Evaluation of hyaluronidase activity in equine and bovine sera and equine synovial fluid samples by use of enzyme zymography

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Objective—To investigate the activities of hyaluronidases in equine sera and synovial fluid samples and sera from fetal and adult bovids and evaluate the extent to which the degradation of hyaluronan is influenced by chondrocytes.

Sample Population—Commercial and noncommercial samples of equine (n = 6) and bovine (6) sera and 16 synovial fluid samples from horses.

Procedure—Hyaluronidase activities in sera and synovial fluid samples were assessed via enzyme zymography (performed at pH 4, 5, 6, or 7). Chondrocytes were isolated from equine cartilage and cultured with or without hyaluronan (1 mg/mL); the degradation of hyaluronan was assessed via agarose gel electrophoresis.

Results—Hyaluronidase activity was detected in equine sera and synovial fluid samples at pH 4, but not at pH 7, and in bovine sera at both pH values. In all samples at pH 4, a major band of activity (molecular weight, approx 60 kd) and some additional higher molecular weight bands were detected; high- and low-molecular-weight activities were detected in bovine sera at pH 7. Hyaluronan in tissue culture medium with or without fetal calf serum was degraded in the presence, but not the absence, of equine chondrocytes.

Conclusions and Clinical Relevance—Hyaluronidase activity was detected in equine sera and synovial fluid at pH 4 and in bovine sera at pH 4 and 7. Primary chondrocytes in monolayer culture can degrade exogenous hyaluronan. Modulating native hyaluronidase activity may offer a new approach to improve the quantity and quality of hyaluronan in articular joints. (*Am J Vet Res* 2005;66:984–990)

Hyaluronan is a ubiquitously expressed, unsulfated glycosaminoglycan comprised of alternating N-acetyl glucosamine and glucuronic acid.[1] It was originally discovered in 1934 in samples of vitreous humor,[2] but determination of the diverse biological activities of this molecule has only recently begun. Hyaluronan varies considerably in chain length and has important natural and therapeutic roles in articular joint health.[3-5] Hyaluronan is a major component of synovial fluid[6] and is responsible for the boundary lubrication, steric hindrance, and cushioning properties of this fluid. In the cartilage matrix, hyaluronan functions as the backbone for aggrecan.[7] Hyaluronan was originally used in the early 1970s as a preventative and therapeutic treatment for degenerative joint conditions in horses after it was discovered that hyaluronan was the component of synovial fluid responsible for its viscosity and that its concentration in synovial fluid decreased with age.[8] Extensive veterinary use led to the suggestion that hyaluronan could be used to treat arthritis in humans, and hyaluronan preparations are now approved for clinical use to reduce the clinical signs of osteoarthritis not only in equine patients[3,9] but also in humans.[10-12] Intra-articular administration of hyaluronan is safe and effective in the relief of pain associated with arthritis in humans.[13] As well as lubricating and protecting articular surfaces and exerting an anti-inflammatory effect, hyaluronan decreases nerve impulses and sensitivity associated with arthritis.[14]

Hyaluronan directly activates cells via specific cell-surface receptors, and the primary receptor for hyaluronan is termed CD44.[15] The interaction between hyaluronan and CD44 is important in chondrogenesis and chondrocyte-matrix interactions during development[16]; a pericellular hyaluronan coat is involved in cell-matrix adhesion and the proliferation and differentiation of mature chondrocytes.[17,18] Hyaluronan concentration and chain length undergo rapid changes in response to injury and disease. In newts, upregulation of the production of hyaluronan occurs in association with the active cell proliferation stage of forelimb regrowth following amputation.[19] Hyaluronan modulates cancer progression[20,21] and functions in injury repair processes in many tissues including lung,[22] cartilage,[23] skin,[24] and blood vessels.[25]

The turnover of hyaluronan is controlled by 2 classes of enzymes: hyaluronan synthases that direct hyaluronan synthesis[26] and hyaluronidases that regulate hyaluronan breakdown.[27,28] In addition, the enzymatic activity of hyaluronidases can be suppressed by the presence of inhibitors of hyaluronidase.[29] The hyaluronan synthase family contains 3 genes that code for enzymes that regulate hyaluronan production,[26,30,31] and there are 6 genes that code for hyaluronidase enzymes in the human and mouse genome.[32] One hypothesis is that some hyaluronidases function lysosomally because most of the known hyaluronidases act optimally at or near pH 4, which is consistent with the acidic environment within lysosomes.[33,34] Hyaluronidases have been found in semen and human rheumatoid synovial fluid as well as in human and mouse sera.[35,36] The sera of several other species, including horses and cattle, have been evaluated previously, but no hyaluronidase detection was reported.[37]

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Hyaluronidases break down hyaluronan into short-chain sugars, typically tetrasaccharides. A decrease in the molecular size of hyaluronan is detected in the synovial fluid of horses with arthritis, and hyaluronan size has been proposed as a marker of joint disease.[6] It has also been proposed that hyaluronan degradation may play a role in **interleukin (IL)**-1–induced resorption of the cartilage matrix and subsequent loss of articular cartilage[38]; furthermore, hyaluronan degradation may occur as a result of CD44-dependent uptake[39] prior to lysosomal degradation.[33] Hyaluronan oligosaccharides as well as long-chain hyaluronan molecules are very biologically active, and the functional effects of the fragments often differ from those of intact molecules. Small fragments of hyaluronan stimulate angiogenesis in chick embryos, whereas longer chains of hyaluronan inhibit that process.[40] The addition of short fragments of hyaluronan to human monocyte–derived dendritic cells resulted in cell maturation as well as the production of the cytokines IL-1β, tumor necrosis factor-α, and IL-12.[41] Soluble hyaluronan fragments stimulate the release of the inflammatory cytokines IL-1, tumor necrosis factor-α, and insulin-like growth factor-1 from macrophages.[42] Compared with findings in synovial fluid from healthy joints, a decrease in synovial fluid hyaluronan concentration is detected in human,[43] canine,[44] and equine[45-47] joints with osteoarthritis.

