Mannosamine Inhibits Aggrecanase-mediated Degradation of the Mechanically Functional Portion of Proteoglycans and of the Physical Properties of Articular Cartilage

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ABSTRACT

The enzymatic processes underlying the degradation of aggrecan in cartilage and the corresponding changes in the biomechanical properties of the tissue are an important part of the pathophysiology of osteoarthritis. Recent studies have demonstrated that the hexosamines glucosamine (GlcN) and mannosamine (ManN) can inhibit aggrecanase-mediated cleavage of aggrecan. The term aggrecanase describes two or more members of the ADAMTS family of metalloproteases whose activity is known to be responsible for much of the aggrecan degradation seen in human joint diseases. In this study we verified that ManN and GlcN inhibit aggrecanase-mediated degradation induced by IL-1α of the mechanical properties of bovine calf articular cartilage. We then correlated the aggrecanase-generated aggrecan species remaining in the tissue with the results of mechanical testing . After 6 days of culture in 10 ng/ml IL-1 plus ManN, mechanical testing of explants in confined compression demonstrated that ManN inhibited the IL-1-induced degradation in tissue equilibrium modulus, dynamic stiffness, streaming potential, and hydraulic permeability, in a dose-dependent fashion, with peak inhibition (~75-100% inhibition) reached by a concentration of 1.35 mM. Results for GlcN were similar but occured at concentrations five times higher (5-10 mM). Western analysis demonstrated that in tissue which was completely protected by ManN from IL-1-induced degradation in physical properties, aggrecanase cleavage was inhibited at the aggrecanase site at the N-terminal end of the aggrecan core protein (the Glu^{373}-Ala^{374} bond in the interglobular domain). However, the proportion of aggrecan remaining in the tissue which was cleaved at aggrecanase sites in the chondroitin sulfate (CS)-rich region (Glu^{1501} and Glu^{1687}) was higher than that of controls. This result suggests that the preservation of mechanical properties by hexosamines in explants was primarily due to inhibition of cleavage at the Glu^{373} site. While the precise mechanism by which hexosamines function in this system is unclear, the present analysis suggests that the mechanical properties examined may be predominantly a function of electrostatic repulsion due to the charged CS chains in the tightly packed repetitive sequences of the CS-1 region.

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Contents

Chapter 1
Introduction

1.1 Cartilage Research

Articular cartilage is a specialized connective tissue which plays an important role in the physiology of joint function. The degeneration of the articular cartilage is in fact the hallmark of the most common human joint disease, osteoarthritis (degenerative joint disease, osteoarthrosis) (Cotran et al). However, the link between the molecular basis of normal cartilage degradation and the mechanical forces which lead eventually to pathophysiology is unclear. This research investigates the degradation due to the enzyme aggrecanase by characterizing the inhibition of aggrecanase by hexosamines, and by examining the effect of aggrecanase action on the mechanical properties of bovine calf articular cartilage tissue.

1.2 Cartilage Components

The physiologic function of cartilage is to provide a low-friction surface for joint articulation and to absorb some of the compressive stresses generated over the joint surfaces by body weight and muscle action. The function of cartilage is supported by a specialized extracellular matrix composed mostly of type II collagen and the proteoglycan aggrecan. The cells present in cartilage, the chondrocytes, are responsible for maintaining this matrix. It is the complex physical structure of the matrix components which is important in providing the mechanical properties of cartilage. Collagen has been shown to be important in providing shear strength to the tissue (Zhu et al), whereas aggrecan is important in providing much of the compressive strength (Frank and Grodzinsky).

1.3 Aggrecan Structure

By far the most abundant proteoglycan by weight in articular cartilage, aggrecan is a macromolecule named for its ability to form large aggregating structures with hyaluronan and link protein. The aggrecan molecule's core protein is divided into three globular domains (G1, G2, and G3), an interglobular domain, and the chondroitin sulfate

(CS) attachment domain (Fig 1-1). At the N-terminal end of the core protein, the G1 region is important for interacting with link protein to form aggregates with hyaluronan. A short region between G1 and G2 is known as the interglobular domain. Following G2 is the extended CS domain in which many serine residues (in particular, serine-glycine pairs) are glycosylated with the sugar chains keratan sulfate and chondroitin sulfate. This domain can be divided into a CS-1 region, where CS attachment sites appear regularly on the core protein (ie, the repeated [**SG**LP**SG**EVLETAAPGVEDI] sequence in humans), and a CS-2 region at the C-terminal end of the domain, where CS attachment sites appear more irregularly. Finally, the core protein is terminated at its C-terminal end by the G3 domain, whose specific function is not yet understood (Heinegård et al, Hardingham and Fosang).

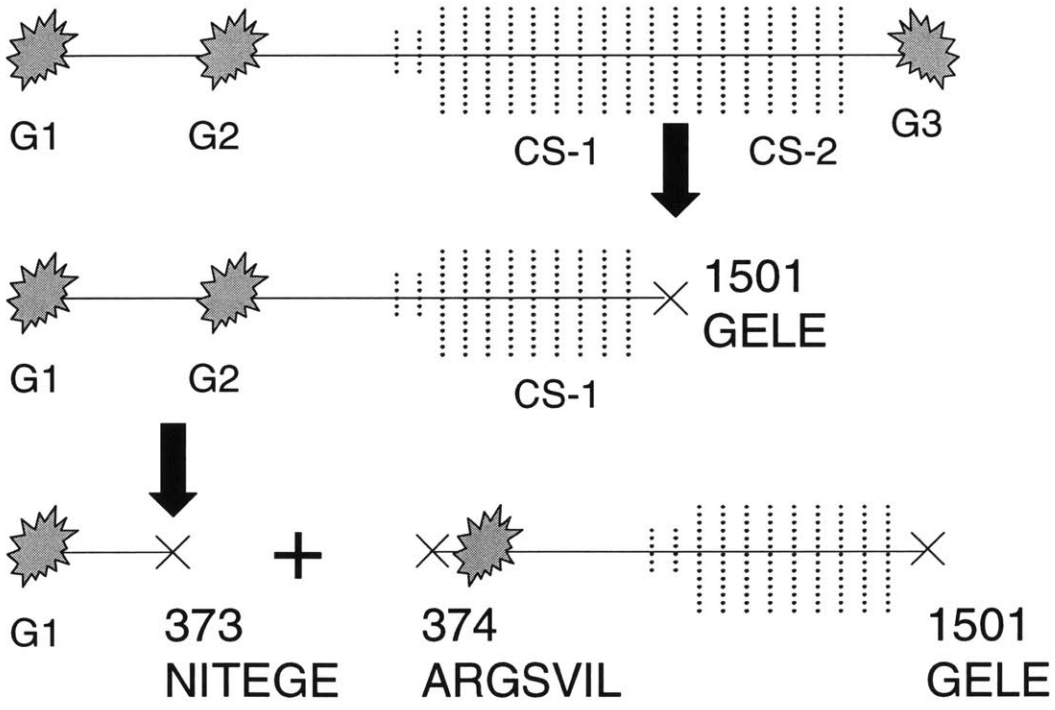

Figure 1-1 Proposed pathway for degradation of aggrecan by aggrecanase in bovine articular cartilage

1.4 Aggrecanase

Because loss of proteoglycans is one of the earliest events in the pathophysiology of osteoarthritis, it is important to identify and characterize the enzymatic processes which lead to the degradation of aggrecan. In particular, the enzyme aggrecanase has been been found to be active in the cartilage tissue and synovial fluid of patients suffering from a number of joint diseases (Lohmander et al, Sandy et al 1992). Aggrecanase has only recently been sequenced and identified as two or more enzymes (ADAMTS 5/11 and ADAMTS 4) of the ADAMTS (a disintegrin-like and metalloprotease [reprolysin type] with thrombospondin type 1 motif) family of proteins (Tortorella et al).

The presence of aggrecanase was proposed in 1991 by the observation that cartilage tissue incubated with IL-1 resulted in cleavage of aggrecan at the $NITEGE^{373}$ - $^{374}ARGSVIL$ peptide bond of the core protein (in the interglobular domain between G1 and G2) (Sandy et al 1991), and that this cleavage could not be reproduced any of the metalloproteases known at the time. This cleavage in the interglobular domain of aggrecan by aggrecanase, which forms the G1-$NITEGE^{373}$ fragment in the tissue, is now well-accepted (Fig. 1-1). In addition to this cleavage site, aggrecanase has been shown to cleave at multiple locations within the CS-2 domain of the core protein (Sandy et al 1995). In human cartilage, these sites include the peptide bonds following the glutamine residues at $SELE^{1545}$, $KEEE^{1714}$, $APTAQE^{1819}$, and $PTISQE^{1919}$.

Since aggrecanase is not available as the purified enzyme, research into its effects relies on cytokine-induced upregulation of aggrecanase activity. In particular, degradation of aggrecan induced by interleukin-1 (IL-1) is known to be due to aggrecanase activity – i.e., only cleavage at the aggrecanase site of the aggrecan core protein is found to occur in the tissue (Arner et al). As a result, a common experimental technique for studying aggrecanase activity is to culture cartilage in IL-1.

1.5 Hexosamines

Glucosamine is sold in large quantities in the United States as a substance which "promotes healthy joint function", as a "natural" remedy which does not require clinical

evidence for any claimed benefit. Several randomized, placebo-controlled clinical trials of glucosamine have shown a benefit to daily consumption of 500 mg GlcN three times per day (Noack et al, Reginster et al). However, all have been sponsored by the pharmaceutical company which markets GlcN in Europe, and the first trial conducted by a neutral investigator demonstrated no significant difference of GlcN over normal therapy in their primary endpoint (Houpt et al). Nevertheless, interest remains high that GlcN may have a benefit in a some patients.

Prior studies by Sandy et al (1998, 1999) have reported that two hexosamines, glucosamine and mannosamine, inhibit aggrecanase activity in chondrosarcoma cell cultures in a dose-dependent manner. This result was also verified in finely-sliced bovine cartilage tissue. Mannosamine, however, was found to be the more potent inhibitor, with maximal inhibition at ~1 mM, while ~5 mM glucosamine was required for full inhibition.

1.6 Specific Aims

The objectives of this study were (1) to quantify the effects of ManN and GlcN on IL-1-induced degradation of the physical properties of bovine cartilage explants, including the equilibrium modulus, dynamic stiffness, streaming potential and hydraulic permeability, and (2) to compare changes in these physical properties with the appearance of specific aggrecan fragments associated with aggrecanase activity in the presence and absence of ManN and GlcN.

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Chapter 2

Effect of Mannosamine on Aggrecanase-mediated Degradation

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Mannosamine Inhibits Aggrecanase-Mediated Changes in the Physical Properties and Biochemical Composition of Articular Cartilage

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Abstract

The enzymatic processes underlying the degradation of aggrecan in cartilage and the corresponding changes in the biomechanical properties of the tissue are an important part of the pathophysiology of osteoarthritis. Recent studies have demonstrated that the hexosamines glucosamine (GlcN) and mannosamine (ManN) can inhibit aggrecanase-mediated cleavage of aggrecan in IL-1-treated cartilage cultures. The term aggrecanase describes two or more members of the ADAMTS family of metalloproteinases whose glutamyl endopeptidase activity is known to be responsible for much of the aggrecan degradation seen in human arthritides. In this study we examined the effect of ManN and GlcN on aggrecanase-mediated degradation of aggrecan induced by IL-1α and the corresponding tissue mechanical properties in newborn bovine articular cartilage. After 6 days of culture in 10 ng/ml IL-1 plus ManN, mechanical testing of explants in confined compression demonstrated that ManN inhibited the IL-1α-induced degradation in tissue equilibrium modulus, dynamic stiffness, streaming potential, and hydraulic permeability, in a dose-dependent fashion, with peak inhibition (~75-100% inhibition) reached by a concentration of 1.35 mM. Aggrecan from explants cultured in IL-1 was found by Western analysis to be almost entirely processed down to the G1-NITEGE^{373} end-product. Addition of ManN or GlcN was found to produce 75-90% inhibition of this cleavage, but the proportion of aggrecan remaining in the tissue which was cleaved at aggrecanase sites in the CS-rich region (Glu^{1501} and Glu^{1687}) was higher than with IL-1 alone. This result suggests that the preservation of mechanical properties by hexosamines in explants is primarily due to inhibition of cleavage at the Glu^{373} site in the interglobular domain. While the precise mechanism by which hexosamines function in this system is unclear, the present analysis suggests that the mechanical properties examined may be predominantly a function of electrostatic repulsion due to the charged CS chains in the tightly packed repetitive sequences of the CS-1 region.

Introduction

A prominent feature of osteoarthritis is the degradation of the cartilage extracellular matrix, including loss of aggrecan and damage to the collagen network, resulting in changes to the mechanical properties of the tissue (1, 2). This degradation is thought to involve an interaction between the mechanical forces applied to the joint and cell-mediated enzymatic degradation. Studies in vitro and in vivo have shown that mechanical forces can regulate both the quantity and quality of extracellular matrix macromolecules synthesized by chondrocytes, and that mechanical overload (impact loading) can lead to long-term degradation of matrix by the remaining viable cells (reviewed in 3). Recently, Bonassar *et al* showed that IL-1 and retinoic acid induced marked degradation of cartilage material properties which accompanied loss of tissue GAG due to aggrecanase. These effects could be partially inhibited by addition of MMP inhibitors (4). More specific inhibitors of aggrecanase activity now enable more detailed studies of the relation between aggrecan fine structure and cartilage biomechanical properties.

The activity of the enzyme aggrecanase has been found to be responsible for IL-1-induced degradation of aggrecan *in vitro* (5-8) and to play an important role in the pathophysiology of arthritic diseases (9, 10). The hallmark of aggrecanase activity has been its cleavage of the aggrecan core protein at specific Glu-X bonds, generating fragments which can be detected with antibodies to the newly-exposed terminal amino acid residues. The initial identification of aggrecanase activity focused mainly on the cleavage of aggrecan at the Glu^{373}-Ala^{374} site, in the interglobular domain of the core protein, which generates the N-terminal fragment G1-$NITEGE^{373}$. However, cleavage at four additional cleavage sites in the chondroitin sulfate (CS)-2 attachment domain of the core protein generates small G3-bearing fragments and complementary G1-bearing fragments (6, 7, 11) which appear to be largely G1-$TAGELE^{1501}$ and a small amount of G1-$TFKEEE^{1687}$. Aggrecanase-generated fragments from these CS-2 cleavage sites also appear to represent the major degradative products present in human osteoarthritis synovial fluids. (11; J. D. Sandy, unpublished data). However, the effects of these site-

specific aggrecan cleavages on the biomechanical properties of the tissue are not known.

