Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes

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Objective—To characterize potential mechanisms of action of glucosamine inhibition of matrix metalloproteinase (MMP) expression and activity in lipopolysaccharide (LPS)-stimulated equine chondrocytes.

Sample Population—Chondrocytes cultured from samples of metacarpophalangeal articular cartilage collected from cadaveric limbs of horses.

Procedure—The effect of glucosamine on MMP activity in conditioned medium from LPS-stimulated cartilage explants was determined by a colorimetric assay with azocoll substrate. Treatments consisted of negative and positive controls, glucose (50mM), and glucosamine (50, 25, 6.25, 3, and 1.5mM). The influence of glucosamine on MMP synthesis was determined in chondrocytes in pellet culture incubated with LPS (20 µg/mL). Concentration of MMP-13 was quantified in spent medium via ELISA; nonspecific MMP activity was determined via azocoll digestion in organomercurial-activated medium. Effects of glucosamine on MMP mRNA concentration in similarly treated chondrocytes were determined by northern blot hybridization with MMP-1, -3, and -13 probes. Statistical analyses were performed with 2-way ANOVA.

Results—Glucosamine had no effect on activated MMP activity but inhibited MMP protein expression, as determined by azocoll digestion (glucosamine, 3 to 50mM) and MMP-13 ELISA (glucosamine, 1.5 to 50mM). Resting mRNA concentrations for MMP-1, -3, and -13 mRNA were significantly lower in cultures exposed to glucosamine at concentrations of 50 and 25mM than those of positive controls.

Conclusions and Clinical Relevance—Glucosamine appears capable of pretranslational, and possibly also translational, regulation of MMP expression; data suggest a potential mechanism of action for chondroprotective effects of this aminomonosaccharide. (*Am J Vet Res* 2003;64:666–671)

Osteoarthritis is a chronic degenerative disease characterized by a loss of structural components from articular cartilage, including collagen type II and proteoglycan.[1-3] Experimental evidence suggests that degradation of the cartilage extracellular matrix is mediated by the **matrix metalloproteinase** (**MMP**) enzyme family; this group includes collagenases 1 and 3 (MMP-1, MMP-13), which primarily degrade the collagen component of the extracellular matrix, and stromelysin 1 (MMP-3), which primarily degrades noncollagenous matrix components and serves to activate latent collagenases.[4-9]

A variety of therapeutic agents have been used for treatment of clinical signs of osteoarthritis. Recently, interest has focused on agents that may arrest cartilage degradation (chondroprotective agents), such as supplements containing glucosamine, which are administered orally. Glucosamine is a 6-carbon amino sugar that is readily absorbed after oral administration, nontoxic, and concentrated in cartilage.[10] In vitro, glucosamine has anabolic effects on cartilage; glucosamine stimulates proteoglycan production in human chondrocytes cultures,[11] prevents repression of β-1,3-glucuronosyltransferase I (a key biosynthetic enzyme in glycosaminoglycan synthesis),[12] and inhibits aggrecanase activity.[13] Additionally, both glucosamine hydrochloride and glucosamine sulfate prevent cartilage degradation induced by **lipolysaccharide** (**LPS**) and recombinant human interleukin-1 in equine cartilage explants.[14,15] Inflammatory mediators, such as nitric oxide and prostaglandins, are inhibited by glucosamine.[12,14,15] Glucosamine also prevents the up-regulation of MMP-3 mRNA expression in interleukin-1 stimulated rat chondrocytes.[12]

The treatment of clinical signs of osteoarthritis with oral administration of glucosamine has been investigated in several clinical studies in humans. A review[16] of 16 randomized controlled clinical trials that involved treatment with glucosamine suggested that this agent is an effective and safe treatment for osteoarthritis. Findings of 4 studies[16] in humans indicate that the efficacy of glucosamine is equal to or greater than that of ibuprofen (a nonsteroidal anti-inflammatory drug). Comparison of the 2 agents revealed that patient pain scores decrease more rapidly during treatment with ibuprofen but that oral administration of glucosamine yields a lower pain score after 8 weeks of treatment.[17] Furthermore, data collected during a 3-year study[18] of humans with osteoarthritis indicate that glucosamine prevented space loss within the knee joint, which suggests that glucosamine does have a chondroprotective effect.

