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INFLUENCE OF GLUCOSAMINE ON MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY IN LPS-STIMULATED EQUINE CHONDROCYTES

presented by

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has been accepted towards fulfillment
of the requirements for

MS degree in Large Animal Clinical Sciences

Major professor

Date 6.25.02

INFLUENCE OF GLUCOSAMINE ON MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY IN LPS-STIMULATED EQUINE CHONDROCYTES.

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Large Animal Clinical Sciences

2002

ABSTRACT

INFLUENCE OF GLUCOSAMINE ON MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY IN LPS-STIMULATED EQUINE CHONDROCYTES

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The objective of this project was to characterize potential mechanisms of action of glucosamine inhibition of MMP expression and activity in lipopolysaccharide (LPS)-stimulated equine chondrocytes. The influence of glucosamine (50 mM, 25 mM, 6.25 mM, 3 mM, and 1.5 mM) on MMP activity in conditioned medium from LPS-stimulated cartilage explants was determined by a colorimetric assay using azocoll as a substrate. The influence of glucosamine on MMP synthesis in equine chondrocytes in pellet cultures was determined using a commercial MMP-13 ELISA and azocoll digestion in organomercurial-activated medium. The effects of glucosamine on MMP mRNA levels in similarly treated equine chondrocytes was determined by Northern blotting using MMP-1, -3, and -13 probes. Statistical analysis was carried out with two-way ANOVA, and P was set at 0.05. Glucosamine had no effect on activated MMP activity. Glucosamine inhibited MMP protein expression, as determined by azocoll digestion at 3 to 50 mM, and as determined by MMP-13 ELISA at 1.5 to 50 mM. Resting mRNA levels for MMP-1, -3, and -13 mRNA were significantly lower in cultures exposed glucosamine at concentrations of 50 mM and 25 mM. These results indicate that glucosamine is capable of pre-translational, and possibly also translational, regulation of MMP expression and provide a potential mechanism of action for previously reported chondroprotective effects of this aminomonosaccharide.

ACKNOWLEDGMENTS

I would like to thank Tonia Peters for her indispensible technical help. I would also like to thank Dr. Venta for his insight and suggestions. Finally, many thanks go to the members of my committee, Dr. Orth, Dr. Lloyd, and Dr. Caron for their intellectual input and service on the committee.

Special thanks go to my wife, Rachael, who stood with me through the rough times and helped make the journey worthwhile.

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LIST OF ABBREVIATIONS

Extracellular Matrix.....ECM

Matrix Metalloproteinase.....MMP

Non-Steroidal Antiinflammatory Drug.....NSAID

Lipopolysaccharide.....LPS

Glucosamine.....GLCN

Tissue Inhibitor of Metalloproteinase.....TIMP

Enzyme-Linked Immunosorbent Assay.....ELISA

Aminophenylmercuric Acetate.....APMA

Phospholipase.....PLA

Nitric Oxide.....NO

Polysulfated Glycosaminoglycan.....PSGAG

Glycosaminoglycan.....GAG

Randomized Controlled Trial.....RCT

Tumor Necrosis Factor-α.....TNF-α

Interleukin.....IL

Toll-Like Receptor.....TLR

INTRODUCTION

Osteoarthritis (OA) causes substantial morbidity in the form of lameness and may seriously curtail quality of life in addition to athletic serviceability of affected horses (Rossdale *et al.* 1985, Todhunter and Lust 1990). It can be initiated by a number of causes and ultimately affects all articular tissues. The hallmark of the disease is the degeneration of articular cartilage, a highly specialized tissue consisting of relatively few cells but having a rich extracellular matrix. Although there are a number of possible causes of matrix degradation, experimental evidence suggests that proteolytic degradation of cartilage by matrix metalloproteinases (MMPs) is a characteristic feature of OA (McIlwraith 1996, Goldring 2000).

Matrix metalloproteinases (MMPs, matrixins) are a family of enzymes that degrade the extracellular matrix of tissues. Several subgroups of MMPs based on substrate preference and structural domains including collagenases, gelatinases, and stromelysins are active in OA (Vu and Werb 2000, Nagase and Woessner 1999, Pozzi *et al.* 2000, Rabbani *et al.* 2000). Since collagen damage is an important initial event in OA (Roberts *et al.*1986, McIlwraith 1996, Goldring 2000), MMPs, particularly collagenases, may serve as a pivotal component of OA initiation and progression. Stromelysin (MMP-3) is also important in that it not only degrades non-collagenous ECM components, including aggrecan, but also serves to activate latent collagenases (Goldring 2000).

Glucosamine therapy for the treatment of OA has been the subject of several clinical studies in human patients. One recent review of 16 randomized control trials utilizing glucosamine for the treatment of human OA reported that glucosamine is both

effective and safe for its treatment of OA (Towheed *et al.* 2001). When glucosamine was compared to an NSAID for treatment of OA, its effectiveness was found to be equivalent in two trials and superior in two. While ibuprofen effects a more rapid decrease in patient pain scores, oral glucosamine therapy yields an ultimately lower pain score after eight weeks of treatment (Barclay *et al.* 1998). Glucosamine has also been shown to prevent knee joint space loss in OA patients during the course of a three-year study (Reginster *et al.* 2001).

The effects of glucosamine on *in vitro* cellular metabolism have received recent attention. Proteoglycan production is stimulated by exogenous glucosamine in human chondrocyte cultures (Bassleer *et al.* 1998) and glucosamine has been shown to prevent the repression of beta-1,3-glucuronosyltransferase I, a key biosynthetic enzyme in glycosaminoglycan synthesis. Importantly, glucosamine prevents the up-regulation of MMP-3 mRNA expression by interleukin-1 beta stimulated rat chondrocytes (Gouze *et al.* 2001). While glucosamine has been shown to inhibit proteoglycan loss, inducible nitric oxide synthase, and MMP activity in equine cartilage explants stimulated with LPS or recombinant interleukin-1 (Fenton *et al.* 2000 a,b), the specific level at which these effects occur remain unclear.

The purpose of the study presented here was to determine the nature of the inhibition of MMP activity attributed to glucosamine HCl in an *in vitro* model of OA. The specific aims were to determine if glucosamine HCl influences 1) the catalytic activity of chondrocyte MMPs, 2) synthesis of MMPs at the protein level, and 3) resting expression of MMP mRNA levels in LPS-stimulated equine chondrocytes.

The following chapters describe the *in vitro* research and results that address these specific aims. Chapter 1 is a review of the current literature relating to the project which provides the necessary background information. Chapter 2 contains a description of the methods, the results, and a discussion of the project. Chapter 3 contains concluding comments regarding the project, the results, and questions that may be addressed through future research.

Chapter 1

LITERATURE REVIEW

Synovial Joint Structure and Function

The two major functions of synovial joints are the facilitation of joint movement and the transfer of weight-bearing loads. Proximal limb joints which have freedom to move in several planes and have the capability for rotational movement largely derive their stability from large muscle masses and tendons surrounding them. Distal limb joints rely more on ligamentous support, joint capsule integrity, and joint contour for stability (Todhunter 1996). Certain joints such as the femorotibial and temporomandibular joints incorporate menisci that lend stability (Mankin and Radin 1997).

Articular Cartilage

The subchondral bone plate beneath articular cartilage is composed of dense cortical bone with haversian system orientation parallel to the joint surface. The hyaline cartilage overlies this subchondral bone plate and is bound to the bone by collagen fibers which originate at right angles to the haversian system (Mankin and Radin 1997). These collagen fibrils impart tensile strength and provide a scaffold within which chondrocytes and proteoglycans are embedded (Walsh *et al.* 1997).

Articular cartilage is organized into four distinct histological zones (Mankin and Radin 1997). The surface *tangential zone* exhibits collagen fibers organized parallel to the articular cartilage surface and elongated chondrocytes whose long axis is also oriented parallel to the articular surface. The deeper *transitional zone* exhibits rounder

chondrocytes with the appearance of random distribution. *Radial zone* chondrocytes are arranged in short, irregular columns. Chondrocytes in the deepest *calcified zone* are surrounded by hydroxyapatite.

Extracellular Matrix of Cartilage

Articular cartilage is composed of 70% water and 30% matrix. The extracellular matrix (ECM) is composed of 50% collagen, 35% proteoglycan, 10% glycoproteins, 3% mineral, and 1% lipid. Chondrocytes account for 1 to 12% of the articular cartilage by volume.

The major contributor to cartilage tensile strength is collagen type II which is highly cross linked in mature hyaline cartilage. Type II collagen has a greater percentage of lysine residue hydroxylation and greater glycosylation than type I collagen in equids (Todhunter *et al.* 1994). Other minor collagen components include collagens type VI, IX, X (physeal and hypertrophic cartilage), XI, XII, and XIV. These minor collagens have important structural and functional properties as they serve to further organize the type II collagen fibrils and mediate ECM-ECM interactions as well as ECM-cell interactions. Type VI collagen is a dimer that forms microfibrils which may link larger collagen fibrils and chondrocyte cell surface receptors. Type IX collagen is a triple helix that has a chondroitin sulfate side chain and mediates interaction between major collagen fibrils and other ECM molecules. Type X collagen is an accessory collagen that is present exclusively within the hypertrophic calcified zone of articular cartilage. Type XI collagen is interspersed between type II fibrils. Type XII collagen is present in small amounts in articular cartilage and other connective tissues. Type XIV collagen is structurally related

to type IX (Todhunter 1996, Mayne and Brewton 1993). Collagen types II, IX, X and XI are found exclusively in cartilage (Walsh *et al.* 1997).

Proteoglycans are largely responsible for the elasticity of hyaline cartilage (Walsh *et al.* 1997). They are composed of a linear protein core to which are attached the glycosaminoglycans chondroitin 6-sulfate, chondroitin-4-sulfate, and keratan sulfate. Proteoglycans include aggrecan, biglycan, decorin, fibromodulin, and lumican. Aggrecan is the major proteoglycan in articular cartilage and contributes the greater part of cartilage resistance to compressive stresses through interaction of highly negatively charged glycosaminoglycan side chains (Goldring 2000). As many as 50 GAG chains (chondroitin 6-sulfate, chondroitin-4-sulfate, and keratan sulfate) may be attached to a single core protein. The electronegativity of these side chains allows the proteoglycan unit to maintain a rigid structure interspersed with water molecules, lending resiliency to the articular cartilage (Mankin and Radin 1997).

Collagen turnover rates have been estimated at 120 years for canine cartilage and 350 years for human cartilage (Akizuki et al. 1987). Overall proteoglycan turnover, however, is approximately 300 days in rabbit and canine cartilage and approximately 1800 days in human cartilage (Maroudas 1980). These data suggest that matrix turnover is generally slow, and collagen turnover in particular is easily slower than the life expectancy of the animal or person.

Chondrocytes

Chondrocytes are interspersed throughout articular cartilage in small groups or as single cells. They are responsible for the synthesis and breakdown of articular cartilage

ECM components. They respond to mechanical and chemical stimuli in the articular cartilage to regulate these diverse activities.

In osteoarthritis the metabolic responses of the chondrocyte to its environment are altered by disruption of the chondrocyte-ECM interaction (Radin *et al.*1991, Pool 1997). Disruption of matrix interactions leads to early clonal growth, increased production of cartilage matrix components, and increased production and release of catabolic cytokines and matrix-degrading enzymes (Goldring 2000). Chondrocyte proliferation leads to local chondron formation. Type II collagen and aggrecan production are increased in early OA but decrease in late-stage OA. Interleukin-1 and IL-1 receptor type 1 are increased serving as autocrine and paracrine catabolic signals. Important proteinases in OA cartilage degradation produced by chondrocytes include matrix metalloproteinases, aggrecanase, plasminogen activator, and cathepsins.

Synovium and Synovial Fluid

Lining the joint is a modified mesenchyme called synovium. The intima is between one and four synoviocytes thick with no basement membrane and overlies a layer of connective tissue. Synoviocytes have both secretory and phagocytic functions, and have been shown to synthesize MMPs and hyaluronan, a major component of synovial fluid.

Synovial fluid is an ultrafiltrate of plasma containing only small proteins. The fluid contains fewer than 500 nucleated cells/μl, composed of 90% mononuclear cells with 10% neutrophils. Hyaluronan is present in normal equine synovial fluid at a concentration of 0.5 mg/ml. Normal human synovial fluid contains hyaluronan at a

concentration of 2 to 3 mg/ml. Fluid exchange between plasma and synovium is governed by Starling forces and driven by motion-assisted lymphatic flow and intrasynovial fluid pressures (Todhunter 1996).

Joint Biomechanics

Diarthrodial joints function with an extremely low coefficient of friction (Palmer and Bertone 1996). Whereas the coefficient of friction of good artificial joints is between 0.1 and 0.5, healthy biologic diarthrodial joints have a much lower 0.002 coefficient of friction (Simon and Radin 1997). Contributing to the low coefficient of friction is the parallel orientation of collagen fibers to the joint surface, the porosity of cartilage, the presence of hyaluronan, and the presence of lubricating glycoproteins (lubricin) within the synovial fluid.

Hyaluronan lends thixotropic flow characteristics to synovial fluid (Mankin and Radin 1997). This means that the more slowly synovial fluid flows the more viscous it becomes. Due to the extremely low friction of normal cartilage-on-cartilage contact, the periarticular soft tissues provide the majority of the frictional resistance to joint movement. It is this peripheral friction which hyaluronan mitigates, serving as a boundary lubricant rather than as a lubricant of the cartilage surface itself (Mankin and Radin 1997).

The glycoprotein lubricin serves as the lubricant of cartilage surfaces against each other during high load, low speed joint movement (Palmer and Bertone 1996). Lubricin decreases the surface tension of joint surfaces, adhering to the cartilage and the synovial surface, to effectively decrease shear stresses. Lubricin prevents the fusion or binding of

surfaces and may also play a role in aiding articular tracking during joint movement (Palmer and Bertone 1996).

