matrix metabolism in experimental investigations of osteoarthritis, cartilage explants or isolated chondrocytes are commonly treated with recombinant IL-1α or IL-1β. Although these studies have proven valuable for studying the basic mechanisms involved in matrix degradation and for evaluating treatments, they were not optimally designed to reflect the native articular environment because no synovial membrane or synovial fluid was present in the culture system. Homeostasis of the articular environment depends on interactions between the cartilage, synovial membrane, and synovial fluid and not just the ability of cartilage to respond to molecular or mechanical cues. Therefore, the effects of soluble protein mediators or mechanical stress on cartilage biosynthesis will likely differ depending on the type of culture system employed in each study. For example, cartilage explants have significantly lower synthetic activity when cultured in the presence of synovial fluid versus their activity in tissue culture medium supplemented with fetal bovine serum. The purpose of the study reported here was to evaluate the effects of IL-1β on proteoglycan metabolism in cartilage explants cultured in the presence of synoviocytes. The objective was to examine a coculture system in which cartilage and synoviocytes share a common medium to determine whether the well-established effects of IL-1β on cartilage matrix catabolism were altered by the presence of synoviocytes. Our hypothesis was that the catabolic effects of IL-1β on articular cartilage would be significantly diminished in cultures containing synoviocytes.

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Materials and Methods

Cartilage and synovium were collected from both femoropatellar joints of 3 horses (2 to 3 years old) that were euthanized (via IV injection of an overdose of pentobarbital) for reasons unrelated to lameness or this study. All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University. Three groups were established for samples collected from each of the 3 horses, and all sample groups were cultured in split-well plates specifically designed for coculture of tissues. The experiment was designed with synoviocytes in monolayer on the bottom of the well and cartilage explants suspended in the medium of the same well by use of a low–protein-binding polyester membrane insert (pore size, 3 μm; Figure 1). The insert has gaps in its perimeter to allow free exchange of medium between the 2 compartments. Three groups, each in triplicate, were established: cartilage only (no synoviocytes), synoviocytes only (no cartilage explants), and coculture (cartilage explants with synoviocytes). Two treatments (ie, with or without IL-1β; 10 ng/mL) were applied to each group for each of the 3 horses. Experiments for each of the 3 horses were performed on independent days.

Tissue procurement and culture—Samples of full-thickness cartilage were removed from the trochlear ridges of the femora, and synovial membrane was removed from the proximal portion of the femoropatellar joints. Careful dissection was performed to minimize inclusion of fat and fibrous joint capsule tissue in the synovial membrane samples. The synovium was digested in DMEM, 0.15% collagenase type 2, and 0.015% DNAse I; 10 mL of digest medium/g of synovium was used, and digestion proceeded for 2 hours at 37°C. The resulting cell slurry was filtered through 44-μm nylon mesh and centrifuged at 300 X g for 10 minutes. Synoviocytes were plated at 1.5 X 10^6 cells/well in DMEM-complete (ie, DMEM with 10% fetal bovine serum, 25mM HEPES, ascorbic acid [50 μg/mL], α-ketoglutaric acid [30 μg/mL], l-glutamine [300 μg/mL], penicillin sodium [100 U/mL], and streptomycin sulfate [100 μg/mL]). Synoviocytes were allowed to adhere to the plate for 18 hours while cartilage explants were held in DMEM-complete. Culture and cartilage samples were maintained at 37°C with 5% CO2 at 90% humidity. Full-thickness cartilage explants (5 X 5 mm) were then added to the synoviocyte cultures. Five explants were placed into each coculture well insert according to the aforementioned experimental design. Cultures were allowed to equilibrate for a further 24 hours, at which time the medium was replaced with DMEM-complete and 2% fetal bovine serum with or without IL-1β (10 ng/mL). This medium (with or without IL-1β) was exchanged at 48 hours after initiation of treatment, and cultures were harvested after 96 hours of treatment. At 48 and 96 hours, exhausted medium was collected and 10% (vol/vol) protease inhibitors were added. Samples of medium were centrifuged at 350 X g for 10 minutes to remove particulate matter and stored at –80°C until analysis. At termination of the study, 1 cartilage explant from each well was fixed in 4% paraformaldehyde for histochemical analysis (toluidine blue assay) and the remaining explants were snap-frozen in liquid nitrogen for GAG and mRNA analyses.

