Effects of polysulfated glycosaminoglycan and hyaluronan on prostaglandin E2 production by cultured equine synoviocytes

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Objective—To investigate effects of the anti-arthritic agents hyaluronan and polysulfated glycosaminoglycan (PSGAG) on inflammatory metabolism in cultured equine synoviocytes.

Sample Population—Synoviocytes cultured from samples obtained from the metacarpophalangeal joints of 4 horses.

Procedure—Equine synoviocytes were grown in monolayer culture. Synoviocytes were stimulated with lipopolysaccharide (LPS) and simultaneously treated with various concentrations of hyaluronan or PSGAG for 48 hours. Three hyaluronan preparations were compared. Prostaglandin E2 (PGE2) concentrations in culture medium were measured, using radioimmunoassay.

Results—The highest concentrations of hyaluronan and PSGAG tested inhibited PGE2 production.

Conclusions and Clinical Relevance—Clinically achievable concentrations of hyaluronan and PSGAG inhibited PGE2 synthesis by cultured equine synoviocytes. This anti-inflammatory action may be a mechanism through which these agents exert anti-arthritic effects. The effect was obtained at concentrations that can be achieved by use of intra-articular, but not systemic, administration of hyaluronan or PSGAG.

Joint disease in horses commonly is characterized by inflammation of the synovium, producing the classic signs of swelling (joint effusion), heat, and pain, causing loss of normal function and manifesting as lameness. Although synovitis often is characterized by leukocytic infiltration of the synovial membrane, synoviocytes can produce pro-inflammatory cytokines, metalloproteinases, and eicosanoids. Of the latter, prostaglandin E2 (PGE2) has been suggested as an important mediator of inflammation and hyperalgesia through its enhancement of vascular permeability, vasodilatory properties, and sensitization of joint nociceptors. Pharmacologic treatment of joint disease typically is directed at alleviating the signs of joint inflammation; nonsteroidal anti-inflammatory drugs (NSAID) and synthetic glucocorticoids are the most commonly used compounds. These drugs act primarily through inhibition of PGE2 production, although glucocorticoids possess other actions, such as inhibition of induction of inducible nitric oxide synthase and the inducible isoinorm of cyclooxygenase (ie, cyclooxygenase 2 [COX-2]).

Pharmacologic intervention also may be directed at limiting damage to, and stimulating repair of, articular cartilage. To this end, glycosaminoglycan preparations such as polysulfated aminoglycan (PSGAG) and hyaluronan are administered intra-articularly or systemically (IV administration in the case of hyaluronan and IM administration in the case of PSGAG). Although these compounds can directly stimulate neosynthesis of cartilage matrix, it is possible that their chondroprotective effects may result indirectly from a diverse range of anti-inflammatory properties. Included in these putative anti-inflammatory actions is inhibition of PGE2 production.

Cultured equine synoviocytes synthesize large amounts of PGE2 in vitro when treated with lipopolysaccharide (LPS). Using the in vitro system of that study, we investigated the effects of various concentrations of hyaluronan and PSGAG on PGE2 production by cultured equine synoviocytes. In light of claims by pharmaceutical companies of benefit of 1 hyaluronan preparation, compared with other preparations, it was of interest to evaluate a therapeutic Streptococcus zooepidemicus-derived hyaluronan preparation and a generic laboratory-grade S zooepidemicus-derived preparation. Furthermore, claims have been made of greater therapeutic effectiveness of bacterial-derived hyaluronan, compared with rooster comb-derived hyaluronan; thus, we believed it pertinent to evaluate a preparation of hyaluronan from this latter source.

The purpose of the study reported here was to investigate possible anti-inflammatory properties of hyaluronan and PSGAG, which are used in the treatment of equine joint disease. Furthermore, the study was intended to reveal information about their mechanism of action, which remains poorly defined.