Although glucosamine appears to have chondroprotective properties, further characterization of

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the specific conditions under which these protective effects occur is required. The purpose of the study reported here was to characterize the mechanisms of action involved in glucosamine inhibition of MMP expression and activity in LPS-stimulated equine chondrocytes (used as an in vitro model of osteoarthritis). Our study was intended to test the hypotheses that glucosamine hydrochloride influences the catalytic activity of MMPs in activated chondrocytes, synthesis of MMPs, and resting MMP-1, MMP-3, and MMP-13 mRNA concentrations in LPS-stimulated equine chondrocytes.

Materials and Methods

Tissue sources—Metacarpophalangeal articular cartilage of normal gross appearance was obtained from 4 horses (2 to 8 years of age) that had died or were euthanatized with an overdose of pentobarbital for reasons other than joint disease. Cartilage was dissected from the subchondral bone and incubated at 25°C for 1 hour in physiologic saline (0.9% NaCl) solution containing penicillin (500 U/mL) and streptomycin (500 mg/mL). Chondrocytes were isolated by sequential digestion with pronase and collagenase, as described.[19] After digestion, cells were separated by centrifugation (300 × *g* for 10 minutes), washed, and resuspended in 10 mL of **Dulbecco's minimal essential medium** (**DMEM**) supplemented with 10% **fetal bovine serum** (**FBS**), penicillin (100 U/mL), and streptomycin (100 mg/mL).

Pellet cultures—Cell concentration was determined by use of a hemocytometer; 1×10^6 cells in 2 mL of supplemented DMEM were placed in 15-mL polypropylene centrifuge tubes. After centrifugation (300 × *g* for 5 minutes), pellets were incubated under standard cell culture conditions (ie, at 37°C in 95% relative humidity with 5% carbon dioxide). Medium was renewed every 3 to 4 days. Pellet cultures were deprived of serum for 3 to 5 days prior to the start of an experiment. Medium from treated pellet cultures was collected to determine activity and concentration of MMPs.

Monolayer cultures—After centrifugation and washing, high-density monolayer cultures were established in supplemented DMEM under standard cell culture conditions. Experiments were performed first-passage high-density cultures, which were deprived of serum for 3 to 5 days prior to the start of an experiment. The RNA of treated monolayer chondrocytes was collected to determine relative amount of MMP RNA.

LPS-conditioned medium preparation—To prepare an MMP-rich medium for subsequent MMP activity assays, metacarpophalangeal joint cartilage in 30 mL of supplemented DMEM was placed in 50-mL polypropylene centrifuge tubes. Lipopolysaccharide was added at a concentration of 160 µg/mL, and cartilage was incubated in standard cell culture conditions for 24 hours. The LPS-conditioned medium was stored at –80°C until used in analyses of MMP activity by colorimetric methods.

Effect of glucosamine on MMP protein synthesis—Pellet cultures were placed in 600 µL of fresh DMEM with penicillin (100 U/mL) and streptomycin (100 mg/mL) without FBS. Treatment media contained glucose at a concentration of 50mM or glucosamine hydrochloride at a concentration of 50, 25, 6.25, 3, or 1.5mM. Lipopolysaccharide was added to a final concentration of 20 µg/mL to all pellet cultures, except those used as negative controls. Positive control samples contained LPS but no glucose or glucosamine hydrochloride. Pellet cultures were incubated in 15-mL polypropylene centrifuge tubes with the caps loosened under standard cell culture conditions for 24 hours. Media was analyzed for MMP activity by azocoll digestion and MMP-13 concentration by ELISA.