GAG content of cartilage and medium samples—Total GAG content of cartilage and media samples was assessed by use of a 1,9-dimethyl-methylene blue dye-binding microwell spectrophotometric assay. Media samples were digested (1:1 [vol/vol]) in 0.05% papain. To determine cartilage GAG content, lyophilized samples were digested (10% [wt/vol]) in 0.05% papain and the optical density at 595 nm was determined with a spectrophotometer. Mixed-isomer shark chondroitin sulfate was used to construct a standard curve. Cartilage GAG content was expressed per microgram of DNA, which was assessed fluorometrically. Calf thymus DNA was used to construct a standard curve.

RNA isolation and analyses—Four explants from each well were rinsed in PBS solution, snap-frozen in liquid nitrogen, and pulverized in a freezer-mill. Total RNA from cartilage and synoviocytes was isolated by use of a monophasic solution of phenol and guanidine isothiocyanate according to the manufacturer’s directions. Further purification of cartilage RNA was achieved by use of RNA purification spin columns. Real-time quantitative PCR assays were then performed to assess changes in transcript levels of MMP-3 and -13 (cartilage and synoviocytes), aggrecan (cartilage only), aggrecan (synoviocytes only), and MMP-2 (cartilage only), MMP-3 (cartilage only), MMP-13 (cartilage only), MMP-2 (synoviocytes only), MMP-3 (synoviocytes only), MMP-13 (synoviocytes only), and MMP-9 (cartilage only). There was significantly more GAG loss attributable to IL-1β compared with untreated cultures. However, as expected, IL-1β treatment resulted in a significant loss of matrix GAG in cartilage-only groups (ie, without synoviocytes), compared with untreated cultures. There was significantly more GAG loss attributable to IL-1β in the cartilage-only cultures, compared with the cocultures (2-sample t test). Data are mean values from 3 horses in 3 independent experiments. *Value significantly (P < 0.05; 1-sample t test) different from that of the control culture within this culture group.
and collagen type II (cartilage only); transcript levels of 18S were assessed as a control. The total RNA was reverse transcribed and amplified by use of a 1-step system with sequence detection software. The primers and dual-labeled fluorescent probes (6-carboxyfluorescein [6-FAM] as the 5' label [reporter dye] and tetramethylrhodamine [TAMRA] as the 3' label [quenching dye]) were designed with specialized software and equine sequences published in GenBank, sequenced in our laboratories, or obtained from another research group.

Histochemical analysis with toluidine blue—One cartilage explant from each well was used for histochemical analysis, yielding triplicate samples per treatment group per horse. Explants were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 6 µm. Cartilage sections were stained with 0.5% toluidine blue and assessed for matrix metachromasia via light microscopy.

Statistical analysis—Differences in outcome variables within a group (cartilage only, synoviocyte only, or coculture) attributable to IL-1β treatment were assessed by use of 1-sample t tests. To assess differences in the magnitude of response to IL-1β treatment between groups, the fold change in outcome variables (attributable to IL-1β treatment) within each group was calculated and then the fold changes between culture groups were compared by use of 2-sample independent-groups t tests. A 2-sample t test analysis was chosen over an ANOVA because the objective was to compare the responses between groups and not to compare all groups (ie, cartilage-only, synoviocyte-only, and coculture groups with or without IL-1β) simultaneously. Differences in GAG concentration in media samples at 48 and 96 hours were assessed by use of a paired t test. Outcome variables analyzed in cartilage were DNA concentration (µg of DNA/mg of cartilage [dry weight]), GAG concentration (µg of GAG/µg of DNA), and mRNA expression of MMP-3, MMP-13, aggrecan, and collagen type IIβ relative to 18S expression. In synoviocytes, mRNA expression of MMP-3 and -13 (relative to 18S RNA) were analyzed. Values of P ≤ 0.05 were considered significant. Statistical analyses were performed by use of computer software.

Results

GAG content of cartilage and medium—There was no significant (P = 0.13) effect of IL-1β on GAG content in cartilage that was cultured with synoviocytes (Figure 2). As expected, GAG concentration in the cartilage-only group (ie, cartilage cultured without synoviocytes) was significantly (P = 0.001) decreased by IL-1β treatment. Subsequently, when the fold changes in GAG concentration attributable to IL-1β in the cartilage-only and coculture groups were compared, there was significantly (P = 0.04) more GAG loss in the cartilage-only group than in the coculture group. There were no significant (P = 0.14) differences in DNA content within groups in response to IL-1β treatment, allowing for normalization of GAG content to DNA content. Glycosaminoglycan concentration in media samples was increased (P ≤ 0.03) by IL-1β treatment at 48 and 96 hours in cartilage-only and coculture groups, compared with media collected from their respective untreated cultures (Figure 3). After 48 or 96 hours, there was no difference (P > 0.8) in media GAG concentration between the 2 IL-1β-treated cultures; however, GAG content within IL-1β-treated culture groups was significantly (P ≤ 0.04) greater at 96 hours, compared with their respective values at 48 hours.