Recently, two enzymes which catalyze aggrecanase-specific cleavage of bovine aggrecan have been cloned and shown to be members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin-1-like motifs) family of peptidases (12, 13). The ADAMTS present in articular chondrocytes does not appear to be regulated at the level of transcription (14). On the other hand, aggrecanase activity has been shown to be modulated by addition of the hexosamines mannosamine (ManN) and glucosamine (GlcN) (15, 16), and it appears that this is due to modulation of downstream control mechanisms such as peptidase activation and/or localization.

The finding that mannosamine and glucosamine inhibit the formation of the G1-NITEGE fragment in bovine cartilage explants treated with IL-1 or retinoic acid (15, 16) also presents an opportunity to study the effects of aggrecanase activation and inhibition on the mechanical properties of the tissue. Therefore, the objectives of this study were (1) to quantify the effects of ManN and GlcN on IL-1-induced degradation of the physical properties of bovine cartilage explants, including the equilibrium modulus, dynamic stiffness, streaming potential and hydraulic permeability, and (2) to compare changes in these physical properties with the appearance of specific aggrecan fragments associated with aggrecanase activity in the presence and absence of ManN and GlcN.

Materials and Methods

Cartilage explantation and culture

Articular cartilage disks, 0.5 mm thick and 3 mm in diameter, were obtained from the femoropatellar groove of one- to two-week-old calves using methods similar to those described in detail previously (17). In brief, 9 mm diameter cylinders of full-thickness cartilage and bone were cored from the articular cartilage and inserted into a sample holder of a sledge microtome. After removing sufficient superficial cartilage to create a flat surface for biomechanical tests (usually less than 500 μm), the next two sequential 0.5 mm thick slices were cut. From each of these slices, a dermal punch was used to

produce 4 - 5 cartilage disks (3 mm in diameter). Groups of cartilage disks were incubated at 37 °C in an atmosphere of 5% CO_2, in wells containing 0.25 ml/disk of a serum-free culture medium (low-glucose DMEM [Gibco, Grand Island, NY] with 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). Medium was collected and replaced every 2-3 days. Treatment groups consisted of four cartilage disks which were incubated in medium alone (controls), medium plus 10 ng/ml human recombinant IL-1α (Genzyme; now R&D Systems, Inc., Minneapolis, MN) without hexosamine, and in media plus 10 ng/ml IL-1α with varying concentrations of added ManN or GlcN (Sigma Chemical Co., St. Louis, MO). An additional group of disks incubated in medium plus hexosamine without IL-1 was included in some experiments. Each experiment had four to seven such treatment groups, allowing disks among different groups to be matched for their location along the surface of a single joint.

Biochemical Analysis

Tissue and medium were analyzed for composition of aggrecan fragments by Western blotting. Aggrecan core fragments from medium (equal portions from medium collected at days 2, 4, and 6) and cartilage tissue (extracted with 4M guanidine·HCl) were isolated by ethanol precipitation and deglycosylation as previously described (15). Portions of media and tissue digests corresponding to 10 μg GAG were loaded on 4-12% polyacrylamide (Novex) gels for Western analysis and probed with polyclonal antisera to the bovine G1 domain, G3 domain (LEC-7) and the C-terminal neoepitopes TAGELE^{1501}, TFKEEE^{1687}, NITEGE^{373}, and ^{374}ARGSV [BC3]. The LEC-7 antiserum was from Dr. Kurt Doege; the TFKEEE^{1687} antiserum was from Merck & Co., Inc. (Whitehouse Station, NJ); and the NITEGE^{373} antiserum was from Dr. John Mort. Relative amounts of immunoreactive fragments were calculated by densitometric scan and calculation of integrated pixel density on NIH Image software (15). Tissue which was analyzed for sulfated glycosaminoglycan (GAG) content was digested with proteinase K and assayed by reaction with dimethylmethylene blue (DMMB) dye.

Physical Property Testing

Upon removal from culture, mechanical and electromechanical properties of cartilage disks were determined using methods similar to Bonassar et al (4). During testing, cartilage plugs were bathed in 0.15 M phosphate-buffered saline (PBS, Gibco) containing 100 U/ml penicillin G at room temperature and pH 7.4. The plugs were subjected to uniaxial confined compression in an electrically-insulating poly(methylmethacrylate) chamber, between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted at the bottom of the chamber. An identical electrode was placed in the surrounding PBS bath. Compression was controlled by a Dynastat mechanical spectrometer (IMASS, Hingham, MA) which was interfaced to a computer to record displacement and load during testing.

The equilibrium confined compression modulus was calculated by linear regression from the relaxed equilibrium stress attained after 3 sequential step compressions to 5%, 10%, and 15% strain. Both dynamic stiffness (defined as the dynamic stress amplitude divided by dynamic strain amplitude) and streaming potential (the dynamic potential amplitude divided by dynamic strain amplitude) were measured during application of a dynamic compression (1% dynamic strain amplitude at 15% static offset strain) at frequencies of 0.01, 0.1 and 1 Hz. The equilibrium modulus, dynamic stiffness, and streaming potential were used to calculate the effective hydraulic permeability and the intrinsic electrokinetic coupling coefficient from a macrocontinuum poroelastic model for uniaxial confined compression of tissue disks (18).

Statistical Analysis

All data are presented as mean ± standard error. Comparisons between groups were made with the Student's t-test (two-tailed with pooled variance estimate). For physical property testing, the null hypothesis was that no difference existed between the properties of cartilage disks treated with IL-1 alone and those treated with IL-1 plus 1.35 mM mannosamine. The extreme studentized deviate (ESD) statistic was used to exclude outlying data points at significance level $\alpha = 0.01$ (19).

Results

Biochemical Analysis

The content of GAG remaining in tissue after 6 days of treatment shows the protective effect of mannosamine on IL-1-induced loss of GAG (Fig. 1). The data are pooled from two experiments (n = 7) and normalized to control values (from disks incubated in medium with no IL-1 or ManN). Disks incubated in IL-1 with no ManN lost ~80% of the tissue GAG compared to controls. This loss of tissue GAG was inhibited in a concentration-dependent fashion, with GAG loss reduced to ~15% by addition of 1.35 mM ManN.

In order to examine the degradative effect of IL-1 and the protective effect of hexosamines on tissue aggrecan, we examined the aggrecan core protein structures present in the tissue after 6 days of treatment (Fig. 2). The Western analysis with an antiserum to the bovine G1 domain showed the presence of 5 major immunoreactive species. On the basis of size and immunoreactivity with antisera to the G3 domain and to aggrecanase-generated and MMP-generated C-terminal neo-epitopes (data not shown), the likely structures of each of the 5 species is shown schematically (A-E). The full-length aggrecan (A) reacts with both anti-G1 and anti-G3 antisera. The only CS-substituted species shown which lack the G3 domain (B) appear to be composed of a minor proportion of G1-KEEE^{1687} and a high proportion of G1-GELE^{1501}. The two forms of "free" G1 domain (D, E) each run as doublets due to variable glycosylation in the interglobular domain, and while the C-terminal of the larger form (D) is unknown, the smaller form (E) is the aggrecanase product which terminates at Glu^{373}.

Densitometric analysis of these representative samples has been used to provide a percentage distribution of the G1 domain among the 5 species in these samples (Fig. 2, percentage values in parentheses). In 6-day control tissue (without IL-1) there was a marked conversion of full-length aggrecan to degraded forms, since in fresh tissue before culture over 80% of the aggrecan was present as full-length (A) and the remainder as the long form of "free" G1 domain (D) (data not shown). In the presence of IL-1 (lane 2), the vast majority of tissue aggrecan was in the form of G1-NITEGE and

no intermediate forms were detected in the tissue. However, in the media, along with more G1-NITEGE, the intermediates G1-TAGELE, G1-TFKEEE and two fragments with the equivalent C-termini but an N-terminal of Ala^{374} were also found (data not shown). With either GlcN or ManN added to IL-1 cultures (lanes 3-4), marked changes in the tissue composition were observed. The accumulation of G1-NITEGE was largely, but not entirely, blocked, and this was reflected in the increased abundance of G1-G3 (A). On the other hand, relative to the no IL-1 control, there was a clear accumulation of the CS-bearing, C-terminally truncated form(s) (B) in the presence of IL-1 and hexosamine.

Physical Properties

For the physical property versus ManN concentration measurements of Figs. 3-7, each experiment included a control group (no IL-1), IL-1 alone, and from 1 to 4 of the IL-1 plus ManN groups shown. Data were normalized to control values and then pooled from the results of 4 independent experiments. As a result, the pooled number of disks tested in each condition varied from 3 to 12. In addition, 2 disks were excluded by outlier analysis.

After 6 days of treatment with IL-1 but no ManN, equilibrium modulus, dynamic stiffness, and streaming potential were reduced to about 20% of control values (Figs. 3-5), while hydraulic permeability was increased to about 8 times that of controls (Fig. 6). For all four mechanical properties, addition of ManN inhibited the IL-1-induced degradation of the properties in a dose-dependent fashion. The maximum protective effect appeared to be reached by 1.35 mM ManN, as higher concentrations of ManN did not result in additional protection. Protection reached 100% for the measurements of equilibrium modulus and hydraulic permeability. However, the dynamic stiffness and streaming potential of cartilage disks incubated with IL-1 were not fully protected by 1.35 mM ManN. This result was most marked in measurements made at the lowest frequency (0.01 Hz), where these properties were found to be about 75% of control values. The difference between disks treated with IL-1 alone and disks treated with IL-1 plus 1.35 mM ManN was statistically significant ($p < 0.001$) for all four properties. Mechanical

properties of disks incubated in ManN without IL-1 were not significantly different from controls.

After 7.5 days in culture, mechanical properties of disks treated with IL-1 and GlcN demonstrated that 10 mM GlcN was able to inhibit the IL-1-induced degradation in equilibrium modulus, dynamic stiffness, and streaming potential (Fig. 7). The inhibition was also found to occur in a dose-dependent fashion similar to that of IL-1 and ManN (data not shown). The concentration of GlcN required for significant inhibition, however, was at least 5 times higher than that of ManN.

Discussion

In this study, mannosamine and glucosamine were found to inhibit IL-1α-induced degradation in the physical properties of bovine calf articular cartilage in a dose-dependent fashion. Western analysis demonstrated that the protective effect of ManN on mechanical properties was accompanied by marked changes in the composition of the aggrecanase-mediated aggrecan fragments remaining in the tissue after six days of culture. Previous work has shown that ManN inhibits aggrecanase cleavage at the Glu^{373}-Ala^{374} site on the aggrecan core protein in rat chondrosarcoma cell cultures and in finely-sliced bovine articular cartilage (16). The present study extends those results by showing that inhibition of aggrecanase activity by mannosamine also inhibits the degradation of cartilage physical properties. As expected, these changes in physical properties in the presence ManN were accompanied by changes in the forms of aggrecanase-generated aggrecan fragments in the tissue and medium.

Treatment of articular cartilage disks with IL-1 for 6 days resulted in release of ~80% of tissue GAG content. As a significant part of articular cartilage tissue's ability to withstand compressive loads is due to the electrostatic repulsion of neighboring charge groups on the CS-GAGs of aggrecan (1, 20), this tissue GAG loss was reflected in an approximately proportional reduction in compressive stiffness. In this work we found that ManN could inhibit ~80% of the IL-1-induced GAG loss after six days in culture.

This inhibition of GAG loss is reflected in consistent protection from IL-1-induced degradation in all of the physical properties which were measured.

The inhibition by mannosamine of the degradation in mechanical properties is clearly significant and dose-dependent, and high levels of mannosamine achieved either complete or partial protection depending on the property measured. While both the intrinsic equilibrium compression modulus and the effective hydraulic permeability remained at control levels in the presence of IL-1 and ManN, the dynamic material properties (dynamic stiffness and streaming potential) were only partially protected (about 75%), with the least protection seen at the lowest frequency. Since varying the frequency of compression produces fluid flow at varying depths into the tissue, the dynamic stiffness can be thought of as a reflection of tissue properties at differing depths into the tissue, where higher frequencies reflect the primarily the properties of the upper surface of the tissue disks. Thus, the frequency-dependence of the normalized data suggests that inhomogeneities exist within the tissue, and that the protective effect of ManN was greatest at the edges and somewhat reduced at the center of the tissue. Immunohistological and tissue transport studies will be required to resolve this issue.

Disks incubated in hexosamine alone exhibited no adverse effects on tissue properties at the highest levels used here. In fact, although not significant, the mechanical properties of disks treated with hexosamine alone are in general slightly higher than those of controls. This could reflect inhibition of the basal level of aggrecanase-mediated degradation of aggrecan which occurs without IL-1 in these disks (21).

Western analysis showed that the protective effect of ManN on IL-1-induced degradation was accompanied by changes in the abundance of the different forms of aggrecanase-generated fragments found in the tissue (Fig. 2) and released into the media during culture (data not shown). These effects are clearly a reflection of the stepwise nature of the degradative pathway, which appears to begin in the CS-attachment region, followed by interglobular domain cleavage (Glu^{373}) (11, 28).

However, the finding that the higher concentrations of ManN generate an increased release of the G1-G3 and CS-domain-cleaved species and a concomitant decrease in G1-NITEGE release suggests that the ManN-dependent inhibition operates primarily on interglobular domain cleavage, while allowing a high level of C-terminal processing to continue.

While the mechanism of inhibition by the hexosamines is presently unclear, a number of possibilities exist (16). For example, mannosamine is a known inhibitor of glycosylphosphatidylinositol (GPI) anchor formation in a range of cell types (22-24). Recently, it has been observed that ManN inhibits GPI anchor synthesis in rat chondrosarcoma cells over the same concentration range as that required for aggrecanase inhibition (J. D. Sandy, unpublished data) and that aggrecanase activity is inhibited by other known inhibitors of GPI anchor synthesis (16). These results would suggest that aggrecanase activity requires GPI anchor formation, most likely because a cofactor or activator enzyme is a GPI-linked protein. In this regard, it is interesting that chondrocytes express glypican, a GPI-anchored heparan sulfate proteoglycan (25) which may serve to localize the ADAMTS at the cell surface through interaction with the thrombospondin motifs.