Evidently, hyaluronan is an important mediator in equine synovial fluid and joint cells, and maintenance of high-quality hyaluronan within joints is beneficial. Because hyaluronidases would be capable of influencing both the quality of cell-secreted hyaluronan and the efficacy of hyaluronan treatments, the purpose of the study reported here was to investigate the presence and activation of hyaluronidases in samples of equine sera and synovial fluid and bovine sera. In addition, the presence of hyaluronidases in fetal and adult bovine sera was assessed because these sera are used extensively in cell-culture experiments for investigation of mechanisms of cartilage degradation and repair.

Materials and Methods

Samples—Noncommercial equine serum samples from adult university-owned healthy horses housed at the Center for Equine Health at the University of California, Davis, were used in this study. The horses were of various breeds (age range, 10 to 25 years). Whole blood samples were taken from the jugular vein and placed immediately into silicone-coated evacuated tubes with no additives. Blood was allowed to clot at room temperature (19°C) for several hours, and serum was removed. To ensure that hyaluronidase activity was not a result of sample preparation, commercial equine serum[a] was used as a control sample.

Equine synovial fluid samples were obtained from Standardbred racehorses in active training at a local racetrack facility. Samples were collected from metacarpophalangeal, middle carpal, and tarsocrural joints as a by-product of therapeutic procedures and were collected and stored at the same time those horses were treated. One synovial fluid sample was collected from each of 16 horses. Samples were frozen at –20°C in silicone-coated evacuated tubes within 2 hours of collection.

Fresh bovine sera obtained from cattle at the dairy at the University of California, Davis, were prepared in the same manner as equine sera. Commercial **fetal calf serum (FCS)**[b,c] and adult bovine sera[b] were analyzed for comparison with the freshly collected sera.

All samples were collected by use of procedures approved by the Animal Care and Use Committee of the University of California, Davis.

Heat inactivation—In some experiments, the commercial bovine sera were heat attenuated at 60°C for 1 hour, 2 hours, or 4 hours to determine whether this procedure, commonly used to denature proteins and enzymes, would inactivate serum hyaluronidases.

Hyaluronidase zymography—The hyaluronidase activity of samples was assessed by use of a previously described zymogram technique.[35-37] This technique involves standard SDS-PAGE methods with hyaluronan present in the gel. Briefly, sodium hyaluronate[d] (170 µg/mL) was added to a 10% SDS-PAGE gel. A 4% stacking gel containing no hyaluronan was used. One microliter of serum or synovial fluid was combined with 1 µL of 2× PAGE-sample buffer (2.5 mL of 0.5M Tris [pH, 6.8], 2.0 mL of 10% SDS, 2.0 mL of glycerol, 0.01 g of bromphenol blue, and 3.2 mL of H_2O) and loaded into the wells. In some instances, 10 µL of serum was loaded onto the gel to identify the most abundant bands. All gels were run with an electrode buffer (pH, 8.3) containing 0.09M Tris, 0.09M boric acid, and 2mM EDTA. Synovial fluid samples from 16 horses and sera from 6 horses and 6 bovids (including the commercial sera) were analyzed. Samples from different animals were never pooled. Electrophoresis was performed in a commercial gel apparatus.[e] Gels were run for approximately 90 minutes on ice (until the dye front approached the bottom of the gel). Gels were then placed in a 0.9% NaCl solution buffered with 3% Triton X-100 and 0.025M Tris and allowed to equilibrate for 1 hour. Gels were subsequently incubated for 4 hours in a 0.2M ammonium acetate-acetic acid buffer solution at pH 4, 5, 6, or 7 and then stained for approximately 18 hours in a 0.005% solution of Stains-All[b] in 50% ethanol. This dye is specific for acidic macromolecules, such as glycosaminoglycans, but is more sensitive than the commonly used alcian blue. After destaining for 2 hours in water, the gels were scanned on a flatbed scanner.

Cell culture—Samples of fresh cartilage were obtained from the metacarpophalangeal joints of horses with no prior history of joint disease that were euthanatized (for reasons not related to this study) at the Veterinary Medical Teaching Hospital of the University of California, Davis. Cartilage samples were collected from 2 joints of 6 horses each. The cartilage was digested by use of a 3-step enzymatic process previously described.[48] Following digestion, primary chondrocytes were plated in monolayers at a high density (3×10^6 cells/mL) in 35-mm tissue culture dishes. Tissue culture medium[c] supplemented with antimicrobials[a] (basal medium) was used for chondrocyte culture. For experiments, the medium was supplemented with hyaluronan[c] (1 mg/mL). All chondrocyte or control sample cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were initially plated with 5% FCS to allow adherence to the plastic tissue culture dishes. After an overnight incubation (approx 18 hours), cells were washed with serum-free medium to remove all residual FCS and incubated for a further 24 hours in serum-free medium. The medium was removed, and hyaluronan (1 mg/mL) was then added to the cultured cells (1 mL/35-mm dish) in either serum-free basal medium, basal medium containing 5% FCS, or basal medium containing a serum-free tissue culture supplement[f] (10 µL/mL). As control samples, cell culture dishes were maintained with hyaluronan-supplemented media, but with no chondrocytes, under identical incubation condi-

tions. Duplicate culture dishes of all treatments were maintained for each experiment in the presence and absence of cells. In the presence of cells, media reached pH 4 after 2 days but remained at neutral pH in the absence of cells. Media were removed after 2, 4, and 6 days of incubation, and the molecular size of hyaluronan present in the media was assessed via agarose gel electrophoresis. Media samples were immediately stored at –20°C until analysis was performed. Experiments were performed on cells derived from 6 horses, and chondrocytes from different preparations were not pooled.