Quantification of preformed MMP activity and MMP synthesis—Azocoll[a] (200 mg) was placed in 10 mL of reaction buffer (Tris buffer [pH, 7.4] or MES buffer [2-N-morpholinoethanesulphonic acid; pH, 5.0]), vortexed briefly, and incubated at 25°C for 2 to 3 hours before being filtered and resuspended.[20] The azocoll suspension was filtered through No. 1 filter paper and resuspended with vigorous agitation in 10 mL of reaction buffer. Test media (200 µL) were placed into centrifuge tubes with 25 µL of 4-**aminophenylmercuric acetate (APMA)** solution (12mM) and 100 µL azocoll suspension. Twenty-five microliters of 1,10-phenanthroline solution (120mM) was added to negative control tubes as an inhibitor of MMP activity. Reaction buffer was added to a final volume of 600 µL; buffer used in treatment tubes contained glucose at a concentration of 50mM or glucosamine at a concentration of 50, 25, 6.25, 3, or 1.5mM. Centrifuge tubes were capped tightly and incubated at 37°C for 72 hours. Reaction tubes were centrifuged (12,000 × *g* for 3 minutes), and absorbance values were obtained at 520 nm. Reactions were performed at pH 7.4 to detect total MMP activity[21,22] and at pH 5.0 to detect stromelysin activity.[22]

To quantify MMP synthesis, test media (200 µL) were placed into centrifuge tubes with 25 µL APMA solution (12mM) and 100 µL azocoll suspension. Reaction buffer (Tris buffer; pH, 7.4) was added to a final volume of 600 µL. Centrifuge tubes were capped tightly and incubated at 37°C for 72 hours. Reaction tubes were centrifuged (12,000 × *g* for 3 minutes), and absorbance values were obtained at 520 nm. Reactions were performed at pH 7.4 to detect total MMP activity.[21,22]

MMP-13 ELISA—Stored media from pellet cultures were assayed for proMMP-13 and MMP-13 by means of a commercially available kit[b] (in accordance with manufacturer's instructions). Briefly, 100 µL of prepared standard and test media (diluted 1:4) were incubated at 25°C for 2 hours in assay wells coated with antiMMP-13 antibody. Wells were washed with the supplied phosphate buffer and incubated at 25°C for 1 hour with 100 µL of antiMMP-13 peroxidase conjugate. Wells were washed and incubated at 25°C for precisely 30 minutes with 100 µL of 3,3'5,5'-tetramethylbenzidine-hydrogen peroxide in 20% (w/v) dimethylformamide. The reaction was stopped by the addition of 1.0M sulfuric acid, and optical density was measured at 450 nm.

Extraction of monolayer RNA—Total RNA was extracted by means of a commercial extraction preparation[c] in accordance with manufacturer's instructions. Briefly, 1 mL of this agent was added to each monolayer well and incubated at 25°C for 5 minutes. The solution was transferred to centrifuge tubes, and 200 µL of chloroform was added. The tubes were agitated vigorously and incubated at 25°C for 10 minutes. After centrifugation (12,000 × *g* for 15 minutes at 4°C), the aqueous phase was removed, and the RNA was precipitated with isopropanol. After centrifugation (12,000 × *g* for 15 minutes at 4°C), the pellets were washed with 75% ethanol solution. Pellets were resuspended in sterile water with 0.1% diethylpyrocarbonate.

Northern blot hybridization—Northern blot hybridization was conducted with a commercially available kit,[d] in accordance with manufacturer's instructions. Briefly, total chondrocyte RNA was resolved on 1.2% agarose-formaldehyde gels (applying 3 µg of RNA). After transfer to nylon membranes (at 25°C for 16 hours), RNA was cross-linked to

the membranes by exposure to UV light. After prehybridization for 1 hour at 50°C, the membranes were hybridized for 16 hours at 50°C in a probe preparation[23] (200 ng of labeled probe/mL). Serial posthybridization washes were performed at 25 and 68°C, with decreasing concentrations of sodium-citrate-based buffer, followed by washing in maleate buffer at 25°C. Detection was accomplished by means of a chemiluminescent method with digoxigenin-11-uridyl triphosphate[e] and CSPD[f] (3,4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan-4-yl}phenyl phosphate) as a substrate for alkaline phosphatase conjugated to digoxigenin antibody Fab fragments. Chemiluminescence was detected by exposure of the membranes to scientific imaging film[g] for 10 minutes at 25°C. Developed film was scanned,[h] and relative MMP band intensities were analyzed by means of computer software.[i] Chemiluminescent bands were standardized against methylene-blue stained 28S ribosomal RNA bands on the nylon transfer membranes. Specific mRNA expression was calculated as the ratio of the intensity of the MMP bands to the intensity of the 28S rRNA bands.