It is also possible that the inhibitory effects of glucosamine and mannosamine may be exerted through a common pathway. It is generally accepted that in cells expressing UDP-GlcNac 2-epimerase activity, there will be conversion of glucosamine to N-acetyl mannosamine and other intermediates of sialic acid biosynthesis which are directly derived from mannosamine (26). Indeed experiments in rat chondrosarcoma cells have shown that a small proportion of [^{3}H]glucosamine is incorporated into sialic acid via this pathway (27). Therefore, if the inhibitory effects of mannosamine are exerted through the sialic acid pathway, then the less potent inhibitory effect of glucosamine might result from the same pathway by a limited epimerase-dependent conversion of glucosamine into sialic acid precursors or sialylated glycoconjugates. However, the mechanism of action of GlcN may be unrelated to that of ManN, and it is of course possible that hexosamine inhibition is mediated by multiple effects on chondrocyte function (16).

Although the precise mechanisms by which hexosamines function in this system are unclear, the present analysis suggests that the mechanical properties examined may be predominantly a function of electrostatic repulsion due to the charged CS chains in the tightly packed repetitive sequences of the CS-1 region of aggrecan, which remains intact after cleavage within the CS-2 region generates the intermediate G1-GELE^{1501} (Fig 2, species B). This idea can now be investigated by evaluating both the mechanical properties and the precise structure of tissue aggrecan at different stages of aggrecanase-mediated degradation *in situ* by the approach used in this study.

Acknowledgments

The authors would like to thank Vivian Thompson and Christine Verscharen for their skilled technical assistance, Dr. Eliot Frank for his experienced advice, and Dr. Elizabeth Arner for performing the Western Analysis to detect G1-GELE^{1501} and ^{374}ARGSV [BC3]. This work was supported by the Whitaker Foundation, NIH grant AR33236, and the Shriners of North America.

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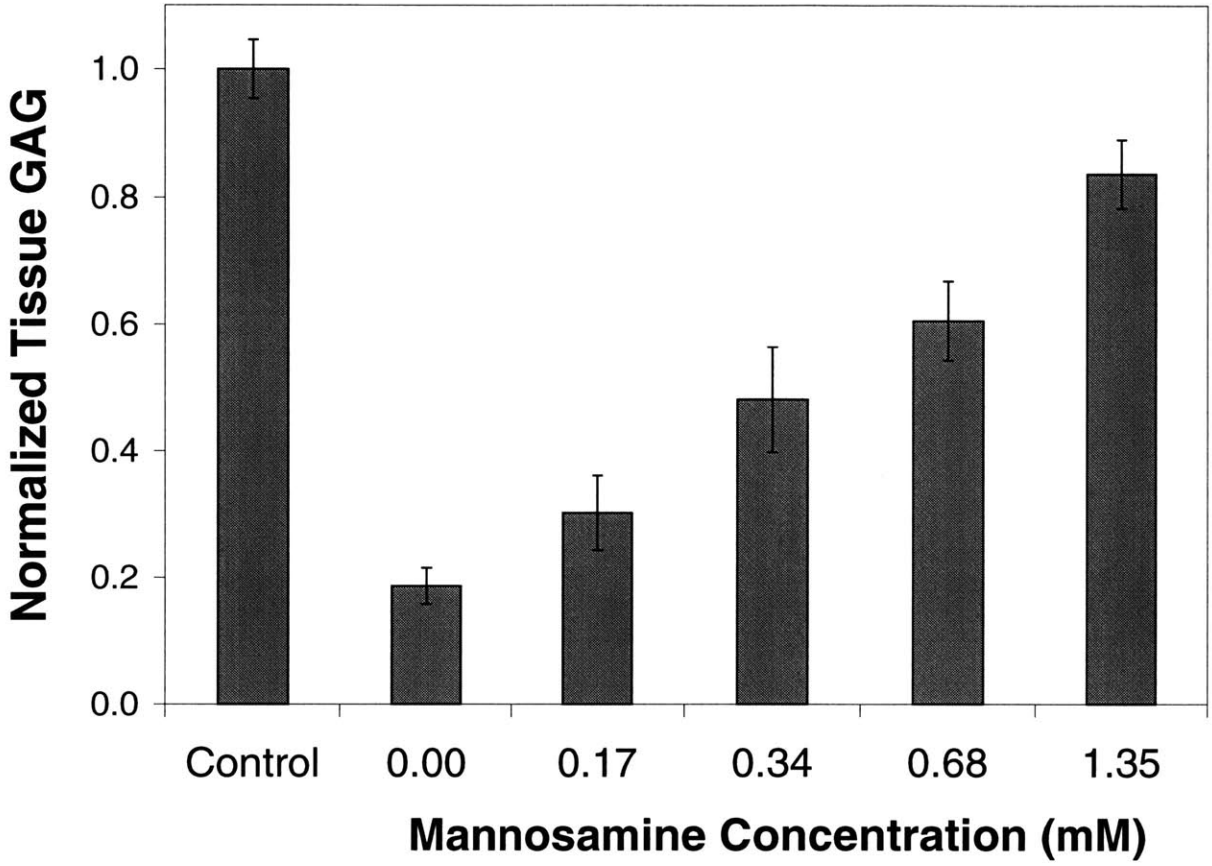

Fig. 1 GAG content of cartilage explants after 6 days of incubation in serum-free medium with 10 ng/ml IL-1α and increasing amounts of mannosamine. The data are normalized to control plugs (control) that were incubated in serum-free medium with no IL-1 or ManN. All values are mean ± sem (n = 7).

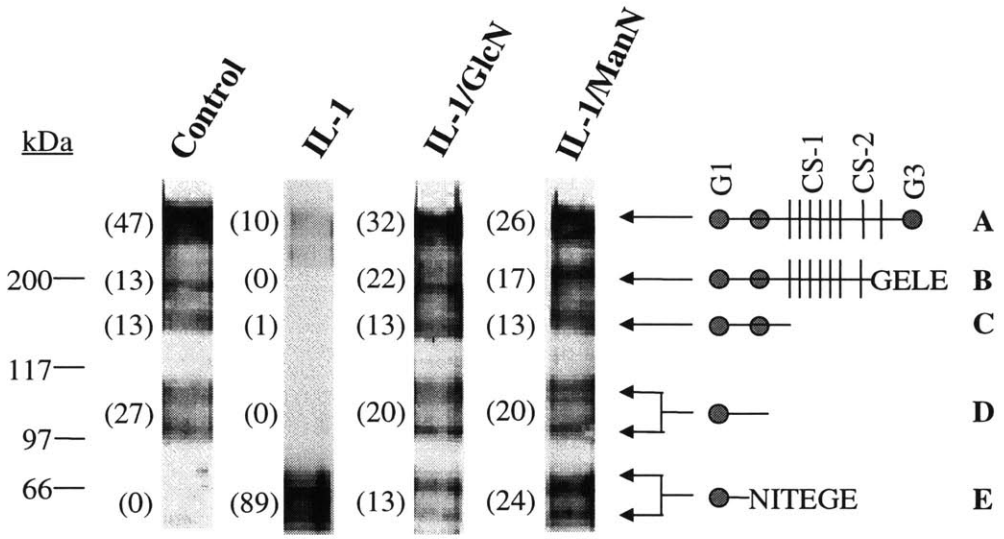

Fig. 2 Western analysis with anti-G1 antiserum of the aggrecan remaining in the cartilage tissue after incubation for 6 days in serum-free medium (control, lane 1), 10 ng/ml IL-1 (IL-1, lane 2), IL-1 plus 10 mM glucosamine (IL-1/GlcN, lane 3), and IL-1 plus 1.35 mM mannosamine (IL-1/ManN, lane 4). Suggested structures are shown on the right (A-E) and the percentage values in parentheses represent the percentage abundance of the G1 immunoreactivity for the G1-bearing species in the adjacent lane.

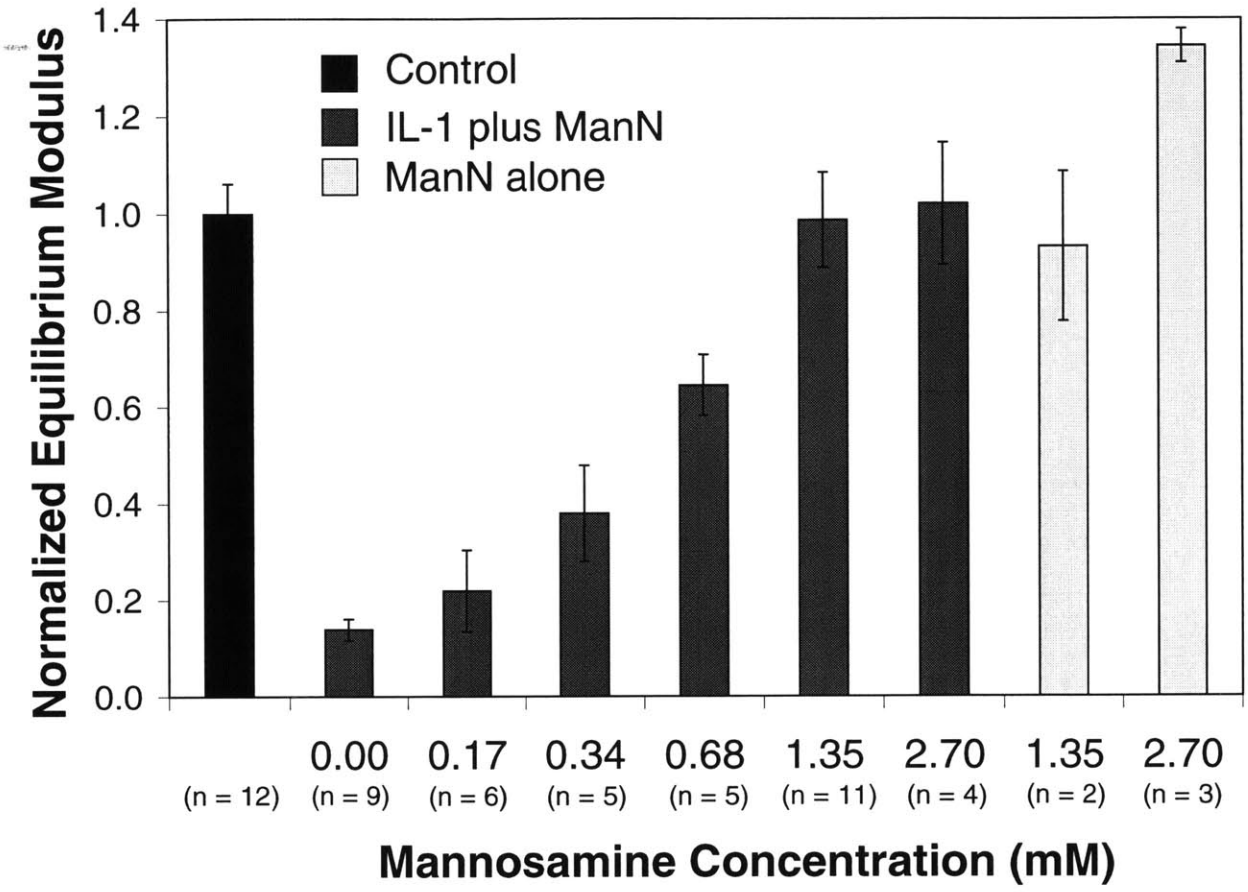

Fig. 3 Equilibrium confined compression modulus of cartilage disks after 6 days of incubation in medium with 10 ng/ml IL-1 plus varying concentrations of ManN, or with ManN alone. All data are normalized to control values and are displayed as mean ± sem.

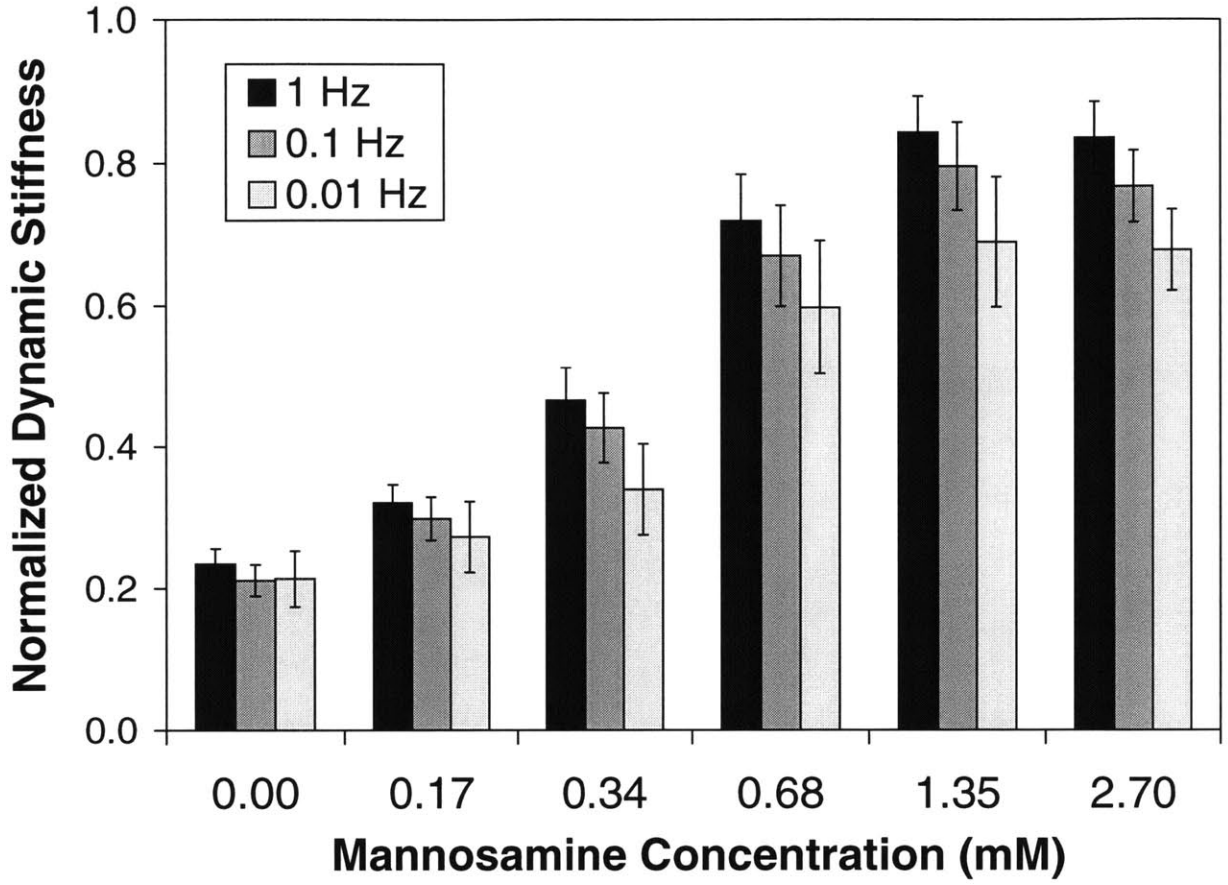

Fig. 4 Dynamic stiffness of cartilage disks at frequencies of 0.01, 0.1, and 1 Hz. Cartilage explants were cultured for 6 days in medium with 10 ng/ml IL-1 plus varying concentrations of mannosamine. All data are normalized to control values and are displayed as mean ± sem with n as in figure 3.

