MECHANISMS AND FUNCTIONAL IMPLICATIONS OF AGGRECAN CATABOLISM IN CARTILAGE AND MENISCAL FIBROCARTILAGE

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MECHANISMS AND FUNCTIONAL IMPLICATIONS OF AGGRECAN CATABOLISM IN CARTILAGE AND MENISCAL FIBROCARTILAGE

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"*Panta Rhei*"

Heraclitus

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Words cannot due justice to what I feel about the people in my life. Sometimes, when I think about these people – my family, friends from home, friends from college, my roommates, random smiles I pass in the hallway, and kindred spirits - my heart nearly explodes. This document is, physically, a static stack of paper or 1s and 0s in a pdf, and describes how some cells remodel their home, the extracellular matrix. What this document can't tell you is how much I have grown – have been remodeled - over the last 4-ish years through *dynamic exchange* with dozens of people. It is with utmost gratitude for these people and great anticipation for the ones I haven't met yet that I bring this push, part of my dream, a life's chapter…to a close.

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LIST OF SYMBOLS AND ABBREVIATIONS

AC	Articular chondrocyte
ADAMTS	A disintegrin and metalloproteionase with thrombospondin-type motifs
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
IGD	Interglobular domain
IL-1α,β	Interleukin-1α,β
IL-1RA	Interleukin-1 receptor antagonist
ITS	Insulin, transferrin, selenium
kDa	KiloDaltons
MFC	Meniscal fibrochondrocyte
MMP	Matrix metalloproteinase
NEAA	Non-essential amino acids
OA	Osteoathritis
DPBS	Dulbecco's phosphate buffered saline
sGAG	Sulfated glycosaminoglycans
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TGF-β	Transforming growth factor β
TGF-βRI	Transforming growth factor β type I receptor
E*	Complex compression modulus
G*	Complex shear modulus
δ	phase angle

SUMMARY

Articular cartilage and fibrocartilage participate in bone-to-bone load transfer and provide low-friction surfaces for smooth joint motion. These tissues are susceptible to traumatic injury and arthritic disease, and their degeneration leads to loss of joint function and pain for the patient. Cells that reside in cartilage and fibrocartilage normally remodel the extracellular matrix in response to biochemical and mechanical signals, and progression of joint disease is characterized by excessive cell-mediated degradation of the tissues. In addition, pathologic changes in tissue composition typically result in loss of tissue material properties. Differences between cartilage and fibrocartilage matrix composition, cell metabolism, and mechanical function suggest that the mechanisms and functional implications of normal and pathologic remodeling may also be different. The goal of this work was to examine tissue-specific responses to catabolic and anabolic stimuli with respect to production and processing of aggrecan, a structural matrix molecule in cartilage and fibrocartilage. Studying matrix remodeling can contribute to our understanding of arthritis, and may give insight into therapeutic approaches for the repair or replacement of degenerative joint tissues.

As an *in vitro* model of cartilage degradation, explanted cartilage and fibrocartilage were subjected to stimulation with a proinflammatory cytokine. Selective protease inhibitors were used to perturb matrix remodeling, antibodies raised to aggrecan neoepitopes were used to characterize protease activity, and compression and torsion tests were used to measure tissue material properties. Time course experiments showed that protease inhibitors delayed, but did not block destructive aggrecan remodeling in

cartilage. In contrast, fibrocartilage cultures treated with a broad-spectrum metalloproteinase inhibitor were protected from degradation as indicated by biochemical assay and mechanical testing. For the final experiments, chondrocytes and fibrochondrocytes were suspended in agarose and stimulated with an anabolic growth factor. The cell types exhibited similar patterns of aggrecan processing, though proteoglycan biosynthesis and construct material properties were substantially higher in the chondrocyte cultures. Collectively, these results reveal intrinsic differences in tissue-specific cellular responses to catabolic and anabolic cytokines that may underlie some aspects of arthritic joint degeneration.

CHAPTER 1

INTRODUCTION

1.1 Motivation

Arthritis includes many conditions of the joints characterized by inflammation, pain, and loss of joint function that affect 66 million people in the U.S. alone[9]. During arthritic degeneration of articular cartilage, the resident cells, chondrocytes, exhibit downregulated synthesis of extracellular matrix (ECM) molecules and upregulation of proteolytic enzymes. Fibrochondrocytes, found in meniscal fibrocartilage, appear to behave in a similar way. The disrupted balance of biosynthetic and proteolytic activity in these tissues can be driven by proinflammatory cytokines like interleukin-1α and β (IL-1α,β). Treatment with IL-1 initiates rapid depletion of proteoglycan, primarily aggrecan, from the tissue, and there is a concomitant loss of mechanical function indicating that aggrecan catabolism is a significant, early event in degeneration. Transforming growth factor-β (TGF-β) is a pleiotropic cytokine and anabolic stimulus implicated in cartilage development, homeostasis, and repair. Chondrocytes stimulated with TGF-β exhibit upregulated aggrecan expression, but also exhibit upregulation of aggrecan-degrading enzymes; the duality in this response suggests an important role for aggrecan catabolism in normal aggrecan turnover. Proteolytic systems responsible for aggrecan degradation, therefore, have been the focus of intense research and drug design efforts.

Metalloproteinases, including matrix metalloproteinases (MMP) and a disintegrin and metollproteinase with thrombospondin motif (ADAMTS) class enzymes have demonstrated efficient, distinct aggrecanolysis activity in *in vitro* and *in vivo* models of

arthritis. ADAMTS-4 and ADAMTS-5 (aggrecanase-1 and -2, respectively) are thought to be primary mediators of pathologic aggrecan catabolism, while MT4-MMP (MMP-17) may be involved in ADAMTS activation[70,78,169,197,211,223]. There is growing evidence, however, for metalloproteinase-independent mechanisms of aggrecan catabolism[199,212,219]. The neutral cysteine proteinase m-calpain has been detected in synovial fluid and cartilage ECM from arthritic joints, and chondrocytes have been shown to secrete this typically intracellular protease[66,217,218,248]. Recently, *in vitro* m-calpain-mediated aggrecanolysis was shown to yield aggrecan cleavage products found in extracts of mature bovine articular cartilage[164]. It is possible that m-calpain cooperates with the metalloproteases to mediate aggrecanolysis in cartilage. Interestingly, the 5'-flanking region of the gene encoding m-calpain contains an activation protein-1 (AP-1) transcription factor binding site[85], and many MMPs have also been shown to be regulated by AP-1[14,125,216,234]. This suggests a convergence, and possible therapeutic target, in the upstream regulation of metalloproteinases and m-calpain expression.

To examine the roles of aggrecanases, MMPs, and m-calpain in chondrocyte- and fibrochondrocyte-mediated aggrecanolysis, two investigative tools were used throughout the studies in this dissertation. First, low-molecular weight (300-450Da) pharmacologic protease inhibitors were used to selectively perturb aggrecanolysis by these enzyme systems. Second, antibodies raised to protease-specific "neoepitopes," the peptide sequences exposed following cleavage of the aggrecan core protein, were used to detect aggrecan fragments in tissue extracts and conditioned media.

Cartilage and fibrocartilage function principally as load-bearing connective tissues, and tissue "functionality" may be quantified by measurement of material

properties, such as complex moduli and tangents of the phase angle (tan δ). Moduli describe a material's resistance to deformation, whereas tan δ is the ratio of viscous (time-dependent) to elastic (time independent) material responses. Sulfated glycosaminoglycans (sGAG), the negatively charged carbohydrate chains on the aggrecan core protein, contribute to the compression moduli and viscoelasticity of cartilage, and may also participate in resisting shear stresses. Dynamic mechanical tests for measuring compression and shear moduli were performed to evaluate the functional implications of aggrecan processing in cartilage and fibrocartilage.

1.2 Research Objectives

The overall objective of this work was to investigate metalloproteinases and m-calpain as comediators of aggrecan turnover in articular cartilage and meniscal fibrocartilage. The central hypothesis of this dissertation is that aggrecanases, MMPs, and m-calpain differentially alter the material properties of articular cartilage and meniscal fibrocartilage by participating in cell-mediated aggrecan turnover.

Specific Aims

The first two aims address mechanisms of pathologic aggrecan processing in cartilage and meniscal fibrocartilage. The third aim addresses mechanisms of aggrecan turnover under anabolic conditions. For specific aims 1 and 2, explanted tissue was stimulated with exogenous IL-1α as a model of cell-mediated ECM degradation in the inflamed joint. Studies in aim 3 used complimentary model systems of TGF-β1-stimulated tissue explants and cell-agarose constructs as models of cell-mediated ECM homeostasis and assembly, respectively, to investigate aggrecan turnover mechanisms in

developing cartilage and fibrocartilage. For all studies, tissue and cell sources were from immature bovine stifle joints.

Specific Aim 1: Analyze the kinetics and functional implications of metalloproteinase-mediated ECM degradation in IL-1-stimulated articular cartilage.

Hypothesis 1: Aggrecanases are primary mediators of aggrecan degradation and loss of tissue compression and shear properties in IL-1-stimulated cartilage.

In vitro stimulation of articular cartilage explants with IL-1 is a well-established model of cell-mediated degradation. For this aim, articular cartilage explants from immature bovine stifle joints were cultured with or without 20ng/mL IL-1α for up to 24 days. Some of the IL-1-stimulated explants were additionally treated with pharmacologic protease inhibitors selective for aggrecanases (including ADAMTS-4 and -5), MMPs, or both aggrecanases and MMPs. Inhibitor doses were determined from a preliminary dose response study. Explant material properties were measured through equilibrium and oscillatory unconfined compression tests and oscillatory torsion tests. The aggrecan and collagen contents of explant digests and conditioned media were quantified by spectrophotometric methods. To investigate aggrecanase- and MMP-mediated aggrecanolysis in IL-1-stimulated explants, conditioned media were probed with antibodies to the aggrecan G1-NITEGE and aggrecan G1-VDIPEN neoepitopes, respectively. Finally, aggrecan G1-NITEGE was immunolocalized in thin sections of explants at days 4 and 20 of the experiment.

Specific Aim 2: Analyze the localization, kinetics, and functional implications of metalloproteinase-mediated ECM degradation in IL-1-stimulated meniscal fibrocartilage.

Hypothesis 2: Aggrecanases and MMPs are primary mediators of ECM degradation and loss of tissue compression and shear properties in IL-1-stimulated fibrocartilage.

Initial work for this aim established regional variations in sGAG density and aggrecanase activity (by immunodetection of aggrecan G1-NITEGE) in freshly isolated meniscal fibrocartilage. In a subsequent experiment, fibrocartilage explants isolated from the middle region midsubstance were stimulated with or without 20ng/mL IL-1α for up to 12 days. Some explants were additionally treated with pharmacologic protease inhibitors selective for aggrecanases, MMPs, or both aggrecanases and MMPs. Explant material properties were measured by oscillatory unconfined compression and oscillatory torsion tests. The aggrecan and collagen contents of explant digests and conditioned media were quantified by spectrophotometric methods. To investigate release of aggrecanase- and m-calpain-generated aggrecan from IL-1-stimulated explants, conditioned media were probed with antibodies to the aggrecan G1-NITEGE and aggrecan-DLS neoepitopes, respectively. Aggrecan retained within the explants was characterized by immunoblots of explant extracts for the aggrecan G1 domain. The results of this aim showed that mechanisms of proteoglycan catabolism and release in meniscal fibrocartilage were distinct from those in cartilage. The work in this aim also revealed that aggrecanases were active in basal cultures of fibrocartilage with only moderate effects on explant material properties. This observation helped motivate the investigation of aggrecan turnover in cartilage and fibrocartilage under less catabolic conditions in aim 3.