Agarose gel electrophoresis—The comparative size and degradation of in vitro hyaluronan samples were assessed by use of an agarose gel electrophoresis method.[49] All gels were run on a 10 × 6-inch DNA subcell, horizontal gel electrophoresis unit.[e] All hyaluronan-containing samples of media were pretreated with protease[g] to remove proteins that alter the electrophoretic mobility of hyaluronan. This proteinase mixture (32 µg/mL) in 0.15M NaCl was mixed with the hyaluronan samples at a ratio of 1:2, respectively.[49] The samples were then incubated at 37°C for 5 hours. A 0.5% agarose gel was prepared by dissolving 0.9 g of agarose[h] in 176.4 mL of water. When the agarose was completely solubilized, 3.6 mL of 50× **Tris-acetate-EDTA (TAE)** buffer (400mM Tris, 50mM sodium acetate, and 9mM EDTA [pH, 7.9]) was added to the solution. The solution was allowed to cool slightly and then poured into the gel electrophoresis chamber, and a comb was added to form gel-loading wells. The gel was allowed to set for 15 minutes and then was submerged in 1× TAE buffer and allowed to equilibrate for approximately 18 hours. Once the gel was equilibrated, sample tissue culture medium (10 µL) was combined with 2M sucrose in TAE buffer (2 µL) and loaded into the gel wells. Gels were run for 13 hours at 50 V in TAE buffer. Gels were then placed in glass dishes containing 0.005% Stains-All in 50% ethanol and kept in the dark for approximately 12 hours. Gels were destained in water for 2 days in the dark; after destaining, the gels were scanned by use of a flatbed scanner.

Results

Hyaluronidase activity in equine sera—Fresh serum samples from 5 horses as well as 1 commercially prepared equine serum sample[c] were assessed for hyaluronidase activity via gel zymography. When 14 µL of serum/well was loaded onto the gel and incubation was performed at pH 4, a major band of approximately 60 kd was detected as well as a considerable amount of higher molecular weight activity (**Figure 1**). To identify the most abundant bands, gels were loaded with 10 µL of serum/well. At this lower loading concentration, the major band of approximately 60 kd was identified as well as activity at approximately 90 kd. Samples of equine serum had no hyaluronidase activity when gels were loaded with 14 µL of synovial fluid/well and incubated at pH 5 and 6 (data not shown) or pH 7.

Hyaluronidase activity in equine synovial fluid—Sixteen equine synovial fluid samples from Standardbred racehorses were assayed for hyaluronidase activity via gel zymography. Synovial fluid samples were obtained from metacarpophalangeal, middle carpal, and tarsocrural joints. All synovial fluid samples analyzed had 1 band of hyaluronidase activity when gels were incubated at pH 4, which correlated to a protein of approximately

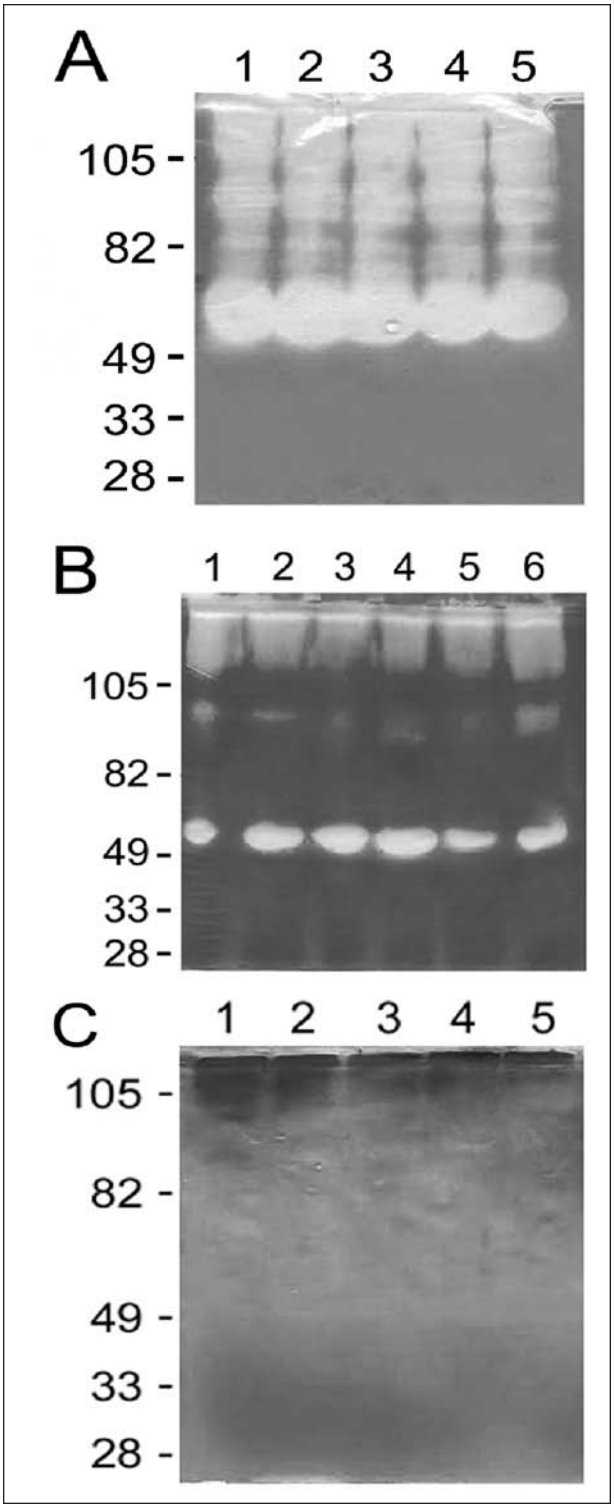

Figure 1—Results of hyaluronan zymography to assess the hyaluronidase activity in samples of serum obtained from 5 horses. Lane numbers are displayed at the top of each gel. A—Lanes 1 to 5 were each loaded with a serum sample (14 µL/well) from one of the 5 horses, and zymography was performed at pH 4. B—Lanes 1 to 5 contained the same stock serum samples as used in panel A but were loaded at 10 µL/well, and lane 6 contained commercial equine serum. Zymography was performed at pH 4. C—Lanes 1 to 5 contained the same stock serum samples as used in panel A but were loaded at 14 µL/well. Zymography was performed at pH 7. Numerals on the left represent molecular weight markers in kilodaltons.