Statistical analyses—Comparison of means for the activity of MMP in conditioned medium, medium concentrations of MMP-13, and relative expression of MMPs were performed by 2-way ANOVA (blocked by horse) followed by a Student-Newman-Keuls multiple comparisons procedure. Values of $P < 0.05$ were considered significant.

Results

Effect of glucosamine hydrochloride on activated MMP activity—Exposure of cartilage explants to LPS at high concentration produced an MMP-rich conditioned medium for use in the azocoll substrate digestion procedures. Substrate digestion by this MMP-rich conditioned media (after MMP activation by an organomercurial agent) was not significantly affected by glucosamine at pH 7.4 (**Fig 1**). Results were comparable at pH 5.0. This finding indicated that glucosamine has no influence on the activity of preformed MMPs.

Effect of glucosamine hydrochloride on MMP synthesis and MMP-13 production—Not unexpectedly, LPS (20 µg/mL) stimulated the synthesis of MMP in equine chondrocyte pellet cultures, as determined by azocoll digestion. The LPS-stimulated positive control

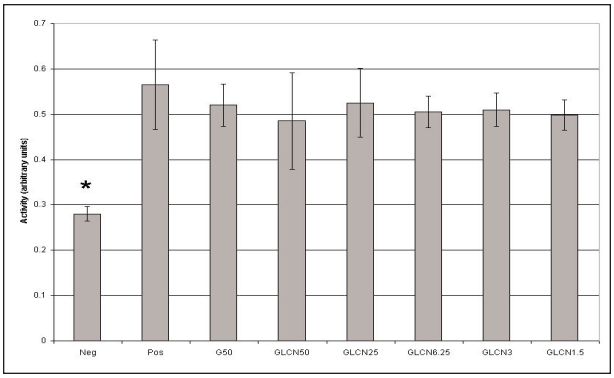

Figure 1—Mean (± SEM) matrix metalloproteinase (MMP) activity, as determined by optical density of azodye released into the LPS-conditioned cartilage explant supernatant at pH 7.4. Neg = O-phenanthroline-treated control sample. Pos = APMA-treated (4-aminophenylmercuric acetate) control sample. G50 = Glucose treatment (50mM). GLCN = Glucosamine treatment at the indicated concentrations (50, 25, 6.25, 3, and 1.5mM). *Indicates significant difference ($P < 0.05$) from positive control.

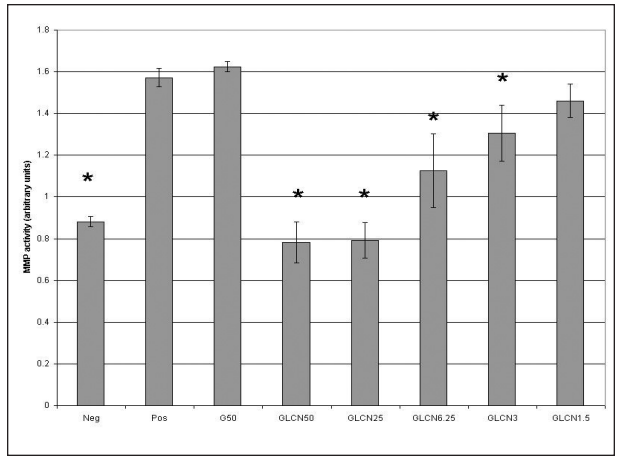

Figure 2—Mean (± SEM) MMP production in equine chondrocyte pellet cultures stimulated with lipopolysaccharide (LPS; 20 µg/mL). Production of MMP was determined by azodye release with azocoll as a digestion substrate (pH 7.4). Neg = Unstimulated chondrocyte control sample. Pos = LPS-stimulated chondrocyte control sample. G50 = Glucose treatment (50mM). GLCN = Glucosamine treatment at the indicated concentrations (50, 25, 6.25, 3, and 1.5mM). *Indicates significant difference ($P < 0.05$) from positive control.