Specific Aim 3: Examine the contributions and functional implications of aggrecanase-, MMP-, and m-calpain-mediated aggrecanolysis in TGF-β-stimulated cartilage and fibrocartilage.

Hypothesis 3: Aggrecanase-, MMP-, and m-calpain-mediated aggrecanolysis are essential to aggrecan turnover and maintenance of tissue material properties in cartilage fibrocartilage.

The contribution of aggrecanolysis to TGF-β-induced aggrecan turnover and ECM assembly in cartilage and fibrocartilage were investigated. Aggrecan turnover was studied first in cartilage and fibrocartilage explants, similar to those prepared in chapters 3 and 4. Explants were cultured under serum-free conditions for up to 10 days in the absence or presence of 5ng/mL TGF-β1. Some of the TGF-β1-stimulated explants were additionally treated with 5μM of an aggrecanase-, MMP-, or calpain-selective inhibitor. In addition, a group of basal (unstimulated) explant cultures was treated with a pharmacologic inhibitor to the kinase domain of TGF-β type I receptor to investigate the participation of endogenous TGF-β in cartilage and fibrocartilage ECM turnover. In a second study investigating *de novo* aggrecan deposition and turnover, cells liberated from articular cartilage and fibrocartilage were suspended in agarose and cultured with or without 10ng/mL TGF-β1 for up to 16 days. Groups of TGF-β1-stimulated cell-agarose constructs were additionally treated with 1μM aggrecanase-, MMP-, or calpain-selective inhibitor. Mitochondrial activity was evaluated at the end of each study by the WST-1 assay. The distributions of proteoglycans were quantified by measurement of sGAG in

tissue extracts, residual tissue digests, and conditioned media. Extracted proteoglycans were immunoblotted for the aggrecan G1, G2, and G3 domains and the NITEGE and DLS neoepitopes. Explant and construct material properties were determined by oscillatory unconfined compression and oscillatory torsion tests.

1.3 Scientific Contribution

ECM turnover describes the cell-mediated processes that underpin tissue development, maintenance, and numerous pathologies. Study of molecular mechanisms linking cytokine stimulation, aggrecan catabolism, and alterations in the material properties of cartilage and fibrocartilage may reveal ways of disrupting or inducing turnover for therapeutic benefit. This work examines tissue-specific mechanisms and functional implications of aggrecan processing in models of destructive and non-destructive aggrecan turnover.

The work in this dissertation demonstrates marked differences in mechanisms of aggrecan turnover between cartilage and meniscal fibrocartilage. Stimulation with either IL-1 or TGF-β triggers tissue-specific patterns of proteoglycan release and retention and the immature meniscus exhibits regional variations in aggrecan processing. The signals regulating aggrecan turnover remain unclear, but tissues (or regions of tissues) that are abundant in high molecular weight aggrecan typically undergo compression *in vivo*. Degenerative joint disease is characterized by aberrant degradation and release of aggrecan from cartilage and subsequent loss of joint function. Elucidating the pathogenesis of cell-mediated degeneration will require understanding the quantitative and tissue-specific relationships between changes in aggrecan structure and loss of tissue function. In addition, identifying the regulatory signals that trigger tissue-specific cell

ECM assembly and turnover will be useful in the design and evaluation of engineered tissue replacements.

CHAPTER 2

BACKGROUND

2.1 Cartilage and Fibrocartilage: Pathology

Degenerative joint disease is increasingly responsible for compromised quality of life and morbidity in the aging U.S. population. Arthritis currently affects 46 million people and costs $86 billion annually in the U.S. alone[10]. The pathogenesis of osteoarthritis (OA), the most prevalent form, is unknown, but traumatic injury to the joint (*i.e.*, injuries causing tears, lesions, sprains, or rupture of load-bearing connective tissues) and advanced age are prognostic for OA. OA is characterized by erosion of the cartilage on the articulating surfaces of bones, loss of joint range of motion, and pain. The mechanisms underlying cartilage degradation in OA are the subject of intense research, and the clinical management of injured joints and arthritis is expected to improve with the development of engineered tissue replacements and pharmacologic inhibitors of tissue destruction.

The knee menisci are fibrocartilage tissues located between the femoral condyles and the tibial plateau and are essential to maintaining healthy joint mechanics [178,237]. Disruption of normal joint mechanics by injurious damage to the menisci leads to rapid onset of OA[50] (Figure 2.1). Alternatively, the menisci undergo age-related degenerative changes in tissue composition and material properties[40,67,104,161]. Meniscal degeneration can make the joint susceptible to tears or it can persist with long-term sequellae characteristic of OA. Thus, identifying mechanisms of meniscal degeneration will be important in the pathogenesis of OA.

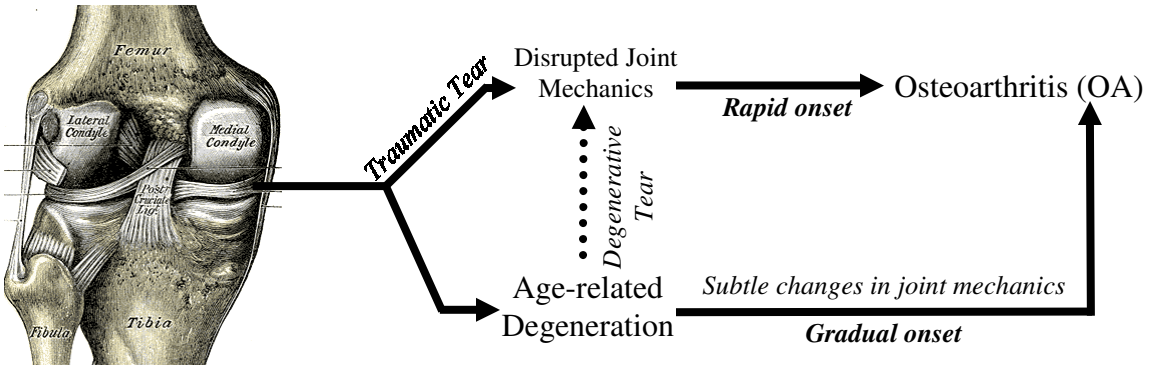

Figure 2.1 Pathways of joint pathology stemming from damage to the meniscal fibrocartilage. The menisci may be injured, leading to abrupt changes in joint mechanics. Alternatively, meniscal fibrocartilage undergoes degenerative changes in ECM composition and material properties with age that predispose the menisci to tears (dotted arrow) or cause gradual adverse changes in joint mechanics. Both injury and degeneration lead to OA and loss of joint function. Image at left is from Gray, 1918, and is in the public domain.

The clinical management of joint injury begins with arthroscopic examination of joint surfaces and the menisci. Magnetic resonance imaging is also used to assess disruptions in joint spaces and some aspects of tissue structure. Focal lesions in the articular cartilage may be treated in a variety of ways, depending on the site and severity of injury. Mosaicplasty, in which fragments of cartilage are harvested from unloaded or peripheral joint surfaces and implanted into the injury site, and autologous chondrocyte implantation, in which cells isolated from periosteal tissues are expanded *ex vivo* and implanted into the injury site, are two procedures developed for repair of focal cartilage lesions. Menisci with tears in the "white" (inner avascular) zone may undergo partial resection to remove damaged tissue. Procedures ancillary to partial menisectomy, such as drilling and rasping, were developed to elicit intrinsic repair processes and enhance recruitment of progenitor cells. Surgical interventions have demonstrated substantial utility in the clinical management of soft tissue injury.

Surgical repair of injured joint tissues does not prevent the onset of degenerative disease. Rather, therapeutic approaches to managing late stage rheumatoid and

osteoarthritis have targeted the cytokines and proteases that drive tissue degeneration. IL-1 receptor antagonist is a competitive inhibitor of IL-1α and IL-1β that binds the IL-1 receptor without triggering intracellular signals. Similarly, soluble tumor necrosis factor-alpha (TNF-α) receptor or anti-TNF-α antibodies sequester TNF-α and thus reduce or block this cytokine's proinflammatory effects. Inhibition of IL-1 and TNF-α, individually or in combination, has demonstrated the potential to interrupt cartilage degeneration in a variety of arthritis models and in human patients[15,16,54,69,103,114,167,243,254,257]. Indeed, Anakinra© and Infliximab© are biologics based on IL-1 receptor antagonist and anti-TNF-α currently in clinical trials. Despite the apparent efficacy of these inhibitors of upstream signaling events, not all patients respond to them, suggesting that other signaling pathways can also drive degenerative joint disease.

2.2 Cartilage and Fibrocartilage: Composition and Function

Articular cartilage is a highly hydrated soft tissue that permits smooth, low friction transfer of compressive loads between bones. A low cell density, dense extracellular matrix (ECM), and minimal vascular supply contribute to the tissue's poor regenerative capacity. The ECM is rich in type II collagen and the large proteoglycan, aggrecan[117]. The N-terminal globular domain (G1) of aggrecan is associated with link protein and a hyaluronic acid (HA) backbone (Figure 2.2). The more C-terminal regions of the aggrecan core protein are heavily glycosylated with chondroitin sulfate and keratan sulfate, forming sulfated glycosaminoglycan (sGAG) – rich regions of the proteoglycan. Entanglement of the HA and collagen networks allows aggrecan to be retained within the ECM, and interactions between the confining collagen network and the hydrated

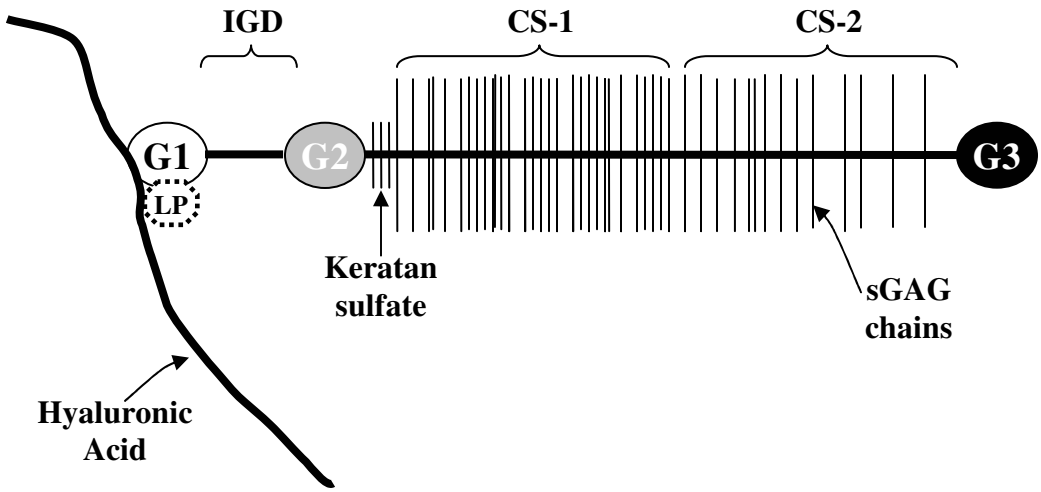

Figure 2.2 Aggrecan complexes with link protein (LP) and hyaluronic acid in the extracellular matrix (ECM). Globular domains of the aggrecan core protein are marked with a G. The interglobular domain (IGD), keratan sulfate domain, and 2 sulfated glycosaminoglycan (sGAG)-rich domains (CS-1 and CS-2) are delineated above.

proteoglycans confer biphasic compressive properties[152]. Compression of cartilage pressurizes interstitial fluid in the ECM, and this pressure is dissipated as the fluid is extruded from the tissue; an osmotic pressure generated by concentrated, negatively charged sGAG side chains on aggrecan core proteins aids rehydration and maintains swelling of the ECM upon unloading[48]. The equilibrium compressive properties of cartilage are dictated almost exclusively by the concentration of sGAG, as repulsive electrostatic interactions between negative charges of juxtaposed sGAG chains provide resistance to compaction of the ECM. The concentration of proteoglycan also influences the shear properties of the tissue[106]. Retention of aggrecan – especially the sGAG-rich portion of the molecule – is essential to the mechanical function of articular cartilage.