60 kd. Four of the 5 samples had an additional band of enzyme activity that corresponded to a protein of approximately 90 kd (**Figure 2**). One of the samples had sanguinous contamination and an additional band of activity that corresponded to a protein of approximately 28 kd. No hyaluronidase activity was detected in synovial fluid samples incubated at pH 5 and 6 (data not shown) or pH 7. Comparison of equine serum and synovial fluid samples side by side on the same gel revealed that the major hyaluronidase activity in synovial fluid was identical in size to the band of activity in serum (**Figure 3**). However, this comparison revealed that serum contained both a greater quantity and greater range of activities than synovial fluid.

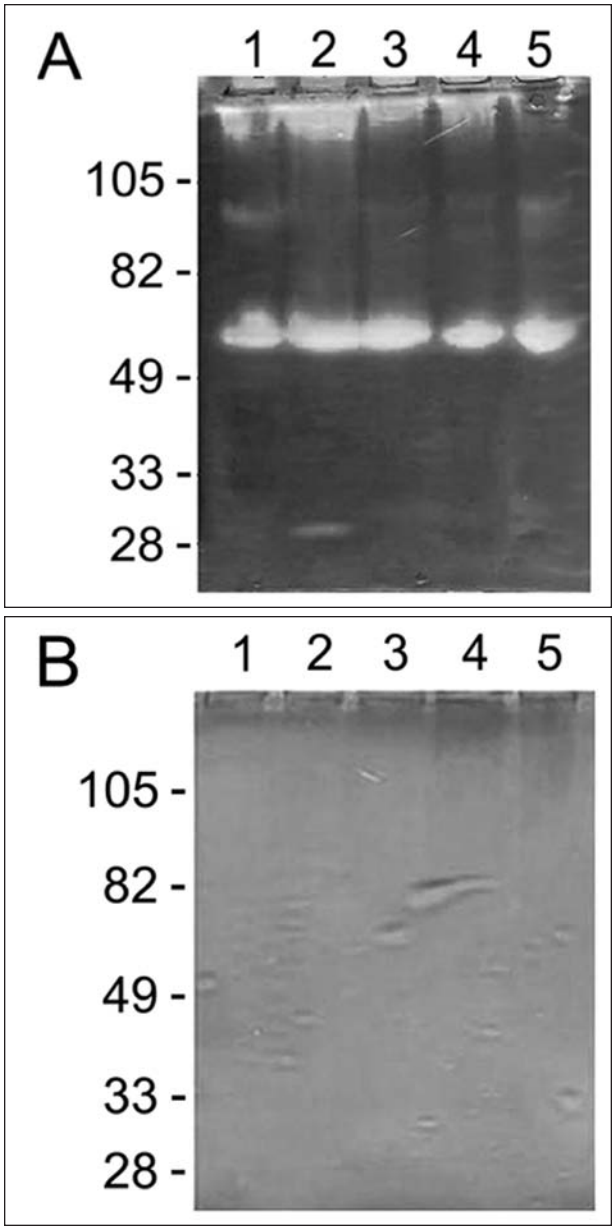

Figure 2—Results of hyaluronan zymography to assess hyaluronidase activity in samples of synovial fluid obtained from 5 horses. Lane numbers are displayed at the top of each gel. A—Results of zymography performed at pH 4. B—Results of zymography performed at pH 7 on identical samples to those used in panel A. In both panels, lane 1 contained metacarpophalangeal joint synovial fluid, lane 2 contained metacarpophalangeal joint synovial fluid with some gross blood contamination, lane 3 contained middle carpal joint synovial fluid, and lanes 4 and 5 contained tarsocrural joint synovial fluid. All samples were loaded at 14 µL of synovial fluid/well. Numerals on the left represent molecular weight markers in kilodaltons.

Hyaluronidase activity in bovine sera—Two different commercially prepared FCS samples,[b,c] a commercially available adult bovine serum sample[c] and fresh bovine sera, from 5 cattle were assessed for hyaluronidase activity via gel zymography. All bovine sera assayed had hyaluronidase activity at pH 4, and there was considerably more activity in these samples than that detected in equine serum samples (**Figure 4**). When loaded at 14 µL of serum/well, there was too much hyaluronidase activity to determine the exact location of major bands when gels were incubated at pH 4 after electrophoresis. At serum concentrations of 10 µg/well in gels incubated at pH 4, the major band of hyaluronidase activity was approximately 60 kd. In gels loaded with 14 µL of serum/well and incubated at pH 7 after electrophoresis, a range of hyaluronidase activity was detected in bovine serum samples. Heat attenuation at 60°C for as long as 4 hours decreased the hyaluronidase activity but did not fully remove it (data not shown).