Materials and Methods

Tissue specimens—Synovial membrane was obtained from the cranial and palmar joint recesses of metacarpophalangeal (fetlock) joints of euthanatized horses. Each experiment (1 therapeutic agent/experiment) used pooled tissue specimens obtained from both metacarpophalangeal joints of the same horse. Inter-individual variation was avoided as much as possible; tissue was obtained from horses < 10 years old (as determined on the basis of examination of dentition). Synovium was only used when examination of the joints, ligaments, and tendons in the distal aspect of each limb did not reveal macroscopic signs of acute or chronic musculoskeletal injury.
the following treatment conditions: unstimulated (medium only), 10 μg of LPS/ml, 10 μg of LPS/ml and 10^-6 M indomethacin, 10 μg of LPS/ml and various concentrations of PSGAG (2, 200, 2,000, 10,000 and 20,000 μg/ml), PSGAG only (20,000 μg/ml), and 10 μg of LPS/ml and a volume of PBS solution equivalent to that of 20,000 μg of PSGAG/ml.

Prostaglandin E2 assay—Radioimmunoassay for determination of PGE2 concentration was conducted in accordance with the general methods described by Salmon, using commercially available antisera raised against PGE2-bovine serum albumin and tritiated PGE2 tracer. Absolute PGE2 concentrations of specimens were calculated by comparison with PGE2 standards of known concentration. Specimens were diluted to ensure that values could be extrapolated from the standard curve and were subsequently corrected for the original dilution. Upper and lower limits of detection of the assay were 10 ng/ml and 50 pg/ml, respectively.

Statistical analysis—Statistical analysis was conducted, using a proprietary statistical software program. For each therapeutic agent, results represented the data of 1 experiment, with 4 replicates in each treatment group (i.e., n = 4). Values were expressed as mean ± SEM. Significant differences in PGE2 synthesis in response to various treatments were determined by use of one-way ANOVA followed by use of the Dunnett test. Significance was assigned at P < 0.05.

Results

Commercially available hyaluronan preparation—Synoviocytes incubated with 10 μg of LPS/ml produced concentrations of PGE2 significantly (P < 0.001) higher than the basal concentrations of unstimulated cells. The LPS-indomethacin positive-control treatment caused a significantly (P < 0.001) reduced concentration of PGE2 synthesis, compared to the unstimulated synoviocytes (Dunnett test, 2-tailed). Values differ significantly (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) from those for unstimulated synoviocytes (Dunnett test, 2-tailed). Values differ significantly (o = P < 0.05, oo = P < 0.01, ooo = P < 0.001) from those for synoviocytes incubated with LPS (Dunnett test, 2-tailed). PBS = Phosphate-buffered saline solution.
with synoviocytes incubated with 10 µg of LPS/ml. Unstimulated synoviocytes incubated with the commercially available preparation at concentrations of 1,000 and 2,000 µg/ml caused decreases in PGE2 synthesis of 21 and 61%, respectively, but these values were not significantly different (Fig 1). The LPS-stimulated cultures incubated with PBS solution equivalent in volume to 1,000 and 2,000 µg of hyaluronan/ml had significantly (P < 0.001) increased PGE2 synthesis, compared with values for unstimulated cells. The LPS-stimulated cells treated with hyaluronan (20, 200, 500 and 1,000 µg/ml) did not have significant changes in the enhanced PGE2 synthesis, compared with values for LPS-stimulated cells not treated with hyaluronan (Table 1). The highest concentration of the commercially available hyaluronan preparation (2,000 µg/ml) caused a significant (P < 0.001) decrease in PGE2 production, compared with PGE2 concentrations for cells incubated with LPS alone.

Streptococcus zooepidemicus hyaluronan preparation—Incubation of synoviocytes with LPS significantly (P < 0.001) increased PGE2 concentrations, compared with values for basal concentrations of unstimulated cells. The LPS-indomethacin positive-control treatment significantly (P < 0.001) reduced PGE2 synthesis, compared with values for LPS alone. Synoviocytes incubated with hyaluronan (1,000 and 2,000 µg/ml) had slight, but not significant, decreases in PGE2 synthesis of 8 and 11%, respectively. Incubation of LPS-stimulated cultures with PBS solution equivalent in volume to 1,000 and 2,000 µg of hyaluronan/ml significantly (P < 0.001) increased PGE2 synthesis, compared with values for unstimulated cells; value for the cells incubated with LPS-PBS solution was similar to that of cells treated with LPS alone (Fig 2). Similar to the commercially available hyaluronan preparation, the S. zooepidemicus hyaluronan preparation at low concentrations did not cause significant effects on LPS-enhanced PGE2 synthesis (Table 1). Similar to the commercially available hyaluronan preparation, however, the highest concentration of S. zooepidemicus hyaluronan preparation (2,000 µg/ml) produced a significant (P = 0.007) reduction (56%) in PGE2 synthesis, compared with that for cells incubated with LPS alone.