Cartilage gene expression—Matrix gene expression in response to IL-1β treatment was different between groups, and aggrecan mRNA was partially protected against the treatment in coculture groups. In cartilage-only groups, IL-1β induced a significant (P ≤ 0.02) decrease in aggrecan and type II collagen gene expression, as expected (Table 1). However, in coculture, aggrecan expression was less affected by IL-1β, and values in the cartilage-only and coculture groups after treatment were significantly (P = 0.03) different from each other. Collagen gene expression in response to IL-1β treatment was not significantly (P = 0.4) different between cartilage-only and coculture groups.

The catabolic genes MMP-3 and -13 were significantly (P ≤ 0.03) increased in response to IL-1β treatment in both culture groups (Table 1). There were no differences (P ≥ 0.07) in cartilage MMP-3 or -13 expression after IL-1β treatment between the cartilage-only and coculture groups.

Synoviocyte gene expression—In synoviocyte-only (ie, no cartilage explants) and coculture groups, expression of MMP-3 and -13 was significantly (P ≤ 0.02) different from each other. Conversely, aggrecan expression in synoviocytes was significantly different (P ≤ 0.001) between the cartilage-only and coculture groups.
increased in an attempt at cartilage self-repair. Despite this effort to reestablish homeostasis, cartilage degeneration continues with a marked loss of proteoglycan content. Treatment of cartilage explants or chondrocytes in monolayer culture with IL-1α or -1β induces a similar loss of aggrecan from the extracellular matrix with a concomitant decrease in proteoglycan synthesis, and such treatments can be used to induce cartilage degradation for osteoarthritis studies. In the present study, the results of IL-1β treatment of cartilage explants that were cultured alone were consistent with findings of previous studies, but when cartilage explants were cultured with synoviocytes, the catabolic effects of IL-1β were diminished. The protection imparted by the presence of synoviocytes was relatively specific for proteoglycan biosynthesis.

In cartilage-synoviocyte cocultures, there was no significant loss of GAG from cartilage after treatment with IL-1β. This was an unexpected finding because the cocultures were exposed to a relatively high concentration of IL-1β (10 ng/mL) for 96 hours, which should have resulted in considerable GAG loss from cartilage matrix. In contrast, and as expected, approximately 51% of GAG content was lost from cartilage explants cultured without synoviocytes but in the presence of IL-1β. These results were supported by toluidine blue histochemistry—

In cartilage-only cultures, matrix metachromasia was markedly diminished throughout cartilage explants by IL-1β treatment, as anticipated (Figure 4). However, in coculture, matrix metachromasia in cartilage explants was intense and evenly distributed throughout the depth of the tissue and was subjectively increased, compared with findings in cartilage-only groups in the absence of IL-1β. In addition, there was little perceivable loss of metachromasia in cocultured cartilage explants treated with IL-1β, compared with findings in cocultured cartilage explants cultured in the absence of IL-1β, which supported the biochemical and molecular findings.

**Discussion**

In osteoarthritis, aggrecan synthesis is initially increased in an attempt at cartilage self-repair. Despite this effort to reestablish homeostasis, cartilage degeneration continues with a marked loss of proteoglycan content. Treatment of cartilage explants or chondrocytes in monolayer culture with IL-1α or -1β induces a similar loss of aggrecan from the extracellular matrix with a concomitant decrease in proteoglycan synthesis, and such treatments can be used to induce cartilage degradation for osteoarthritis studies. In the present study, the results of IL-1β treatment of cartilage explants that were cultured alone were consistent with findings of previous studies, but when cartilage explants were cultured with synoviocytes, the catabolic effects of IL-1β were diminished. The protection imparted by the presence of synoviocytes was relatively specific for proteoglycan biosynthesis.

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**Table 1—Cartilage matrix and synoviocyte gene expression in equine cartilage-only or synoviocyte-only cultures and cartilage-synoviocyte cocultures.**

<table>
<thead>
<tr>
<th>mRNA analysis</th>
<th>Cartilage or synoviocytes only</th>
<th>Cartilage with synoviocytes</th>
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<tbody>
<tr>
<td></td>
<td>Without IL-1β</td>
<td>With IL-1β</td>
</tr>
<tr>
<td>Cartilage aggrecan</td>
<td>27.8 X 10³</td>
<td>11.1 X 10³**</td>
</tr>
<tr>
<td>Cartilage Col IIB</td>
<td>1.26 X 10³</td>
<td>0.811 X 10³**</td>
</tr>
<tr>
<td>Cartilage MMP-3</td>
<td>0.360 X 10³</td>
<td>69.1 X 10³*</td>
</tr>
<tr>
<td>Cartilage MMP-13</td>
<td>0.112 X 10³</td>
<td>32.0 X 10³*</td>
</tr>
<tr>
<td>Synoviocyte MMP-3</td>
<td>283.1 X 10³</td>
<td>484.9 X 10³***</td>
</tr>
<tr>
<td>Synoviocyte MMP-13</td>
<td>0.128 X 10³</td>
<td>0.326 X 10³*</td>
</tr>
</tbody>
</table>

Data are presented as mean (SE) of cDNA copy number of gene/ng of RNA/copy of 18S from 3 horses in 3 independent experiments.