Hyaluronic acid degradation by chondrocytes—An agarose gel electrophoresis method was used to assess the degradation of hyaluronan in tissue culture media in the presence and absence of equine chondrocytes. Medium containing either a serum-free tissue culture supplement (10 µL/mL), 5% FCS, or no additives was incubated in 35-mm tissue culture plates at 37°C for 6 days in the absence of cells or in the presence of a confluent monolayer of equine chondrocytes for 2, 4, or 6 days (**Figure 5**). In the absence of cells, hyaluronan had a broad size range when incubated in tissue culture medium alone or in the presence of FCS or serum-free tissue culture supplement. In the presence of chondrocytes, hyaluronan was degraded to

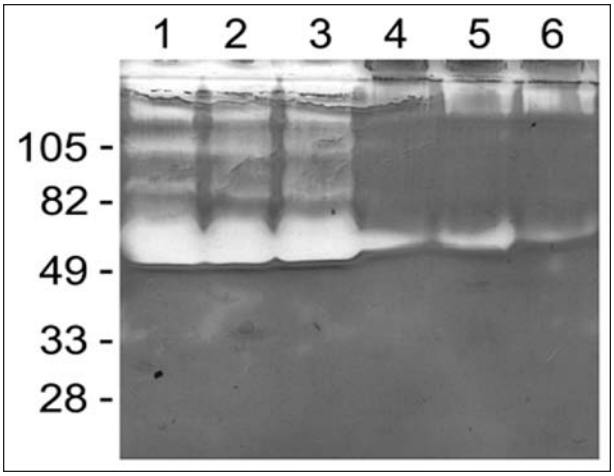

Figure 3—Comparison of hyaluronidase activity in samples of equine serum and synovial fluid assessed hyaluronan zymography at pH 4. Lane numbers are displayed at the top of the gel. Lanes 1 to 3 contained equine sera, lanes 4 and 5 contained samples of metacarpophalangeal joint synovial fluid, and lane 6 contained a sample of tarsocrural joint synovial fluid. Lanes were loaded at 10 µL of sample/well. Numerals on the left represent molecular weight markers in kilodaltons.

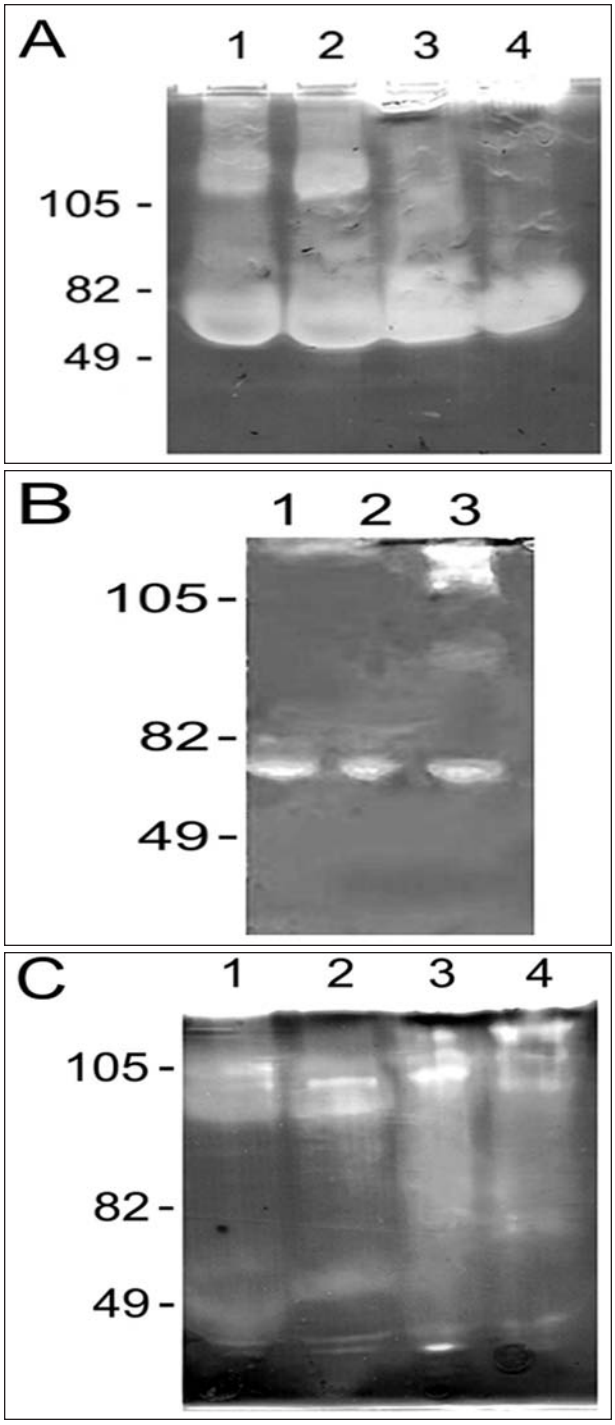

Figure 4—Results of hyaluronan zymography to assess hyaluronidase activity in samples of serum from fetal and adult bovids. Lane numbers are displayed at the top of each gel. A—Results of zymography performed with 14 µL of serum/well at pH 4. Lane 1 contained commercial fetal calf serum (FCS),[c] lane 2 contained commercial FCS,[b] lane 3 contained commercial adult bovine serum,[b] and lane 4 contained noncommercial adult bovine serum. B—Results of zymography performed with 1 µL of serum/well at pH 4 to visualize the major band of hyaluronidase activity. Lane 1 contained commercial FCS,[c] lane 2 contained commercial FCS,[b] and lane 3 contained commercial adult bovine serum. C—Results of zymography performed with 14 µL of serum/well at pH 7. Lane 1 contained commercial FCS,[c] lane 2 contained commercial FCS,[b] lane 3 contained commercial adult bovine serum,[b] and lane 4 contained noncommercial adult bovine serum. Numerals on the left represent molecular weight markers in kilodaltons.