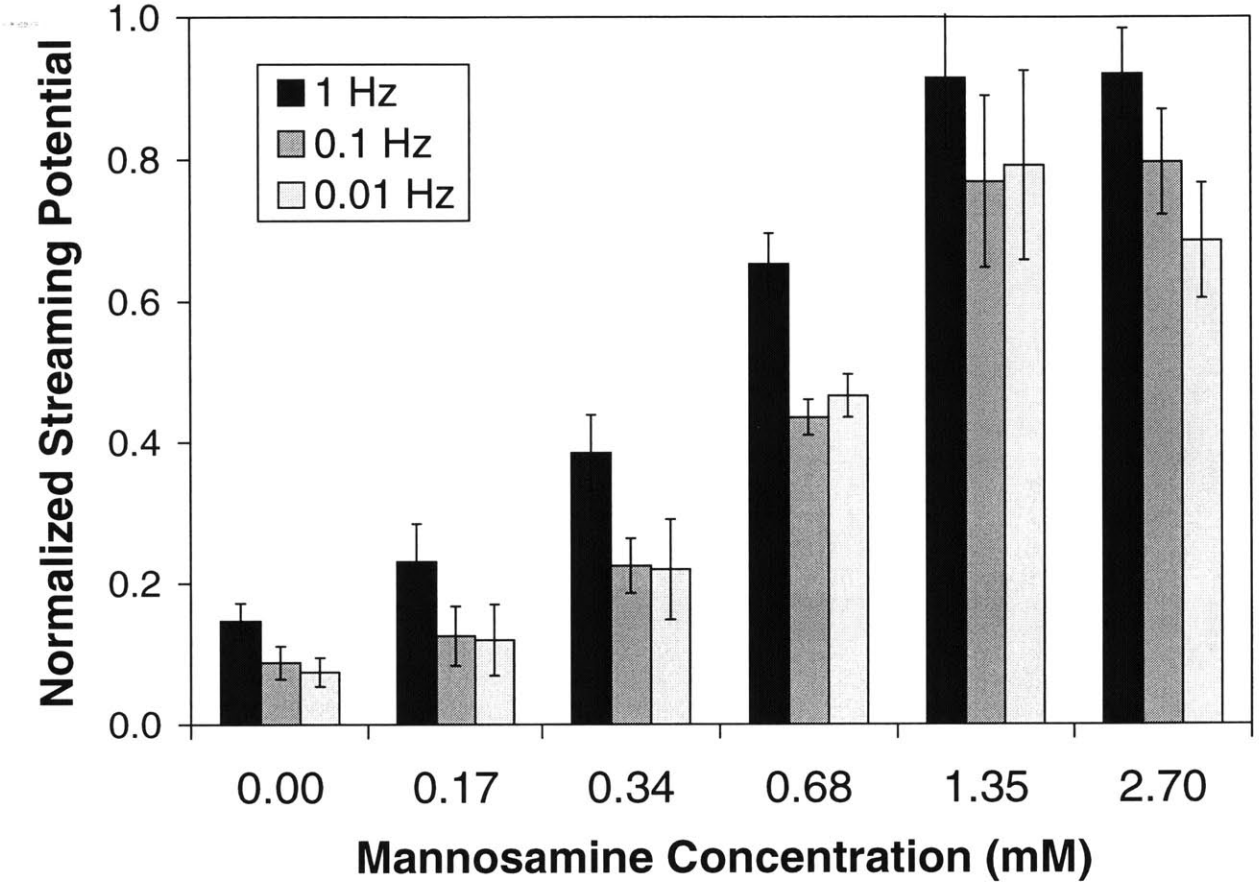

Fig. 5 Streaming potential of cartilage disks at frequencies of 0.01, 0.1, and 1 Hz. Cartilage explants were cultured for 6 days in medium with 10 ng/ml IL-1 plus varying concentrations of mannosamine. All data are normalized to control values and are displayed as mean ± sem with n as in figure 3.

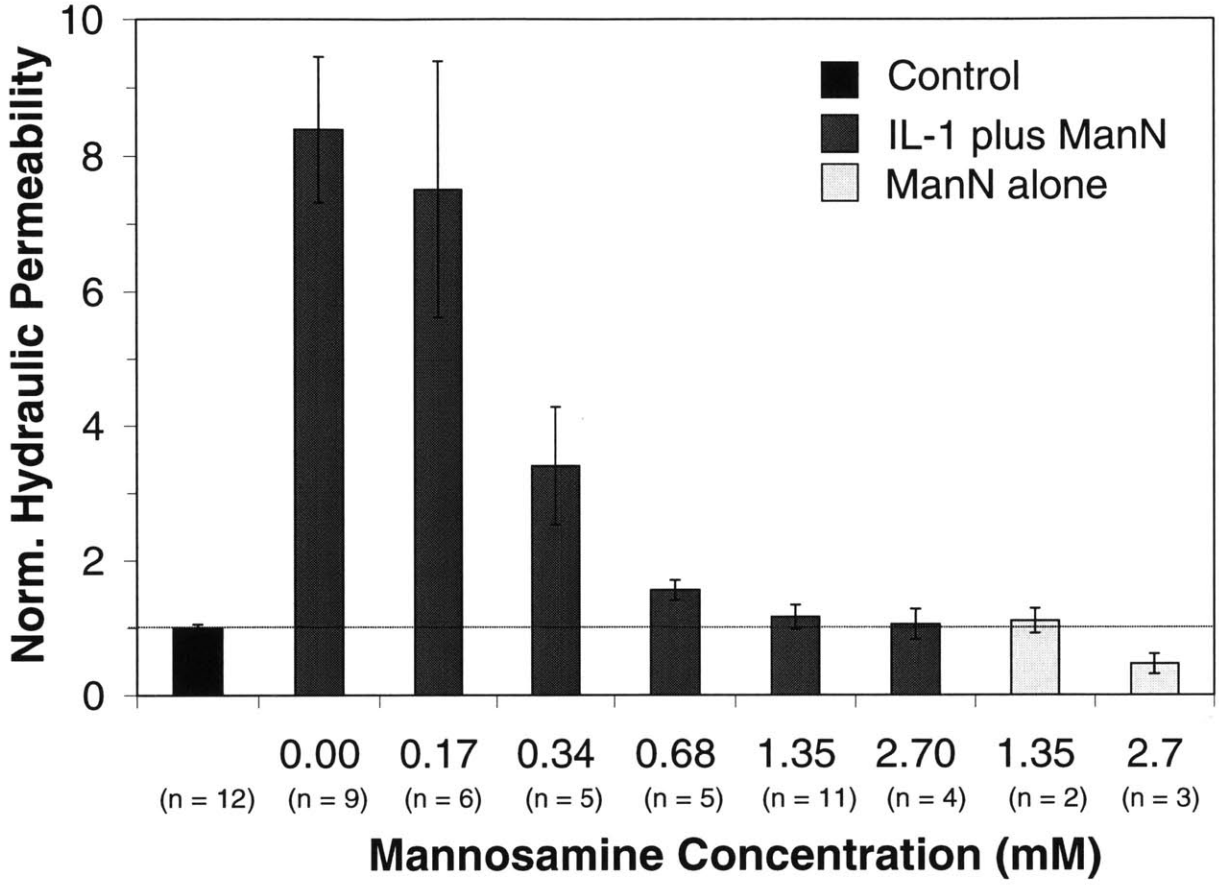

Fig. 6 Effective hydraulic permeability of cartilage disks after 6 days of incubation in medium with 10 ng/ml IL-1 plus varying concentrations of ManN, or with ManN alone. All data are normalized to control values and are displayed as mean ± sem.

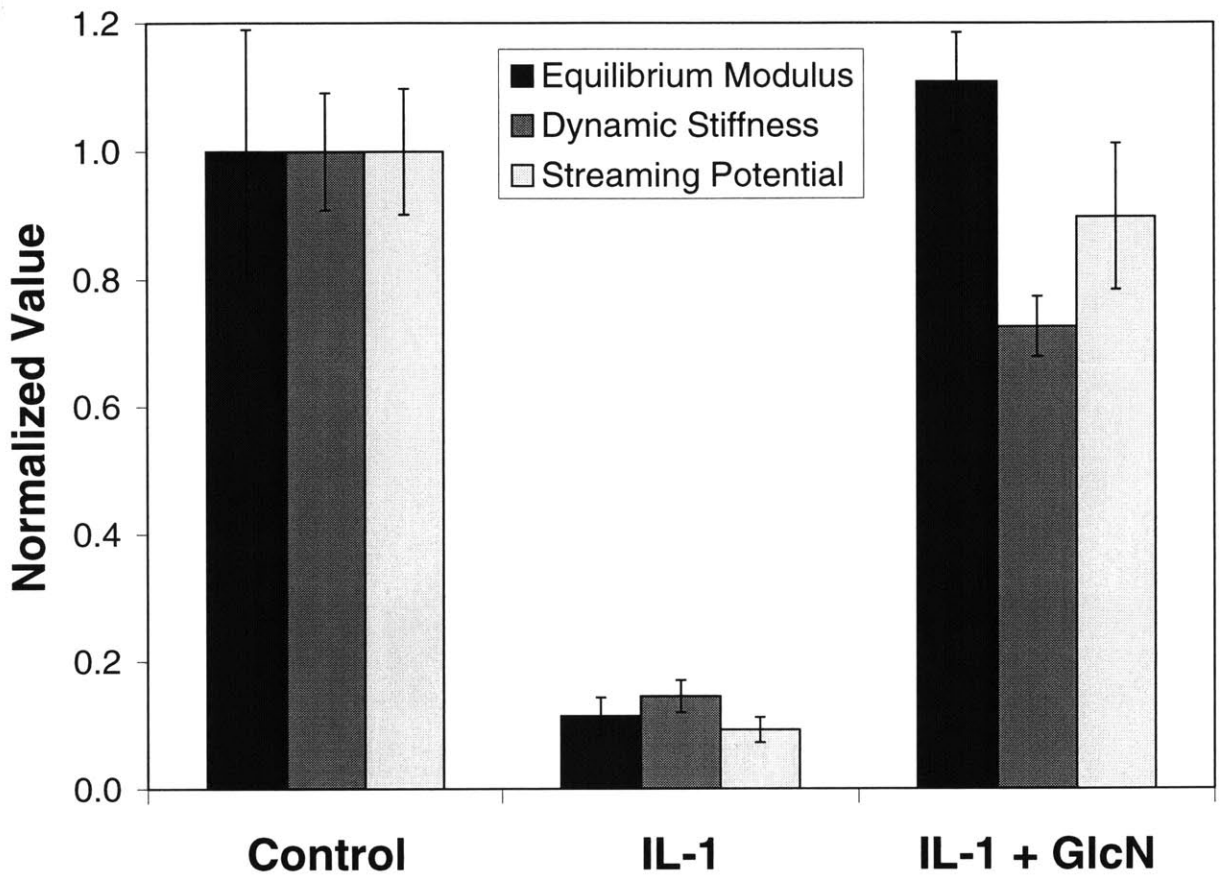

Fig. 7 Physical properties of cartilage disks after 6 days of incubation in medium with 10 ng/ml IL-1 plus 10 mM GlcN, compared to cartilage incubated with IL-1 and no GlcN. Results for equilibrium confined compression modulus, dynamic stiffness at 1 Hz, and streaming potential at 1 Hz are shown. The data are normalized to control values and are presented as mean ± sem with n = 4 for control and IL-1 and n = 3 for IL-1 + GlcN.

Addendum

In total, seven separate experiments were performed as described above to measure the mechanical properties of cartilage explants after incubation for 6 days in IL-1 plus 1.35 mM ManN. The results of these experiments (Fig. A) demonstrate that the average level of protection from IL-1-induced degradation by 1.35 mM ManN varies widely among joints (each experiment used the joint of a different animal). In four experiments, there was complete protection from IL-1-induced degradation of equilibrium compression modulus. On the other hand, in three experiments, the average level of protection was about 60-75%.

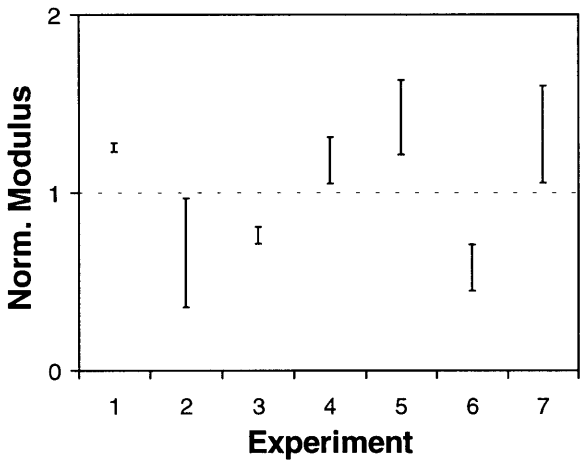

Figure A Normalized equilibrium compression modulus for each experiment (mean ± SEM) Cartilage explants were incubated for 6 days in 10 ng/ml IL-1 plus 1.35 mM ManN. N was 2, 2, 3, 4, 4, 5, and 4 for experiments 1 through 7, respectively.