Aside from the boundary lubrication described above which functions in low speed motion, there are two mechanisms currently thought to function in high speed motion joint lubrication. Squeeze film lubrication occurs when fluid is forced out of the high load areas of the porous cartilage, increasing pressure and assisting in load carrying. Hydrodynamic lubrication occurs when joint surfaces move tangentially to one another forming a wedge of fluid ahead of the motion that acts to support and separate the cartilage. Two relatively new hydrodynamic-based theories have addressed additional properties of diarthrodial joint biomechanics. Elastohydrodynamic lubrication takes into account cartilage deformation, which increases surface area of the loaded cartilage surface, thereby increasing load-bearing capacity. Microelastohydrodynamic lubrication theory proposes that natural irregularities in the cartilage surface are smoothed out during weight bearing due to lubrication at the peaks and lack of weight bearing in trough area. This increase in surface area would serve to increase the weight bearing area of cartilage (Palmer and Bertone 1996).

Natural lubrication theory considers the biomechanics of the cartilage as well as the viscoelastic principles listed above. In this theory, cartilage becomes less permeable as load increases due to decreased space in the ECM. As this occurs, fluid is forced out of the cartilage ECM into the joint, thereby increasing lift and separation between the cartilage surfaces (Palmer and Bertone 1996).

Collagen and proteoglycans within the cartilage ECM retain fluid within the matrix and offer resistance to interstitial fluid flow, which contributes to the cartilages

ability to withstand high compressive loads. Carboxyl and sulfate groups of cartilage glycosaminoglycans have a fixed negative charge. Water within the ECM neutralizes this charge. During weight bearing water is forced out of the ECM, thereby increasing the negative charge density as the GAGs come into closer contact. These electrical properties contribute to the ability of cartilage to resist compressive loads (Simon and Radin 1997).

Additional support during compressive loading is offered by the subchondral bone plate and trabecular bone, which expand to support and distribute loads during weight bearing as well as absorbing concussive forces through micromotion (Simon and Radin 1997).

Alteration of the function of any component of the synovial joint can create imbalances in the normal biomechanics of movement, resulting in cartilage breakdown. Osteoarthritic processes can result in cartilage ECM compositional changes which may alter the biomechanical attributes described above (McIlwraith 1996).

Matrix Metalloproteinases and Their Inhibitors

MMP Family

Matrix metalloproteinases (MMPs, matrixins) are a family of enzymes that degrade the extracellular matrix of tissues as required during embryonic development, morphogenesis, reproduction, and tissue remodeling. Most MMPs are synthesized as proenzymes and secreted as inactive pro-MMPs. MMPs are transcriptionally regulated by growth factors, cytokines, and cellular transformation (Nagase and Woessner 1999), and their proteolytic activities are controlled by activators of pro-MMPs, endogenous inhibitors, alpha-macroglobulins, and tissue inhibitors of matrix metalloproteinases

(TIMPs). These enzymes are dependent on Ca^{2+} and Zn^{2+} for activity. Several MMP subgroups exist that are based on primary substrate preference and structural domains, including collagenases which are most active against fibrillar collagen, gelatinases which are most active against denatured collagens, stromelysins that primarily degrade non-collagen components of the extracellular matrix, and membrane-type MMPs (MT-MMPs) that are transmembrane enzymes (Vu and Werb 2000). In addition to primary preferences, most MMPs have a wide range of available substrates (Table 1). In addition to ECM turnover, MMPs also serve to modify the biological effects of ECM components by changes in structure and unmasking of biologically active domains (Nagase and Woessner 1999). MMPs also serve a role in many pathological processes including arthritis, cancer, cardiovascular disease, corneal ulceration (Nagase and Woessner 1999, Pozzi *et al.* 2000, Rabbani *et al.* 2000).

MMPs in OA

Several matrix metalloproteinases are involved in cartilage ECM degradation including collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10, -11), gelatinases (MMP-2, -9), and membrane-type (MMP-14) (Goldring 2000, review). Levels of TIMPS are also elevated in synovial fluid of arthritis patients however there may be a local imbalance of MMP to TIMP at areas of continued degradation (Dean *et al.* 1989). Since collagen damage may be an initial event in osteoarthritis disease progression (Roberts 1986, McIlwraith 1996), MMPs, particularly collagenases, may serve as a focal component of OA initiation and progression. While proteoglycan degradation is also observed early in OA progression, collagen damage may carry more serious and

permanent consequences due to its slow rate of turnover (Aigner and McKenna 2002). Stromelysin (MMP-3) is also important in that it not only degrades non-collagenous cartilage ECM components, including aggrecan, but also serves to activate latent collagenases (Goldring 2000). Collagenases that degrade type II collagen, the primary collagenous cartilage component, include MMP-1, -8, and -13. Evidence of MMP-13 expression in cartilage and its effectiveness at degrading type II collagen suggests a major role for this enzyme in OA progression (Knauper *et al.* 1996, Mitchell *et al.* 1996, Billinghurst *et al.* 1997, Cawston *et al.* 1999, Dahlberg *et al.* 2000).

Matrix metalloproteinases-1 and -13 have similar substrate specificities, with MMP-1 having a slightly broader range of substrates. MMP-1 and -13 both degrade collagens type I, II, III, VII, and X, as well as gelatins and aggrecan. Additionally, MMP-1 degrades tenascin and link protein (Martel-Pelletier *et al.* 2001). Although similar substrate preferences are apparent for these two collagenases, human MMP-13 has been shown to have a higher catalytic efficiency for collagen type II than human MMP-1 (Jeffrey 1998). The roles of MMP-1 and MMP-13 in OA progression also appear to differ, with MMP-1 associated with superficial cartilage and MMP-13 associated with the intermediate and deep cartilage zones. This suggests that MMP-1 may be associated with acute tissue destruction during inflammatory phases, whereas MMP-13 may have a role in remodeling (Martel-Pelletier *et al.* 2001). Activation of both collagenases occurs extracellularly.

Table 1. Matrix metalloproteinases and their preferred substrates. Adapted from Martel-Pelletier *et al.* 2001

MMP	Enzyme Name(s)	Preferred Substrates
MMP-1	Collagenase-1, interstitial collagenase	Collagen types I, II, III, VII, X, gelatins, aggrecan, tenascin, link protein
MMP-2 (MMP-5)	Gelatinase A, gelatinase 72 kDa	Gelatins, collagens I, II, III, IV, V, VII, X, XI, type IV collagenase, fibronectin, laminin, aggrecan, elastin, tenascin, vitronectin
MMP-3 (MMP-4) (MMP-6)	Stromelysin-1	Aggrecan, gelatin, fibronectin, laminin, link protein, elastin, collagen types I, III, IV, V, VIII, IX, X, procollagenase-1 (activation), vitronectin, tenascin, decorin
MMP-7	Matrilysin	Aggrecan, fibronectin, vitronectin, tenascin, laminin, gelatin, collagen type IV, elastin, procollagenase-1 (activation), link protein
MMP-8	Neutrophil collagenase	Collagen types I, II, III, VIII, X, aggrecan, link protein
MMP-9	Gelatinase B, gelatinase 92 kDa	Gelatins, collagen types I, III, IV, V, VII, X, XI, XIV, aggrecan, elastin, vitronectin
MMP-10	Stromelysin-2	Aggrecan, fibronectin, laminin, collagen types I, III, VI, V, VIII, IX, gelatin, elastin, laminin
MMP-11	Stromelysin-3	Fibronectin, laminin, collagen type IV, aggrecan, gelatin, alphaI-antitrypsin, serpin
MMP-12	Metalloelastase	Elastin
MMP-13	Collagenase-3	Collagen types I, II, III, VII, X, aggrecan, gelatins
MMP-14	MT1-MMP	Pro-MMP-2 (activation), collagen types I, II, III, dermatan sulfate, laminin, fibronectin, gelatin, vitronectin
MMP-15	MT2-MMP	
MMP-16	MT3-MMP	Pro-MMP-2 (activation)
MMP-17	MT4-MMP	
MMP-18	Collagenase-4	
MMP-19	RASI-1	
MMP-20	Enamelysin	Enamel matrix
MMP-21	X-MMP	
MMP-22	C-MMP	
MMP-23	CA-MMP, MIFR-1	
MMP-24	MTS-MMP	
MMP-25	MT6-MMP	
MMP-26	Endometase	
MMP-27	Epilysin	
MMP-28	GeneBank AAG41981.1	

TIMPs

Tissue inhibitors of matrix metalloproteinases (TIMPs) are a family of proteins which regulate the activity of MMPs by formation of a 1:1 MMP:TIMP complex. Mammalian TIMPs are two-domain molecules and belong to one of four subgroups (TIMP-1 through TIMP-4). The N-terminal domain consists of approximately 125 amino acids and the C-terminal domain contains approximately 65 amino acids, with each domain being stabilized by three disulfide bonds (Williamson *et al.* 1990). The N-terminal domain is capable of MMP inhibitory activity when separated from the C-terminal domain (Murphy *et al.* 1991). TIMP inhibitory activity is mediated through a conformational change in the MMP. This is brought about by TIMP binding to the MMP active site, which causes disruption of amino acid residue ionic interactions (Brew *et al.* 2000, review).

The four known TIMPs are evolutionarily related. They are the products of gene duplication events (Brew *et al.* 2000, review). TIMPs bind tightly to most MMPs (Murphy and Willenbrock 1995), however there appears to be specificity in their interactions with MMPs and with other proteins. For example, TIMP-2 plays a critical role in the activation of proMMP-2 in the presence of MT1-MMP (Strongin *et al.* 1995), while TIMP-1 does not have this ability (Brew *et al.* 2000, review). As another example, TIMP-3, but not any other TIMP, binds tightly to ECM components (Pavloff *et al.* 1992, Yang *et al.* 1992)

In addition to active MMP inhibition, TIMPs have been shown to aid in pro-MMP activation (Strongin *et al.* 1995), matrix binding (Pavloff *et al.* 1992, Yang *et al.* 1992),

inhibition of angiogenesis (Murphy *et al.* 1993), induction of apoptosis (Baker *et al.* 1999), and promote cell growth (Bertaux *et al.* 1991).

In Vitro Assessment of MMPs

Options for detection of enzymes in a fluid medium include various activity assays that detect active enzyme indirectly, or Western blotting and enzyme linked immunosorbent assay (ELISA) which detect proenzymes or active enzymes.

Measurement of MMP activity can be performed using zymography (Lein *et al.* 1997), utilizing substrate digestion within a gel matrix, or colorimetric substrate degradation assays which rely on dye liberation upon substrate degradation (Chavira *et al.* 1984, Vachova and Moracova 1993).

Recently, commercially available ELISA-based detection systems have become available (Lein *et al.* 1997) and have been validated for use in detecting and quantifying MMPs from venous blood of healthy human adults. These systems detect both inactive and active enzyme directly through antibody-based binding that is coupled to a colored reporter substrate.

Detection of MMP mRNA may be performed using either Northern blotting or polymerase chain reaction (PCR) methods. Collagenases, gelatinases, and stromelysins have been detected using the northern blot and reverse transcriptase-PCR methods (Maquoi *et al.* 2002, Han *et al.* 2002). Reverse transcriptase-PCR has a higher sensitivity than northern blotting for the detection and quantitation of MMP mRNA (Fehr *et al.* 2000).

Azocoll-based MMP detection

MMP activity may be detected using the colorimetric substrate azocoll, an insoluble ground collagen which liberates a red azodye when degraded by proteolytic action (Chavira *et al.* 1984). Azocoll digestion has been used to verify the presence of MMPs in experimental laboratory samples and fluids from clinical patients. Increased azocoll digestion has been correlated with the presence of collagenases, gelatinases, and stromelysins in purified MMP samples (Ko *et al.* 2000) and wound fluids (Trengove *et al.* 1999, Ashcroft *et al.* 1997). The technique has been applied to many fields of MMP research, including dentistry (Broverman *et al.* 1998) and rheumatology (Okada *et al.* 1990). The azocoll assay has proven to be a rapid and reliable method of characterizing the relative MMP content of experimental fluid samples.

Total matrix metalloproteinase activity against azocoll may be measured at pH 7.0 since both stromelysin and gelatinases are active at neutral pH (Okada *et al.* 1990, Nagase 1998). Azocoll reactions run under acidic conditions favor the activity of stromelysin (MMP-3) since this enzyme's peak activity occurs at pH 5.5 (Nagase 1998). Inhibition of proteolytic activity with the chelating agent 1,10-phenanthroline indicates activity is the result of MMPs (Young and Grinnell 1994).

In vitro MMP Activation

In vitro -activity assays require activation of MMPs. This is often accomplished through use of (4-aminophenyl)mercuric acetate (APMA), an organomercurial compound which causes a disruption of the pro-MMP, resulting in an unstable intermediate which rapidly converts by autocatalysis to the stable active MMP form (Nagase *et al.* 1990).

The activation of MMPs with APMA has been utilized in many studies involving explant and cultured cells. Differentiation between latent and active enzyme may be discerned through APMA activation of latent MMPs in both explant and cultured chondrocytes (Beekman *et al.* 1998, van Lent *et al.* 2002, Yasuda *et al.* 2002). Localization of matrix degradation in time may be correlated with the activation of MMPs through use of APMA and subsequent MMP activity assay (Milner *et al.* 2001). APMA has been used to discern the effects of PSGAG on proteoglycan degradation in normal and chronic OA cartilage (Sevalla *et al.* 2000). In addition to studies utilizing supernatant from cultured and explant chondrocytes, APMA may also be used to assay MMP activity in synovial fluid samples of osteoarthritic and rheumatoid arthritic joints (Yoshihara *et al.* 2000).

Osteoarthritis

Osteoarthritis (OA) is a progressive joint disease that affects approximately 12% of the population of the United States. The incidence increases with advancing age (Hawker 1997). The disease is characterized by gradual loss of articular cartilage and, in human patients, has traditionally been considered a noninflammatory process, although most animal models as well as numerous human patients exhibit signs of local inflammation and synovitis. Inflammatory cytokine concentrations are consistently elevated in OA (Goldring 2000). The disease is often defined by the presence of clinical signs (pain, decreased range of motion) together with radiographic signs (osteophytosis, joint space narrowing) (Felson 1997). The view of OA as a disease of articular cartilage

has given way to a more global understanding which takes into account the effects of the total joint environment (Sokoloff 1987, McIlwraith 1996).