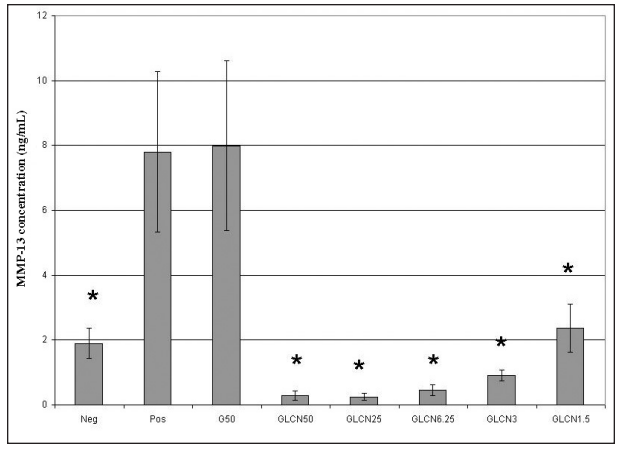

Figure 3—Mean (± SEM) MMP-13 concentration in media supernatant of equine chondrocyte pellet cultures stimulated with LPS (20 µg/mL). Concentration of MMP-13 was determined by means of a commercially available ELISA. *See* Figure 2 for key.

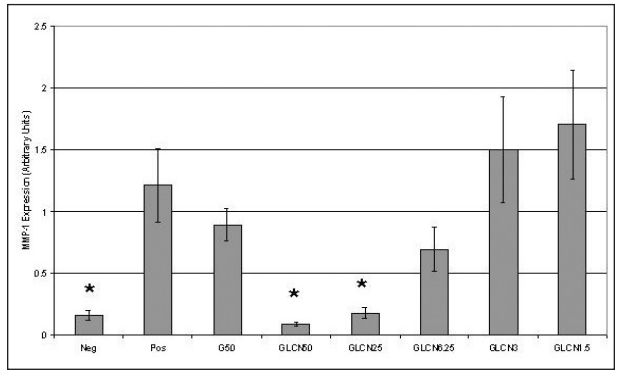

Figure 4—Mean (± SEM) relative MMP-1 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 µg/mL). Concentration of MMP-1 mRNA is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. *See* Figure 2 for key.

samples had almost a 2-fold increase in MMP synthesis, compared with that of unstimulated negative controls (**Fig 2**). This effect was significantly reduced by glucosamine at all concentrations tested except the lowest (1.5mM). This effect was paralleled by a significant reduction in MMP-13 levels in medium supernatant at all glucosamine concentrations tested, as determined by ELISA (**Fig 3**). The LPS-stimulated positive control samples had an approximately 4-fold increase in MMP-13 content, compared with that of unstimulated negative controls. Glucosamine treatment (3 to 50mM) resulted in MMP-13 concentrations lower than those observed in the unstimulated negative control samples, while glucosamine treatment at 1.5mM was associated with MMP-13 concentration similar to those in the unstimulated negative controls.

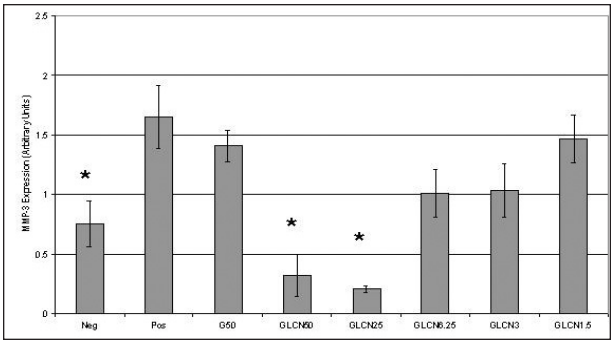

Figure 5—Mean (± SEM) relative MMP-3 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 µg/mL). Concentration of MMP-3 mRNA is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. *See* Figure 2 for key.