Meniscal fibrocartilage shares structural and functional features with articular cartilage, including low cell density, a dense ECM, a complex mechanical loading environment, and poor regenerative capacity. There are, however, important differences in ECM composition and organization, cell morphology, and mechanical properties

between the tissues. The menisci of the knee are semilunar-shaped structures composed primarily of collagen type I. Other collagens, including types II and VI, are also present and appear to be associated with the collagen type I fibers. The small proteoglycans, decorin and biglycan, which have roles in collagen fiber assembly, are found in the meniscus[140], and the aggrecan G1 domain was recently immunolocalized along collagen fibers in the canine meniscus[230]. Collagen fibers, especially at the surface and outer regions of menisci, are oriented circumferentially and confer the tensile strength required for bearing hoop stresses generated during physiologic loading[56]. The inner portion of the meniscus bears primarily compressive loads and is richer in sGAG and intact aggrecan, similar to articular cartilage[156]. A primary function of the menisci is to minimize stress concentrations at the articulating surfaces of the adjacent bones. The concave triangular cross section and relatively high compliance of healthy menisci afford conformity and congruency with the pseudo-spherical femoral condyles and the planar tibial plateau. The menisci also reduce stress concentrations on surrounding cartilage by dissipating energy[143,237,238]. Fibrocartilage exhibits a biphasic response to loading in which energy is dissipated through the extrusion of water. The role of aggrecan in generating an energy absorbing, compliant fibrocartilage ECM is unknown, and this appears to be quite different from that in articular cartilage.

2.3 Regulation of ECM Turnover in Cartilage and Fibrocartilage

Chondrocytes and fibrochondrocytes are sensitive to physical and biochemical stimuli which, in concert, regulate the balance between anabolic and catabolic activity in ECM turnover. Oscillatory compressive loading, for example, can stimulate chondrocytes to secrete ECM proteins[189]. Chondrocyte metabolism is also regulated by insulin-like

growth factor (IGF) and its binding proteins (IGFBPs) and members of the transforming growth factor β (TGF−β) family; IGF has generally anabolic effects [142], whereas TGF-β can have anabolic or catabolic effects[79,151]. Inflammatory cytokines such as interleukin-1 α or β (IL-1α,β) and tumor necrosis factor α (TNF-α) depress synthesis of ECM molecules and upregulate expression/activity of proteases responsible for ECM degradation[190,227]. Mechanisms and functional consequences of aggrecan degradation in articular cartilage have been studied *in vitro*, and IL-1 is commonly used to induce arthritis-like changes in tissue properties, cell signaling, and protease activity. IL-1 stimulation of bovine cartilage explants triggers loss of sGAG and tissue biophysical properties[22,23,170]. The cytokine has been immunolocalized in osteoarthritic (OA) and rheumatoid arthritic (RA) tissue[42,222], and serves as a potent paracrine, autocrine, and intracrine signal for initiation and progression of the catabolic cascade[226,241]. Static compressive loading of surface-zone cartilage has also been shown to downregulate ECM synthesis, and, interestingly, this response can be blocked by IL-1 receptor antagonist[153].

In explant culture, meniscal fibrochondrocytes respond to static and dynamic compressive stimulation by upregulation of MMPs and the small proteoglycan decorin, and increasing general biosynthetic activity; interaction between IL-1 signaling and mechanical stimulation is also reported in this model system[207,228]. The mechanisms of aggrecan turnover in meniscal fibrocartilage are largely unknown, though disrupted proteoglycan metabolism has been observed in long-term explant cultures[232].

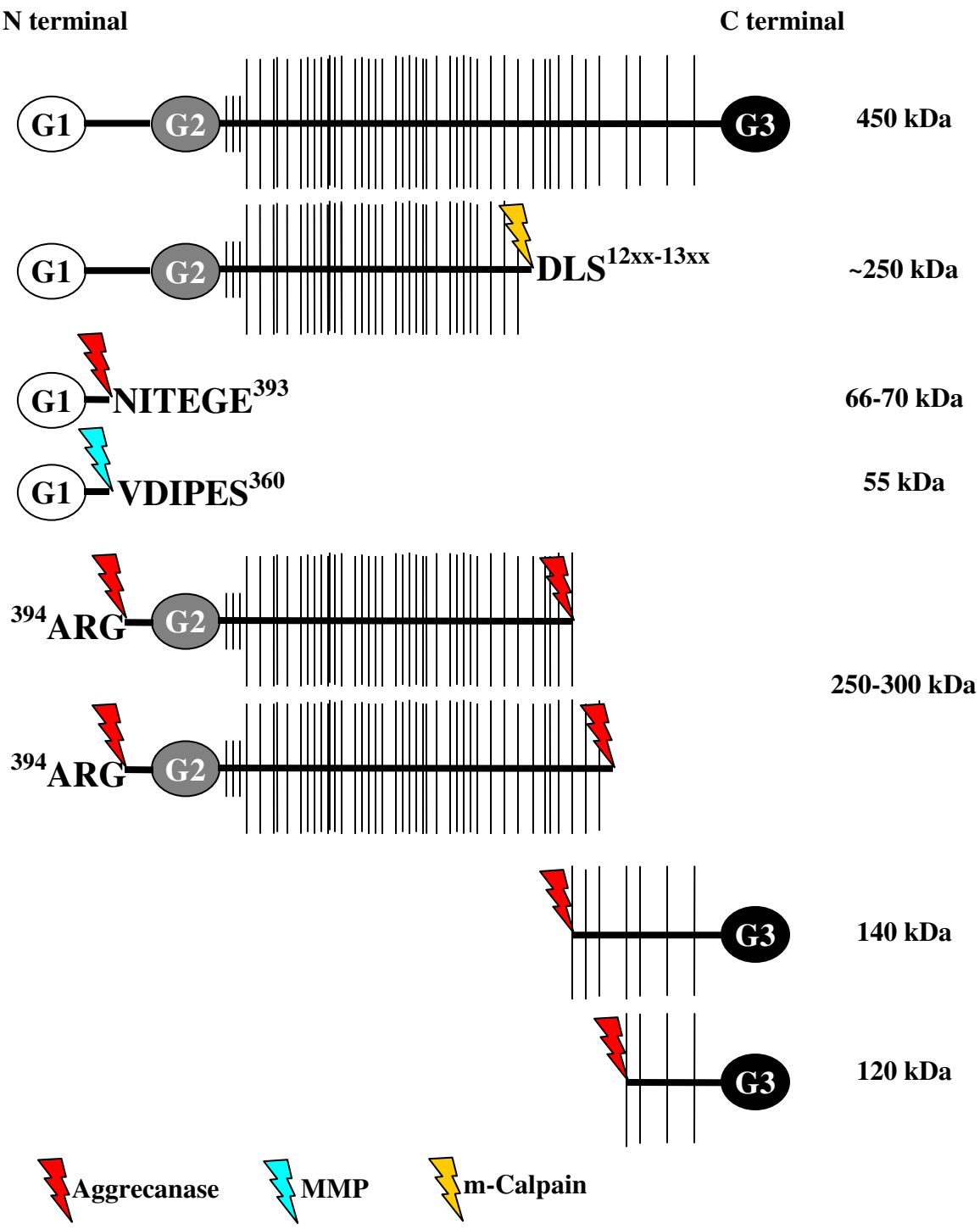

Figure 2.3 A subset of aggrecan species detected in articular cartilage and synovial fluid. Bolts indicate enzyme-specific sites of proteolytic attack. Neoepitope sequences are given with terminal residue numbers based on the sequence of bovine aggrecan. The DLS neoepitope can be exposed at multiple points within the CS-1 domain of aggrecan. Approximate molecular weights (from migration in SDS-PAGE) are shown to the right of each fragment.

2.4 Mechanisms of Aggrecan Catabolism

Proteases responsible for ECM degradation in cartilage degeneration include MMP and ADAMTS class enzymes. There are 23 known MMPs, and collectively they are capable of hydrolyzing every type of extracellular matrix and basement membrane molecule. The collagenases (MMP-1, -8, -13), the gelatinases (MMP-2, -9), stromelysin-1 (MMP-3), and membrane-type MMP-14 and -17 are expressed as zymogens by chondrocytes constitutively or upon stimulation with proinflammatory cytokines[12,20,70,115,162]. Regulation of MMP activity is achieved post-translationally by removal of pro-domains and extracellular abundance of tissue inhibitors of metalloproteinases (TIMPs). MMPs generally have broad substrate specificity, as MMPs-1, -2, -3, -8, -13, and -14 are capable of degrading multiple collagens (including types I and II) as well as proteoglycans, including aggrecan. MMP-mediated aggrecanolysis, indicated by generation of the aggrecan G1-VDIPES neoepitope (Figure 2.3), does not contribute substantially to release of sGAG from IL-1-stimulated cartilage explants. ADAMTS-1, -4, -5, -8, and -15 demonstrate more specific activity, efficiently cleaving at multiple sites on the aggrecan core molecule[39,154,182] (Figure 2.3). ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) have been studied extensively in the context of cartilage degradation, and are responsible for up to 90% of the pathologic aggrecan catabolism observed in cartilage explants over 72 hours of IL-1 stimulation[223]. Interestingly, MMP-17 was recently shown to participate in post-translational modification of ADAMTS-4, indicating a possible downstream convergence point in regulation of MMP- and ADAMTS-mediated aggrecan catabolism[70]. ADAMTS-4 is translated as a protein that undergoes furin-mediated removal of a pro-domain within the