Chondrocytes, fibroblast-like synovial cells, and macrophage-like synovial cells serve as sources of cytokines that induce a cascade of cartilage digestion as chondrocytes secrete matrix-degrading proteases (Goldring 2000). The inciting cause is generally considered to be mechanical in nature, focusing on the extracellular matrix and the effects of chondrocyte metabolism on this matrix, although pre-existing subchondral bone or synovial pathology may also predispose the joint to OA development (McIlwraith 1996, Burton-Wurster *et al.* 1999, Goldring 2000).

Articular cartilage degradation is characterized by a decrease in proteoglycan content, changes in proteoglycan structure, increased cartilage water content, and changes in collagen structure. Differences in individual joint predisposition to development of OA has been linked to the molecular structure of matrix components (Cole *et al.* 2002). Structurally, the cartilage becomes weaker in both compression due to proteoglycan changes and in tension due to collagen changes (Kempson *et al.* 1971, Kempson *et al.* 1973, Wirth *et al.* 1980). Damage to the collagen component of ECM may be the earliest event in OA degradation. Proteoglycan content of cartilage in early OA may be unchanged which indicates that the collagen damage could be the initial event in disease progression (Roberts 1986, McIlwraith 1996). However, early loss of matrix aggrecan is also prominent event in OA progression (Mankin *et al.* 1971). While proteoglycan degradation is also observed early in OA progression, collagen damage may carry more serious and permanent consequences due to its slow rate of turnover (Aigner and McKenna 2002).

Drugs in OA Treatment

The goals of OA treatment are to prevent the onset and progression of cartilage degradation and to alleviate the effects of disease symptoms, particularly pain associated with joint use (Lozada and Altman 1997). Drugs that primarily alleviate the pain associated with OA, such as NSAIDS, are useful in that they increase mobility but may not prevent progression of the disease itself. These drugs may speed progression of OA by allowing increased use of damaged joints, thereby increasing cartilage destruction (Blot *et al.* 2000). In addition, NSAID toxicities are commonly reported, although these drugs are popular and usually safe if used in an appropriate manner. Similarly, corticosteroids are used intra-articularly for their antiinflammatory and analgesic activities. These drugs also have side effects associated with their use.

In contrast to drugs whose primary functions are antiinflammatory and analgesic activities, chondroprotective drugs serve to slow, prevent, or reverse OA progression. These drugs may also have some analgesic and antiinflammatory actions associated with their use, secondary to their primary chondroprotective role.

Symptomatic Drugs

Corticosteroids

Corticosteroids modulate inflammatory cascades at several levels. Their effects are mediated through cytoplasmic glucocorticoid receptors. Once bound, the receptor-glucocorticoid complex localizes to the nucleus where it regulates gene transcription through binding to glucocorticoid response elements in the promoter region of many

genes (Barnes and Addock 1993) or by direct interactions with transcription factors (Yang-Yen *et al.* 1990). Glucocorticoids may also modify RNA translation, protein synthesis, and protein secretion through mechanisms other than DNA interactions (Chrousos *et al.* 1993, Schleimer 1993).

Corticosteroids interrupt the arachidonic acid inflammatory cascade through induction of lipocortins, which inhibit the actions of PLA_2, preventing the conversion of cell membrane phospholipid into arachidonic acid (May and Lees 1996). Corticosteroids inhibit both the cyclooxygenase and lipoxygenase inflammatory pathways, and have other effects independent of arachidonic acid metabolism, such as decreased vascular permeability (Carnuccio *et al.* 1987), inhibition of nitric oxide synthesis (Radomski *et al.* 1990), inhibition of collagenases, and inhibition of chemotactic factors (Schleimer 1993). Corticosteroids are typically used intra-articularly in the treatment of osteoarthritis, but can have several side effects such as retarded chondrocyte metabolism, inhibition of normal bone metabolism, and potentiation of infection (Trotter 1996a).

Nonsteroidal Antiinflammatory Drugs

Nonsteroidal antiinflammatory drugs inhibit cyclooxygenase, the enzyme responsible for conversion of arachidonic acid to prostaglandins (May and Lees 1996). Two isoforms of cyclooxygenase exist, the constitutively expressed COX-1 and the inducible COX-2 (Clements and Harold 1997). The inhibition of constitutive COX-1 is responsible for many NSAID-linked toxicities, including gastrointestinal ulceration, renal papillary necrosis, and vascular thrombosis (May and Lees 1996). In addition to cyclooxygenase regulation, certain NSAIDS, including ketoprofen, inhibit the

lipoxygenase pathway (Dawson *et al.* 1982). NSAIDS may alleviate OA symptoms through pathways other than cyclooxygenase and lipoxygenase inhibition, including inhibition of neutrophil activation (Abramson *et al.* 1985).

Chondroprotective Drugs

Hyaluronan

Hyaluronan is a nonsulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. It is an integral component of synovial fluid and articular cartilage in normal joints (Todhunter 1996), and exogenous hyaluronan may be administered intravenously or intra-articularly (Howard and McIlwraith 1996, review). Exogenous hyaluronan has been hypothesized to act in many ways in the abatement of OA clinical signs including acting as a joint lubricant (Swann *et al.* 1974), an antiinflammatory agent (Howard and McIlwraith 1996, review), an enzyme inhibitor (through steric hindrance) (Ogston and Sherman 1961, Forrester and Wilkinson 1981), and a free radical scavenger (Sato *et al.* 1988).

Polysulfated Glycosaminoglycans

Polysulfated glycosaminoglycans are a normal component of articular cartilage ECM. Exogenous PSGAGs may be administered intramuscularly and intra-articularly. The proposed mechanisms of action of exogenous PSGAGs in OA have included degradative enzyme inhibition, antiinflammatory action, and enhanced synthesis of hyaluronic acid, glycosaminoglycans, and collagen (Trotter 1996b).

Pentosan Polysulfate

Pentosan polysulfate is a plant-derived polysulfated polysaccharide that has been administered intramuscularly, subcutaneously, orally, intravenously, and intra-articularly. Proposed mechanisms of action in OA include improved incorporation of proteoglycans into the cartilage ECM, stimulation of hyaluronan synthesis by synovium, and inhibition of proteoglycan loss from the ECM, by degradative enzymes (Little and Ghosh 1996).

Glucosamine

The amino sugar glucosamine (2-amino-2-deoxy-alpha-D-glucose), together with galactosamine, is one of the major hexosamine sugars present in animal cells (Kelly 1998). The only difference between glucosamine and glucose is the replacement of the glucose carbon-2 OH group with an NH_3 group. It is synthesized by the enzyme glucosamine synthetase, which transfers an amide group from glutamine to fructose-6-phosphate, to yield glucosamine-6-phosphate. This formation of glucosamine-6-phosphate is the rate-limiting step in amino sugar biosynthesis. Glucosamine is then acetylated to form N-acetylglucosamine, followed by conversion to either N-acetylgalactosamine or N-acetylmannosamine. This pathway provides the amino sugars necessary for the synthesis of macromolecules, including glycolipids, glycoproteins, glycosaminoglycans, hyaluronate, and proteoglycans (Kelly 1998). Glucosamine is the hexosamine component of the glycosaminoglycan keratan sulfate and the proteoglycan backbone hyaluronic acid (Barclay et al. 1998).

The pharmacokinetics of glucosamine have been described in dogs and in humans (Setnikar et al. 1986, Setnikar et al. 1993). After oral administration glucosamine is

completely ionized in the stomach and is readily absorbed. Approximately 87% of the administered dose is absorbed from the canine gastrointestinal tract. Glucosamine in plasma exists as free glucosamine and is not bound to plasma proteins. It is therefore unlikely that glucosamine will interact with other drugs pharmacokinetically. A single 2000 mg oral dose of glucosamine in a beagle dog achieves short-term plasma concentrations between 15 and 20 μg/ml (Liang *et al.* 1999), and intravenous administration can achieve much higher transient peaks of at least 150 μg/ml (Du *et al.* 2001). The free glucosamine is rapidly and selectively concentrated within organs, particularly liver (incorporation into plasma globulins) and kidney (urinary excretion), as well as the ECM of tissues such as cartilage.

Glucosamine is distributed to all connective tissues after intravenous or oral administration, including the matrix of muscle, bone, and skin. Cartilage uptake of glucosamine is greater than that of other musculoskeletal tissues, with approximately 2.5 times more glucosamine concentrated in cartilage compared to muscle at both 2 hours and 144 hours after intravenous administration in dogs (Setnikar *et al.* 1986). This margination of glucosamine reached its peak between 30 and 60 minutes after intravenous glucosamine administration and was maintained until at least 144 hours.

Clearance of glucosamine is primarily through urinary excretion by the kidneys and secondarily as CO_2 in expired air and in feces.

Glucosamine Clinical Trials

Many clinical trials have utilized glucosamine as a treatment for OA since the early 1980s (da Camara and Dowless 1998). Most trials support glucosamine therapy with few minor side effects but many of the studies are plagued by several problems rendering the usefulness of the results unclear. These trials have consistently enrolled small numbers of patients, utilized short treatment duration, failed to report inclusion criteria or severity of disease indices, had short wash-out periods for other OA medications before the onset of the studies, included variable periods of bed rest, lacked baseline studies for reported chondroprotective effects, had variable or unreported doses and routes of administration, and utilized non-blinded scoring (da Camara and Dowless 1998). Furthermore, the studies have largely relied upon industry support for funding and may be subject to conflict of interest problems (McAlindon *et al.* 2000). One recent review of 16 randomized controlled trials (RCTs) utilizing glucosamine for the treatment of osteoarthritis, reported that glucosamine is both effective and safe for the treatment of osteoarthritis (Towheed *et al.* 2001). In 13 RCTs that compared glucosamine to a placebo, glucosamine was found to be superior to placebo in 12. In the 4 RCTs in which glucosamine was compared to a non-steroidal anti-inflammatory drug (NSAID) glucosamine was found to be superior to NSAID in 2 and equivalent in 2. This review indicated that while glucosamine appears to be safe and effective for the treatment of OA, further research is needed to confirm effectiveness and toxicity. Furthermore, the effectiveness of different preparations by different manufacturers is unknown. There is, in fact, a wide variability in the dose and form of glucosamine within marketed glucosamine products (da Camara and Dowless 1998) and no study to date has compared

the effectiveness of these different preparations in a clinical trial. Another review of 17 controlled trials involving glucosamine reported poor study quality scores (McAlindon *et al.* 2000). Combined results showed a moderate benefit for glucosamine and a large benefit for chondroitin. However, the utility of these benefit magnitudes remains unclear due to study design inconsistencies as well as dependence on industry support for funding.

One recent clinical trial has addressed some of the fundamental flaws of earlier trials by using a larger number of patients over a longer trial period (Reginster *et al.* 2001). This trial utilized 212 patients, dividing them equally and randomly into placebo-treated and glucosamine-treated groups. Investigators were blinded to treatment assignments. Patients were allowed access to NSAID medication as needed for rescue analgesia with a washout period of at least 5 half-lives before symptom assessment. Glucosamine was administered as a once daily oral dose of 1500 mg for 3 years. Symptoms were scored on the Western Ontario and McMaster Universities (WOMAC) index and mean joint-space of the medial femorotibial joint was assessed by digital image analysis. Patients were examined upon enrolment, at 1 year, and at 3 years. Patients in the glucosamine treatment group showed a significant improvement in WOMAC index scores compared to a slight worsening of index scores in placebo-treated patients. Mean joint-space width of the medial femorotibial joint in glucosamine-treated patients showed no significant decrease while placebo-treated patients exhibited progressive joint-space narrowing. The authors of this study concluded from the results that glucosamine could be a disease-modifying agent in osteoarthritis.

Overall, clinical trial data support the clinical use of glucosamine for treatment of OA. In general, analgesic effects take much longer than those of NSAIDS, however the maximal effect may be greater. Additionally, where NSAIDS do not appear to prevent the progression of disease indices such as joint space narrowing, glucosamine has a beneficial effect. These findings support glucosamine as a chondroprotective substance that provides symptomatic relief with long-term use.

Glucosamine Side Effects

There is no currently established LD_{50} for glucosamine since no mortality has been observed in laboratory animals, even at the extremely high doses of 5000 mg/kg after oral administration (Kelly 1998). This is well below the dose of 500 mg orally three times daily that has been used in most human clinical studies (Barclay *et al.* 1998). Glucosamine has been administered to arthritic human patients with a variety of diseases such as cardiovascular disease, liver disease, diabetes, pulmonary disease, and depression with no adverse effects on the course of the disease or interference with the pharmacologic treatments of these diseases (Kelly 1998). In comparison to NSAID therapy for osteoarthritis, glucosamine has been reported to have a lower incidence of side effects (Wright 2001). Adverse side effects when they do occur are generally related to the gastrointestinal system (mild abdominal pain, nausea, diarrhea, constipation) (Kelly 1998, Reginster *et al.* 2001). Individual reports of other side effects such as immediate hypersensitivity reactions, photosensitization, moderate systolic hypertension, proteinuria, and elevated creatine phosphokinase levels in blood have also been reported (Matheu *et al.* 1999, Danao-Camara 2000).

Glucosamine Mechanisms of Action: Research to Date

Proteoglycan production is stimulated by exogenous glucosamine in human chondrocyte cultures maintained in a 3-dimensional culture system (Bassleer *et al.* 1998). The chondrocytes were collected from osteoarthritic femoral head cartilage and maintained in DMEM media for up to 16 days in a gyratory shaker under standard culture conditions. This effect was noted for glucosamine concentrations of 10 μg/ml and 100 μg/ml, but was not demonstrated at 1 μg/ml. Additionally, the stimulatory effect of 100 μg/ml glucosamine was still present 4 days after removal of glucosamine from the culture medium.