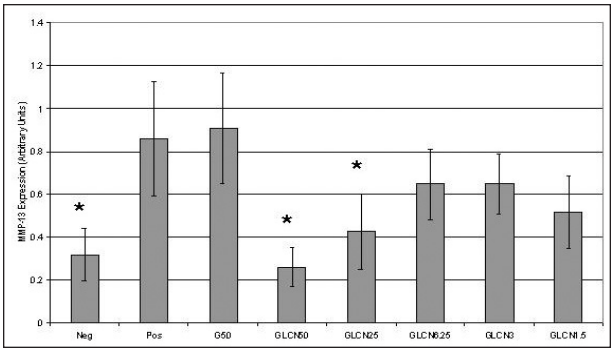

Figure 6—Mean (± SEM) relative MMP-13 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 µg/mL). Concentration of MMP-13 mRNA is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. *See* Figure 2 for key.

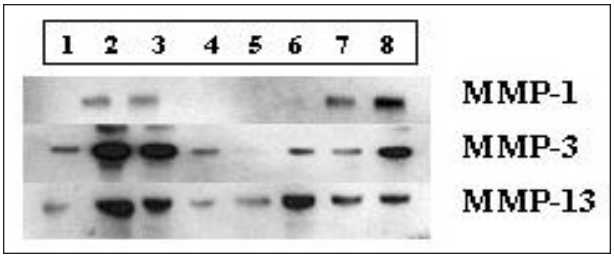

Figure 7—Representative autoradiographs of northern blots with probes for MMP-1, -3, and -13 obtained with a commercially available DNA labeling and detection kit. Lane 1 = Negative control sample. Lane 2 = Positive control sample. Lane 3 = Glucose treatment (50mM). Lanes 4-8 = glucosamine treatment (50, 25, 6.25, 3, and 1.5mM, respectively).

Effect of glucosamine on MMP-1, -3, and -13 mRNA expression—Lipopolysaccharide (20 µg/mL) significantly stimulated expression of MMP-1, -3, and -13, resulting in concentrations that were approximately 3- to 5-fold higher than resting levels (**Fig 4–6**). Induction of messenger RNA of these 3 MMPs was reduced by glucosamine. This effect was statistically significant at the 50 and 25mM levels for all 3 MMPs. Chemiluminescent detection of all 3 MMPs in total chondrocyte RNA via northern blot hybridization was obtained (**Fig 7**).

Discussion

Our data supported the findings of other studies,[13-15] which suggest that glucosamine has chondroprotective properties; furthermore, the results of our study have provided preliminary information on the mechanism of action of glucosamine with regard to inhibition of MMP synthesis. The range of glucosamine concentrations used in our study was similar to that used in other in vitro studies,[12-15,24,25] as well as that used in a study[26] involving IV glucosamine infusion in rats. Although glucosamine is absorbed readily after oral administration and is selectively concentrated in cartilage,[10] the concentration range used in our study exceeded concentrations achieved in vivo after oral administration of glucosamine at recommended doses in dogs.[27] For comparison, a single oral dose of glucosamine (2,000 mg) administered orally in Beagles achieves short-term plasma glucosamine concentration of 15 to 20 µg/mL.[27] It is noteworthy that the experimental protocol of the study reported here involved simultaneous administration of LPS and glucosamine. Prevention of cartilage degradation by glucosamine treatment prior to irreversible extracellular matrix structural damage may require lower doses than those needed to mitigate the profound chondrocyte stimulation observed in our study. Therefore, glucosamine concentrations required to achieve chondroprotective effects in vivo may be much lower than those required in our study. The minimal effective dose of glucosamine that is capable of modulating MMP expression remains to be determined.

Results of our experiments involving azocoll digestion with APMA-activated conditioned medium indicated that glucosamine hydrochloride had negligible effects on the activity of preformed MMP. Matrix metalloproteinase activity assays were performed in conditions of pH 5.0 or 7.4 to favor the activity of stromelysin and total MMP activity, respectively.[21,22] The fact that glucosamine did not inhibit MMP activity at either pH condition used in our study indicated that the activity of the well-characterized MMPs were not notably inhibited by glucosamine.