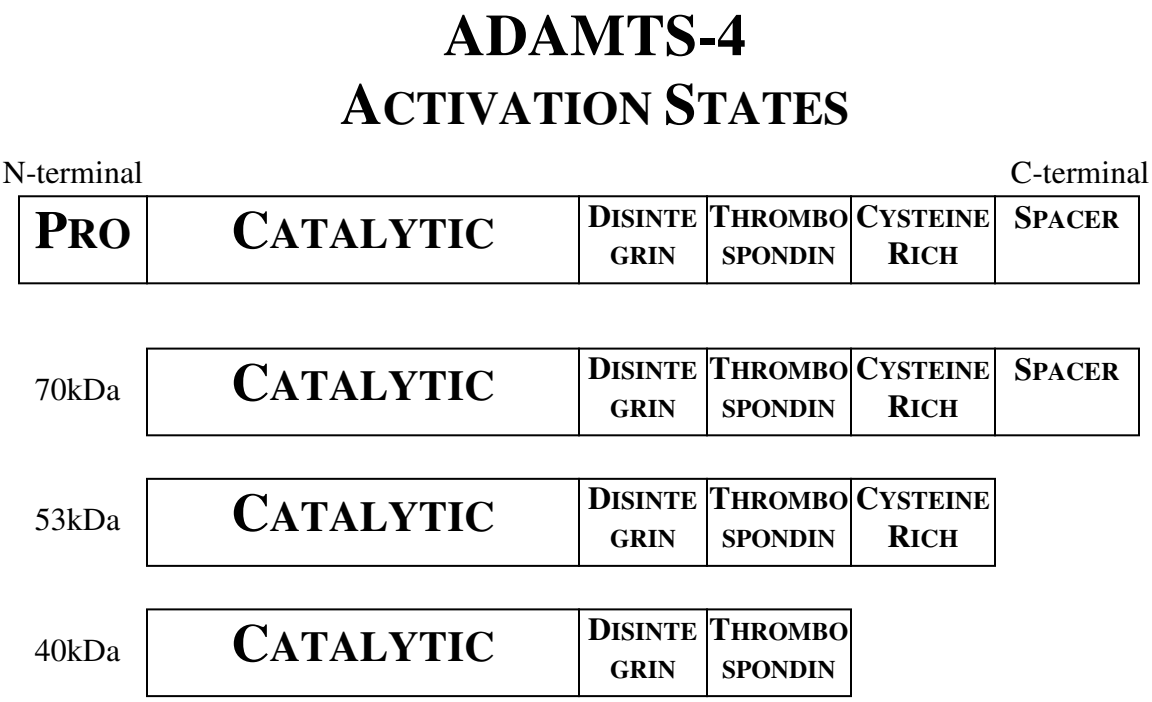

Figure 2.4 ADAMTS-class proteases undergo proteolytic processing, which modulates their activity. Full length ADAMTS-4 (Aggrecanase-1, 100kDa) is first activated by furin-mediated removal of the N-terminal pro-domain. The enzyme's activity is enhanced further through C-terminal trimming by MMPs and/or autocatalysis. The 40kDa species is thought to be the minimal structure for retaining proteolytic activity. Adapted from [70].

cell[233] (Fig. 2.4). The resulting secreted form is capable of cleaving aggrecan only within the sGAG-rich region; alternatively, this form can complex with glycosylphosphatidyl-inositol (GPI) – anchored MMP-17 at the cell surface and undergo truncation of a C-terminal spacer domain. This mechanism generates "activated" forms of ADAMTS-4 that cleave within the aggrecan interglobular domain (IGD) at the classical aggrecanase site, thereby accelerating depletion of sGAG from the ECM[70,71,111]. MMPs and ADAMTSs are potent mediators of aggrecan catabolism in models of cartilage degeneration.

m-Calpain is a neutral cysteine endopeptidase that is also involved in proteolysis of the aggrecan core protein. The enzyme is calcium dependent and generally thought be ubiquitous and confined to the cytosol. Several studies, however, implicate m-calpain in

normal and pathologic extracellular aggrecan catabolism by demonstrating extracellular localization and secretion by chondrocytes, potent aggrecanolytic activity, and regulation by proinflammatory cytokines[30,66,100,213,214,217,218]. Figure 2.3 depicts one product of m-calpain-mediated aggrecanolysis, but others have been detected, including a fragment bearing the C-terminal neoepitope GVA**719** and migrating at ~120kDa on SDS-PAGE. m-Calpain is a likely candidate for the unidentified enzyme responsible for post-translational cleavage of an aggrecan precursor in the endoplasmic reticulum[3], and accumulation of m-calpain-generated aggrecan fragments in mature bovine articular cartilage suggests a role for this enzyme in normal ECM turnover[164]. m-Calpain activity is governed by local calcium concentration and abundance of the endogenous inhibitor calpastatin, and the cell can regulate these parameters by shifting localization of the enzyme[92,93]. It is unclear how, where, and whether m-calpain is activated for aggrecan catabolism in articular cartilage.

2.5 Cell-agarose Constructs as Model Systems

Three-dimensional hydrogel scaffolds, such as agarose and alginate, have demonstrated utility in preservation of chondrocyte phenotype and are being explored as therapeutic devices in cartilage repair[28,34,86,157]. In addition to preserving critical microenvironmental cues for maintaining cell-type specific behavior, cell-hydrogel constructs are more amenable to proteoglycan analysis than native tissues since the entrapping collagen network of native tissues is essentially absent. Research applications of these constructs have generally focused on chondrocyte biosynthetic activity and mechanotransduction, and less is known about specific effectors of catabolism in these systems. Ragan and coworkers showed that chondrocytes in alginate suspension

synthesize and retain predominantly intact aggrecan in the pericellular and interterritorial spaces, with minimal aggrecanase or MMP activity through several weeks of *in vitro* cultivation; interestingly, there is some evidence of m-calpain activity in that model system, including a ~120kDa G1-positive aggrecan fragment localized in the further-removed matrix[179]. IL-1 stimulation of chondrocytes in alginate beads triggers release of proteoglycans and upregulation of pro-MMP-2 and -9[13,41]. There is little known about the catabolic activity of fibrochondrocytes in the cell-hydrogel platform. Three-dimensional cell-scaffold constructs can recapitulate some aspects of the native tissue microenvironment, and are useful models for evaluating extracellular events regulating aggrecan catabolism.

CHAPTER 3

METALLOPROTEINASE-MEDIATED AGGRECAN CATABOLISM IN ARTICULAR CARTILAGE DEGRADATION[§]

3.1 Introduction

Articular cartilage provides a low-friction surface for joint motion, and disease or damage to the tissue causes chronic pain and loss of joint function. The dense, highly hydrated extracellular matrix (ECM) of articular cartilage is composed primarily of water, type II collagen, and aggrecan. The aggrecan core protein bears a large number of sulfated glycosaminoglycans (sGAG) which are either chondroitin sulfate or keratan sulfate. Aggregation of aggrecan monomers on hyaluronan chains entangled in the collagen network results in a high matrix fixed charge density and generates an osmotic swelling pressure that resists compression during joint loading[48]. Aggrecan has also been shown to contribute to the shear properties of the tissue[106]. Progressive tissue degeneration *in vivo* is marked by release of aggrecan from the cartilage ECM and loss of compression and shear properties.

Proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 alpha and beta (IL-1α,β), stimulate chondrocytes to degrade cartilage aggrecan and collagen through the production of activated aggrecanases (such as ADAMTS-4 and -5) and collagenases (such as MMP-13), respectively. Many cartilage

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[§] From Wilson CG, Palmer AW, Zuo F, Eugui E, Wilson S, Mackenzie R, Sandy JD, Levenston ME. (2006) *Matrix Biology*, DOI:10.1016/j.matbio.2006.11.001.

explant studies have shown that aggrecanolysis precedes collagenolysis[19,118,175,257], and it has been suggested[175] that aggrecan protects the collagen network from proteolytic attack.

Proinflammatory cytokines also shut down synthesis of matrix molecules, exacerbating their disruption of homeostatic ECM remodeling. These findings are physiologically important because IL-1 can be detected in degenerative cartilage[226], and is present in synovial fluid at concentrations ranging from tens of picograms to nanograms per milliliter[61,94]. IL-1, therefore, has been implicated in the progression of arthritis, and *in vitro* stimulation of explanted cartilage with IL-1 has demonstrated utility as a model of the catabolic events leading to cartilage destruction. The IL-1 signaling axis is further regulated by the physiologic antagonist, IL-1 receptor antagonist (IL-1RA). Local and systemic delivery of IL-1RA alone and in combination with other inflammatory cytokine antogonists (*e.g.,* soluble tumor necrosis factor receptor) can reduce cartilage and bone resorption in animal models of rheumatoid arthritis[15,16]. Clinical studies, however, showed that less than half of the treated patients responded to IL-1RA treatment[68], indicating that cytokine inhibitors are not universally efficacious.

Matrix remodeling in articular cartilage is mediated in part by the MMPs. Several MMPs, including the collagenases MMP-1, -8, and -13, MMP-3 (stromelysin-1), the gelatinases MMP-2 and -9, and membrane type MMP-14 and -17, are expressed in articular cartilage. Active MMPs readily degrade type II collagen and aggrecan[13,115], and exogenous MMPs were shown to modulate the composition and material properties of bovine cartilage explants[23]. The primary substrate for MMPs on the aggrecan core protein is within the interglobular domain (IGD) at the VIPEN$^{\mathbf{360}}$—$^{\mathbf{361}}$FFG bond (the equivalent substrate in bovine aggrecan is VDIPES$^{\mathbf{360}}$—$^{\mathbf{361}}$FFG)[57]. Importantly, MMP-mediated

aggrecan catabolism appears to operate independent of aggrecanases and is thought to be a quantitatively minor mechanism of aggrecan degradation in injured or osteoarthritic cartilage[62,127,195,200].

Chondrocytes express aggrecanases of the ADAMTS family of enzymes (including ADAMTS-1, -4, -5, -8, -9, and -15). Proinflammatory stimuli may upregulate transcription of aggrecanase genes[12,115], and there is mounting evidence for substantial post-translational processing of the enzymes that alter aggrecanase activity and specificity[174]. In human chondrosarcoma cells and bovine cartilage explants, MMP-17 (MT4-MMP) appears to be responsible for C-terminal truncation of ADAMTS-4, a process which converts the enzyme from one which can cleave only the sGAG-rich region to one which can also cleave the interglobular domain (IGD)[70,169]. Aggrecanase activity within the IGD is marked by scission of the NITEGE$^{\mathbf{392}}$—$^{\mathbf{393}}$ARGSVI bond [197]. ADAMTS-4 and -5 (aggrecanase-1 and -2, respectively) appear to mediate the bulk of destructive sGAG release from osteoarthritic human cartilage explants[8], and ADAMTS-5 is primarily responsible for destructive aggrecanolysis *in vivo* in the mouse[211].