Glucosamine has also been shown to inhibit nitric oxide production at 2.5 and 25.0 mg/ml in equine antebrachiocarpal and middle carpal explant cultures (Fenton *et al.* 2000 a,b). Cultures were treated with either LPS (10 μg/ml) or rhIL-1β (50 ng/ml). Nitric oxide production was inhibited by glucosamine at 0.1, 0.5, 1, and 2 mM in cultured rat macrophages stimulated with 1 μg/ml LPS (Meininger *et al.* 2000). Glucosamine (4.5 g/l) has also been shown to inhibit nitric oxide production in IL-1β (25 and 250 units/ml) stimulated rat chondrocytes grown in monolayer culture (Gouze *et al.*2001). Nitric oxide is an important mediator of OA progression. Nitric oxide, produced by equine chondrocytes in response to lipopolysaccharide and interleukin-1 (Frean *et al.* 1997, Fenton *et al.* 2000a), decreases proteoglycan synthesis, and may also up-regulate activation of MMPs (Taskiran *et al.* 1994, Murrell *et al.* 1995). Osteoarthritic cartilage shows enhanced production of nitric oxide (Del Carlo and Loeser 2002). Despite these findings, the role of nitric oxide in the pathogenesis of osteoarthritis is not fully

characterized. NO has been shown to acutely prevent bovine chondrocyte catabolism when exposed to 20 U/ml rhIL-1 (Stefanovic-Racic *et al.* 1996), and chondrocyte cell death in human articular chondrocytes grown in alginate bead and monolayer cultures has been shown to require both reactive oxygen species and NO (Del Carlo *et al.* 2002).

Beta-1,3-glucuronosyltransferase I is a key biosynthetic enzyme in glycosaminoglycan synthesis. Glucosamine at 2 and 4.5 mg/ml prevented the repression of beta-1,3-glucuronosyltransferase I expression in rat articular chondrocyte monolayer cultures stimulated with IL-1β (Gouze *et al.* 2001). Glucosamine at 1 mg/ml was not effective in preventing repression of this enzyme. Glucosamine at 2 and 4.5 mg/ml also increased proteoglycan synthesis in rat articular chondrocytes cultured in alginate bead suspension and stimulated with IL-1β. The same study showed that 4.5 mg/ml glucosamine prevented the up-regulation of MMP-3 mRNA expression by interleukin-1β stimulated rat chondrocytes grown in monolayer culture.

Glucosamine (0.25, 2.5, and 25.0 mg/ml) inhibited cartilage proteoglycan loss and gelatinase/collagenase activities in equine cartilage explants stimulated with LPS (10 μg/ml) or rhIL-1β (Fenton *et al.* 2000 a,b). These effects were shown for both glucosamine HCl and for glucosamine-3-sulfate, but reportedly not for N-acetyl-glucosamine (Fenton *et al.* 2000b).

These activities support the role of glucosamine as a chondroprotective agent and support further investigation into its actions. Induction of chondrocyte proteoglycan production, prevention of cartilage proteoglycan loss, inhibition of NO production, inhibition of gelatinase/collagenase activity, inhibition of MMP production, and

protection of GAG-synthesis enzymes are all mechanisms which may be responsible for the observed chondroprotective properties of exogenous glucosamine.

In Vitro Chondrocyte Culture Systems

In vitro, chondrocytes demand high seeding density and minimal cell-substratum interactions in order to maintain their chondrocytic phenotype. Cell culture systems designed to meet these goals have included cartilage explants, monolayer cultures, cell suspension cultures, agarose gel cultures, and pellet cultures (Solursh 1991, Stewart *et al.* 2000). Cartilage explants maintain the local ECM by transferring an entire island of cartilage to culture without further processing. Suspension cultures rely on constant agitation to maintain isolated chondrocytes in a three-dimensional organization. Agarose gel and alginate bead cultures use a substrate to maintain isolated chondrocytes in a three-dimensional organization.

Monolayer cultures maintain isolated chondrocytes in a single confluent layer. However, cells eventually dedifferentiate from the chondrocytic to a fibroblastic cell phenotype as they lose collagen type II and aggrecan expression, and begin to express collagen type I (Stewart *et al.* 2000).

Monolayer cultures have been used repeatedly in the study of chondrocyte metabolism. Recent experiments in cartilage tissue engineering have also relied on *in vitro* monolayer culture propagation of chondrocytes prior to implantation *in vivo*. Recently, rabbit autologous chondrocytes have been cultured in monolayer cultivation systems under standard cell culture conditions (37 C, 5% CO_2) and used for chondral defect resurfacing (Bacenkova *et al.* 2001). Monolayer cultures have also been used in

the experimental infection of rabbit, sheep, cattle, and human chondrocytes with cell markers for the purposes of cell tracking after knee joint chondral defect repair (Hirschmann *et al.* 2002). First-passage porcine chondrocytes grown in monolayer have been shown to produce cartilage *in vivo* after transplantation (Passaretti *et al.* 2001).

Despite their utility, monolayer cultures of chondrocytes can be phenotypically unstable. Cells grown in monolayer culture over long periods of time begin to express collagen type I with decreasing collagen type II and aggrecan expression (Velikonja *et al.* 2001). Recently, cartilage oligomeric matrix protein and collagen type IX down-regulation have been identified as earlier phenotypic markers of chondrocyte dedifferentiation (Zaucke *et al.* 2001). Differences in collagen type II expression have been attributed to a decrease in transcription factor Sp1 binding activity in dedifferentiated chondrocytes (Dharmavaram *et al.* 1997).

Pellet cultures are formed by gentle centrifugation of isolated chondrocytes within culture tubes. Pellet cultures allow chondrocytes to form an extracellular matrix (Xu *et al.* 1996) and are easily adaptable to laboratory protocols requiring a high number of replicates with closely controlled cell numbers in each replicate.

Pellet cultures may be used to maintain cartilage-like appearance and normal metabolism of chondrocytes (Solursh 1991). Extracellular matrix gene expression profiles of pelleted horse chondrocytes have been shown to be similar to tissue levels. This includes similar expression of collagen type II, collagen type I, and aggrecan (Stewart *et al.* 2000).

Lipopolysaccharide in In Vitro Osteoarthritis Models

Several models have been used to recreate aspects of osteoarthritis in *in vitro* cartilage systems. Two popular methods of inducing an OA-like state is the use of either bacterial lipopolysaccharide (LPS) or interleukin-1 of animal or human origin. Bacterial lipopolysaccharide and recombinant human interleukin-1 initiate similar metabolic responses in cultured equine chondrocytes (Fenton *et al.* 2000 a,b). LPS is efficient in the induction of cartilage damage and is at least 10 times more potent in horses than most other mammals (MacDonald and Benton 1996). In addition to mimicking septic arthritis and reactive arthritis conditions, LPS is a useful and economical model for the study of osteoarthritis.

LPS mediates IL-1 expression by chondrocytes (MacDonald and Benton 1996), which in turn stimulates expression of MMPs associated with osteoarthritis (Fenton *et al.* 2000 a,b). Mammalian cells respond to LPS stimulation by releasing several inflammatory cytokines including TNF-α, IL-6, and IL-1β (Ulevitch *et al.* 1995), initiating conditions similar to those encountered in OA cartilage such as enhanced MMP activity, proteoglycan release, and nitric oxide production (Fenton *et al.* 2000b). Mammalian cells respond to pathogen-associated molecules through a family of Toll-like receptors (Taro *et al.* 2001, Axtelle and Pribble 2001). *E.coli* LPS, which was used in this study, signals through the Toll-like receptor 4 (TLR4) complex (Pulendran *et al.* 2001). This receptor includes an intracellular region belonging to the IL-1R family and therefore uses the same signaling pathways as IL-1 (Bowie and O'Neill 2000). In light of these studies LPS is an appropriate stimulus for the induction of chondrocyte responses similar to those observed in OA.

References

Abramson S, Korchak H, Ludewig R, *et al.* Modes of action of aspirin-like drugs. Proc Natl Acad Sci USA 1985;82:7227-7231.

Aigner T, McKenna L. Molecular pathology and pathobiology of osteoarthritic cartilage. Cell Mol Life Sci 2002;59:5-18.

Akizuki S, Mow VC, Muller F, et al. Tensile properties of human knee joint cartilage: II. Correlations between weight bearing and tissue pathology and the kinetics of swelling. J Orthop Res 1987;5:173-186.

Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer RW, Schultz GS, Ferguson MW. Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. Cell Tissue Res 1997;290:581-591.

Axtelle T, Pribble J. IC14, a CD14 specific monoclonal antibody, is a potential treatment for patients with severe sepsis. J Endotoxin Res 2001;7:310-314.

Bacenkova D, Rosocha J, Svihla R, Vasko G, Bodnar J. Repair of chondral defects of the knee using a combination of autologous chondrocytes and osteochondral allograft- an animal model. Part I: in vitro culture of autologous chondrocytes. Acta Chir Orthop Traumatol Cech 2001;68:363-368.

Baker AH, George SJ, Zaltsman AB, Murphy G, Newby AC. Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. Br J Cancer 1999;79:1347-1355.

Barclay TS, Tsourounis C, McCart GM. Glucosamine. Ann Pharm 1998;32:574-579.

Barnes PM, Addock I. Antiinflammatory actions of steroids: Molecular mechanisms. Trends Pharmacol Sci 1993;14:436.

Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-434.

Beekman B, Verzijl N, de Roos JA, TeKoppele JM. Matrix degradation by chondrocytes cultured in alginate: IL-1 beta induces proteoglycan degradation and proMMP synthesis but does not result in collagen degradation. Osteoarthritis Cartilage 1998;6:330-340.

Bertaux B, Hornebeck W, Eisen AZ, Dubertret L. Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. J Invest Dermatol 1991;97:679-685.

Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne A, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chen J, Van Wart H, Poole AR. Enhanced cleavage of type II collagen by collagenase in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534-1545.

Blot L, Marcelis A, Devogelaer JP, Manicourt DH. Effects of diclofenac, aceclofenac and meloxicam on the metabolism of proteoglycans and hyaluronan in osteoarthritic human cartilage. Br J Pharmacol 2000;131:1413-1421.

Bowie A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for proinflammatory interleukins and microbial products. J Leukocyte Biol 2000; 67:508.

Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophysi Acta 2000;1477:267-283.

Broverman RL, Nguyen KH, da Silveira A, Brinkley LL, Macauley SP, Zeng T, Yamamoto H, Tarnuzzer RW, Schultz GS, Kerr M, Humphreys-Beher MG. Changes in the expression of extracellular matrix (ECM) and matrix metalloproteinases (MMP) of proliferating rat parotid acinar cells. J Dent Res 1998;77:1504-1514.

Burton-Wurster N, Farese J, Todhunter R, Lust G. Site-specific variation in femoral head cartilage composition in dogs at high and low risk for development of osteoarthritis: insights into cartilage degeneration. Osteoarthritis Cartilage 1999;7:486-497.

Carnuccio R, Di Rosa M, Guerrasio B, *et al.* Vasocortin: A novel glucocorticoid-induced anti-inflammatory protein. Br J Pharmacol 1987;90:443.

Cawston T, Billington C, Cleaver C, Elliott S, Hui W, Koshy P, *et al.* The regulation of MMPs and TIMPs in cartilage turnover. Ann NY Acad Sci 1999;878:120-129.

Chavira R Jr, Burnett TJ, Hageman JH. Assaying proteinases with azocoll. Analytical Biochemistry 1984;136:446-450.

Chrousos GP, Detera-Wadleigh SD, Karl M: Syndromes of glucocorticoid resistance. Ann Intern Med 1993;119:1113.

Clements PJ, Harold EP. Nonsteroidal Antirheumatic Drugs. In Textbook of Rheumatology, 5th edn. Kelley WN, Harris ED, Ruddy S, Sledge C, Eds. WB Saunders, Philadelphia, 1997;pp.707-740.

Cole AA, Kuettner KE. Molecular basis for differences between human joints. Cell Mol Life Sci 2002;59:19-26.

da Camara CC, Dowless GV. Glucosamine sulfate for osteoarthritis. Rheumatology 1998; 32:580-587.

Dahlberg L, Billinghurst RC, Manner P, Nelson F, Webb G, Ionescu M, Reiner A, Tanzer M, Zukor D, Chen J, Van Wart HE. Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase I (matrix metalloproteinase I). Arthritis Rheum 2000;43:673-682.

Danao-Camara T. Potential side effects of treatment with glucosamine and chondroitin. Arthritis Rheum 2000;43:2853.

Dawson W, Boot JR, Harvey J, Walker JR. The pharmacology of benoxaprofen with particular reference to effects on lipoxygenase product formation. Eur J Rheumatol Inflamm 1982;5:61-68.

Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF Jr. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J Clin Invest 1989;84:678-685.

Del Carlo M Jr, Loeser RF. Nitric oxide-mediated chondrocyte cell death requires the generation of additional reactive oxygen species. Arthritis Rheum 2002;46:394-403.

Dharmavaram RM, Liu G, Mowers SD, Jimenez SA. Detection and characterization of SP1 binding activity in human chondrocytes and its alterations guring chondrocyte dedifferentiation. J Biol Chem 1997;272:26918-26925.

Du J, Adebowale A, Liang Z, Eddington ND. Bioavailability and disposition of the dietary supplements, FCHG49 glucosamine and TRH122 chondroitin sulfate in dogs after single and multiple dosing. Amer Assoc Pharm Scientists Proc 2001.

Fehr JE, Trotter GW, Oxford JT, Hart DA. Comparison of Northern blot hybridization and a reverse transcriptase-polymerase chain reaction technique for measurement of mRNA expression of metalloproteinases and matrix compounds in articular cartilage and synovial membrane from horses with osteoarthritis. Am J Vet Res 2000;61:900-905.

Felson DT. Epidemiology of the Rheumatic Diseases. In Arthritis and Allied Conditions: A Textbook of Rheumatology, 13th ed. Koopman WJ, Ed. Williams and Wilkins, Baltimore, Maryland 1997; pp 3-34.

Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-265.

Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-451.

Forrester JV, Wilkinson PC. Inhibition of leukocyte locomotion by hyaluronic acid. J Cell Sci 1981;48:315-330.

Frean S, Bryant C, Froling I, Elliott J, Lees P. Nitric oxide production by equine articular cells in vitro. Equine Vet J 1997;29:98-102.

Ghosh P. Vascular mechanisms in osteoarthritis. Best Pract Res Clin Rheum 2001;15:693-710.

Goldring MB. The role of the chondrocyte in osteoarthritis. Arthritis Rheum 2000;43:1916-1926.

Gouze J, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, Fournel-Gigleux S, Ouzzine M. Interleukin-1 beta down-regulates the expression of glucuronyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis. Arthritis Rheum 2001;44:351-360.