These results suggest that in addition to a wide variability among joints of the effect of ManN on IL-1 induced degradation, the response may be bimodal. Should the response of the cartilage tissue in different animals be found to be bimodal, this has interesting implications for the mechanism of action of ManN. Further studies characterizing histology between ManN-protected and ManN-unprotected groups may provide some insight into this phenomenon.

In addition, we performed experiments as described in the Methods to determine whether glucosamine also inhibits aggrecanase-mediated degradation of bovine articular cartilage in a dose-dependent fashion.

3 mm diameter by 0.5 mm thick explant disks were obtained from bovine calf femoropatellar groove articular cartilage, and incubated for 7.5 days in either serum-free media (controls), media plus 10 ng/ml IL-1a, or media plus IL-1 and varying concentrations of GlcN. After culture, the disks were subjected to mechanical testing in confined compression.

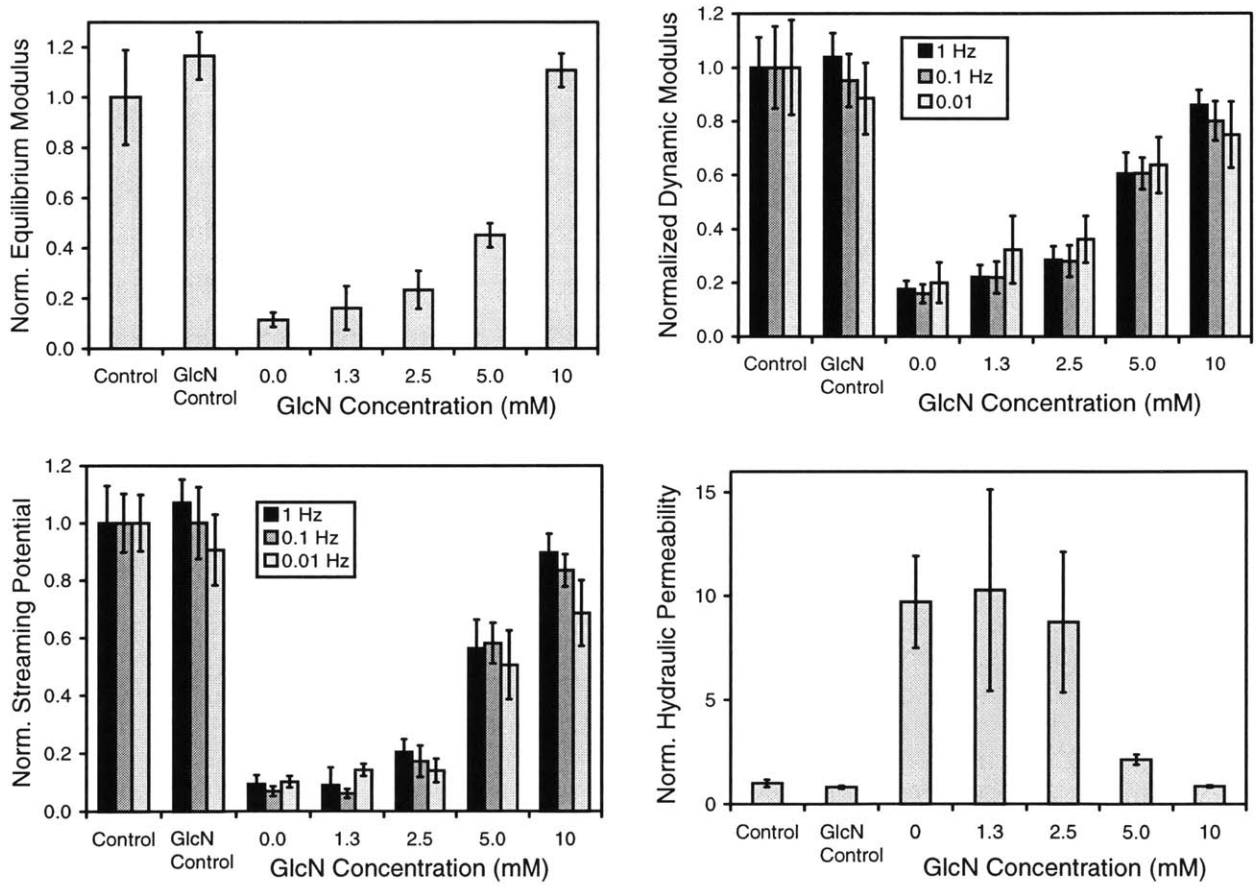

Figure B. Electromechanical properties of cartilage disks incubated for 7.5 days with IL-1 plus varying concentrations of GlcN demonstrate dose-dependent inhibition of IL-1-induced degradation by GlcN. GlcN controls were incubated in 10 mM GlcN without IL-1. Data are mean ± sem with n = 4 except for the IL-1 + 10 mM GlcN group which has n = 3.

These results demonstrate that glucosamine, like mannosamine, can inhibit aggrecanase-mediated degradation in the mechanical properties of bovine articular cartilage in a dose-dependent fashion. An important difference, however, is that the concentration of GlcN required for this effect is an order of magnitude higher than that of ManN.

Chapter 3
Mechanical Properties of the Chondroitin Sulfate-2 Domain of Aggrecan

3.1 Introduction

The preceding results demonstrated that inhibition of aggrecan cleavage can protect the mechanical properties of cartilage from the degradation induced by interleukin-1. The results also suggested that mechanical properties were preserved despite some cleavage of the CS-2 domain of aggrecan, implying that the CS-2 domain of the molecule might not be important in providing the mechanical strength of the tissue. Recent studies have demonstrated that in normal mature human cartilage, ~60% of the aggrecan present has been truncated at or near the TASELE^{1539} aggrecanase cleavage site in the CS-2 domain (Sandy et al 2000). Thus the mechanical function of the CS-2 domain could have important implications for normal joint physiology.

However, the Western analysis shown in the preceding results is subject to confounding since the amount of G1-GELE vs full-length aggrecan in the tissue is not the only difference between the control and IL-1 plus ManN groups. In order to investigate further the possibility of examining the mechanical function of the CS-2 domain, an additional set of experiments were conducted to specifically examine this hypothesis.

We hypothesized that control tissue, tested on day 0, would allow us to measure the mechanical properties of cartilage tissue in which the aggrecan was present mostly the full-length molecule. Similarly, we hypothesized that IL-1-treated tissue would be composed mostly of G1-NITEGE. And finally, we hypothesized that IL-1 plus ManN-treated tissue which showed complete protection from IL-1-induced degradation of mechanical properties would be composed of mostly full-length aggrecan with some G1-GELE but no G1-NITEGE.

3.2 Methods

We obtained 3 mm diameter by 0.5 mm thick cylindrical explant disks of articular cartilage from the femoropatellar groove of bovine calves. These disks were distributed to one of four experimental groups, with ten disks per group:

- Controls Cultured in media only, for less than 24 hrs.
- IL-1 Cultured for 6 days in medium plus 10 ng/ml IL-1a
- IM3.5 Cultured for 3.5 days in medium plus IL-1 plus 1.35 mM ManN
- IM6 Cultured for 6 days in medium plus IL-1 plus 1.35 mM ManN

For all groups, disks were cultured in serum-free medium consisting of low-glucose DMEM plus antibiotics. Medium was changed every third day.

After culture, five disks from each group were subjected to electromechanical testing in confined compression. If mechanical properties of the tissue incubated in IL-1 plus ManN were found to be completely protected from IL-1-induced degradation, a Western analysis was performed on the five tested disks from each group, plus the five remaining untested disks (Western analysis was performed by Vivian Thompson and John D. Sandy, PhD, of Shriners Hospital for Children, Tampa, FL). Relative amounts of G1-bearing aggrecan species in the tissue were quantified by densitometric scanning of the Western blots. Identification of aggrecan species present in the tissue as G1-G3, G1-GELE^{1501}, and G1-NITEGE^{373} was confirmed by immunoreactivity with anti-serum to the G3 domain, the GELE^{1501} neoepitope, and the NITEGE^{373} neoepitope, respectively (Western analysis for confirmation of GELE^{1501} was performed by Elizabeth C. Arner, PhD, of DuPont Pharmaceuticals Co.).

3.3 Results

Mechanical Testing

After six days of culture in IL-1 plus 1.35 mM ManN, mechanical testing confirmed that for this joint, ManN was completely protective against IL-1-induced degradation of mechanical properties. Explant disks treated with IL-1 for 6 days lost ~80% of their compressive strength compared to controls incubated in medium with no IL-1 (Figure 3-1). On the other hand, the compressive strength of disks incubated for six days in IL-1 plus 1.35 mM ManN was not significantly different from that of control disks. Mechanical testing for dynamic stiffness demonstrated similar results (data not shown).

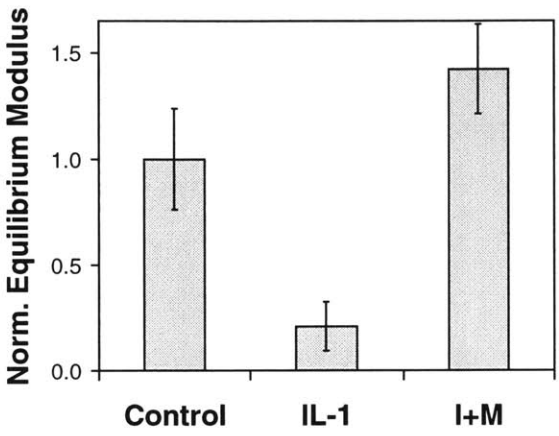

Figure 3-1 Equilibrium compression modulus of cartilage explants incubated for six days in serum-free media (control), 10 ng/ml IL-1 (IL-1), or IL-1 plus 1.35 mM ManN (I+M). Data are normalized to control values and are mean ± SEM (n = 4-5).

Western Analysis

The G1-bearing aggrecan species in control tissue, analyzed on day 0, were present as ~80% full-length aggrecan, and ~20% present as a species with an unidentified but non-aggrecanase-mediated C-terminal sequence (Figure 3-2). In disks incubated for six days in IL-1, almost all the G1-bearing aggrecan had been completely cleaved to the G1-NITEGE end-product. After six days' incubation in IL-1 plus 1.35 mM ManN, however, G1-bearing aggrecan was again mostly full-length aggrecan, but with an increased amount of aggrecan cleaved in the CS-2 domain (~30%).

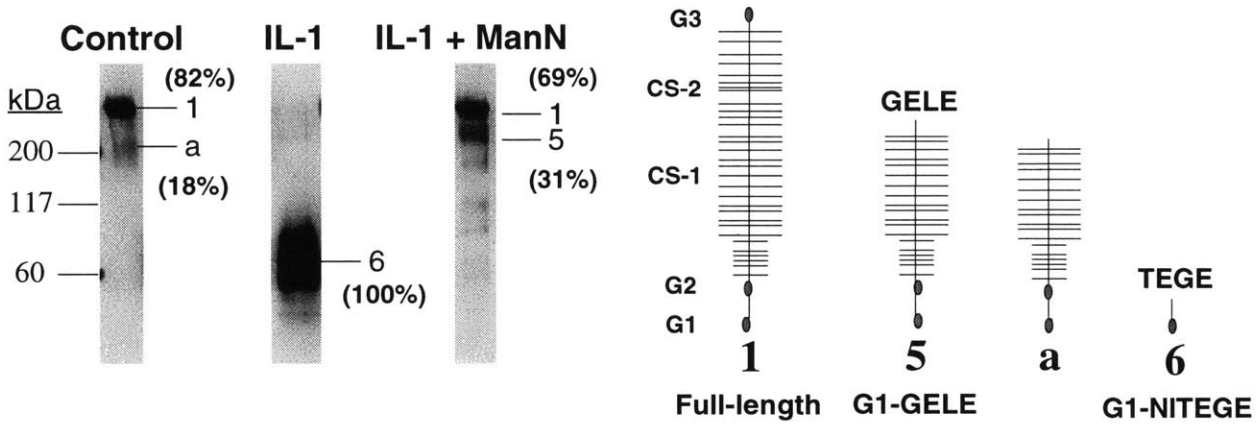

Figure 3-2 Western analysis with anti-G1 antiserum of the aggrecan remaining in the cartilage tissue after culture in serum-free media for less than 24 hrs. (Control, lane 1); in 10 ng/ml IL-1 for six days (IL-1, lane 2); and in IL-1 plus 1.35 mM ManN for six days (IL-1 + ManN, lane 3). Structures of the aggrecan species labeled 1, 5, a, and 6 on the Western blots are shown schematically on the right. The percentage values in parenthesis represent the percentage abundance of the G1 immunoreactivity for the adjacent G1-bearing aggrecan species.

3.4 Discussion

Our results demonstrated that despite complete preservation of mechanical properties of tissue incubated in IL-1 and ManN, aggrecanase-mediated cleavage in the CS-2 domain of the aggrecan core protein did occur. Since this tissue had approximately a 10% decrease in aggrecan CS-2 domain as compared to control tissue, one might have expected to see a loss of compressive strength, in proportion to the amount of GAG present on the CS-2 domain. Thus, the increase in aggrecan cleaved in the CS-2 domain without any decrease in mechanical properties suggests that the compressive strength of aggrecan may be primarily due to the electrostatic interactions among chondroitin sulfate chains attached to the CS-1 domain.

However, several factors currently limit the conclusions which can be drawn from this experiment. First, densitometric analysis of Western blots gives only semi-quantitative estimates of relative abundance. Second, confounding factors which may affect the mechanical properties of the cartilage during culture must be accounted for. Indeed, the cartilage explants swell during six days of cartilage, so the water content of explants tested at day 0 and at day 6 are likely to be different. However, water content should not significantly affect measurements of the equilibrium modulus. It is also possible that ManN has additional effects on the tissue besides inhibition of aggrecanase. Most importantly, since the abundance of aggrecanase cleaved in the CS-2 domain was only 10% higher in the treatment group than in controls, it is unlikely that this experiment could demonstrate that the mechanical properties of explants treated with IL-1 plus ManN are not less than those of control plugs with enough statistical power for a meaningful conclusion to be drawn.