Rooster-comb hyaluronan preparation—Unstimulated synoviocytes had a higher basal PGE2 synthesis than unstimulated synoviocytes in the other 2 experiments involving the use of hyaluronan. Incubation with LPS produced a comparatively weaker, nonsignificant increase in PGE2 concentration, although mean absolute PGE2 concentration (724 ng/ml) was similar to that produced by use of LPS in the other 2 experiments involving hyaluronan (Fig 3). Incubation of LPS-stimulated cultures with PBS solution equivalent in volume to hyaluronan (1,000 and 2,000 µg/ml) enhanced PGE2 synthesis similar to that of cells treated with LPS alone. In contrast to the generic S. zooepidemicus and commercially available hyaluronan preparation, rooster comb hyaluronan (1,000 µg/ml) enhanced synthesis of PGE2 (increase of 83%) in unstimulated cells, which was not

Table 1—Effect of various concentrations of 3 hyaluronan preparations on the percentage change in prostaglandin E2 synthesis by lipopolysaccharide (LPS)-stimulated synoviocytes obtained from the metacarpophalangeal joints of clinically normal horses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Commercial</th>
<th>Streptococcus zooepidemicus</th>
<th>Rooster comb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
<td>LPS+Hyaluronan (20)</td>
<td>LPS + Hyaluronan (200)</td>
</tr>
<tr>
<td>LPS</td>
<td>NA</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>LPS+HA (20)</td>
<td>NA</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>LPS+HA (200)</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>LPS+HA (500)</td>
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<td>8</td>
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<tr>
<td>LPS+HA (1,000)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>LPS+HA (2,000)</td>
<td>65</td>
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Positive values reflect increased PGE2 synthesis, negative values reflect decreased PGE2 synthesis, compared with values for synoviocytes incubated with LPS alone.

Values are significantly (a, P < 0.01; b, P < 0.001) different from those for synoviocytes incubated in LPS alone (Dunnett test, 2-tailed).

NA = Not applicable.
Incubation of synoviocytes with LPS caused PGE2 synthesis by cultured human synoviocytes; macrophage-like synovial cells cultured from synovium obtained from osteoarthritic humans had a decrease in interleukin-1α-induced PGE2 production when treated with hyaluronan at concentrations of 0.5 and 1 mg/ml. This decrease in PGE2 synthesis also was dependent on hyaluronan molecular weight. Lapine chondrocytes stimulated with interleukin-1 similarly had a decrease in PGE2 synthesis when treated with hyaluronan at similar concentrations to those used on human cells; in that study, hyaluronan had a concentration-dependent (0.5, 1, and 2 mg/ml) and molecular weight-dependent (0.5, 1, and 2 kd) inhibition of PGE2 synthesis. The exact molecular weight of each hyaluronan preparation used in the study reported here is not known. However, the commercially available hyaluronan preparation used in our study is of lower viscosity than other commercially available hyaluronan preparations licensed for intra-articular administration.

Analysis of the data in the study reported here failed to confirm the findings of other investigators. None of the 3 hyaluronan preparations, at concentrations up to 1,000 µg/ml, exerted inhibitory actions on LPS-induced PGE2 synthesis. However, at the highest concentration (2,000 µg/ml), 2 of the 3 preparations (commercially available preparation and S. zooepidemicus preparation) significantly inhibited but, unlike indomethacin, did not abolish PGE2 production. The reason for the failure of the rooster comb hyaluronan preparation to inhibit PGE2 synthesis at each concentration is not known. It may have been attributable to a low molecular weight of that preparation. Indeed, low concentrations of this preparation failed to inhibit and actually increased PGE2 synthesis beyond that caused by LPS alone.