Han Z, Chang L, Yamanishi Y, Karin M, Firestein GS. Joint damage and inflammation in c-Jun N-terminal kinase 2 knockout mice with passive murine collagen-induced arthritis. Arthritis Rheum 2002;46:818-823.

Hawker G. Update on the epidemiology of the rheumatic diseases. Curr Opin Rheumatol 1997;9:90-94.

Hirschmann F, Verhoeyen E, Wirth D, Bauwens S, Hauser H, Rudert M. Vital marking of articular chondrocytes by retroviral infection using green fluorescent protein. Osteoarthritis Cartilage 2002;10:109-118.

Howard RD, McIlwraith CW. Hyaluronan and its use in the treatment of equine joint disease. In Joint Disease In the Horse, McIlwraith and Trotter, eds. WB Saunders, Philadelphia 1996;pp 257-269.

Jeffrey JJ. Interstitial collagenases. In Matrix Metalloproteinases, Parks WC and Mecham RP, Eds. Academic Press, 1998.

Kelly GS. The role of glucosamine sulfate and chondroitin sulfates in the treatment of degenerative joint disease. Alt Med Review 1998;3:27-39.

Kempson GE, Freeman MAR, Swanson SAV. The determination of a creep modulus for articular cartilage from indentation tests of the human femoral head. J Biomech 1971;4:239-250.

Kempson GE, Muir H, Pollard C, Tuke M. The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. Biochim Biophys Acta 1973;297:456-472.

Kerin A, Patwari P, Kuettner K, Cole A, Grodzinsky A. Molecular basis of osteoarthritis: biomechanical aspects. Cell Mol Life Sci 2002;59:27-35.

Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. J Biol Chem 1996;271:1544-1550.

Ko R, Okano K, Maeda S. Structural and functional analysis of the Xestia c-nigrum granulovirus matrix metalloproteinase. J Virol 2000 74:11240-11246.

Liang Z, Leslie J, Adebowale A, Ashraf M, Eddington ND. Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatives with ultraviolet HPLC. J Pharm Biomed Analysis 1999;20:807-814.

Lein M, Nowak L, Jung K, Koenig F, Lichtinghagen R, Schnorr D, Loening SA. Analytical aspects regarding the measurement of metalloproteinases and their inhibitors in blood. Clin Biochem 1997;30:491-496.

Little C, Ghosh P. Potential use of pentosan polysulfate for the treatment of equine joint disease. In Joint Disease In the Horse, McIlwraith and Trotter, eds. WB Saunders, Philadelphia 1996;pp 281-291.

Lozada CJ, Altman RD. Management of Osteoarthritis. In: Arthritis and Allied Conditions: A Textbook of Rheumatology, 13th edn. Koopman W, Ed. Williams and Wilkins, Baltimore, Maryland, 1997;pp 2013-2025.

MacDonald MH, Benton HP. Cellular responses and receptor mechanisms in joint disease: a bacterial lipopolysaccharide-induced model of articular damage. In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 447-467.

Mankin HJ, Radin EL. Structure and function of joints. In Arthritis and Allied Conditions: A Textbook of Rheumatology, WJ Koopman, ed. Williams and Wilkins, Baltimore 1997.

Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. J Bone Joint Surg 1971;53A:523-537.

Maquoi E, Munaut C, Colige A, Lambert C, Frankenne F, Noel A, Grams F, Krell HW, Foidart JM. Stimulation of matrix-metalloproteinase-9 expression in human fibrosarcoma cells by synthetic matrix metalloproteinase inhibitors. Exp Cell Res 2002;275:110-121.

Maroudas A: Metabolism of cartilagenous tissues: A quantitative approach. In Studies In Joint Disease, vol. 1 Maroudas A, Holborow EJ, eds. Tunbridge Wells, England, Pitman, 1980;pp 59-86.

Martel-Pelletier J, Welsch D, Pelletier J-P. Metalloproteases and inhibitors in arthritic diseases. Best Pract Res Clin Rheum 2001;15:805-829.

Matheu V, Gracia Bara MT, Pelta R, Vivas E, Rubio M. Immediate-hypersensitivity reaction to glucosamine sulfate. Allergy 1999;54:643.

May SA, Lees P. Nonsteroidal anti-inflammatory drugs. In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 223-236.

Mayne R, Brewton RG. Extracellular matrix of cartilage: Collagen. In Joint Cartilage Degradation: Basic and Clinical Aspects. Woessner JF, Howell DS, eds. Marcel Dekker, Inc. New York 1993;p 82.

McAlindon TE, LaValley MP, Gulin GP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA 2000;283:1469-75.

McCarty MF. Enhanced synovial production of hyaluronic acid may explain rapid clinical response to high-dose glucosamine in osteoarthritis. Med Hypoth 1998;50:507-510.

McCarty MF, Russell AL, Seed MP. Sulfated glycosaminoglycans and glucosamine may synergize in promoting synovial hyaluronic acid synthesis. Med Hypoth 2000;54:798-802.

McIlwraith, CW. General pathobiology of the joint and response to injury. In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 40-69.

McIlwraith CW, Trotter GW, eds. Joint Disease in the Horse. 1996; WB Saunders, Philadelphia.

Meininger CJ, Kelly KA, Li H, Haynes TE, Wu G. Glucosamine inhibits inducible nitric oxide synthase. Biochem Biophys Res Commun 2000;279:234-239.

Milner JM, Elliott SF, Cawston TE. Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways. Arthritis Rheum 2001;44:2084-2096.

Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 1996;97:761-8.

Murphy AN, Unsworth EJ, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. J Cell Physiol 1993;157:351-358.

Murphy G, Houbrechts A, Cockett MI, Williamson RA, O'Shea M, Docherty AJ. The N-terminal domain of tissue inhibitor of metalloproteinases retains metalloproteinase inhibitory activity. Biochemistry 1991;30:8097-8102.

Murphy G, Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymol 1995;248:496-510.

Murrell G, Jang D, Williams R. Nitric oxide activates metalloproteinase enzymes in articular cartilage. Biochem Biophys Res Commun 1995;206: 15-21.

Nagase H, Enghild JJ, Suzuki K, Salvesen G. Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acid. Biochemistry 1990;29:5783-5789.

Nagase H. Stromelysins 1 and 2. In: Matrix Metalloproteinases, Parks WC and Mechum RP, eds. Academic Press, San Diego1998.

Nagase H, Woessner J Jr.. Matrix Metalloproteinases. Journal of Biological Chemistry 1999; 274:21491-21494.

Ogston AG, Sherman TF. Effects of hyaluronic acid upon diffusion of solutes and flow of solvent. J Physiology 1961;156:67-74.

Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G, Nagase H. Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur J Biochem 1990;194:721-30.

Palmer JL, Bertone AL. Joint biomechanics in the pathogenesis of traumatic arthritis. In: Joint Disease in the Horse, Mc Ilwraith CW and Trotter GW, eds.;WB Saunders, Philadelphia 1996;pp 104-119.

Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, Yaremchuk M. Cultured chondrocytes produce inectable tissue-engineered cartilage in hydrogel polymer. Tissue Eng 2001;7:805-815.

Pavloff N, Staskus PW, Kishnani NS, Hawkes SP. A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. J Biol Chem 1992;267:17321-17326.

Poole AR. Cartilage in health and disease. In Arthritis and Allied Conditions: a Textbook of Rheumatology. Koopman WJ, ed.. Baltimore: Williams and Wilkins; 1997;pp. 255-308.

Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA. Elevated matrix metalloproteinase and angiotensin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. PNAS 2000;97:2202-2207.

Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. J Immunol 2001;167:5067-5076.

Rabbani SA, Harakidas P, Guo Y, Steinman D, Davidsen SK, Morgan DW. Synthetic inhibitor of matrix metalloproteinases decreases tumor growth and metastases in a syngeneic model of rat prostate cancer *in vivo*. Int J Cancer 2000;87:276-282.

Radin El, Burr DB, Caterson B, Fyhrie D, Brown TD, Boyd RD. Mechanical determinants of osteoarthritis. Semin Arthritis Rheum 1991;21:12-21.

Radomski MW, Palmer RMJ, Moncada S *et al.* Glucocorticoids inhibit expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci USA 1990;87:10043

Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, Giacovelli G, Henrotin Y, Dacre JE, Gossett C. Long-term effects of glucosamine sulphate on osteoarthritis progression: A randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-256.

Roberts S, Weightman B, Urban J, Chappel. Mechanical and biochemical properties of human articular cartilage in osteoarthritic femoral heads and in autopsy specimens. J Bone Joint Surg 1986;68B:278-288.

Rossdale PD, Hopes R, Digby NJ, Offord K. Epidemiological study of wastage among racehorses, 1982 and 1983. Vet Rec 1985;116:66-69.

Sato H, Takahashi T, Ide H. Antioxidant activity of synovial fluid, hyaluronic acid, and two subcomponents of hyaluronic acid. Arthritis Rheum 1988;31:63-71.

Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.

Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.

Sevalla K, Todhunter RJ, Vernier-Singer M, Budsberg SC. Effect of polyslfated glycosaminoglycan on DNA content and proteoglycan metabolism in normal and osteoarthritic canine articular cartilage explants. Vet Surg 2000;29:407-414.

Schleimer RP: An overview of glucocorticoid anti-inflammatory actions. Eur J Clin Pharmacol 1993;45 (Suppl 1):S3.

Simkin PA. Synovial Physiology. In: Arthritis and Allied Conditions: A Textbook of Rheumatology, WJ Koopman, ed. Williams and Wilkins, Baltimore 1997.

Simon SR, Radin EL. Biomechanics of Joints. In: Textbook of Rhematology, 5th edn, WN Kelley, ED Harris, S Ruddy, CB Sledge, eds. WB Saunders, Philadelphia 1997.

Sokoloff L. Aging and degenerative diseases affecting cartilage. In. Cartilage Vol. 3. Hall BK, ed. New York: Academic Press, 1987:110-141.

Solursh M. Formation of cartilage tissue in vitro. J Cell Biochem 1991;45:258-260.

Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH. The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. J Immunol 1996;156:1213-1220.

Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J Bone Min Res 2000;15:166-174.

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-Kda type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem 1995;270:5331-5338.

Swann DA, Radin EL, Nazimiec M, Weissner PA, Curran N, Lewinnek G. Role of hyaluronic acid in joint lubrication. Ann Rheum Dis 1974;33:318-326.

Taro K, Osamu T, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S. Lipopolysaccharide stimulates the MyD88-Independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 2001;167:5887-5894.

Taskiran D, Stefanovic-Racic M, Georgescu M, Evans C. Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. Biochem Biophys Res Commun 1994;200:142-148.

Todhunter RJ, Lust G. Pathophysiology of synovitis: Clinical signs and examination in horses. Compend Cont Educ Pract Vet 1990;12:980-992.

Todhunter RJ. Anatomy and physiology of synovial joints. In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 1-28.

Todhunter RJ, Wootton JAM, Lust G, et al. Structure of equine type I and type II collagen. Am J Vet Res 1994;38:425-431.

Towheed TE, Anastassiades TP, Shea B, Houpt J, Welch V, Hochberg MC. Glucosamine therapy for treating osteoarthritis (Cochrane Review). Cochrane Database Syst Rev 2001;1:CD002946.

Trengove NJ, Stacey MC, MacAuley S, Bennett N, Gibson J, Burslem F, Murphy G, Schultz G. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 1999;7:442-452.

Trotter GW. Intra-articular corticosteroids. In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 237-256.

Trotter GW. Polysulfated glycosaminoglycan (Adequan). In Joint Disease In the Horse, McIlwraith CW and Trotter GW, eds. WB Saunders, Philadelphia 1996;pp 270-280.

Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995;13:437.

Vachova L, Moracova . Two microassays for determination of a wide range of proteolytic activities using Azocoll as a substrate. Biochem Mol Biol Int 1993;30:311-318.

van Lent PL, Holthuysen AE, Sloetjes A, Lubberts E, van Den Berg WB. Local overexpression of adeno-viral IL-4 protects cartilage from metalloproteinase-induced destruction during immune-complex-mediated arthritis by preventing activation of pre-MMPs. Osteoarthritis Cartilage 2002;10:234-243.

Velikonja NK, Wozniak G, Malicev E, Knezevic M, Jeras M. Protein synthesis of human articular chondrocytes cultured in vitro for autologous transplantation. Pflugers Arch 2001;442(Suppl 1):R169-170.

Vu TH, Werb Z. Matrix metalloproteinases:effectors of development and normal physiology. Genes Development 2000;14:2123-2133.

Walsh DA, Sledeg CB, Blake DR. Biology of the normal joint. In Textbook of Rheumatology, 5th ed. Kelley WN, Harris ED Jr., Ruddy S, Sledge CB, Eds. Philadelphia, PA: WB Saunders 1997.

Williamson RA, Marston FA, Angal S, Koklitis P, Panico M, Morris HR, Carne AF, Smith BJ, Harris TJ, Freedman RB. Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP). Biochem J 1990;268:267-274.

Wirth CR, Argello FA, Mow VC, Roth V. Variation of tensile properties of human patellar cartilage with age and histological indices. Orthop Trans 1980;4:177-178.

Wright IM. Oral supplements in the treatment and prevention of joint diseases: a review of their potential application to the horse. Eq Vet Educ 2001;13:135-139.

Xu C, Oyajobi OB, Frazer A, Kozaci LD, Russell RGG, Hollander AP. Effects of growth factors and interleukin-1 alpha on proteoglycan and type II collagen turnover in bovine nasal and articular chondrocyte pellet cultures. Endocrinology 1996;137:3557-3565.

Yang TT, Hawkes SP. Role of the 21-Kda protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. Proc Natl Acad Sci USA 1992;89:10676-10680.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M. Transcriptional interference between c-jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 1990 62:1205.

Yasuda T, Poole AR. A fibronectin fragment induces type II collagen degradation by collagenase through an interleukin-1-mediated pathway. Arthritis Rheum 2002;46:138-148.

Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, Okada Y. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis 2000;59:455-461.

Young PK, Grinnell F. Metalloproteinase activation cascade after burn injury: a longitudinal analysis of the human wound environment. J Invest Derm 1994;103:660-664.

Zaucke F, Dinser R, Maurer P, Paulsson M. Cartilage oligomeric matrix protein (COMP) and collagen type IX are sensitive markers for the differentiation state of articular primary chondrocytes. Biochem J 2001;358(Pt 1):17-24.