MMPs, aggrecanases, and their post-translational activation mechanisms are obvious targets for clinical intervention in arthritis, and many natural and synthetic inhibitors have been investigated for potential therapeutic use[32,37]. Indeed, a broad spectrum metalloproteinase inhibitor was found to reduce aggrecan depletion and loss of material properties in IL-1-stimulated cartilage explants[22]. Inhibitors of glycophosphatidyl inositol-anchor formation, including mannosamine and glucosamine, interfere with MMP-17-mediated activation of ADAMTS-4 and reduce IL-1-induced sGAG release and loss of material properties in cartilage explants[170]. Synthetic

aggrecanase inhibitors delayed sGAG and collagen release from IL-1-stimulated nasal cartilage, and preservation of aggrecan using an aggrecanase inhibitor protected the collagen network from proteolytic attack[175]. Collectively, these studies suggest that inhibitors of aggrecanases specifically or metalloproteinases generally (ADAMTSs and MMPs) can attenuate cell-mediated aggrecan catabolism and loss of tissue function associated with arthritic disease.

While several reports have shown that metalloproteinase inhibitors can abrogate IL-1-induced cartilage degradation, non-metalloproteinase pathways can also be quantitatively important. For example, Sugimoto and coworkers demonstrated that a broad spectrum inhibitor of MMPs and aggrecanases perturbed, but did not block, loss of aggrecan from IL-1-stimulated cartilage explants, and the authors concluded that IL-1 was stimulating hyaluronidase activity[212]. In other work, it was shown that depolymerization of hyaluronic acid may contribute to extrusion of aggrecan from diseased or injured tissue[219]. The effects of aggrecan depletion by metalloproteinase-independent pathways on changes on the material properties of cartilage, however, have not been characterized. Studies coupling analysis of molecular level changes in extracellular matrix with tissue level changes in matrix mechanical property are useful for evaluating the therapeutic potential of metalloproteinase inhibitors and permit investigation of the relationships between matrix composition, structure, and function. The objective of the current study was to examine the time–course of ECM catabolism and loss of mechanical properties in IL-1-stimulated articular cartilage explants treated with selective or non-selective metalloproteinase inhibitors. These studies show that inhibition of MMPs and/or aggrecanases does not effectively block IL-1-induced ECM

destruction and support the idea that other enzymes, such as hyaluronidases, participate in aggrecan degradation and loss of tissue function.

3.2 Materials & Methods

Inhibitor Selectivity Assays

The potencies and selectivities of the inhibitors for various MMPs were determined (by Rebecca Mackenzie, Roche-Palo Alto) using recombinant mouse or human MMPs (R&D Systems, Minneapolis, MN) and the fluorogenic peptide substrate MCA-Pro-Leu-Gly-Leu-DAP(DNP)-Ala-Arg-NH2 (Bachem, Heidelberg, Germany). Recombinant enzymes were activated with 1mM aminophenylmercuric acetate (Sigma), and reacted with the substrate in the presence of an aggrecanase-selective inhibitor (RO3310769, Roche-Palo Alto, Palo Alto, CA), a MMP-selective inhibitor (RO1136222, Roche-Palo Alto), or a non-selective metalloproteinase inhibitor (RO4002855, Roche-Palo Alto). The inhibitors were tested at concentrations ranging from 0-200μM. Reaction rates were measured by detection of the cleaved substrate's fluorescent signal (excitation λ = 334nm, emission λ = 390nm) over 20min at 37°C. The results are reported as IC_{50}, the inhibitor concentration causing 50% of the maximal reduction in the rate of substrate cleavage.

The potencies and selectivities of the inhibitors for ADAMTS-4 were determined via an enzyme linked immunosorbent assay (ELISA). Recombinant human ADAMTS-4 was produced in Sf9 cells and purified by column chromatography as previously described[187]. Aggrecan substrate was isolated from young adult bovine nasal cartilage by extraction in 4M guanidine hydrochloride and subsequent fractionation by CsCl density

gradient centrifugation. Microplates were treated with 660ng aggrecan/well overnight at 4°C, and blocked with 1% BSA for 1h at room temperature. Recombinant ADAMTS-4 in reaction buffer containing 50mM Tris-HCl, 1% glycerol, 10mM $CaCl_2$, pH 7.5 was added with varying concentrations of inhibitors. The digestions were carried out at room temperature for 2.5h. Reaction products bound to the plate were incubated with anti-NITEGE primary antibody diluted 1:3000 over 2h at room temperature. The extent of digestion was then measured by reaction of an alkaline phosphatase-conjugated secondary antibody with the chemiluminescent substrate disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan)-4-yl)phenyl phosphate (CSPD, Applied Biosystems, Bedford, MA). The results are reported as IC_{50}, the inhibitor concentration causing 50% of the maximal reduction in abundance of NITEGE-positive aggrecan fragments.

Tissue Culture

Articular cartilage was harvested aseptically from the femoral condyles and femoropatellar grooves of 1-2 week old calves (Research 87, Marlborough, MA) using a 4mm diameter biopsy punch. Full thickness explants were then cut to 2mm thick on a custom sizing block. The explants were cultured in 5% CO_2, 95% humidity and 37ºC for 72 hrs in the presence of serum-free media consisting of high glucose DMEM (Invitrogen, Carlsbad, CA), 10mM non-essential amino acids (Invitrogen), 50ug/mL gentamicin (Invitrogen, Carlsbad, CA), and 50µg/mL ascorbic acid (Sigma, St. Louis, MO). In a dose-response study, explants were stimulated with 20ng/mL recombinant human IL-1α (Peprotech, Rocky Hill, NJ) and treated with 0.5, 5, or 50µM aggrecanase-, MMP-, or non-selective inhibitors. Stock solutions of the compounds were prepared with

dimethylsulfoxide (DMSO), and final concentrations of DMSO in the culture media did not exceed 0.2%. At doses below 1%, DMSO has been shown to have no effects on cartilage explant proteoglycan synthesis or viability[209].

Explants were cultured for up to 24 days with or without 20ng/mL *rh*IL-1α. Some explants were additionally treated with 20μM of the aggrecanase-selective inhibitor, 5μM of the MMP-selective inhibitor or 5μM of the non-selective inhibitor. The four-fold higher dose of aggrecanase-selective inhibitor was chosen, based on the dose-response study and aggrecanase inhibition assay data, to yield similar inhibition of IL-1-induced sGAG release as the non-selective inhibitor. Media were collected and replenished every two days, and conditioned media were stored at -20ºC. Inhibitors were added to the media with each media change. Explants harvested at days 0, 4, 8, 12, 16, 20 and 24 days of culture (n = 5/treatment/time point) were stored frozen until mechanical testing in Dulbecco's phosphate buffered saline (DPBS) with a proteinase inhibitor cocktail (Calbiochem, San Diego, CA) including ethylenediaminetetraacetic acid (EDTA), 4-(2-aminoethyl)benzenesulfonylfluoride, leupeptin, and aprotinin. In a second study, explants harvested at days 0, 4, 8, and 20 of culture (n = 4/treatment/time point) were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned to 4μm for immunostaining. The kinetics of sGAG release to the media were similar between these two studies (not shown).

Biochemistry

Following mechanical testing, explants were lyophilized, weighed dry, and digested overnight in 0.0125 mg/mg tissue proteinase K (Invitrogen) at 60°C. Explant digests and conditioned media were spectrophotometrically assayed for sGAG content by

the dimethylmethylene blue (DMMB) dye-binding method[53] and for hydroxyproline content by the chloramine-T/para-dimethylaminobenzadehyde reaction[247]. Collagen content was calculated from the hydroxyproline content assuming a collagen:hydroxyproline mass ratio of 8:1[180].

Immunodetection

Tissue sections were deparaffinized using a Leica autostainer and loaded into Sequenza immunostaining racks (ThermoShandon, Waltham, MA). Sections were deglycosylated with 0.1U/mL chondroitinase ABC (Sigma) for 1hr at 37°C and blocked in 2% normal goat serum, 0.1% gelatin, 1% bovine serum albumin, and 0.05% Tween-20 for 1h at room temperature. Primary antibody or species-matched non-immune IgG diluted to 10μg/mL was then applied to sections for 1hr at room temperature. A goat anti-rabbit-Alexa Fluor 488 secondary antibody (Molecular Probes) was used for detection and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes). Images were captured through individual fluorescein isothiocyanate (FITC) and DAPI filters using a Ziess Axiovert 200 epifluorescent microscope with a CCD camera. All images were captured with the same exposure time, and the FITC and DAPI images were combined using image analysis software (Carl Zeiss MicroImaging, Thornwood, NY).

To assess MMP and aggrecanase activity on endogenous substrate in the explants, conditioned media from the second experiment were analyzed by immunoblot with antibodies to the VDIPEN[360] neoepitope (JSCVDI) or the NITEGE[392] neoepitope (JSCNIT). Antibodies raised against the VDIPEN neoepitope have been previously shown to detect the bovine VDIPES sequence [128]. Pooled media samples from days 2 and

4, 6 and 8, or 10 and 12 of the experiment were treated with ice cold ethanol/5mM sodium acetate to precipitate proteoglycans, and precipitates were deglycosylated by sequential digestion with protease-free chondroitinase ABC (Sigma) and Keratanases I (Sigma) and II (Associates of Cape Cod, East Falmouth, MA) as previously described[200]. Equal quantities of sGAG (5 μg) were loaded into 4-12% gradient Tris-glycine gels (Invitrogen). Following electrophoresis and transfer to nitrocellulose, aggrecan fragments were identified with primary antibodies at 1μg/mL. Blots were developed using a secondary antibody conjugated with alkaline phosphatase followed by exposure to the fluorescent substrate ECF (Amersham, Piscataway, NJ). Bands were visualized using a Fuji FLA3000 Phospho-imager.

Mechanical Testing

Explants were thawed to room temperature in DPBS with protease inhibitors and weighed wet before testing in torsional shear on a CVO120 rheometer (Bohlin, East Brunswick, NJ) and in unconfined compression on an ELF3200 uniaxial loading frame (Enduratec, Minnetonka, MN). After application of a 10% compressive strain and relaxation for 12min, each explant was first tested in oscillatory torsion with 0.25% shear strain applied at 0.01-10Hz to yield a frequency-dependent dynamic shear modulus G*. Following a 12min reequilibration to the free-swelling state, each explant was then tested in compression through 4 steps of stress relaxation (5% each, 10min/step) to determine the equilibrium modulus E and through oscillatory compression about a 10% offset of ±1.5% strain at 0.001-1Hz.to determine the frequency-dependent dynamic compression modulus E*.

Table 3.1 Inhibitors demonstrated differential selectivity for MMPs and aggrecanases. Inhibitor selectivities, indicated by concentrations of half maximal inhibition (IC_{50}, in nM), were determined by recombinant enzyme-fluorescent substrate assay (MMPs) and ELISA (ADAMTS-4).

	Aggrecanase Inhibitor RO-3310769	MMP Inhibitor RO-1136222	Non-Selective Inhibitor RO-4002855
ADAMTS-4	8	7200	1.5
MMP-1	6500	1800	260
MMP-8	5600	4	1.7
MMP-13	5720	0.61	2.4
MMP-2	NT	0.22	0.28
MMP-9	NT	NT	7.5
MMP-3	NT	0.52	3.4
MMP-7	16000	1200	2400
MMP-14	710	0.32	0.87
MMP-17	>10,000	50	0.6

Statistics

Differences between treatment groups at a given day were evaluated by one-way analysis of variance and Dunnett's post-hoc test using the IL-1 treated group as a control and significance at $p<0.05$.