A possible explanation for the increased PGE2 concentrations measured in cultures treated with rooster comb hyaluronan is that there may be pro-inflammatory contaminants in the preparation, as has been suggested elsewhere. On the basis of this supposition, it could be suggested that the enhanced PGE2 synthesis obtained in our study may have been countered only by higher concentrations of hyaluronan (1,000 µg/ml) offsetting the PGE2-stimulatory effect of potential pro-inflammatory contaminants. However, it would appear that enhanced PGE2 synthesis was not an artifact resulting from contaminated PBS solvent, because the same stock of PBS solution was used throughout the study. Furthermore, LPS-stimulated cells incubated with a volume of PBS solution equivalent to that for hyaluronan concentrations of 1,000 and 2,000 µg/ml had similar PGE2 synthesis to those cells stimulated with LPS alone.

The reduction in PGE2 production in response to 2 hyaluronan preparations (commercially available and S. zooepidemicus preparations) at the highest concentrations used (2,000 µg/ml) as a result of mere dilution of the culture medium to 80% of its original concentration can be discounted. In control cells treated with LPS in medium diluted with a volume of PBS solution equivalent to that for hyaluronan at 1,000 and 2,000 µg/ml, PGE2 concentrations were similar to those obtained in LPS-stimulated cultures maintained in full medium.

Using unstimulated hamster kidney cells, Dietmar...
reported that PSGAG at concentrations of 0.03, 0.1, 0.3, and 1% reduced PGE2 concentrations in culture supernatant in a concentration-dependent manner at 16 hours (44 to 52%), and 48 hours (33 to 67%) after initiation of incubation. The PSGAG used in that study is a preparation licensed for use in humans; that preparation, similar to the preparation licensed for use in horses, contains 250 mg of PSGAG/ml. Conversion of units of concentration used in our study (µg/ml) to those used by Dietmar (percentage) indicates that reductions in LPS-induced PGE2 synthesis in cultures of equine synoviocytes were obtained at PSGAG concentrations of 0.02, 0.2, 1, and 2%, similar to those used with hamster kidney cells. The lower concentrations used in the study reported here (ie, 0.002 and 0.0002%) did not inhibit LPS-induced PGE2 synthesis.

We were unable to account for the increase above baseline values of PGE2 synthesis by LPS-stimulated cells treated with 20 µg of PSGAG/ml. Results of experiments had little intra-experiment variation, and conditions were standardized with regard to number of cells and population. Therefore, it is unlikely that these potentially variable factors accounted for the increase in PGE2 concentration. Thus, the implication is that the increase in this treatment group may have been the result of a possible synergistic effect of LPS and PSGAG on PGE2 synthesis at that concentration of the compound. However, this is unlikely in view of the remainder of the data.

Similar to our observations of decreased PGE2 synthesis in response to hyaluronan, decreased PGE2 concentrations were obtained in cultures treated with concentrations of PSGAG similar to those estimated to be obtainable by intra-articular injection, but not at concentrations that can be achieved after IM administration.

Synoviocytes for each experiment were cultured from tissue of specific horses, which varied among experiments. All other experimental conditions were standardized, and batches of reagents and equipment were common to all experiments. Therefore, it is likely that the differences in absolute baseline and LPS-stimulated PGE2 concentrations measured in each experiment resulted from variation among horses, despite efforts to standardize selection of synovium from nonarthritic joints in horses of similar age.