Chapter 2

INFLUENCE OF GLUCOSAMINE ON MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY IN LPS-STIMULATED EQUINE CHONDROCYTES.

Summary

Objective: To characterize potential mechanisms of action of glucosamine inhibition of MMP expression and activity in LPS-stimulated equine chondrocytes.

Design: The influence of glucosamine and suitable controls on MMP activity in conditioned medium from LPS-stimulated cartilage explants was determined by a colorimetric assay using azocoll as a substrate. Treatments consisted of negative and positive controls, glucose (50 mM), and glucosamine (50 mM, 25 mM, 6.25 mM, 3 mM, and 1.5 mM). The influence of glucosamine on MMP synthesis was determined using equine chondrocytes in pellet culture incubated with LPS (20 μg/ml). MMP 13 was quantified in spent medium using a commercial ELISA, and non-specific MMP activity was determined using azocoll digestion in organomercurial-activated medium. The effects of glucosamine on MMP mRNA levels in similarly treated equine chondrocytes was determined by Northern blotting using MMP-1, -3, and -13 probes. Statistical analysis was carried out with two-way ANOVA, and P was set at 0.05.

Results: Glucosamine had no effect on activated MMP activity. Glucosamine inhibited MMP protein expression, as determined by azocoll digestion at 3 to 50 mM, and as determined by MMP-13 ELISA at 1.5 to 50 mM. Resting mRNA levels for MMP-1, -3,

and -13 mRNA were significantly lower in cultures exposed to glucosamine at concentrations of 50 mM and 25 mM.

Conclusion: These results indicate that glucosamine is capable of pre-translational, and possibly also translational, regulation of MMP expression and provide a potential mechanism of action for previously reported chondroprotective effects of this aminomonosaccharide.

Introduction

Osteoarthritis (OA) is a well-known and expensive problem that causes substantial morbidity in the form of lameness and may seriously curtail quality of life in addition to athletic serviceability of affected horses (Rossdale *et al.* 1985, Todhunter and Lust 1990). It can be initiated by a number of causes and ultimately affects all articular tissues. The hallmark of the disease is the degeneration of articular cartilage, a highly specialized tissue consisting of relatively few cells but having a rich extracellular matrix. Although there are a number of possible causes of matrix degradation in OA, abundant experimental evidence suggests that proteolytic degradation of cartilage by matrix metalloproteinases (MMPs) is a predominant event (McIlwraith 1996, Goldring 2000).

Matrix metalloproteinases (MMPs, matrixins) are a family of enzymes that degrade the extracellular matrix of tissues. There are several subgroups of MMPs based on substrate preference and structural domains including collagenases, gelatinases, and stromelysins, which are active in OA (Vu and Werb 2000, Nagase and Woessner 1999, Pozzi *et al.* 2000, Rabbani *et al.* 2000). Since collagen damage is an important initial

event in OA (McIlwraith 1996, Goldring 2000, Roberts *et al.* 1986), MMPs, particularly collagenases, may serve as a pivotal component of OA initiation and progression. Another MMP, stromelysin (MMP-3) is also important in that it not only degrades non-collagenous extracellular matrix components, including the principal aggregating proteoglycan of cartilage (aggrecan), but also serves to activate latent collagenases (Goldring 2000).

Oral administration of glucosamine as a symptomatic treatment of OA has been the subject of several clinical studies in human patients. One recent review of 16 randomized controlled clinical trials utilizing glucosamine suggested that glucosamine is both an effective and safe treatment for the disease (Towheed *et al.* 2001). When glucosamine was compared to an NSAID (ibuprofen), it was equally effective in two trials and superior in two other trials. While ibuprofen effects a more rapid decrease in patient pain scores, oral glucosamine therapy yields an ultimately lower pain score after eight weeks of treatment (Barclay *et al.* 1998). A chondroprotective effect has been supported by data indicating that glucosamine prevents knee joint space loss in OA patients over the course of a three-year study (Reginster *et al.* 2001).

Certain effects of glucosamine on *in vitro* cellular metabolism have received recent attention. Proteoglycan production is stimulated by exogenous glucosamine in human chondrocyte cultures (Bassleer *et al.* 1998) and glucosamine prevents the repression of beta-1,3-glucuronosyltransferase I, a key biosynthetic enzyme in glycosaminoglycan synthesis. Importantly, glucosamine prevents the up-regulation of MMP-3 mRNA expression by interleukin-1 beta stimulated rat chondrocytes (Gouze *et al.* 2001). While glucosamine inhibits proteoglycan loss, inducible nitric oxide synthase,

and MMP activity in equine cartilage explants stimulated with LPS or human and equine recombinant interleukin-1 (Fenton *et al.* 2000a,b), the specific level(s) at which these effects occur require further characterization.

The purpose of the study presented here was to determine the nature of the inhibition of MMP activity attributed to glucosamine HCl in an *in vitro* model of OA. The specific aims were to test the hypothesis that glucosamine HCl influences 1) the catalytic activity of chondrocyte MMPs, 2) synthesis of MMPs at the protein level, and 3) resting MMP-1, MMP-3, and MMP-13 mRNA levels in LPS-stimulated equine chondrocytes.

Materials and methods

Tissue Sources

Grossly normal metacarpophalangeal articular cartilage was obtained from horses between 2 and 8 years of age that had died or were euthanized for reasons other than joint disease. Cartilage was dissected from the subchondral bone and incubated in penicillin (500 U/ml) and streptomycin (500 mg/ml) (25 C, 1 hour). Chondrocytes were isolated by sequential digestion with pronase and collagenase as previously described (Caron *et al.* 1996). After digestion, the cells were separated by centrifugation (300 X g, 10 minutes), washed, and re-suspended in 10 ml DMEM, with 10% FBS, penicillin, and streptomycin.

Pellet Cultures

Cell concentration was determined using a hemocytometer and aliquots of 1 X 10^6 cells were transferred to 15 ml polypropylene centrifuge tubes in 2 ml DMEM with 10%

FBS, penicillin, and streptomycin. Following centrifugation (300 X g, 5 min), pellets were incubated under standard cell culture conditions (37 C, 95% RH, 5% CO_2). Medium was exchanged 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 as described below.

Monolayer Cultures

After centrifugation and washing, high-density monolayer cultures were established in DMEM with 10% FBS, penicillin, and streptomycin under standard cell culture conditions. Experiments were performed using first-passage, high-density cultures which were deprived of serum for 3 to 5 days prior to the start of an experiment. RNA from treated monolayer chondrocytes was collected to determine relative amount of MMP RNA present as described below.

LPS-Conditioned Medium Preparation

To prepare an MMP-rich medium for subsequent MMP activity assays, metacarpophalangeal joint cartilage was placed in 50 ml polypropylene centrifuge tubes in 30 ml DMEM with 10% FBS, penicillin, and streptomycin. LPS was added at a concentration of 160 μg/ml and cartilage was incubated under standard cell culture conditions for 24 hours. LPS-conditioned medium was stored at -80 C until analysis for MMP activity by colorimetric methods as described in more detail below.

Effect Of Glucosamine On MMP Protein Synthesis

Pellet cultures were placed in 600 µl fresh DMEM with penicillin and streptomycin. Treatment media contained glucose at a concentration of 50 mM or glucosamine HCl at a concentration of 50 mM, 25 mM, 6.25 mM, 3 mM, or 1.5 mM. LPS was added at a final concentration of 20 µg/ml to all pellet cultures except negative controls. Positive controls contained LPS with no treatment. Pellet cultures were incubated under standard cell culture conditions for 24 hours with loose caps. Media was analyzed for MMP activity by azocoll digestion as well as for MMP-13 by ELISA as described below.

Quantification Of Pre-Formed MMP Activity And MMP Synthesis

Azocoll (Calbiochem, San Diego, CA) (200 mg) was placed in 10 ml reaction buffer (Tris, pH 7.4 or MES, pH 5.0), vortexed briefly, incubated at 25 C for 2 to 3 hours, filtered, and re-suspended (Chavira *et al.* 1984). The azocoll suspension was filtered through number 1 filter paper and re-suspended in 10 ml reaction buffer with vigorous agitation. Test media (200µl) was placed into centrifuge tubes along with 25 µl APMA (12 mM solution) and 100 µl azocoll suspension. 25 µl 1,10-phenanthroline (120 mM solution) was added to negative control tubes as an inhibitor of MMP activity. Reaction buffer (Tris, pH 7.4 or MES, pH 5.0) was added to a final volume of 600 µl. Treatment tube reaction buffer contained glucose at a concentration of 50 mM or glucosamine at a concentration of 50 mM, 25 mM, 6.25 mM, 3 mM, or 1.5 mM. Centrifuge tubes were capped tightly and incubated at 37 C for 72 hours. Reaction tubes were centrifuged (12,000 X g, 3 minutes) and absorbance read at 520 nm. Reactions were carried out at

pH 7.4 in order to detect total MMP activity (Okada *et al.* 1990, Nagase 1998) and at pH 5.0 in order to favor stromelysin activity (Nagase 1998).

Preparation of treatment-containing buffers consisted of weighing the appropriate amount of glucose or glucosamine for 50 ml of pre-made buffer (Tris or MES). The sugars were then dissolved in buffer at room temperature by vigorous agitation. Each treatment-containing buffer was then adjusted to proper pH (pH = 7.4 for Tris; pH = 5.0 for MES) individually and stored at 4 C.

To quantify MMP synthesis, test media (200µl) was placed into centrifuge tubes along with 25 µl APMA (12 mM solution) and 100 µl azocoll suspension. Reaction buffer (Tris, 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 X g, 3 minutes) and absorbance read at 520 nm. Reactions were carried out at pH 7.4 in order to detect total MMP activity (Okada *et al.* 1990, Nagase 1998).

MMP-13 Elisa

Stored media from pellet cultures was assayed for proMMP-13 and MMP-13 using a commercially available kit (Amersham Biosciences, Inc., Piscataway, NJ) using manufacturer's directions. Briefly, 100 µl prepared standard and test media (diluted 1:4) were incubated in assay wells coated with anti-MMP-13 antibody (2 hours, 25 C). Wells were washed with supplied phosphate buffer and incubated for 1 hour at 25 C with 100 µl anti-MMP-13 peroxidase conjugate. Wells were washed and incubated with 100 µl 3,3'5,5'-tetramethylbenzidine/hydrogen peroxide in 20% (w/v) dimethylformamide for

exactly 30 minutes at 25 C. The reaction was stopped by the addition of 1.0 M sulfuric acid and optical density measured at 450 nm.

Extraction Of Monolayer RNA

Total RNA was extracted, using a commercial extraction preparation (TriReagent, Molecular Research Center, Cincinnati, OH) and following manufacturer's instructions. Briefly, 1 ml of this agent was added to each monolayer well and incubated (25 C, 5 minutes). The solution was transferred to centrifuge tubes and 200 µl chloroform was added. The tubes were agitated vigorously and incubated at 25 C for 10 minutes. After centrifugation (4 C, 12,000 X g, 15 minutes) the aqueous phase was removed and the RNA precipitated with isopropanol. After centrifugation (4 C, 12,000 X g, 15 minutes) the pellets were washed with 75% ethanol. Pellets were resuspended in sterile water treated with 0.1% diethylpyrocarbonate.

Northern Blot Hybridization

Probe preparation was as previously described (Tung *et al.* 2002). Briefly, amplification of cDNA was accomplished, using RT-PCR with 2 µg of total RNA from a chondrocyte culture that had been stimulated (6 h) with 1 µg/ml of LPS using standard protocols. Specific sets of equine oligonucleotide primers for MMP-13 genes were synthesized based on published human cDNA sequences and were used at a final concentration of 5 pM/µl. The cDNA fragments were ligated directly to PCR 2.1 TOPO vector (TOPO TA Cloning Kit; Invitrogen) and the sequencing performed manually. The vector was subcloned into competent *E.coli* (TOPO TA Cloning Kit; Invitrogen). Clones

of MMP-1 and MMP-3 were prepared in pBluescript vector. The transformed bacteria were propagated in NZY broth at 37 C and 260 rpm in an orbital shaker overnight. The plasmid was isolated using standard protocols. Briefly, cell lysis in NET buffer/lysis solution was performed, followed by phenol/chloroform extraction and ethanol precipitation. Precipitate was re-suspended in 1 ml sterile water with 1 µl RNAse A and stored at –80 C.

Restriction endonuclease digest was carried out to free the MMP segment of interest from the plasmid. MMP-1 and MMP-3 plasmid digestion was carried out as follows: 400 µl purified plasmid, 160 µl Eco RI (20 U/µl), 240 µl Xho 1 (400 U/µl), 400 µl React-2 buffer, and 40 µl RNAse (DNAse free) were brought to a final volume of 4 ml with sterile water in a 15 ml polypropylene centrifuge tube and incubated overnight at 37 C. MMP-13 plasmid digestion was carried out as follows: 400 µl purified plasmid, 200 µl Eco RI (20 U/µl), 200 µl Hind III (10 U/µl), 400 µl React-2 buffer, and 40 µl RNAse (DNAse free) were brought to a final volume of 4 ml with sterile water in a 15 ml polypropylene centrifige tube and incubated overnight at 37 C. MMP fragments were then purified by ammonium acetate/ethanol precipitation and re-suspended in 1 ml sterile water. The MMP fragments were isolated by 1.2 % agarose gel purification, retaining the band of interest, followed by extraction using a commercial gel purification kit (Qiaquick Kit; Qiagen). MMP-1 bands were approximately 1.7 kb, MMP-3 bands were approximately 1.6 kb, and MMP-13 bands were approximately 1.3 kb. Conditions of fragment extration from agarose included solubilization in 3 volumes of gel buffer with sodium acetate and isopropanol at 50 C, followed by spin column extraction with 3 spin column tubes per gram of gel. MMP fragment DNA was eluted from the columns using

50 µl Qiagen EB buffer per tube and stored at – 80 C. Concentration was determined by running 8 µl purified fragment on a 1.2% agarose gel and estimating the amount based on band density.