3.3 Results

Selective and non-selective (NS) metalloproteinase inhibitors were used to perturb the catabolic cascade and progressive loss of tissue function in a well-established bovine cartilage explant model. Inhibitor selectivities, determined by recombinant enzyme-fluorescent substrate assays and ELISA, are summarized in Table 3.1 as concentrations of half-maximal inhibition (IC_{50}). The MMP-selective inhibitor effectively blocked ($IC_{50}<50$nM) the collagenases MMP-8 and MMP-13, the gelatinase MMP-2, MMP-3, and the membrane-type MMPs-14 and -17, but it had weaker activity ($IC_{50}>1200$nM) against MMP-1, MMP-7, and ADAMTS-4. The aggrecanase-selective inhibitor was ineffective ($IC_{50}>5600$nM) against most MMPs, partially effective

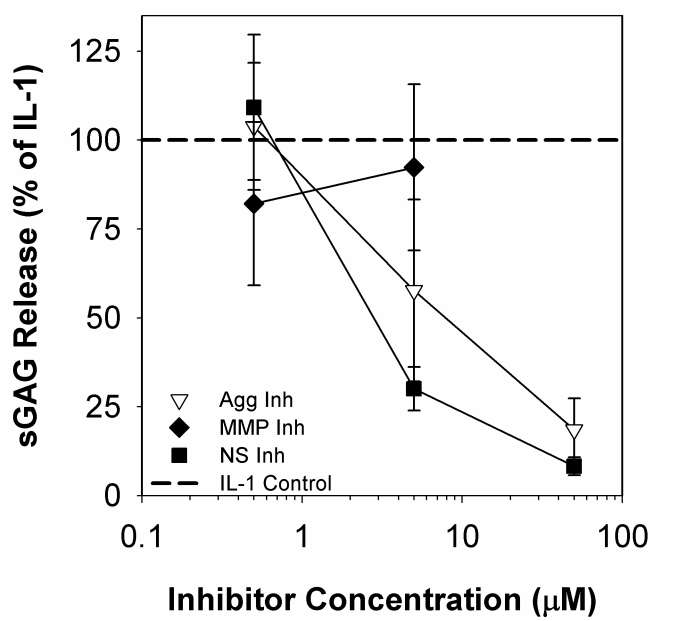

Figure 3.1 Metalloproteinase inhibitors demonstrated dose-dependent reduction in IL-1-stimulated sGAG release. Articular cartilage explants were stimulated with 20ng/mL *rh*IL-1α and treated with 0.05, 5, or 50μM inhibitor for 8 days. Cumulative sGAG release was measured by the DMMB-dye binding assay. Data are mean +/- SEM, n = 5. Cytotoxicity was observed at 50μM for the MMP-selective inhibitor.

(IC_{50}~710nM) against MMP-14 and highly inhibitory (IC_{50}~8nM) against ADAMTS-4. The non-selective metalloproteinase inhibitor was highly inhibitory (IC_{50}<7.5nM) to MMPs-2,3,8,9,13,14, and 17 and ADAMTS-4 and partially effective (IC_{50}>260nM) against MMPs-1 and 7.

As predicted from the selectivity assay IC_{50} data, the inhibitors exhibited different effects on IL-1-induced aggrecan release (as indicated by sGAG release to media) from explanted bovine cartilage (Figure 3.1). The aggrecanase-selective and non-selective compounds demonstrated dose-dependent inhibition of IL-1-induced aggrecan release in the 0.5-50μM range, and this was not due to cytotoxic effects as shown by Live/Dead staining (not shown). The MMP-selective inhibitor had no effect on sGAG release over 8 days at 0.5-5μM and was cytotoxic at 50μM. Based on these results, subsequent

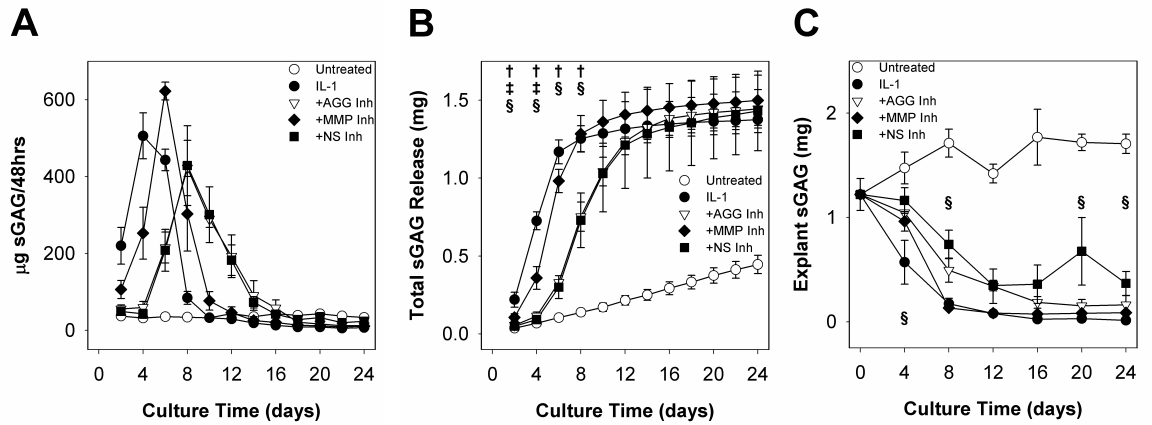

Figure 3.2 Metalloproteinase inhibitors delayed, but did not block, IL-1-stimulated proteoglycan degradation. sGAG content of conditioned media (**A, B**) and explant digests (**C**) was measured by the DMMB dye-binding assay. Data are mean +/- SEM, n = 5. In **B** and **C**, †, ‡, § = $p<0.05$ vs. IL-1 for AGG Inh, MMP Inh, and NS Inh, respectively.

degradation-inhibitor studies were carried out with inhibitor concentrations of 5µM for the MMP- and non-selective inhibitors and a concentration of 20µM for the aggrecanase-selective inhibitor. The aggrecanase inhibitor was used at a four-fold higher concentration than the non-selective inhibitor to account for differences in inhibitor potency against ADAMTS-4 (in substrate assays) and IL-1-induced sGAG release (in explant studies).

Time courses of sGAG release to the media (per 48h in Figure 3.2A, cumulative in Figure 3.2B) and explant sGAG content (Figure 3.2C) were used to assess aggrecan degradation. As expected, IL-1α treatment caused rapid (peak rates in days 2-4) and extensive aggrecan release with nearly complete depletion (~93%) by day 8. The MMP-selective inhibitor delayed the peak rate of IL-1-induced release (from days 2-4 to days 4-6) but did not affect the extent of depletion (~93%) at day 8. The aggrecanase inhibitor caused longer delays (from days 2-4 to days 6-8), but it had no significant effect on the extent of depletion after 24 days (Figure 3.2C). The non-selective inhibitor caused a delay which was almost identical to the aggrecanase inhibitor, and it reduced the extent of depletion by about 25% relative to IL-1.

To characterize IL-1-induced aggrecan degradation in the presence of selective- and non-selective metalloproteinase inhibitors, conditioned media were immunoblotted for fragments of aggrecan core protein (Figure 3.3). Conditioned media of IL-1-stimulated tissue, but not of untreated tissue, contained aggrecan species migrating at 50kDa and 65-70kDa corresponding to the G1-VDIPES and G1-NITEGE fragments, respectively. Treatment with an aggrecanase- or non-selective inhibitor abrogated release of the NITEGE-positive fragment through day 12, and an MMP-selective inhibitor delayed release of this fragment 2-4 days (Figure 3.3). In contrast, release of the G1-VDIPES fragment was blocked by the MMP- and non-selective inhibitors. The aggrecanase-selective inhibitor (20μM) also reduced release of the MMP-generated fragment, but much less potently than the MMP- and non-selective inhibitors.

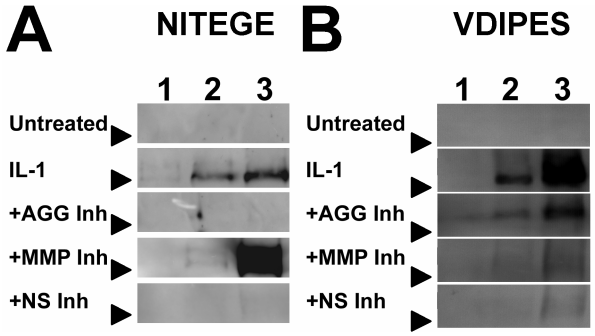

Figure 3.3 Selective and non-selective metalloproteinase inhibitors perturb release of aggrecan fragments from IL-1-stimulated articular cartilage. Aggrecan cleavage in conditioned media was detected by immunoblot for the NITEGE (**A**) and VDIPES (**B**) neoepitopes. Lanes 1, 2, and 3 contain media pooled from days 2 and 4, 6 and 8, and 10 and 12, respectively. Arrows indicate migration of 64kDa (**A**) or 50kDa (**B**) markers.

IL-1-induced aggrecan catabolism was further characterized by immunofluorescent detection of aggrecan cleavage fragments within the explants (Figure 3.4). The NITEGE fragment was localized in IL-1-stimulated tissue at days 4 and (to a lesser extent) 20. Treatment with an aggrecanase- or non-selective inhibitor yielded weaker interterritorial staining with intense intra- or peri-cellular staining. The MMP-

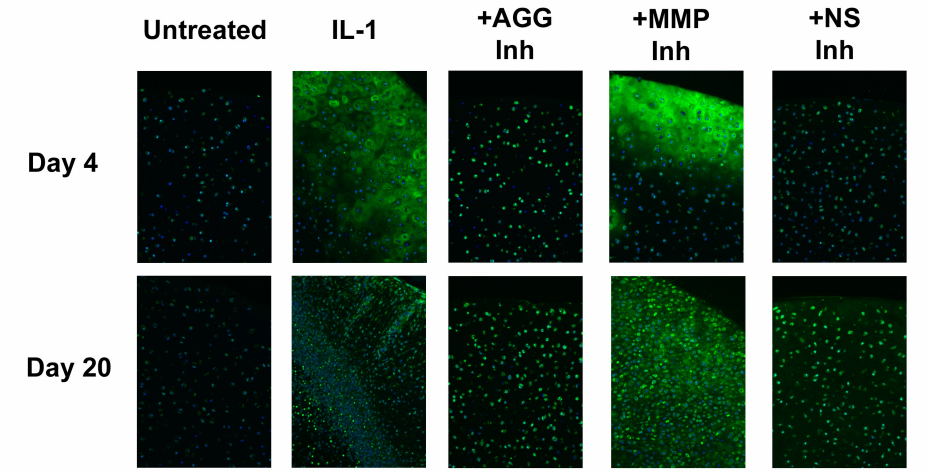

Figure 3.4 Selective and non-selective metalloproteinase inhibitors perturb aggrecanase-mediated aggrecanolysis in IL-1-stimulated articular cartilage. Aggrecan cleavage fragments were localized by immunofluorescent detection of the NITEGE neoepitope. NITEGE-positive regions are green, and cell nuclei are blue. Non-immune IgG-treated negative controls showed no background staining. Original magnification = 10x.