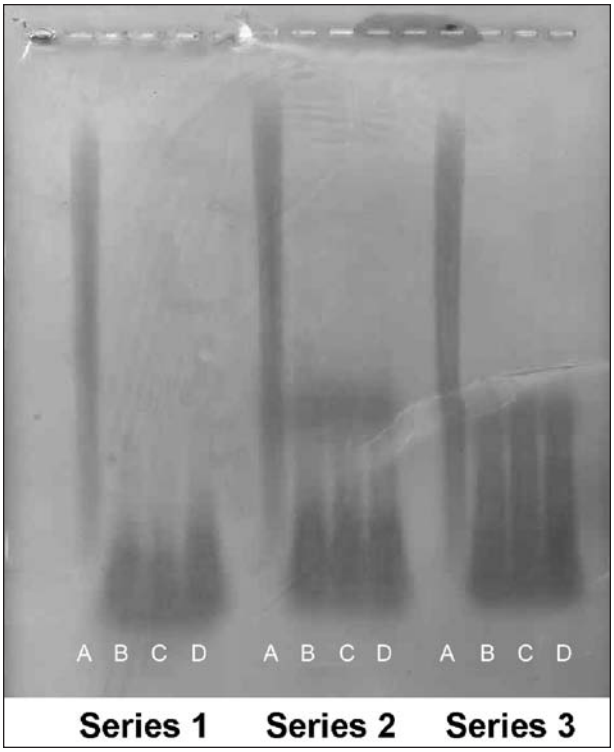

Figure 5—Results of agarose gel electrophoresis to evaluate the degradation of hyaluronan in tissue culture medium. Experiments were performed under 3 conditions: in medium containing hyaluronan (1 mg/µL) and serum-free tissue culture supplement (series 1), in medium containing hyaluronan (1 mg/mL) and 5% FCS (series 2), and in medium containing hyaluronan (1 mg/mL) alone (series 3). In each series, lane A was loaded with medium that had been incubated for 6 days with additives in the absence of chondrocytes, lane B was loaded with medium that had been incubated for 2 days with chondrocytes, lane C was loaded with medium that had been incubated for 4 days with chondrocytes, and lane D was loaded with medium that had been incubated for 6 days with chondrocytes.

polysaccharides of lower molecular weights. This degradation occurred when chondrocytes were incubated in medium alone as well as in media containing FCS or serum-free tissue culture supplement.

Discussion

In a previous study,[37] hyaluronidase activity was not detected in samples of equine or bovine serum. In the study reported here, hyaluronidase activity has been identified in equine and bovine sera as well as in equine synovial fluid; the presence of hyaluronidase activity in equine samples is important with respect to the progression and treatment of osteoarthritis in horses. In addition, the presence of hyaluronidase activity in bovine sera has implications for the interpretation of results of studies investigating hyaluronan activity and degradation in tissue culture systems in which adult or FCS is frequently used as a supplement for cell survival and attachment.

When hyaluronidase activity in hyaluronan-containing zymograms was assessed at pH 4, the major band detected had a molecular weight of approximately 60 kd in all serum and synovial fluid samples analyzed in our study. This is consistent with the presence of Hyal 1, which is the major serum hyaluronidase and is often referred to as plasma hyaluronidase.[50] Activity consistent

with the presence of Hyal 1 has previously been detected in sera from several species including humans, mice, rats, hamsters, and dogs.[37] In addition to this major activity, additional hyaluronidase activities were identified in those species, which is consistent with our observations of multiple bands of hyaluronidase activity in equine and bovine sera. In human sera, hyaluronidase activity (assessed via gel zymography) was approximately 50 times as great as that in human synovial fluid.[35] Similarly, in the present study, considerably more hyaluronidase activity was detected in equine sera than in equine synovial fluid. Synovial fluid is an ultrafiltrate of blood, and our data indicate that the major hyaluronidases present in synovial fluid do not differ in molecular size from those in sera and would be expected to originate from the serum.

In equine sera, hyaluronidase activity was detectable only at acidic pH and no activity was found at neutral pH. However, in bovine sera, some activity was observed at neutral pH. Previous studies[35,50] have revealed that most of the serum hyaluronidases are active only in an acidic environment, but an additional neutral form of the enzyme has been detected in some sera.[35]

There are several commercial hyaluronan products used for the treatment of osteoarthritis in horses.[51] These products vary with respect to the concentration and molecular weight of hyaluronan as well as to viscosity of the preparation and its protein concentration.[51] It is believed that the most beneficial products are of high molecular weight and high viscosity because these would most closely mimic the hyaluronan within joint fluid. The presence of hyaluronidases in equine synovial fluid, if capable of degrading hyaluronan injected in joints for therapeutic purposes, could influence the efficacy of treatment with such products. Therefore, we were interested in determining the extent of degradation of a commercial preparation of hyaluronan in synovial fluid samples when incubated at 37°C. Because the hyaluronidases that were detected in equine synovial fluid were acidic forms, we were also interested in determining how the degradation of hyaluronan would be influenced by the presence of chondrocytes, which generate a local acidic environment as a result of the secretion of lactic acid.[52] Our experiments revealed that chondrocytes were capable of stimulating the degradation of hyaluronan in the presence and absence of FCS. Fetal calf serum added to hyaluronan-containing medium without chondrocytes was not capable of degrading hyaluronan even after 6 days, indicating that the neutral hyaluronidase activity in FCS in hyaluronan zymograms of the present study was not sufficient to degrade hyaluronan in tissue culture medium. The initial hypothesis was that chondrocytes would lower the pH of the medium enough to activate acidic hyaluronidases in the FCS. However, results of our study indicated that the presence of chondrocytes alone, in the absence of FCS and in the absence of a serum-free tissue culture supplement, was sufficient to degrade hyaluronan added to the tissue culture medium at a concentration of 1 mg/mL; this finding suggests that there is a source of enzyme activity from the cells themselves. Chondrocytes obtained from human knee cartilage and from stifle joints of 1- to 2-year-old calves contain hyaluronidase activity,[34] and reverse transcription-polymerase chain reaction analysis of chondrocytes revealed expression of messenger RNAs encoding the Hyal 1, Hyal 2, and Hyal 3 hyaluronidase isoenzymes.[34] Enzyme activity was detected only at pH ≤ 4.5. In a recent study[53] to investigate the activity of cathepsin K on the surface of articular cartilage, it was determined that the surface pH of human arthritic cartilage with severe lesions was as low as pH 4. If this is the case, it would follow that in damaged cartilage, both membrane-bound and secreted hyaluronidases could be active at the articular surface, contrary to our expectation.