Results of MMP synthesis experiments (the azocoll digestion of pellet culture medium and MMP-13 ELISA) indicated that glucosamine modulated expression of MMPs at or before protein translation. This

effect was significant (compared with results of the positive control samples) in the glucosamine concentration range of 3 to 50mM, as determined by azocoll digestion assay, and was mirrored by significant reductions in MMP-13 concentration in culture supernatant samples at all 5 concentrations of glucosamine tested. As part of our investigations, ELISA kits were also tested for detection of equine MMP-1 and -3, but because the human antibodies did not cross-react with these forms of MMPs, the kits could not be validated for use in horses. The results of culture supernatant enzyme activity and MMP-13 concentration were concordant; results differed at the 1.5mM level, which may be explained by differences between the detection methods used or by a difference between total MMP and MMP-13 production. The data did suggest a real difference between MMP-13 levels and levels of other MMPs. Matrix metalloproteinase-13 ELISA results indicated that glucosamine treatment at the 3mM level yielded an 88% reduction in MMP-13 content, compared to positive controls, in contrast to the 17% reduction in total MMP content at the 3mM level, as determined by the azocoll digestion assay.

Expression of MMPs in equine chondrocytes was investigated in this study, cells that had been propagated in high-density, short-term, confluent monolayer cultures with a brief period of serum-free incubation. Although growth in stationary monolayers does not closely mimic the environment of chondrocytes in vivo, the culture system provides a uniform distribution of chondrocytes of a specific phenotype, facilitates quantification of biosynthetic parameters, and permits the subjective assessment of cytotoxicity by inspection. The purpose of the brief period of serum withdrawal was to reduce serum component stimulation of MMP expression and any confounding effects that serum components might have had on LPS stimulation and glucosamine treatment.

Relative mRNA expression of all 3 MMPs was significantly inhibited by glucosamine at 50 and 25mM. This result indicated that glucosamine inhibits MMP mRNA synthesis or enhances its degradation pretranslationally. Similar to protein expression data, MMP-13 mRNA levels appeared to be lower than those of the other MMPs assayed; however, this difference was not significant. Investigation of the differential regulation of the MMPs' gene expression was beyond the scope of our study. Gene expression regulation by glucosamine has been proposed by results of studies[12,24,28] that indicate a role of glucosamine in altering mRNA expression of several key enzymes involved in progression of osteoarthritis. Glucuronosyltransferase I, an enzyme involved in glycosaminoglycan biosynthesis, is repressed by interleukin-1β in rat chondrocyte cultures.[12] This phenomenon was prevented by the addition of 21mM glucosamine to the culture medium. Additionally, the same study reported that glucosamine prevented interleukin-1β-induced up-regulation of MMP-3 mRNA in rat chondrocytes. Similarly, inclusion of glucosamine (2 mmol/L) in culture medium for 4 days has been shown to enhance DNA binding of the Sp1 transcription factor to the PAI-1 promoter site in rat glomerular mesangial cells.[24] Moreover, it has recently been shown that glucosamine is capable of inhibiting interleukin-1 effects through inhibition of the NF κB pathway.[28] In combination with our data, these findings indicate that glucosamine hydrochloride is capable of altering gene expression.

The precise molecular level at which glucosamine inhibits LPS-induction of MMP mRNA synthesis remains unknown. Possible mechanisms include inhibition of signal transduction pathways, regulation of transcription factors, promoter efficiency, transcription itself, or posttranscriptional processing that results in premature MMP mRNA degradation. Results of several studies indicate that regulation of MMP expression occurs via transcription factor activator protein-1,[29] Nmp4 gene splice variants,[30] and Sp family transcription factors.[31] Additionally, Ras-responsive elements have been detected in promoter regions,[32] which suggests MMP expression may be regulated through a number of signaling pathways that involve regulation of gene expression. The means by which glucosamine may influence these regulatory pathways remains to be elucidated.

[a]Calbiochem, San Diego, Calif.
[b]Amersham Biosciences Inc, Piscataway, NJ.
[c]TriReagent, Molecular Research Center, Cincinnati, Ohio.
[d]DIG, Roche Molecular Biochemicals, Indianapolis, Ind.
[e]DIG high prime DNA labeling and detection starter kit II, Roche Molecular Biochemicals, Indianapolis, Ind.
[f]Roche, Indianapolis, Ind.
[g]Eastman Kodak, Rochester, NY.
[h]Epson scanner, Epson, Long Beach, Calif.
[i]Scion image beta 4.02, Scion Corp, Frederick, Md.

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