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Appendix

The following manuscript entitled “Biosynthetic Response and Mechanical Properties of Articular Cartilage after Injurious Compression,” by Bodo Kurz, Moonsoo Jin, Parth Patwari, Michael W. Lark, and Alan J. Grodzinsky, has been submitted to the Journal of Orthopaedic Research.

BIOSYNTHETIC RESPONSE AND MECHANICAL PROPERTIES OF ARTICULAR CARTILAGE AFTER INJURIOUS COMPRESSION

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Summary

Traumatic joint injury is known to produce osteoarthritic degeneration of articular cartilage. To study the effects of compressive overload on the degradation and repair of cartilage *in vitro*, we developed a model that allows strain-, strain rate-, and stress-controlled loading of cartilage explants. The influence of strain rate on both cartilage matrix biosynthesis and mechanical properties was assessed after single compressive overloads. Loading with a strain rate of 0.01 s^{-1} resulted in no significant damage to the cells or to the extracellular matrix, although peak stresses reached levels of about 12 MPa. However, compression with strain rates of 0.1 s^{-1} and 1 s^{-1} caused peak stresses of approximately 18 and 23 MPa, respectively, and resulted in significant decreases in both proteoglycan and collagen biosynthesis. The mechanical properties of the explants (compressive and shear stiffness) were also reduced with increasing strain rate, indicating damage to the collagen network of the injured tissue. Additionally, cell viability decreased with increasing strain rate, and the remaining viable cells lost their ability to exhibit an increase in biosynthesis in response to low-amplitude dynamic mechanical stimulation. This latter decrease in reparative response was most dramatic in the tissue compressed at the highest strain rates. We conclude that strain rate (like peak stress or strain) is an important parameter in defining mechanical injury, and that cartilage injuriously compressed at high strain rates can lose its characteristic anabolic response to low-amplitude cyclic mechanical loading.

INTRODUCTION

Traumatic joint injury has been demonstrated to be a risk factor for development of secondary osteoarthritis (2), but the precise mechanism by which this occurs is unknown. Studies of *in vivo* animal models have demonstrated that high-impact loads to the knee joint can induce cartilage degradation (11,23,24). Attempts to model this process *in vitro* have led to the investigation of the effects of compressing cartilage tissue using loading conditions sufficient to produce acute injury (3,6,7,15,17,22,25).

To simulate a rapid impact injury having a known impact energy, one approach has been the use of a drop-tower apparatus (6,19). However, such an approach does not allow one to study or control separately the displacement or stress waveform applied to the cartilage during compression. Thus, recent studies have used systems capable of controlling load or displacement during injury. In these experiments, the controlled variable in defining the injury has been either peak stress (3,25) or final strain (9,15). Chen et al (1) used a repetitive impact model in which a desired peak stress could be achieved using two different loading rates; they found that the level of injury depended on which loading rate was used.

Since the pathway leading from joint injury to OA is not well understood, it is important to quantify parameters of tissue damage as well as measures of cell metabolism and biosynthesis. Previous studies *in vitro* have therefore focused on tissue swelling, compressive strength (9) and, most recently, denatured collagen strand neoepitopes (1) to quantify specific damage to the collagen network during injury. Previous studies have also demonstrated that compression affects the chondrocytes themselves. Specifically, injurious compression may increase cell death and decrease biosynthesis (1,7,9,15,25).

In contrast, moderate (low-amplitude) dynamic compression of cartilage over a range of frequencies can increase chondrocyte biosynthesis of matrix macromolecules (12,23,21) and upregulate expression of aggrecan and type II collagen (18). We hypothesized that a further consequence of injurious mechanical compression would be

the inability of chondrocytes to respond in a stimulatory fashion to moderate dynamic compression, i.e., the failure of a potential reparative response associated with moderate loading.

Therefore, the objectives of this study were (1) to quantify the relationship between strain rate and peak stress on certain biomechanical and biosynthetic measures of tissue injury, (2) to examine the ability of chondrocytes to recover from an injury by quantifying the response of chondrocytes to subsequent low-amplitude dynamic compression, and (3) to quantify the shear stiffness of injured tissue as an additional parameter demonstrating damage to collagen network integrity.

MATERIALS AND METHODS

Articular cartilage explants

Articular cartilage disks were obtained from the femoropatellar groove of 1-2 week old calves as previously described (20). In brief, cartilage-bone cylinders (9 mm in diameter) were drilled perpendicular to the cartilage surface and placed in a microtome holder. After creating a level surface by removal of the most superficial ~100 μm, the next 2 mm of articular cartilage were sliced by the microtome to produce two 1 mm thick slices. Four explant disks (3 mm in diameter x 1mm thick) were punched out of each slice and equilibrated for 24 hrs. in culture medium (low glucose DMEM supplemented with 10% FBS, 10 mM HEPES buffer, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a 37 °C, 5% CO_2 environment. The four 3-mm diameter disks from each 9-mm diameter slice were distributed to four groups: 0.01 s^{-1}, 0.1 s^{-1}, and 1 s^{-1} strain rates, and controls.

Mechanical injury

Mechanical injury was applied to groups of four cartilage explants one day after dissection (Fig. 1). The explants were placed in chambers during (radially unconfined) compression by an incubator-housed loading device, as described previously (4,9).

Controlled displacement ramps to 50% final strain were applied to the four disks simultaneously at rates of 0.01 s^{-1}, 0.1s^{-1}, or 1 s^{-1} (corresponding to ramp velocities of 0.01 mm/s, 0.1 mm/s, and 1 mm/s, respectively), and held at the final strain such that the total time of compression was five minutes for each group. After injury, the explants were maintained in culture for an additional 0.25 or 3 days, and biochemical or mechanical measurements were made as described below.

Biochemical and biosynthesis studies

Wet weight measurements were taken before injury and at 0.25 and 3 days after injury. For radiolabel incorporation, the explants were placed in fresh culture medium containing 10 μCi/ml $^{35}SO_4^{-2}$ and 20 μCi/ml ^{3}H-proline for 6 hrs. After culture with radiolabel, the explants were washed three times (20 min. each) in washing buffer, digested overnight in a protease K solution, and aliquots of the digest were subjected to scintillation counting to measure radiolabel incorporation, with corrections for spillover and dilution quenching. Scintillation counts were expressed as pMol incorporation per hour per mg wet weight before injury, and normalized to the radiolabel incorporation of uninjured control tissue. The GAG content of the digested samples, as well as the GAG content of the conditioned medium, was measured using the dimethylmethylene blue (DMMB) dye-binding assay.

In separate experiments, the biosynthetic response of injured cartilage to low-amplitude dynamic mechanical stimulation was measured three days after injury. Explant disks from each of the four sample groups (0.01, 0.1, and 1 s^{-1} strain rates and uninjured controls) were subjected for 12 hrs. to either dynamic compression (3% dynamic strain amplitude at 0.1 Hz superimposed on a 10% static offset strain) or static compression alone at the same static offset strain (10%). That is, in each group half the explants were subjected to static compression at the same static offset compression as that used for dynamic compression, to serve as controls. During the 12-hr. dynamic compression, the cartilage disks were incubated with fresh culture medium containing 10 μCi/ml $^{35}SO_4^{-2}$ and 20 μCi/ml ^{3}H-proline. The incorporation rates of the dynamically compressed explants were normalized to their corresponding static controls.

Two additional experiments were performed to assess cell viability 3 days following injury. Following culture, thin slices (about 200 μm thick) of cartilage explants were made with a scalpel blade and stained with an ethidium bromide (EtBr) and fluorescein diacetate (FDA) solution (red/green viability assay).

Measurement of mechanical properties

The mechanical properties of the explants were measured either 6 hrs. or 3 days post-compression. The four explants in each injury or control group were simultaneously subjected to uniaxial unconfined compression in a polysulfone chamber as described above. The equilibrium compressive stiffness was calculated from linear regression of the equilibrium stress attained after three sequential compression ramps to 20%, 23%, and 26% strain. Dynamic compressive stiffness was then measured during application of a sinusoidal compression (3% dynamic strain amplitude) superimposed on the 26% static offset strain, at frequencies ranging from 0.1 to 1 Hz. Similarly, the equilibrium shear modulus was measured by simultaneous application of simple shear strain to all four explants in each group (3 sequential steps of 1% shear strain at an axial compressive offset of 26%) (4). Subsequent application of 3% amplitude sinusoidal shear strain enabled measurement of dynamic shear modulus in the 0.1 - 1 Hz frequency range.

Statistics

Control and experimental groups were compared by paired t-test and significant at $p < 0.05$ unless otherwise specified.

RESULTS

Single compressive overload

Representative stress-strain curves during injurious compression of cartilage disks at three different strain rates (to 50% final strain and held for a total of five minutes) are shown in Fig. 2. The peak stress produced by this compression ranged from ~12 MPa

at a strain rate of 0.01 s^{-1} up to ~23 MPa at 1 s^{-1} (Fig. 3). By contrast, the stress after holding the compression for four minutes decreased with increasing strain rate from ~2.3 MPa at 0.01 s^{-1} to ~1 MPa at 1 s^{-1} (Fig. 3).

Biosynthetic activity and cell viability after a single compressive load

^{35}S-Sulfate ^{3}H-proline incorporation in disks subjected to a strain rate of 0.01 s^{-1} were not significantly different from that in uncompressed control disks at 6 hrs. and 3 days post-compression (Fig. 4A). Cartilage disks subjected to strain rates higher than 0.01 s^{-1} showed decreased proline and sulfate incorporation in a manner dependent on strain rate. The decrease in biosynthetic activity compared to controls was significant for strain rates of both 0.1 s^{-1} and 1 s^{-1}. After injury at a strain rate of 1 s^{-1}, radiolabel incorporation was reduced to ~45% of control levels for proline and ~30% of control levels for sulfate. No significant differences were observed between radiolabel incorporation values at 6 hrs. and 3 days after injury at any applied strain rate.

Cell viability was measured three days after injurious loading in two separate experiments, and the data pooled (n = 6-8 disks at each strain rate). Viability (mean $\pm$ SEM) was 97 $\pm$ 0.6 % in control disks. This decreased to 88 $\pm$ 5 % at 0.01 s^{-1}, 75 $\pm$ 6 % at 0.1 s^{-1}, and 67 $\pm$ 9 % at 1 s^{-1}. The decrease in cell viability was significant for strain rates of 0.1 s^{-1} and 1 s^{-1}, but not at 0.01 s^{-1}. These results were used to re-calculate the day-3 radiolabel incorporation data of Fig. 4A to estimate the biosynthetic activity per viable cell. The radiolabel incorporation per viable cell at three days post-injury decreased with increasing strain rate, reaching significance for ^{35}S-sulfate at 1 s^{-1} strain rate (Fig 4B).

The biosynthetic activity of explants three days after a single compressive overload was also assessed during a subsequent 12-hr. low-amplitude dynamic compression to examine the ability of the chondrocytes to respond to mechanical stimulation after injury. The data are normalized to cartilage disks which had received the same initial injurious compression, but then received only the 10% static offset compression, with no dynamic compression (Fig 5). In uninjured controls, dynamic compression increased ^{3}H-

proline incorporation by ~40% and ^{35}S-sulfate incorporation by ~25%. In contrast, injuriously compressed disks showed reduced ability to respond to dynamic stimulation, and the response decreased with increasing strain rate of injury. After an injury at a strain rate of 1 s^{-1}, dynamic compression no longer stimulated biosynthesis above the 10% offset compression baseline, and biosynthesis was significantly less than dynamically stimulated uninjured control disks.

Tissue mechanical properties after a single injurious compression

After compressive overload at 1 s^{-1}, the cartilage appeared grossly damaged in approximately half of the disks. Damaged disks appeared elliptical in shape and were often fissured. In contrast, no gross damage was seen after injury at 0.1 or 0.01 s^{-1}. The increase in tissue wet weight 6 hours after injury increased with strain rate and was significant for injury at each strain rate (Fig. 6). However, differences in wet weight among conditions were no longer apparent 3 days after injury.

Compressive and shear stiffness of cartilage injured at 0.01 s^{-1} was not significantly different than controls, but then decreased with increasing strain rate (Fig. 7). Equilibrium axial stiffness in unconfined compression (Fig 7A) tended to decrease with increasing strain rate, although the differences were not significant. However, the dynamic stiffness in the 0.01 to 1 Hz frequency range (shown at 0.5 Hz in Fig. 7A) was significantly decreased after injury at 0.1 and 1 s^{-1}. Both the equilibrium and dynamic shear stiffness of the explants decreased with increasing strain rate, down by 50% after injury at 1 s^{-1}. There were no significant differences between measured stiffness values at 6 hours or 3 days after injury (data not shown).

Glycosaminoglycan content of the tissue 3 days after injurious compression decreased with increasing strain rate, although differences were not significant, from 48 ± 4 μg/mg in uninjured controls, to 47 ± 2 μg/mg after injury at a strain rate of 0.01 s^{-1}, to 45 ± 2 μg/mg at 0.1 s^{-1}, and to 43 ± 2 μg/mg at 1 s^{-1} (mean ± SEM; n = 8). Cumulative GAG loss to the medium by 3 days after injury increased with increasing strain rate. After

injury at 1 s^{-1}, cumulative GAG loss to the medium was greater than that of controls by 2.1 ± 0.5 μg/mg.

DISCUSSION

Our results demonstrated that a single compressive overload altered both the mechanical properties of calf cartilage matrix and various measures of chondrocyte behavior in a manner that was dependent on the strain rate of the overload. In particular, this study demonstrated that injury affects not only the basal biosynthetic activity of chondrocytes, but also the ability of subsequent low-amplitude dynamic compression to upregulate biosynthetic activity. Furthermore, the measurement of mechanical properties of the injured explants in shear as well as unconfined compression extended the quantification of damage to the collagen network associated with macroscopic material properties.