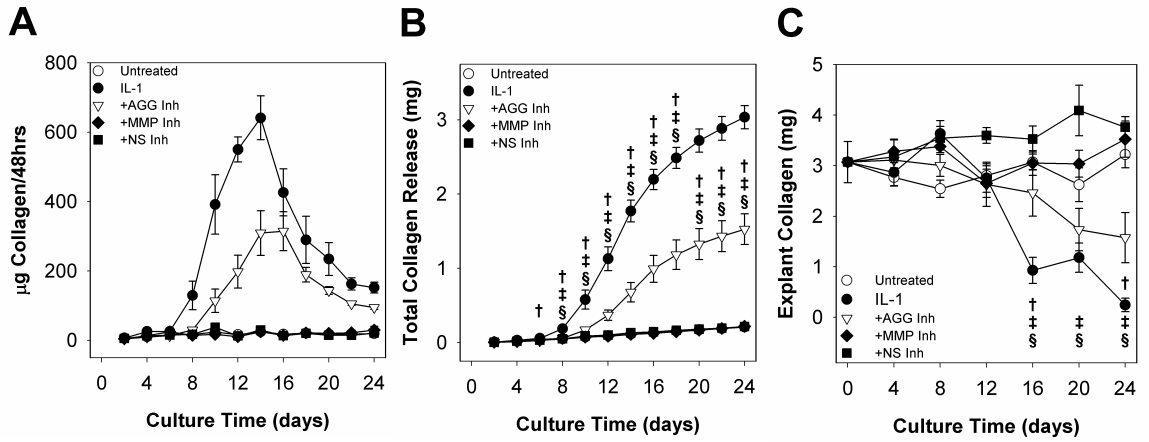

Figure 3.5 MMP inhibitors block, and an aggrecanase inhibitor reduces, IL-1-induced collagen degradation. Hydroxyproline content of conditioned media (**A, B**) and explant digests (**C**) was measured by the chloramine-T/pDAB reaction; collagen content was calculated assuming a collagen:hydroxyproline mass ratio of 8:1. Data are mean +/- SEM, n = 5. In **B** and **C**, †, ‡, § = $p<0.05$ vs. IL-1 for AGG Inh, MMP Inh, and NS Inh, respectively.

selective inhibitor did not substantially reduce or alter the spatial distribution of NITEGE staining, and appeared to have inhibited release of the fragment at later times.

Hydroxyproline release to the conditioned media (Figure 3.5A&B) and explant hydroxyproline content (Figure 3.5C) were measured to examine collagen degradation. As observed in previous studies of IL-1-stimulated cartilage, IL-1-induced collagen release began after nearly complete depletion of aggrecan. Cell-mediated collagen destruction approached completion by day 24, with approximately 8% of t = 0 collagen content remaining. The MMP-selective and non-selective metalloproteinase inhibitors completely blocked collagen release from the tissue throughout the 24 days, indicating a central role for MMPs in degradation of the collagen network. Interestingly, the aggrecanase-selective inhibitor delayed and reduced hydroxyproline release to the media and loss from the explant by 50%. These results are consistent with a previously proposed model in which aggrecan protects the collagen network from proteolytic attack in bovine nasal cartilage[175].

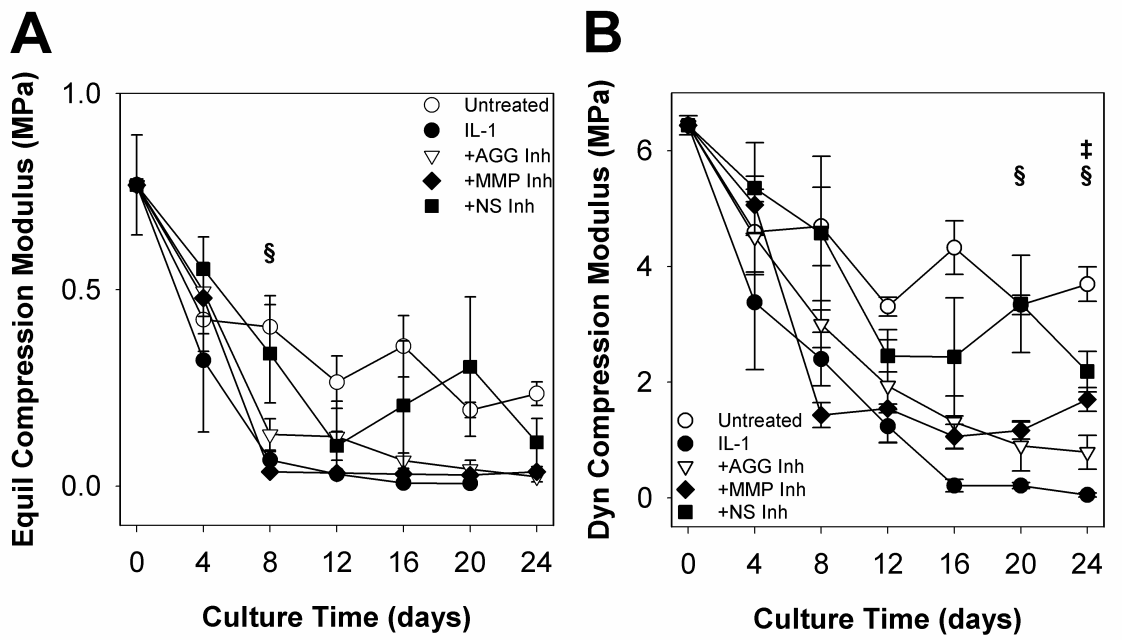

Figure 3.6 Selective and non-selective metalloproteinase inhibitors reduce, but do not block, IL-1-stimulated loss of compression properties. Equilibrium (**A**) and dynamic (**B**) compression moduli were measured by stress relaxation and oscillatory loading (0.1Hz) tests, respectively. Equilibrium moduli were below detection by 24 days of IL-1 treatment. Data are mean +/- SEM, n = 5. ‡, § = $p<0.05$ vs. IL-1 for MMP Inh and NS Inh, respectively.

To evaluate the functional implications of perturbing metalloproteinase activity in IL-1-stimulated articular cartilage, explants were subjected to compression and shear testing. IL-1-stimulated tissue underwent substantial loss (91% and 84% reduction from t = 0 and untreated control explants, respectively) of the equilibrium compression modulus by day 8 (Figure 3.6A), with kinetics similar to proteoglycan depletion. By day 16, the dynamic compression (Figure 3.6B) and shear (Figure 3.7A) moduli were dramatically reduced (by 97% and 96%, respectively, from t = 0 explants, by 95% and 92% from day 16 untreated controls). Treatment with the MMP-selective inhibitor did not prevent loss of the equilibrium modulus, but reduced loss of the dynamic compression and shear moduli after day 8. The aggrecanase-selective inhibitor delayed, but did not block loss of

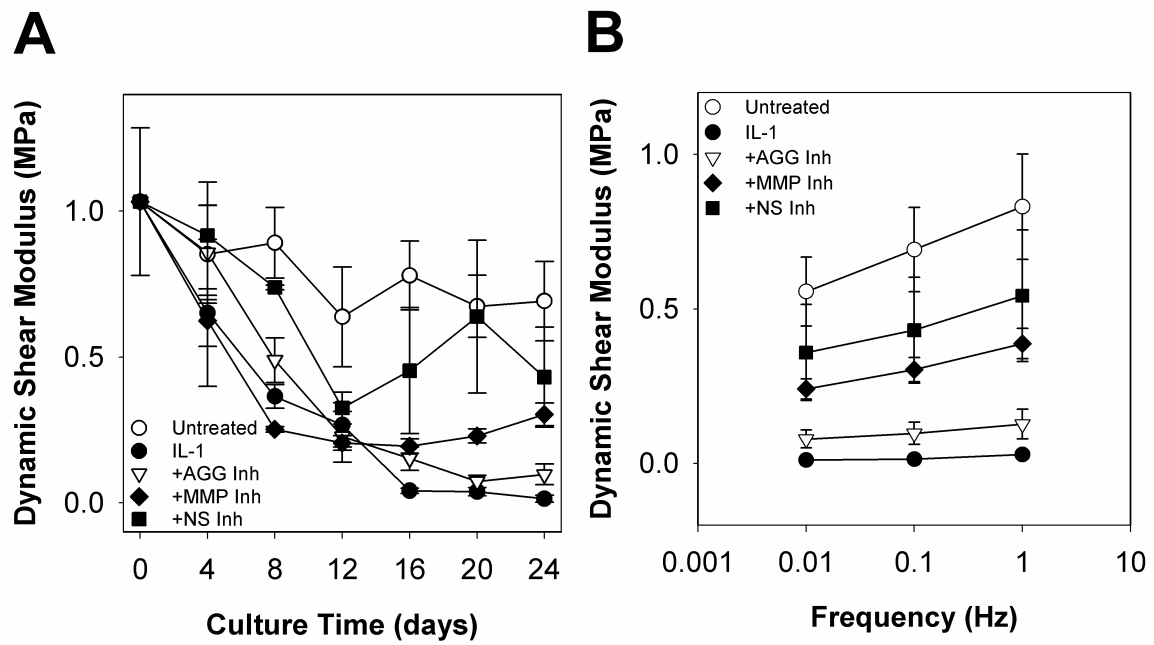

Figure 3.7 Selective and non-selective metalloproteinase inhibitors reduce, but do not block, IL-1-stimulated loss of shear properties. Dynamic shear moduli as a function of culture time (**A,** at 0.1Hz) and frequency (**B**, at day 24) were measured by oscillatory torsion tests. Data are mean +/- SEM, n = 5.

equilibrium properties and reduced loss of the dynamic properties. The non-selective metalloproteinase inhibitor was able to delay and reduce, but did not block, loss of compression properties, and conferred the greatest protection of tissue function in IL-1-stimulated tissue (with 47%, 59%, and 62% of same-day untreated explants' E, E*, and G*, respectively). The protective effects of inhibitor treatments on dynamic shear modulus were comparable across all frequencies tested (Figure 3.7B). Similarly, protection of the dynamic compression modulus by each inhibitor was comparable for all frequencies tested (not shown).

3.4 Discussion

Selective proteinase inhibitors have demonstrated utility in the investigation of cartilage degeneration mechanisms and may have clinical use in the management of arthritis. The efficacy of these inhibitors is most often assessed by biochemical outcomes,

and the studies presented here extend previous work that examines the functional consequences of targeted perturbations in cell-mediated degradation[22,23]. In the present study, treatment of immature bovine cartilage with metalloproteinase inhibitors delayed or reduced IL-1-induced matrix degradation. These inhibitors were shown to potently and selectively target MMP or aggrecanase activity, or to broadly inhibit metalloproteinase activity *in vitro*. In control experiments (not shown), explants treated with metalloproteinase inhibitors (but not exposed to IL-1) had DNA, aggrecan, and collagen contents and basal levels of aggrecan release similar to untreated controls, indicating that these inhibitors do not strongly influence cell proliferation or normal matrix metabolism. The compounds used to study protease activity in these experiments appear to inhibit their targets without disrupting physiologic processes, though it is possible that they have non-specific effects on cell metabolism. It is also noteworthy that the doses required to modify aggrecanolysis in explants were 2-3 orders of magnitude higher than the IC_{50} values measured in the metalloproteinase enzyme assays. This discrepancy in concentrations may be attributed to cellular metabolism of the compounds, transport limitations (e.g., partitioning) or other factors that influence inhibitor stability or delivery. In addition, the inhibitor selectivity assays were done with recombinant enzymes and artificial fluorescent substrates which may not model the natural process accurately. Nonetheless, the results of the explant studies support the specificity and relative activity of the inhibitors suggested by the selectivity assays. Complementary experiments with small interfering RNA, knockout animals, or dominant negative mutants perturbing protease activity may be required to more definitively establish the roles of individual enzymes.