MMP DNA probes were labeled by random primer labeling incorporating digoxigenin-11-UTP using a commercially available kit (DIG High Prime DNA Labeling and Detection Kit II; Roche Molecular Biochemicals, Indianapolis, Indiana). For labeling reactions, DNA was added at a concentration of 62.5 ng/µl to DIG High Prime in a 4:1 ratio. DIG-labeled probe concentration was estimated using DIG detection compared to a known pre-labeled DNA control. DIG-labeled probe was added to DIG Easy Hyb granules to obtain to appropriate concentration for northern blot hybridization.

Northern blot hybridization was conducted using a commercially available kit by following manufacturer's instructions (DIG, Roche Molecular Biochemicals, Indianapolis, IN). Briefly, total chondrocyte RNA was resolved on 1.2% agarose-formaldehyde gels, using 3 µg of RNA. After overnight transfer to nylon membranes at 25 C, RNA was cross-linked to the membranes by exposure to UV light. After prehybridization for 1 hour at 50 C the membranes were hybridized overnight at 50 C with labeled probe in DIG Easy Hyb (MMP-1 = 75 ng/ml, MMP-3 = 200 ng/ml, MMP-13 = 120 ng/ml). Serial post-hybridization washes were carried out at 25 C and 68 C, with decreasing concentrations of sodium-citrate-based buffer, followed by washing in maleate buffer at 25 C. Detection was accomplished using a chemiluminescent method with digoxigenin-11-uridyl triphosphate (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Molecular Biochemicals, Indianapolis, IN) and CSPD (3,4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo{3.3.1.1^{3,7}]decan-4-yl} phenyl

phosphate) (Roche, Indianapolis, IN) as a substrate for alkaline phosphatase conjugated to digoxigenin antibody Fab fragments. Chemiluminescence was detected by exposure to scientific imaging film (Eastman Kodak, Rochester, NY) for 10 minutes at 25 C. Developed film was scanned (Epson scanner, Adobe Photoshop) and relative MMP band intensities were analyzed using available software (Scion Image Beta 4.02, Scion Corp, Frederick, MD). Nylon membranes were stained in methylene blue (0.03% w/v methylene blue in 0.3 M sodium acetate, pH 5.2) for 45 seconds, de-stained in water for 2 minutes, and dried between sheets of filter paper. The 28S ribosomal band densities were then analyzed. 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 Analysis

Sample sizes were determined using calculations based on desired resolution of differences between treatments and expected variability in data based on previous studies. Comparison of means for the activity of MMP in conditioned medium, medium concentrations of MMP-13, and relative expression of MMPs were performed using a two-way ANOVA (blocked by horse) followed by a Student-Newman-Keuls multiple comparisons procedure. A P value < 0.05 was considered significant.

Results

Effect Of Glucosamine On MMP Activity And MMP-13 Production

Substrate digestion by pre-formed, organomercurial-activated MMP was not significantly affected by glucosamine (Fig. 1 and 2).

In pellet cultures, LPS (20 μg/ml) significantly stimulated the synthesis of MMP as determined by azocoll digestion. This effect was significantly reduced by glucosamine at all concentrations tested except the lowest (1.5 mM) (Fig. 3). This effect was paralleled by a significant reduction in MMP-13 levels at all glucosamine concentrations tested (Fig. 4).

Effect Of Glucosamine On Relative MMP-1, MMP-3 And MMP-13 mRNA Expression

LPS (20 μg/ml) significantly stimulated expression of MMP-1, MMP-3, and MMP-13. This effect was significantly reduced by glucosamine at 50 mM and 25 mM for all three MMPs (Fig. 5, Fig. 6, and Fig. 7).

Discussion

These data support the findings of prior studies suggesting chondroprotective properties of glucosamine (Fenton *et al.* 2000a,b, Sandy *et al.* 1998) and offer further information on the mechanism of action of glucosamine with respect to inhibition of MMP synthesis.

Glucose was used as a sugar moiety control for the glucosamine HCl molecule. The only difference between these two molecules is the addition of an amine group in the case of the glucosamine (Fenton *et al.* 2000a). Previous studies of a similar nature have

been conducted using glucose as a control (Fenton *et al.* 2000a,b). No effects were observed for MMP synthesis, NO levels, or proteoglycan release, indicating that the non-aminated monosaccharide is devoid of chondroprotective effects.

The glucosamine dose range used in this study correlates well with doses used in prior *in vitro* studies (Gouze *et al.* 2001, Fenton *et al.* 2000a,b, Sandy *et al.* 1998, Goldberg *et al.* 2000, Shikhman *et al.* 2001). Moreover, one *in vivo* study utilizing intravenous glucosamine infusion in rats measured circulating glucosamine concentrations within this dose range (Virkam and Yki-Jarvinen 1999). Although glucosamine is absorbed readily after oral administration and is selectively concentrated in cartilage (Setnikar *et al.* 1986), the dose range used in this study may not approximate *in vivo* levels achievable with exogenous oral glucosamine supplementation. A single 2000 mg oral dose of glucosamine in a beagle dog achieves short-term plasma concentrations between 15 and 20 μg/ml (Liang *et al.* 1999). Although ultimate cartilage concentrations of glucosamine may be higher than those in plasma due to selective concentration, they may not achieve the levels required to demonstrate *in vitro* effects. Additionally, this study utilizes a model in which glucosamine treatment and stimulus (LPS treatment) occur simultaneously. Treatment with glucosamine prior to irreversible ECM structural damage may require lower doses than those needed to mitigate severe concurrent chondrocyte stimulation. Therefore, glucosamine concentrations required *in vivo* to achieve chondroprotective effects may be much lower than those required in this *in vitro* osteoarthritis model.

Results of our experiments using azocoll digestion with APMA-activated, conditioned medium, indicate that glucosamine HCl has negligible effects on the activity

of pre-formed MMP. Matrix metalloproteinase activity assays were carried out at pH 5.0 and at pH 7.4 to favor the activity of stromelysin and total MMP activity, respectively (Okada *et al.* 1990, Nagase 1998).

The observation that glucosamine did not inhibit MMP activity at either pH tested indicates that the activity of the well-characterized MMPs are not significantly inhibited by glucosamine.

Results of MMP synthesis experiments (pellet culture medium azocoll digestion and MMP-13 ELISA) indicate that glucosamine modulates expression of MMPs at or before protein translation. This effect was significant over the dose range of 3 to 50 mM as determined by azocoll digestion assay and was mirrored by significant reductions in MMP-13 production at all five doses of glucosamine tested. ELISA kits were tested for detection of MMP-1 and MMP-3 as well, however the human antibodies did not cross-react with the equine forms of these MMPs and the kits could therefore not be validated for equine use. These results are supportive of one another, and the difference in significance at the 1.5 mM level may be explained by differences between the detection methods used or due to real differences in total MMP production compared to MMP-13 production. The data does suggest a real difference between MMP-13 levels and levels of other MMPs, as glucosamine treatment at the 1.5 mM level yielded a much larger reduction in MMP-13 content compared to positive controls (70%) than did results of the azocoll digestion assay (8%) (Figs. 3 and 4).

Relative mRNA expression of all three MMPs was significantly inhibited by glucosamine at 50 mM and 25 mM. This result indicates that glucosamine inhibits MMP production or enhances MMP degradation at a pre-translational level. 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.

Recent studies have supported the role of glucosamine in altering mRNA expression of several key enzymes involved in OA progression. Glucuronosyltransferase I, an enzyme involved in glycosaminoglycan biosynthesis is repressed by IL-1β in rat chondrocyte cultures. This phenomenon is prevented by the addition of 21 mM glucosamine to the culture medium (Gouze *et al.* 2001). Additionally, the same study reported that glucosamine prevented IL-1β-induced upregulation of MMP-3 mRNA in rat chondrocytes. Similarly, inclusion of 2 mmol/l glucosamine 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 (Goldberg *et al.* 2000). Moreover, it has recently been shown that glucosamine is capable of inhibiting IL-1 effects through inhibition of the NF κB pathway (Gouze *et al.* 2002). These studies support our finding that glucosamine HCl is capable of altering gene expression. Specifically, in this study glucosamine mitigates the LPS-induced induction of relative MMP mRNA expression in cultured equine chondrocytes.

The precise level of glucosamine action on decreased MMP mRNA production may be due to inhibition of signal transduction pathways, regulation of transcription factors, promoter efficiency, transcription itself, or post-transcriptional processing resulting in premature MMP mRNA degradation. Several studies have indicated regulation of MMP expression through the transcription factor activator protein-1 (Fahmi *et al.* 2002), Nmp4 gene splice variants (Torrungruang *et al.* 2002), and Sp family transcription factors (Liang *et al.* 2002). Additionally, Ras-responsive elements have

been demonstrated in promoter regions (Futamura *et al.* 2001), suggesting MMP expression may be regulated through a number of signaling pathways involving regulation of gene expression. It remains to be determined how glucosamine may influence these regulatory pathways.

References

Axtelle T, Pribble J. IC14, a CD14 specific monoclonal antibody, is a potential treatment for patients with severe sepsis. J Endotoxin Res 2001;7:310-314.

Barclay TS, Tsourounis C, McCarty GM. Glucosamine. Ann Pharm 1998;32:574-579.

Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-434.

Bowie A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for proinflammatory interleukins and microbial products. J Leukocyte Biol 2000;67:508.

Caron JP, Tardif G, Martel-Pelletier J, DiBattista JA, Geng C, Pelletier JP. Modulation of matrix metalloproteinase 13 (collagenase 3) gene expression in equine chondrocytes by interleukin 1 and corticosteroids. Am J Vet Res 1996;57:1631-1634.

Chavira R Jr, Burnett TJ, Hageman JH. Assaying proteinases with azocoll. Anal Biochem 1984;136:446-450.

Fahmi H, Pelletier JP, Di Battista JA, Cheung HS, Fernandes JC, Martel-Pelletier J. Peroxisome proliferator-activated receptor gamma activators inhibit MMP-1 production in human synovial fibroblasts likely by reducing the binding of the activator protein 1. Osteoarthritis Cartilage 2002;10:100-108.

Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-451.

Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-265.

Futamura M, Kamiya S, Tsukamoto M, Hirano A, Monden Y, Arakawa H, Nishimura S. Malolactomycin D, a potent inhibitor of transcription controlled by the Ras responsive element, inhibits Ras-mediated transformation activity with suppression of MMP-1 and MMP-9 in NIH3T3 cells. Oncogene 2001;20:6724-6730

Goldberg HJ, Scholey J, Fantus GI. Glucosamine activates the plasminogen activator inhibitor 1 gene promoter through Sp1 DNA binding sites in glomerular mesangial cells. Diabetes 2000;49:863-871.

Goldring MB. The role of the chondrocyte in osteoarthritis. Arthritis Rheum 2000;43:1916-1926.

Gouze J, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, Fournel-Gigleux S, Ouzzine M. Interleukin-1 beta down-regulates the expression of glucuronyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis. Arthritis Rheum 2001;44:351-360.

Liang L, Major T, Bocan T. Characterization of the promoter of human extracellular matrix metalloproteinase inducer (EMMPRIN). Gene 2002;282:75-86.

Liang Z, Leslie J, Adebowale A, Ashraf M, Eddington ND. Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatives with ultraviolet HPLC. J Pharm Biomed Analysis 1999;20:807-814.

McIlwraith CW. General pathobiology of the joint and response to injury. In McIlwraith CW and Trotter GW, Eds. Joint Disease In the Horse. Philadelphia, PA: WB Saunders 1996:40-69.

Nagase H. Stromelysins 1 and 2. In Parks WC and Mechum RP, Eds. Matrix Metalloproteinases. San Diego, CA: Academic Press 1998:43-47.

Nagase H, Woessner J Jr. Matrix Metalloproteinases. J Biol Chem 1999;274:21491-21494.

Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G, Nagase H. Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur J Biochem 1990;194:721-30.

Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA. Elevated matrix metalloproteinase and angiotensin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. PNAS 2000;97:2202-2207.

Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. J Immunol 2001;167:5067-5076.

Rabbani SA, Harakidas P, Guo Y, Steinman D, Davidsen SK, Morgan DW. Synthetic inhibitor of matrix metalloproteinases decreases tumor growth and metastases in a syngeneic model of rat prostate cancer in vivo. Int J Cancer 2000;87:276-282.

Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, Giacovelli G, Henrotin Y, Dacre JE, Gossett C. Long-term effects of glucosamine sulphate on osteoarthritis progression: A randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-256.

Roberts S, Weightman B, Urban J, Chappel. Mechanical and biochemical properties of human articular cartilage in osteoarthritic femoral heads and in autopsy specimens. J Bone Joint Surg 1986;68B:278-288.

Rossdale PD, Hopes R, Digby NJ, Offord K. Epidemiological study of wastage among racehorses, 1982 and 1983. Vet Rec 1985;116:66-69.

Todhunter RJ, Lust G. Pathophysiology of synovitis: Clinical signs and examination in horses. Compend Cont Ed Pract Vet 1990;12:980-992.

Towheed TE, Anastassiades TP, Shea B, Houpt J, Welch V, Hochberg MC. Glucosamine therapy for treating osteoarthritis (Cochrane Review). Cochrane Database Syst Rev 2001;1:CD002946.

Sandy J, Garnett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 can be inhibited by glucosamine. Biochem J 1998;335:59-66.

Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.

Shikhman AR, Kuhn K, Alaaeddine N, Lotz M. N-Acetylglucosamine prevents IL-1β-mediated activation of human chondrocytes. J Immunol 2001;166:5155-5160.

Taro K, Osamu T, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S. Lipopolysaccharide stimulates the MyD88-Independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 2001;167:5887-5894.

Torrungruang K, Alvarez M, Shah R, Onyia JE, Rhodes SJ, Bidwell JP. DNA binding and gene activation properties of the Nmp4 nuclear matrix transcription factor. J Biol Chem 2002;277:16153-16159.

Tung JT, Arnold CA, Fenton J, Alexander L, Yuzbasiyan-Gurkan V, Venta P, Orth MW, Richardson DW, Caron JP. Equine recombinant interleukin-1β: Effects on equine cartilage metabolism. Can J Vet Res 2002;66:19-24.

Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995;13:437.

Virkam A, Yki-Jarvinen H. Allosteric regulation of glycogen synthase and hexokinase by glucosamine-6-phosphate during glucosamine-induced insulin resistance in skeletal muscle and heart. Diabetes 1999;48:1101-1107.

Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Development 2000;14:2123-2133.

Whitelock JM, Paine ML, Gibbins JR, Kefford RF, O'Grady RL. Multiple levels of post-transcriptional regulation of collagenase (matrix metalloproteinase 1) in an epithelial cell line. Immunol Cell Biol 1993;71(Pt 1):39-47.

Figure 1

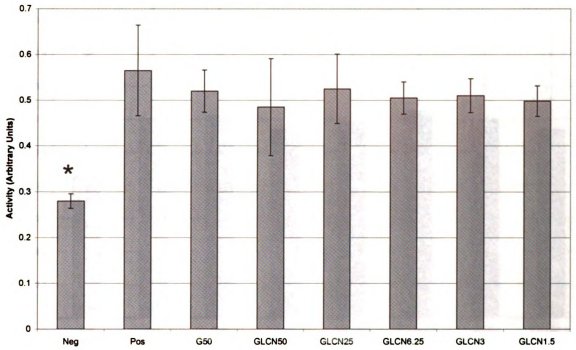

Figure 1. Mean (±SEM) MMP activity as determined by azodye release into the supernatant at pH 7.4. Neg = o-phenanthroline-treated; Pos = APMA-treated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 2

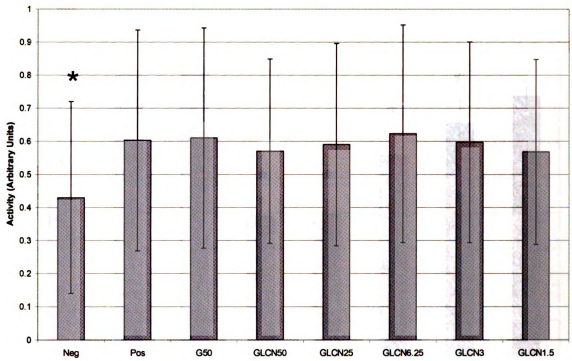

Figure 2. Mean (±SEM) MMP activity as determined by azodye release into the supernatant at pH 5.0. Neg = o-phenanthroline-treated; Pos = APMA-treated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 3

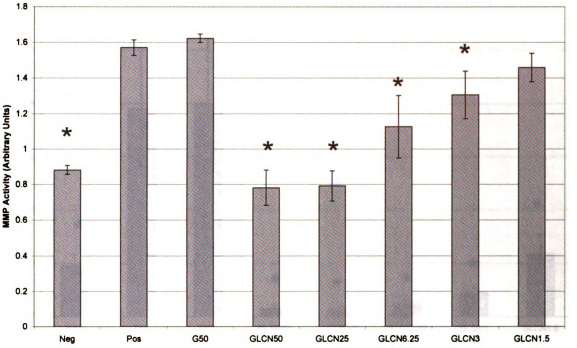

Figure 3. Mean (±SEM) MMP production of equine chondrocyte pellet cultures stimulated with LPS (20 μg/ml). MMP production was determined by azodye release using azocoll as a digestion substrate at pH 7.4. Neg = unstimulated; Pos = LPS-stimulated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 4

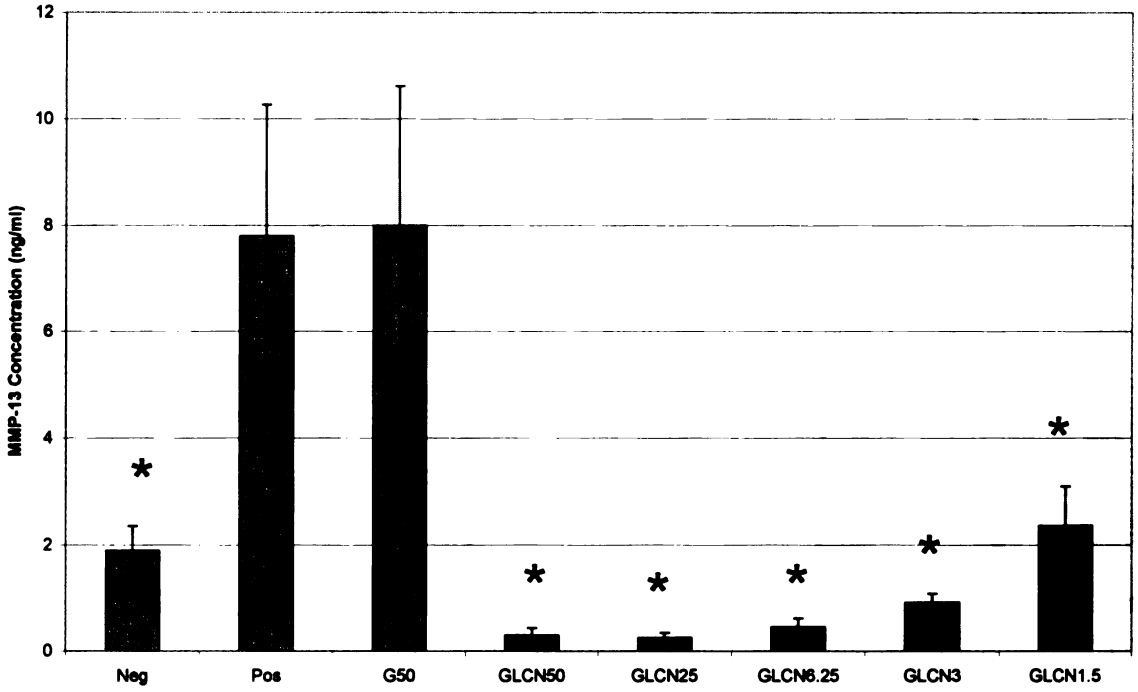

Figure 4. Mean (±SEM) MMP-13 concentration in media supernatant of equine chondrocyte pellet cultures stimulated with LPS (20 μg/ml). MMP-13 concentration was determined using a commercially available ELISA. Neg = unstimulated; Pos = LPS-stimulated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 5

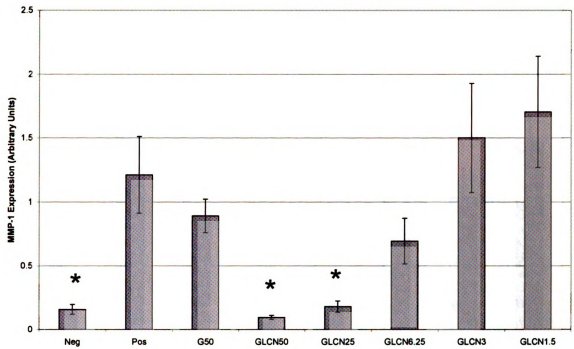

Figure 5. Mean (±SEM) relative MMP-1 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 μg/ml). MMP-1 mRNA concentration is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. Neg = unstimulated; Pos = LPS-stimulated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 6

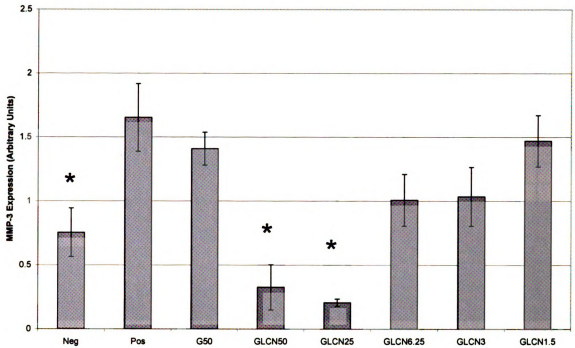

Figure 6. Mean (±SEM) relative MMP-3 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 μg/ml). MMP-3 mRNA concentration is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. Neg = unstimulated; Pos = LPS-stimulated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

Figure 7

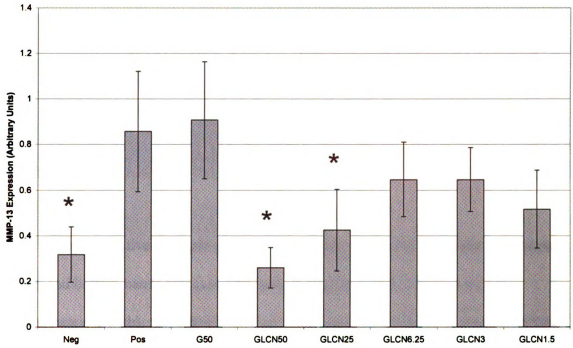

Figure 7. Mean (±SEM) relative MMP-13 mRNA expression of equine chondrocyte monolayer cultures stimulated with LPS (20 μg/ml). MMP-13 mRNA concentration is expressed as the ratio of the intensity of northern hybridization bands to the intensity of the 28S rRNA bands on methylene-blue stained nylon membranes. Neg = unstimulated; Pos = LPS-stimulated; G50 = glucose 50 mM; GLCN = glucosamine at the indicated mM concentrations. * Indicates statistical significance at $p < 0.05$ compared to positive control.

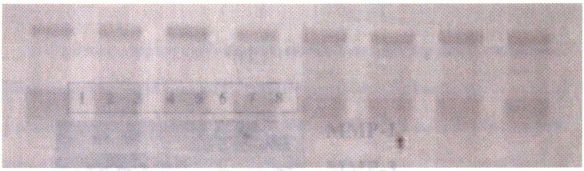

Figure 8. Representative example of a methylene-blue stained nylon transfer membrane. The top and bottom bands represent 28s (approximately 4.7 kb) and 18 s rRNA (approximately 1.9 kb), respectively. Nylon membranes were stained in methylene blue (0.03% w/v methylene blue in 0.3 M sodium acetate, pH 5.2) for 45 seconds, destained in water for 2 minutes, and dried between sheets of filter paper.

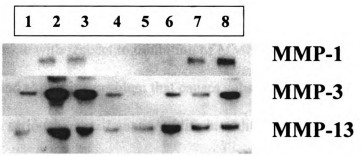

Figure 9. Representative autoradiographs of northern blots with probes for MMP-1, -3, and –13 after detection with the DIG system. Lane 1 = Negative control; 2 = Positive control; 3 = Glucose 50 mM; 4 = GLCN 50 mM; 5 = GLCN 25 mM; 6 = GLCN 6.25 mM; 7 = GLCN 3 mM; 8 = GLCN 1.5 mM.

Chapter 3

CONCLUSION

This study supports the findings of prior studies suggesting chondroprotective properties of glucosamine and offers further information on the mechanism of action of glucosamine with respect to inhibition of MMP synthesis. Osteoarthritis (OA) is a well-known and expensive problem that causes substantial morbidity in the form of lameness and may seriously curtail quality of life in addition to athletic serviceability of affected horses. The hallmark of the disease is the degeneration of articular cartilage. Although there are a number of possible causes of matrix degradation in OA, abundant experimental evidence suggests that proteolytic degradation of cartilage by matrix metalloproteinases (MMPs) is a predominant event. This study has shown the following:

1. Glucosamine does not significantly affect organomercurial-activated MMP activity.
2. Glucosamine significantly reduces the expression of MMPs from LPS-stimulated equine chondrocytes as determined by a substrate digestion assay, and this effect is paralleled by a significant reduction in MMP-13 levels.
3. Glucosamine significantly reduces the upregulation of mRNA in LPS-stimulated equine chondrocytes.

Taken together, these results indicate that glucosamine is capable of pre-translational, and possibly also translational, regulation of MMP expression and provide a potential mechanism of action for previously reported chondroprotective effects of glucosamine.

Results of our experiments using azocoll digestion with APMA-activated, conditioned medium, indicate that glucosamine HCl has negligible effects on the activity of pre-formed MMP. Matrix metalloproteinase activity assays were carried out at pH 5.0 and at pH 7.4 to favor the activity of stromelysin and total MMP activity, respectively. The observation that glucosamine did not inhibit MMP activity at either pH tested indicates that the activity of the well-characterized MMPs are not significantly inhibited by glucosamine.

Results of MMP synthesis experiments (pellet culture medium azocoll digestion and MMP-13 ELISA) indicate that glucosamine modulates expression of MMPs at or before protein translation. This effect was significant over the dose range of 3 to 50 mM as determined by azocoll digestion assay. Additionally, MMP-13 production was significantly inhibited at all five doses of glucosamine tested. Due to the inability to validate MMP-1 and MMP-3 kits for detection of equine MMPs we were unable to specifically demonstrate inhibition of expression of these metalloproteinases as well. These results are supportive of one another, and the difference in significance at the 1.5 mM level may be due to real differences in total MMP production compared to MMP-13 production. Alternately, this difference may be explained by differences between the detection methods used.

Relative mRNA expression of all three MMPs was significantly inhibited by glucosamine at 50 mM and 25 mM. This result indicates that glucosamine inhibits MMP production or enhances MMP degradation at a pre-translational level.

Numerous studies have indicated a potential role for glucosamine in altering several cell signaling pathways. While it remains to be seen which pathways are affected

and in what ways, we feel confident that glucosamine does regulate cellular metabolism through these interrelated pathways. Based on other recent research, glucosamine may modulate MMP expression through pathways involving the transcription factor activator protein-1, Nmp4 gene splice variants, Sp family transcription factors, Ras-responsive elements, and the NF-kappa B pathway. MMP expression may be regulated by glucosamine through a number of these pathways as well as others yet to be demonstrated.

This project has demonstrated a definite role for glucosamine in inhibiting MMPs in an osteoarthritis chondrocyte model. Considering the excellent bioavailability of orally administered glucosamine, its tropism for cartilage, and nearly nonexistent toxicity, a strong case can be made for the inclusion of glucosamine in an OA treatment regimen. While glucosamine alone may not prove to be the proverbial "silver bullet" in medical management of OA, it may potentiate other treatments and decrease the necessity of using treatments with high side effect rates. Glucosamine may also play a role in delaying the onset and slowing the progression of OA.

Future work will be required to validate the usefulness of glucosamine further, and to demonstrate the precise mechanisms of action. The knowledge that glucosamine downregulates resting mRNA levels for MMP-1, -3, and -13 mRNA can help guide future investigations into these mechanisms, which may include inhibition of signal transduction pathways, regulation of transcription factors, promoter efficiency, transcription itself, or post-transcriptional processing resulting in premature MMP mRNA degradation. Additionally, this study design may be used to investigate the effects of other potential

chondroprotective agents, and to investigate their effects on the expression of other factors involved in OA.