These results demonstrated the importance of specifying multiple parameters (i.e., strain, strain rate, and peak stress) when quantifying the injury to the cartilage of a single, controlled, compressive load. When cartilage was compressed to a final strain of 50% at three different strain rates varying over two orders of magnitude (0.01, 0.1, and 1 s^{-1}), the resulting peak stresses varied only from approximately 12 to 23 MPa. Despite this, there was little measured effect on the tissue when compressed at 0.01 s^{-1} but significant injury to the tissue when compressed at 1 s^{-1}. This result might be expected if one hypothesized that injury were related to peak power delivered to the tissue, or any related combination of peak strain and strain rate (or peak stress and stress rate). Furthermore, recent studies by Chen et al (1) using a cyclical impact loading system demonstrated the effect of two different loading waveforms (i.e., two different peak stress rates) on tissue loaded to a given final stress. Although such repetitive cyclical loading protocols may highlight the additional effects of fatigue on loading-induced injury, it is expected that certain results will generalize to single-impact models.

A single injurious compression was found to have significant effects on the biosynthetic activity of the cartilage. Injury at strain rates higher than 0.01 s^{-1} produced a decrease in radiolabeled proline and sulfate incorporation, compared to free-swelling controls, at both 6 hrs. and 3 days after compression. These results are consistent with past studies of the effects of single injurious compression on cartilage (7,25). However, as demonstrated qualitatively by Torzilli et al. (25), a decrease in observed radiolabel incorporation (normalized to DNA content or wet weight) after injury could be due either to a decrease in cellular biosynthetic activity or to a reduction in the number of viable cells. We therefore quantified the cell viability of the tissue 3 days after loading in order to estimate the proteoglycan and total protein synthesis per viable cell. The results suggest that in addition to marked cell death after injury, the biosynthetic activity of the remaining viable cells can still be markedly reduced at 3 days after injury. In contrast with these observations, Jeffrey et al (7) reported that synthetic activity on a per cell basis recovered and exceeded control levels by three days for most impacts. These differences are most likely due to differences in the methods for quantification of cell viability after cell isolation in their study, which used enzymatic digestion of cartilage (we have observed in our lab that enzymatic digestion of tissue after injury can result in the loss of large numbers of chondrocytes which, while initially viable, were too fragile to survive digestion [unpublished data]).

Another consideration is that the red/green or toluidine blue viability assays may be an imperfect tool for assessment of cell death in such situations. Previous research has identified that injurious compression can cause apoptotic cell death (9), and it is unclear whether agents which assess membrane integrity (such as ethidium bromide or propidium iodide) will underestimate cell death due to apoptosis. In addition, the technique used here involving staining of tissue sections may introduce some bias in cell counting due to the difficulty in identifying red-staining nuclei and green-staining cells corresponding to focal planes of identical thickness. Nevertheless, we feel that these estimates are more accurate than those obtained by cell isolation.

In order to characterize further the effect of injurious compression on cellular biosynthetic response, we investigated the ability of injured chondrocytes to respond to dynamic compression. Normal chondrocytes respond to moderate or low-amplitude dynamic compression by upregulating biosynthetic activity (12,13,21), a property which may be an integral part of their ability to maintain a healthy tissue capable of withstanding compressive loads. We found that chondrocytes injured at the higher strain rates lost their ability to respond to dynamic mechanical stimulation. It is important to note that biosynthesis after moderate dynamic compression in these injured plugs was compared to biosynthesis in control plugs which were injured using identical conditions, but not subjected to dynamic compression. Therefore, cell viability associated with injury is equivalent in the tested and control plugs. Furthermore, previous studies (8,21) have shown that the moderate dynamic compression protocol used in this experiment does not alter cell viability. In the context of the present study, the 0.1 Hz, 3% amplitude dynamic compression corresponds to a strain rate of $< 0.01\ s^{-1}$; this strain rate showed no significant decrease in cell viability here.

We also attempted to characterize further the effects of injurious compression on the cartilage tissue matrix itself. Many prior studies have noted that cartilage tissue swells in response to injury. Since the swelling of cartilage is essentially a result of the electrostatic repulsion forces of the charged proteoglycans and opposed by the collagen network, swelling of the tissue after injury is thought to be due to damage to the collagen network (10), which would decrease the restraining forces opposing the swelling pressure within the tissue. As expected, our results show swelling of the tissue soon (6 hrs.) after injury, and that swelling increased with increasing strain rate. In this study, unlike the studies of others (6,9), swelling had increased to approximately the same level in control and injured explants by three days after injury.

In addition to the swelling of the tissue, we quantified the effects of damage to the extracellular matrix on the mechanical properties of the tissue in both shear and compression. The shear stiffness of the tissue, in particular, is known to be very sensitive to the strength and integrity of the collagen network (26). Thus it is interesting

that both the equilibrium and dynamic shear stiffness of the explants injured at 1 s^{-1} were significantly lowered, to about 50% of the stiffness of controls. At 0.1 s^{-1}, the shear stiffness was slightly lower than that of controls but the difference was not significant. The equilibrium and dynamic stiffness of the tissue in unconfined compression showed similar trends, except that dynamic stiffness decreased with increasing strain rates even more than the equilibrium stiffness. Prior studies have quantified damage to the collagen network after injury by comparing stiffness of the tissue in confined and unconfined compression (7,11). The present results are consistent with those studies in demonstrating damage to collagen network is the primary cause for decreased mechanical properties. Along with molecular correlates such as denatured collagen neoepitopes (4), measurement of tissue properties in shear compression offers an improved methodology for quantifying the relationship of damage to the collagen network on the macroscopic material properties of the tissue.

Different compression geometries, protocols, and specimen preparation (i.e., whether or not the cartilage is left on the subchondral bone) must all be kept in mind when comparing results among prior studies. In the presence of bone, several studies have demonstrated that much higher stresses are required to produce the same level of injury as in cartilage taken off the bone (5,15). These studies may more accurately simulate certain conditions of the joint tissue *in vivo*. As a result, the precise strains, strain rates, and stresses reported in our study, performed with tissue removed from the bone and subjected to unconfined compression, should not be directly compared to the magnitudes of loading conditions in vivo. Our experimental design and related model systems used by others are rather intended to focus on mechanisms relating controlled mechanical parameters to observed changes in cells and matrix.

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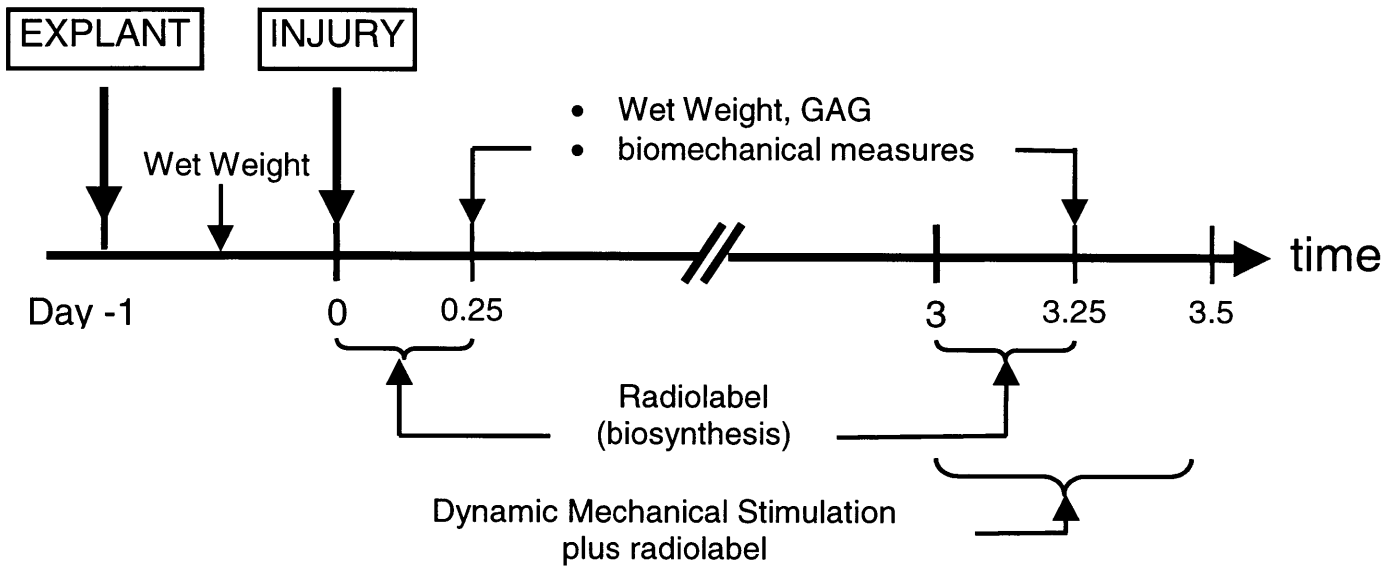

Fig. 1 Schematic timeline of the experimental design for study of the effects of injurious compression on biomechanical properties, biochemical changes, and subsequent response to moderate dynamic compression. Measurements at day 0 and 3 were made with separate groups of explants.

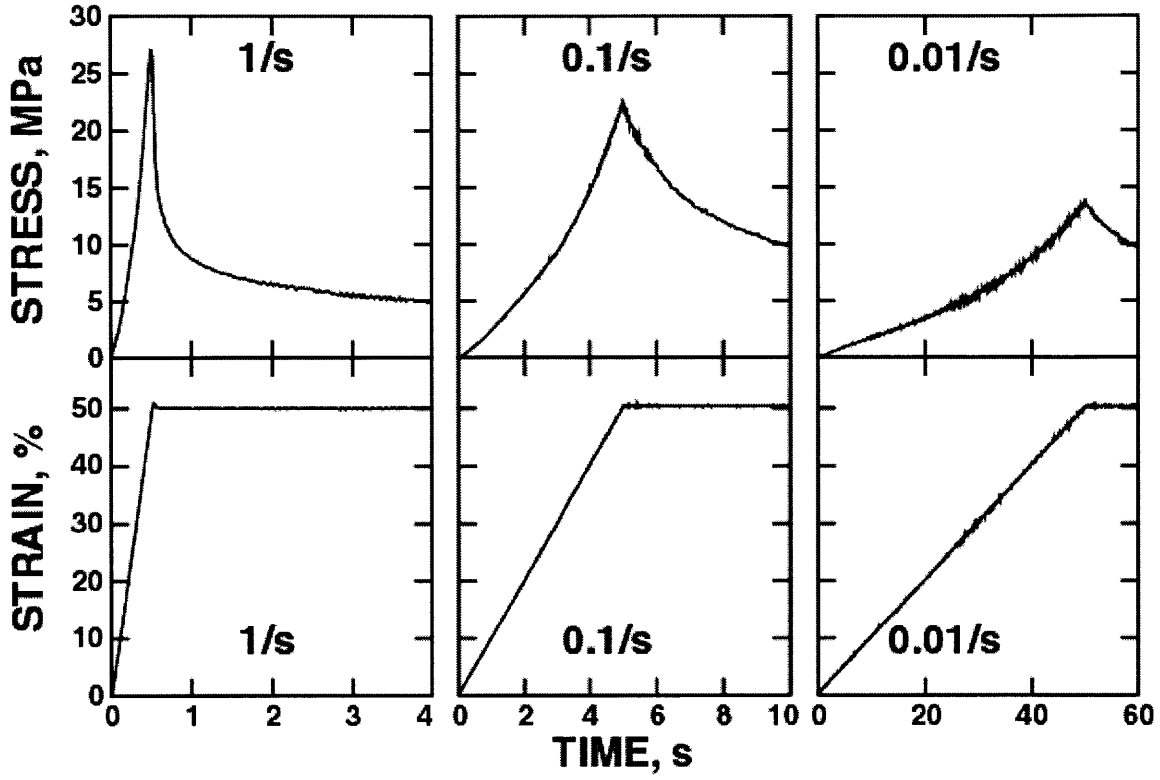

Fig. 2 Representative waveforms of applied strain and resulting stress versus time for the initial 4-60 s of a single five-minute injurious compression protocol. Four cylindrical explant disks (1 mm thick x 3 mm diameter) per group were compressed simultaneously at strain rates of 0.01, 0.1, and 1 s^{-1} until a final strain of 50% was achieved. Stress is computed as the total load divided by the uncompressed cross-sectional area of four disks.

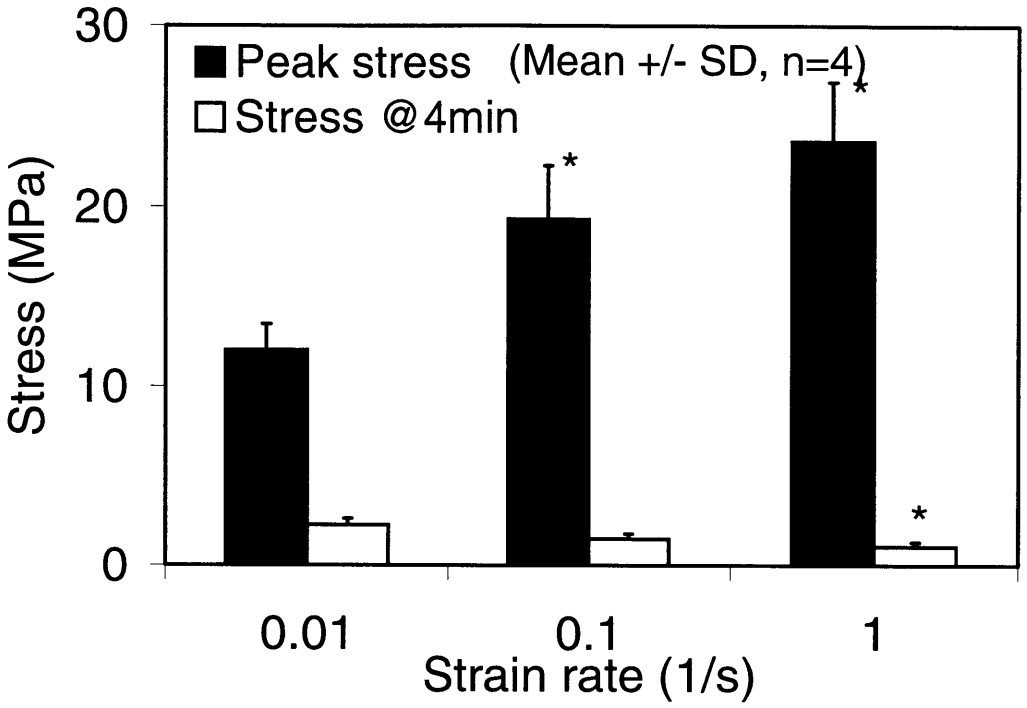

Fig. 3 Peak stress and stress after four minutes of stress-relaxation while held at the final strain of 50%. Data corresponds to mean ± SD of four separate experiments at each strain rate, with each experiment involving simultaneous compression of four cartilage disks.