In the study reported here, we measured reductions in the concentration of PGE2 in culture medium obtained from monolayer cultures of equine synovial cells. However, these experiments did not address the exact mechanism of action of hyaluronan and PSGAG at the cellular and molecular levels. Exposure of many cell types to LPS results in induction of COX-2, with consequent synthesis of PGE2 by that inducible isoform of cyclooxygenase. Mechanisms of action of NSAID and corticosteroids in inhibiting inflammatory eicosanoid synthesis are established (inhibition of the actions of COX-2 and phospholipase A2 respectively, and, in the case of corticosteroids, inhibition of induction of the COX-2 isoenzyme as well). Given the dissimilarity in molecular structure of hyaluronan and PSGAG to corticosteroids and NSAID, it may be considered unlikely that hyaluronan and PSGAG exert their PGE2 concentration-lowering effects through inhibition of these enzymes. The possibility that hyaluronan inhibits the release of PGE2 into culture medium, rather than inhibiting the synthesis of PGE2, is worthy of consideration. Some investigators have suggested that the diverse actions of hyaluronan on cellular metabolism may be a consequence of its high viscosity in solution; it has been suggested that hyaluronan could physically retard the release of PGE2 from cells, as opposed to inhibiting synthesis.11,17,21 Similarly, it has been suggested that hyaluronan may prevent mobilization of eicosanoid precursors. Release of 14C-labeled arachidonic acid from human synovial fibroblasts in response to bradykinin and calcium ionophore is inhibited by hyaluronan in both a molecular weight- and concentration-dependent manner.24 However, authors of that study concluded that inhibition of arachidonic acid release was not associated with viscosity, because methylcellulose, a liquid similarly viscous to hyaluronan, did not inhibit arachidonic acid release.

Binding interactions of polysaccharide preparations, such as pentosan polysulfate and hyaluronan, with cell membrane molecules have received attention. Thrombospondin has been suggested as a receptor through which pentosan polysulfate may act as a matrix metalloproteinase inhibitor.27 Cellular receptors for hyaluronan have been identified, and it has been suggested that adhesion and migration of cells may be modulated through these hyaluronan receptors.26-29 Also, the capacity of hyaluronan to form complexes with phospholipids has been described recently, with the suggestion that administration of hyaluronan into the joint space may provide a source of binding sites for phospholipids and their metabolites (found in appreciable amounts in synovial fluid and synovial tissues of arthritic joints), which may stimulate chondrocyte catabolism. Consequently, hyaluronan may exert a chondroprotective effect by binding these pro-inflammatory (or pro-chondrodestructive) molecules. The observation in the study reported here of reduced PGE2 synthesis after incubation with hyaluronan may be explained by the binding capacity of hyaluronan, on the basis that the production of eicosanoids such as PGE2 results from metabolism of cell membrane phospholipids. Hyaluronan may bind these phospholipids in stable complexes, rendering them less susceptible to breakdown. This hypothesis has not been investigated.

Using a model of osteoarthritis in which a carpal chip was created surgically, Kawcak et al1 measured PGE2 and protein concentrations in synovial fluid of horses treated by IV administration of hyaluronan. Compared with concentrations for untreated control horses, synovial fluid concentrations of PGE2 and protein were decreased in hyaluronan-treated joints. Furthermore, histologic examination of the synovial membranes of surgically explored joints revealed decreased synovial vascularity and cellular infiltration in horses treated by use of IV administration of hyaluronan, compared with surgically explored joints of untreated control horses.1 The authors of that study concluded that IV administration of hyaluronan had a positive effect in ameliorating inflammatory synovitis, although the mechanism of action of hyaluronan remains unknown. The data from our in vitro study
warrant discussion because of results of the aforementioned in vivo study. Our calculations of the plasma and synovial fluid concentrations of hyaluronan that may theoretically be obtained following IV injection suggest that the decreased synovial fluid concentrations of PGE$_2$ observed by Kawcak et al$^2$ were obtained in response to concentrations of hyaluronan far less than those (1,000 to 2,000 µg/ml) required to decrease PGE$_2$ concentrations in culture medium in our experiments. Although equine synovial fibroblasts are a potential source of PGE$_2$, it is likely that leukocytes infiltrating the synovial membrane in horses with synovitis are another important source of PGE$_2$ and other pro-inflammatory mediators. Therefore, it may be that the decreased synovial fluid concentrations of PGE$_2$ reported by Kawcak et al$^2$ are a consequence of hyaluronan's effects on leukocytes that infiltrated the synovium. On the basis of reported inhibitory effects of hyaluronan on leukocyte migration$^{12,13}$ and the identification of hyaluronan receptors on leukocytes,$^{34,36}$ it may be that the anti-inflammatory effects of hyaluronan in that in vivo experimental model of equine joint disease reside in the drug's actions on leukocytes as well as on the synovial membrane. In both instances, it would be necessary to assume that hyaluronan can exert its actions without penetrating the membrane, because this is unlikely, given its high molecular weight.

References