*Value significantly (P < 0.05; 1-sample t test) different from that of the culture that was not treated with IL-1β in the same group.
†Value significantly (P < 0.05; 2-sample t test) different from that of the cartilage-only or synoviocyte-only culture treated with IL-1β.

Col IIB = Collagen type IIB.
dine blue histochemical assessment of matrix metachromasia. Analysis of medium GAG content in cartilage-only and cartilage-synoviocyte cultures with and without IL-1β revealed that IL-1β treatment resulted in a significant increase in medium GAG content in both groups. This suggests that the lack of GAG loss from the cartilage matrix in response to IL-1β is a result of protected or increased GAG synthesis rather than diminished loss of GAG from cartilage in cocultures. Glycosaminoglycan synthesis analysis by use of radiolabeled sulfur incorporation methods was not performed in the present study in lieu of our goal to assess mRNA expression of genes with known roles in matrix synthesis and degradation.

In our study, results of the analysis of aggrecan gene expression supported the GAG biochemical data. Although aggrecan mRNA expression was significantly decreased by IL-1β in the cartilage-only and coculture groups, the effect was significantly less (approx 90% less) in the cartilages than in cartilage-only cultures. Taken together with the cartilage GAG content data, the results imply that in the presence of synoviocytes, aggrecan biosynthesis in cartilage is protected or maintained rather than stimulated. Further studies to determine mRNA half-life and GAG biosynthetic rate would be required to fully evaluate the mechanism by which synoviocytes protect GAG synthesis in cartilage from the effects of IL-1β. This protective effect of synoviocytes on cartilage gene expression was not evident for collagen type IIB, MMP-3, or MMP-13. In assessments of these variables, IL-1β induced alterations in gene expression patterns that were anticipated (i.e., decreased collagen type IIB and increased MMP-3 and -13 expressions).

Synoviocyte mRNA expression subsequent to IL-1β treatment was also disparately affected by culture-group type. In synoviocyte-only culture and coculture, synoviocyte MMP-3 and -13 mRNAs were significantly increased in response to IL-1β exposure, as would be expected. However, compared with the synoviocyte-only group, synoviocyte MMP-3 mRNA was increased by IL-1β to a significantly lesser extent in coculture. It is well known that IL-1α and -1β upregulate MMP-3 expression in articular chondrocytes and synovium and that MMP-3 induces GAG loss from cartilage. Interestingly, hyaluronate can inhibit IL-1β-induced upregulation of MMP-3. Hyaluronate was not measured in the medium samples collected in our study, but if it were a mechanism to explain our findings, then presumably synoviocytes, cartilage, or both in cocultures would have to secrete hyaluronate to a greater extent than synoviocytes alone because the amelioration of IL-1β–induced upregulation of synoviocyte MMP-3 transcription was greater in the coculture group than it was in the synoviocyte-only group. Alternatively, an unidentified molecule may be released from cartilage, which reduces the impact of IL-1β on synoviocyte MMP-3 transcription.

Identification and characterization of mediators secreted from synoviocytes and cartilage in cocultures treated with IL-1β may elucidate novel therapeutic targets for patients with rheumatoid or osteoarthritis. Microarray and proteomic approaches to identify such target molecules are under investigation. Similar experiments using IL-1β–conditioned medium from synoviocyte cultures to identify novel cytokines involved in cartilage catabolism have been performed. The results of the present study suggest that by use of a coculture system, mediators that protect cartilage from IL-1β–induced catabolism may also be identified. Interleukin-1 receptor antagonist protein is a naturally occurring antagonist to IL-1β and has proven effective at protecting cartilage from IL-1–induced catabolism. However, by use of a coculture system similar to that used in the present study, Haupt et al. did not detect an IL-1β–induced increase in IL-1 receptor antagonist protein mRNA synthesis in synoviocytes. This suggests that there are mediators in addition to IL-1 receptor antagonist protein that are capable of protecting cartilage from IL-1β–induced proteoglycan loss.

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the interleukin-1beta-induced expression of matrix metallopro-