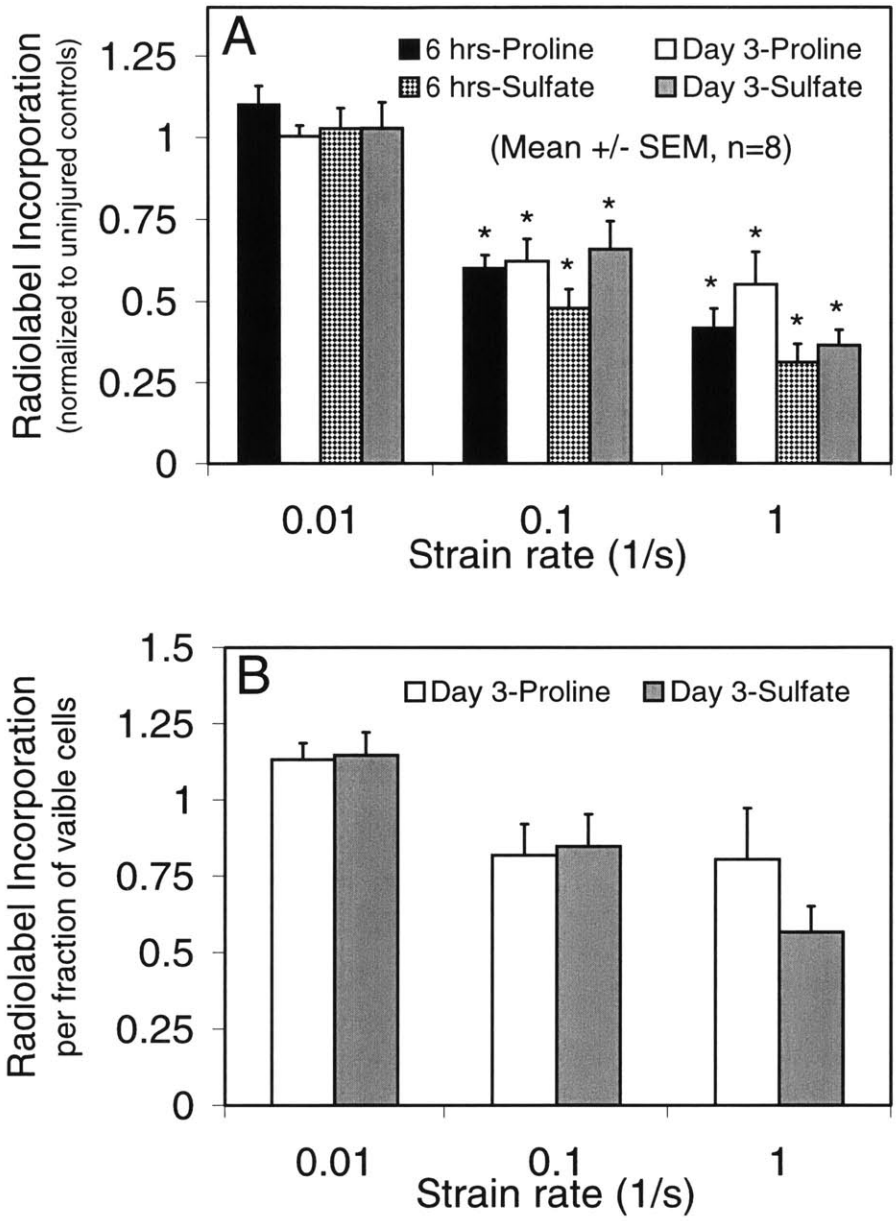

Fig. 4 (A) Biosynthetic activity of cartilage explants under free-swelling conditions at 0.25 and 3 days after single injurious compression to a final strain of 50% at strain rates of 0.01/s, 0.1/s, and 1/s. The data for 35S-sulfate and 3H-proline incorporation correspond to two separate experiments at each condition (four explants per experiment). The values are normalized to the mean radiolabel incorporation of uncompressed control explants. (B) Radiolabel incorporation data of Fig. 4A re-normalized to the mean percentage of viable cells (mean ± SEM, n = 8; *$p < 0.05$) compared to uninjured controls.

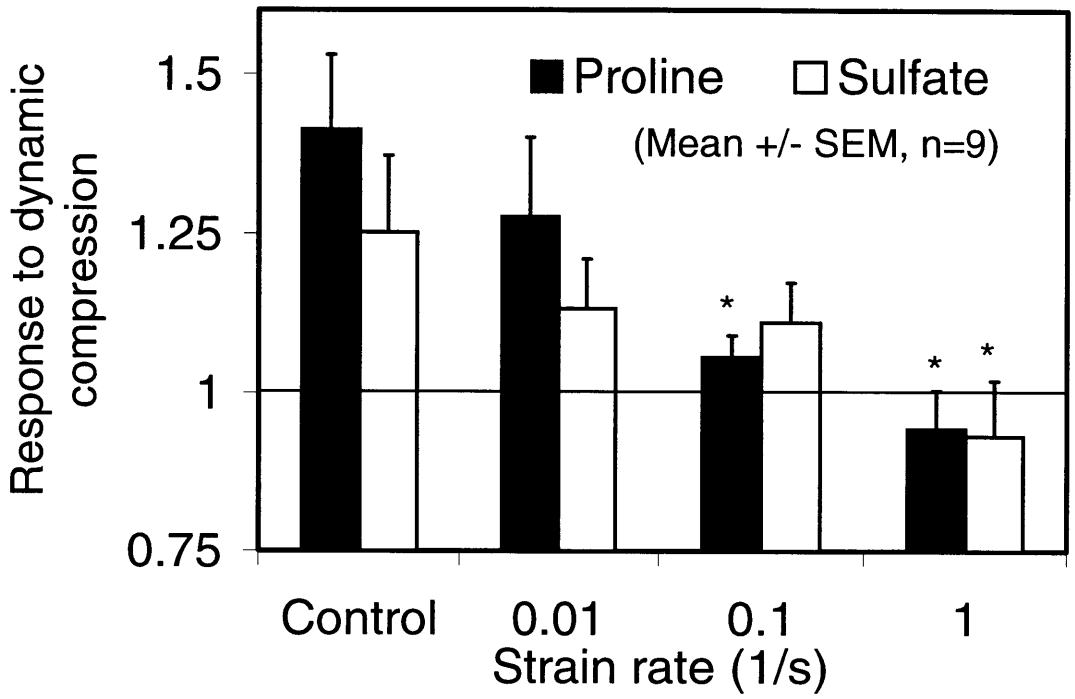

Fig. 5 35S-sulfate and 3H-proline incorporation in cartilage disks during a twelve-hour dynamic compression (3% dynamic strain amplitude at 0.1 Hz, superimposed on a 10% static offset compression), three days after an injurious compression at strain rates of 0 (control), 0.01 s^{-1}, 0.1 s^{-1}, and 1 s^{-1}. The data are normalized to the radiolabel incorporation of explants which were held at the same 10% static offset and subjected to the same injurious compression (dotted line). (mean ± SEM, n = 12, $p < 0.05$)

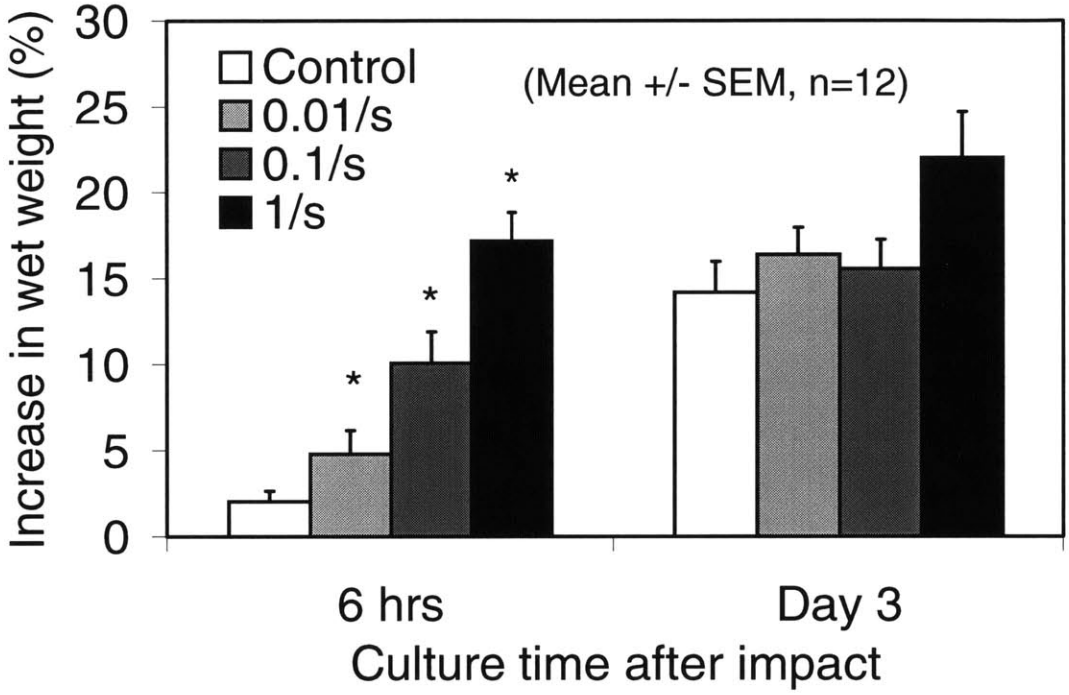

Fig. 6 Percentage increase of cartilage explant wet weight at 6 hours and 3 days after a single injurious compression at strain rates of 0.01/s, 0.1/s, and 1/s. (mean ± SEM, n = 12, $p < 0.05$)

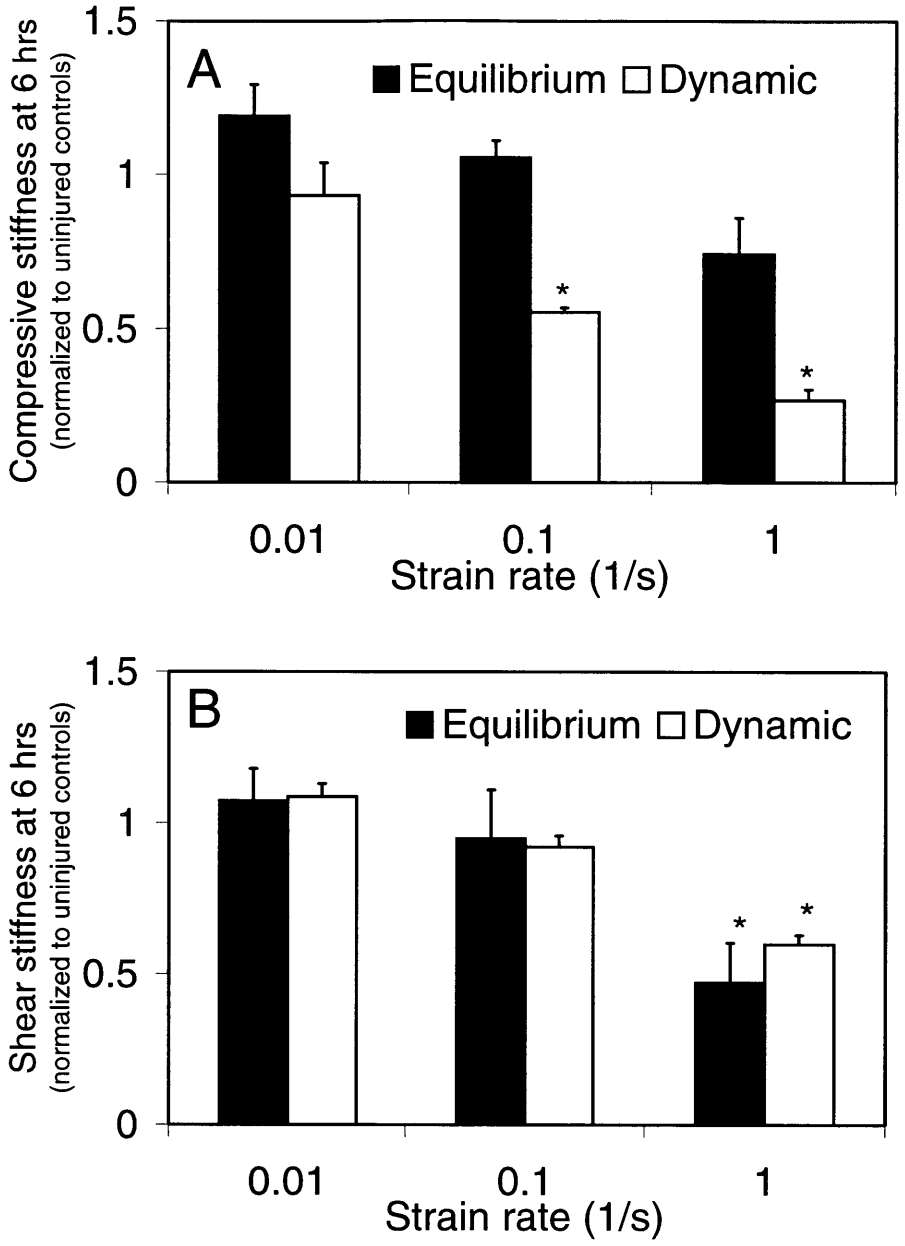

Fig. 7 (A) Equilibrium stiffness and dynamic stiffness (at 0.5 Hz) of cartilage explants in uniaxial unconfined compression at 6 hours after a single injurious compression at strain rates of 0.01/s, 0.1/s, and 1/s. (B) Equilibrium shear stiffness and dynamic shear stiffness (at 0.5 Hz) of cartilage explants at 6 hours after a single injurious compression at strain rates of 0.01/s, 0.1/s, and 1/s. The data are normalized to the mechanical properties of uncompressed control explants (mean ± SEM, $n = 3$, $p < 0.05$).