In the present study, hyaluronidase activity was detected in equine sera and synovial fluids and in bovine sera and findings indicated that equine chondrocytes are capable of degrading hyaluronan present in tissue culture medium. Recently, endogenous inhibitors of hyaluronidases that are members of the inter-α-trypsin family of enzyme inhibitors have been identified.[54] The manipulation of hyaluronidase concentrations in joints may represent an alternative therapeutic approach to maintaining high concentrations of beneficial hyaluronan in those joints.

a. Equitech-Bio Inc, Kerrville, Tex.
b. Sigma Chemical Co, St Louis, Mo.
c. DME/F12 tissue culture medium, Gibco-BRL, Grand Island, NY.
d. Hyvisc, Anika Therapeutics, San Francisco, Calif.
e. Mini-PROTEAN II gel apparatus, Biorad, Hercules, Calif.
f. ITS+ tissue culture supplement, Collaborative Biomedical Products, Bedford, Mass.
g. Pronase, Calbiochem, La Jolla, Calif.
h. Seakem HGT agarose, Cambrex Corp, East Rutherford, NJ.

References

1. McDonald JA, Camenisch TD. Hyaluronan: genetic insights into the complex biology of a simple polysaccharide. *Glycoconj J* 2002;19:331–339.
2. Meyer KPJ. The polysaccharide of vitreous humor. *J Biol Chem* 1934;107:629–634.
3. Kawcak CE, Frisbie DD, Trotter GW, et al. Effects of intravenous administration of sodium hyaluronate on carpal joints in exercising horses after arthroscopic surgery and osteochondral fragmentation. *Am J Vet Res* 1997;58:1132–1140.
4. Barr AR, Duance VC, Wotton SF, et al. Influence of intra-articular sodium hyaluronate and polysulphated glycosaminoglycans on the biochemical composition of equine articular surface repair tissue. *Equine Vet J* 1994;26:40–42.
5. Listrat V, Ayral X, Patarnello F, et al. Arthroscopic evaluation of potential structure modifying activity of hyaluronan (Hyalgan) in osteoarthritis of the knee. *Osteoarthritis Cartilage* 1997;5:153–160.
6. Tulamo RM, Houttu J, Tupamaki A, et al. Hyaluronate and large molecular weight proteoglycans in synovial fluid from horses with various arthritides. *Am J Vet Res* 1996;57:932–937.
7. Watanabe H, Cheung SC, Itano N, et al. Identification of hyaluronan-binding domains of aggrecan. *J Biol Chem* 1997;272:28057–28065.
8. Butler J, Rydell NW, Balasz, EA. Hyaluronic acid in synovial fluid. VI. Effect of intra-articular injection of hyaluronic acid on the clinical symptoms of arthritis in track horses. *Acta Vet Scand* 1970;11:139–155.
9. Lindholm AC, Swensson U, de Mitri N, et al. Clinical effects of betamethasone and hyaluronan, and of defocalized carbon dioxide laser treatment on traumatic arthritis in the fetlock joints of horses. *J Vet Med A Physiol Pathol Clin Med* 2002;49:189–194.

10. Wang CT, Lin J, Chang CJ, et al. Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A meta-analysis of randomized controlled trials. *J Bone Joint Surg Am* 2004;86:538–545.

11. Conrozier T, Bertin P, Mathieu P, et al. Intra-articular injections of hylan G-F 20 in patients with symptomatic hip osteoarthritis: an open-label, multicentre, pilot study. *Clin Exp Rheumatol* 2003;21:605–610.

12. Kelly MA, Goldberg VM, Healy W, et al. Osteoarthritis and beyond: a consensus on the past, present, and future of hyaluronans in orthopedics. *Orthopedics* 2003;26:1064–1079.

13. Waddell DD, Cefalu CA, Bricker DC. An open-label study of a second course of hylan G-F 20 for the treatment of pain associated with knee osteoarthritis. *Curr Med Res Opin* 2003;19:499–507.

14. Moreland LW. Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action. *Arthritis Res Ther* 2003;5:54–67.

15. Jiang H, Peterson RS, Wang W, et al. Requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells. *J Biol Chem* 2002;277:10531–10538.

16. Knudson CB. Hyaluronan and CD44: strategic players for cell-matrix interactions during chondrogenesis and matrix assembly. *Birth Defects Res C Embryo Today* 2003;69:174–196.

17. Cohen M, Klein E, Geiger B, et al. Organization and adhesive properties of the hyaluronan pericellular coat of chondrocytes and epithelial cells. *Biophys J* 2003;85:1996–2005.

18. Ishida O, Tanaka Y, Morimoto I, et al. Chondrocytes are regulated by cellular adhesion through CD44 and the hyaluronic acid pathway. *J Bone Miner Res* 1997;12:1657–1663.

19. Mescher AL, Munaim SI. Changes in the extracellular matrix and glycosaminoglycan synthesis during the initiation of regeneration in adult newt forelimbs. *Anat Rec* 1986;214:424–431.

20. Liu N, Gao F, Han Z, et al. Hyaluronan synthase 3 overexpression promotes the growth of TSU prostate cancer cells. *Cancer Res* 2001;61:5207–5214.

21. Simpson MA, Wilson CM, McCarthy JB. Inhibition of prostate tumor cell hyaluronan synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. *Am J Pathol* 2002;161:849–857.

22. Turino GM, Cantor JO. Hyaluronan in respiratory injury and repair. *Am J Respir Crit Care Med* 2003;167:1169–1175.

23. Kiani C, Chen L, Wu YJ, et al. Structure and function of aggrecan. *Cell Res* 2002;12:19–32.

24. Rilla K, Lammi MJ, Sironen R, et al. Changed lamellipodial extension, adhesion plaques and migration in epidermal keratinocytes containing constitutively expressed sense and antisense hyaluronan synthase 2 (Has2) genes. *J Cell Sci* 2002;115:3633–3643.

25. Hellstrom M, Mattsson C, Hellstrom S, et al. Hyaluronan is differently located in arteries and veins. An immunohistochemical study in the rat. *Cells Tissues Organs* 2003;173:227–233.

26. Spicer AP, Seldin MF, Olsen AS, et al. Chromosomal localization of the human and mouse hyaluronan synthase genes. *Genomics* 1997;41:493–497.

27. Csoka TB, Frost GI, Stern R. Hyaluronidases in tissue invasion. *Invasion Metastasis* 1997;17:297–311.

28. Kreil G. Hyaluronidase—a group of neglected enzymes. *Protein Sci* 1995;4:1666–1669.

29. Mio K, Stern R. Inhibitors of the hyaluronidases. *Matrix Biol* 2002;21:31–37.

30. Recklies A, White C, Melching L, et al. Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma. *Biochem J* 2001;354:17–24.

31. Spicer AP, Olson JS, McDonald JA. Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem* 1998;273:1923–1932.

32. Csoka AB, Frost GI, Stern R. The six hyaluronidase-like genes in the human and mouse genomes. *Matrix Biol* 2001;20:499–508.

33. Hua Q, Knudson CB, Knudson W. Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. *J Cell Sci* 1993;106:365–375.

34. Flannery CR, Little CB, Hughes CE, et al. Expression and activity of articular cartilage hyaluronidases. *Biochem Biophys Res Commun* 1998;251:824–829.

35. Fiszer-Szafarz B, Litynska A, Zou L. Hyaluronidases: electrophoretic multiple forms in somatic tissues and body fluids. Evidence for conserved hyaluronidase potential N-glycosylation sites in different mammalian species. *J Biochem Biophys Methods* 2000;45:103–116.

36. Meyers SA, Rosenberger AE. A plasma membrane-associated hyaluronidase is localized to the posterior acrosomal region of stallion sperm and is associated with spermatozoal function. *Biol Reprod* 1999;61:444–451.

37. Fiszer-Szafarz B, Szafarz D, Vannier P. Polymorphism of hyaluronidase in serum from man, various mouse strains and other vertebrate species revealed by electrophoresis. *Biol Cell* 1990;68:95–100.

38. Sztrolovics R, Recklies AD, Roughley PJ, et al. Hyaluronate degradation as an alternative mechanism for proteoglycan release from cartilage during interleukin-1beta-stimulated catabolism. *Biochem J* 2002;362:473–479.

39. Culty M, Nguyen HA, Underhill CB. The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. *J Cell Biol* 1992;116:1055–1062.

40. Rooney P, Wang M, Kumar P, et al. Angiogenic oligosaccharides of hyaluronan enhance the production of collagens by endothelial cells. *J Cell Sci* 1993;105:213–218.

41. Termeer CC, Hennies J, Voith U, et al. Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J Immunol* 2000;165:1863–1870.

42. Noble PW, Lake FR, Henson PM, et al. Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. *J Clin Invest* 1993;91:2368–2377.

43. Marshall KW. Intra-articular hyaluronan therapy. *Curr Opin Rheumatol* 2000;12:468–474.

44. Arican M, Carter SD, May C, et al. Hyaluronan in canine arthropathies. *J Comp Pathol* 1994;111:185–195.

45. Gaustad G, Larsen S. Comparison of polysulphated glycosaminoglycan and sodium hyaluronate with placebo in treatment of traumatic arthritis in horses. *Equine Vet J* 1995;27:356–362.

46. Fuller CJ, Barr AR, Sharif M, et al. Cross-sectional comparison of synovial fluid biochemical markers in equine osteoarthritis and the correlation of these markers with articular cartilage damage. *Osteoarthritis Cartilage* 2001;9:49–55.

47. Hilbert BJ, Rowley G, Antonas KN. Hyaluronic acid concentration in synovial fluid from normal and arthritic joints of horses. *Aust Vet J* 1984;61:22–24.

48. Tesch AM, MacDonald MH, Kollias-Baker C, et al. Effects of adenosine on bacterial lipopolysaccharide- and interleukin 1-induced nitric oxide release from equine articular chondrocytes. *Am J Vet Res* 2002;63:204–210.

49. Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Anal Biochem* 1994;219:278–287.

50. Frost GI, Csoka TB, Wong T, et al. Purification, cloning, and expression of human plasma hyaluronidase. *Biochem Biophys Res Commun* 1997;236:10–15.

51. Uden PC, Lavoie LM. Laboratory evaluation of commercial hyaluronate sodium products. *J Equine Vet Sci* 1997; 17:123–125.

52. Lee RB, Urban JP. Functional replacement of oxygen by other oxidants in articular cartilage. *Arthritis Rheum* 2002;46:3190–3200.

53. Konttinen YT, Mandelin J, Li TF, et al. Acidic cysteine endoproteinase cathepsin K in the degeneration of the superficial articular hyaline cartilage in osteoarthritis. *Arthritis Rheum* 2002;46:953–960.

54. Mio K, Stern R. Inhibitors of the hyaluronidases. *Matrix Biol* 2002;21:31–37.