Biochemical analysis of conditioned media and explant digests revealed that inhibition of MMPs and ADAMTSs delayed and/or reduced IL-1-induced aggrecan release, but did not prevent it. The aggrecanase- and non-selective inhibitors delayed IL-1-induced release of aggrecan to conditioned media and attenuated generation of the NITEGE neoepitope in immunoblots of conditioned media and in the tissue as shown by immunohistochemistry. These data are consistent with previous reports indicating that aggrecanases are the primary downstream effectors of the cell-mediated catabolic response to IL-1[7,127,223]. The less profound effects seen with the MMP-inhibitor may have been due to an inhibition of MMP-mediated activation of aggrecanases. A mechanism by which MMP-17 cleaves ADAMTS-4 at the cell surface appears to be active in IL-1-stimulated bovine cartilage explants[169], and inhibition of this mechanism with the MMP-selective inhibitor may have resulted in reduced levels of fully activated enzyme[60,70]. The MMP- and non-selective inhibitors prevented IL-1-induced generation of the VDIPES neoepitope, indicating that MMPs also degrade aggrecan in this model. Interestingly, stimulation with a lower dose (2ng/mL) of IL-1 permits a more robust reduction in proteoglycan release by the compounds used in this study (not shown). More potent inhibitors of aggrecanase activity, such as tissue inhibitors of metalloproteinases (TIMP)-3, may also confer long-term protection of the cartilage ECM.

The data from these studies suggest the existence of non-metalloproteinase-mediated pathways for aggrecan release in cartilage explants treated with IL-1. Chondrocyte-derived hyaluronidases may be responsible, although there is conflicting evidence for upregulated expression and activity of hyaluronidases in response to inflammatory cytokines[35,59]. Chondrocytes also express non-metalloproteinase enzymes

such as cathepsin-B[63] and m-calpain[164] which have been shown to cleave the aggrecan core protein, and these enzymes may be upregulated in response to IL-1 treatment.

The delayed aggrecan release observed in tissue treated with aggrecanase-selective and non-selective inhibitors may be due to differential selectivity of these inhibitors for various aggrecanase activation states or enzymes. ADAMTS-4 undergoes post-translational processing to at least 4 activation states, each with distinct substrate specificity and matrix-binding properties that could influence inhibitor potency [71,111,225]. As a result, the aggrecanase inhibitors used in this study may efficiently block aggrecanase activity in the aggrecan IGD and yet permit less destructive activity (at sites in the CS-2 domain) characteristic of lower activation states. In addition, the selectivity of these inhibitors for different aggrecanases in the ADAMTS family of enzymes has not been fully characterized. The ELISA used to establish inhibitor selectivity for aggrecanases tested the inhibitor potency against recombinant ADAMTS-4 (and with similar potency against ADAMTS-5, data not shown), but ADAMTS-1, -8, -9, and -15 are also capable of generating the classical aggrecanase cleavage products. While the non-selective inhibitor is a more potent inhibitor of aggrecanases, a higher dose of the aggrecanase-selective inhibitor was used in these experiments to account for the difference in potency.

Treatment with the MMP-selective inhibitor delayed IL-1-induced release of the G1-NITEGE neoepitope by several days and inhibited release of the fragment. The specific role of MMPs in release of the proteoglycan aggregate is unclear, but may be related to destruction of the collagen network. Blockade of collagen degradation may be sufficient to preserve hyaluronan entanglement and thereby inhibit its diffusion (and

diffusion of aggregable aggrecan fragments) from the ECM. Link protein, which stabilizes the interaction between aggrecan and hyaluronan, is also a substrate for MMPs including matrilysin and stromelysin-1 and -2[158]. The compounds used in this study may, then, interfere with MMP-mediated cleavage of link protein and release of aggrecan.

Analysis of collagen content in explant digests and conditioned media confirmed the role of MMPs in collagenolysis. MMP-selective and non-selective metalloproteinase inhibitors completely blocked release of collagen to the media and depletion of collagen from the tissue. IL-1 upregulates expression of MMP-1, -3, and -13 in bovine cartilage[58], and IL-1-induces exhaustive collagen degradation over a month or less of treatment [159]. Significantly, the aggrecanase-selective inhibitor also conferred some protection of the collagen network. Using a different small-molecule aggrecanase inhibitor, Pratta and coworkers observed a similar result and hypothesized that aggrecan molecules can prevent MMPs from reaching their substrates on collagen fibers, perhaps by steric exclusion[175]. Treatment of IL-1-stimulated cartilage with the aggrecanase-selective inhibitor reduced cumulative collagen release by 50% through day 24 of the experiment, and delayed but did not prevent aggrecan release over the same period. Generation of the G1-NITEGE fragment, however, was reduced in this group, indicating that alternative paths of aggrecan processing had occurred to release the aggrecan. Several enzymes (e.g., m-calpain) truncate aggrecan at C-terminal sites in the sGAG-rich region and leave an intact IGD, yielding a "trimmed" aggrecan that could contribute to partial protection of the collagen network.

Mechanical testing in compression and shear revealed that IL-1-induced reductions in explant material properties are attenuated by inhibition of metalloproteinase

activity. Compression and shear moduli are indicators of tissue mechanical function and depend on the abundance and integrity of ECM constituents[181,205,256]. Whereas IL-1-stimulated tissue retains compression properties approximately 0-4% of the initial (t = 0) values by day 24, treatment with the non-selective metalloproteinase inhibitor was effective at preserving 15% and 42% of the initial equilibrium and dynamic compression moduli, respectively. These data indicate that MMPs and aggrecanases mediate part of the IL-1-induced loss of cartilage compression properties, and further suggest that other enzyme systems or mechanisms of ECM catabolism may participate. The MMP-selective inhibitor attenuated IL-1-induced loss of the dynamic compression modulus, but the aggrecanase-selective inhibitor did not confer significant protection of either compression property by day 24. These data are consistent with the ideas that equilibrium behavior of cartilage is governed by the abundance of aggrecan and the dynamic loading behavior is influenced by the integrity of both aggrecan aggregates and the collagen network[120]. In preventing degradation of the collagen network, the MMP-selective inhibitor partly preserves the tissue's response to dynamic loading. The aggrecanase-selective inhibitor fails to sufficiently protect the aggrecan or collagen and does not prevent loss of equilibrium or dynamic properties. Indeed, destruction of the collagen network, rather than the aggrecan aggregate, is considered the "point of no return" in cartilage degeneration. Of note, the equilibrium and dynamic compression moduli of untreated controls fell to 31% and 54%, respectively, of initial values after 24 days of *in vitro* culture. These changes are attributed in part to collagen network damage at the cut surfaces sustained during explant preparation.

Compounds that inhibit MMP activity protected dynamic material properties of IL-1-stimulated tissue, whereas an aggrecanase inhibitor did not. The MMP-selective and non-selective metalloproteinase inhibitors reduced the loss of the dynamic compression modulus over 24 days of IL-1 stimulation and treatment with the non-selective inhibitor also reduced the loss of the dynamic shear modulus. The aggrecanase-selective inhibitor delayed (by ~4 days) and reduced loss of the dynamic compression modulus, but did not substantially alter loss of the equilibrium compression or dynamic shear moduli. These data are consistent with previously published findings that the dynamic material properties of cartilage depend upon collagen content[5]. Recent work in our lab, however, also demonstrated significant correlations between aggrecan contents and the dynamic shear and compression properties in IL-1-stimulated cartilage[165], and the improved retention of explant aggrecan content by a non-selective metalloproteinase inhibitor observed here may contribute to protection of dynamic material properties. Trends in the dynamic shear properties generally followed those observed in the dynamic compression moduli and revealed similar sensitivities of these properties to IL-1-induced degradation.

The results of this study indicate that selective or broad inhibition of metalloproteinase activity perturbs cell-mediated ECM degradation induced by IL-1-stimulation of bovine cartilage explants. Disruption of the metalloproteinases was insufficient to prevent exhaustive depletion of aggrecan aggregates in this model system and may allow alternative pathways of aggrecan processing to proceed. In addition, these studies demonstrate that non-metalloproteinase mechanisms of aggrecan depletion can mediate IL-1-induced loss of tissue mechanical properties. Identification of these

pathways may reveal new therapeutic requirements for clinical management of cartilage degradation.

CHAPTER 4

METALLOPROTEINASE-MEDIATED AGGRECAN CATABOLISM IN MENISCAL FIBROCARTILAGE

4.1 Introduction

Knee menisci have essential roles in load transfer and distribution during joint motion, and partial or complete meniscectomy often initiates degeneration of the adjacent articular cartilage[102,121,146,150,172,184,185,237]. Studies of magnetic resonance images from asymptomatic knees revealed a prevalence of degenerative changes (*e.g.*, tears) in the menisci, and meniscal degeneration appears to increase with age[21,67,122,206]. In addition, meniscal degeneration was strongly associated with meniscal extrusion and articular cartilage erosion[17,40]. Taken together, these findings suggest that meniscal degeneration is an early event in the onset of arthritis. The causes of age-related meniscal tearing and extrusion are not well understood, and may be related to changes in the fibrocartilage extracellular matrix (ECM) composition, ultrastructure, and material properties.

The complex ECM structure and composition of the menisci allow for the distribution of shear, tensile, and compressive loads. The fibrocartilage ECM is rich in collagen types I, II, and VI, and is highly organized with circumferentially- and radially-oriented collagen fibers[110,139]. The circumferentially-oriented fibers are organized into high tensile strength structures that bear hoop stresses during physiologic loading, and radially-oriented bundles and sheaths stabilize these structures[55,108]. Proteoglycans such as aggrecan, decorin, and biglycan are also found in meniscal fibrocartilage[141,204,230]. In articular cartilage, aggrecan confers compressive stiffness through an osmotic swelling pressure generated by its attached sulfated glycosaminoglycan (sGAG) chains[188].

Valiyaveettil and coworkers immunolocalized aggrecan G1 (the hyaluronan-binding globular domain of aggrecan) along collagen fibers in the canine meniscus and proposed that aggrecan dissipates compressive loads in fibrocartilage[230], and compressed regions of bovine deep flexor tendon were enriched in high molecular weight aggrecan[236]. The function of aggrecan in meniscal fibrocartilage mechanics is unclear, but aggrecan may contribute to the tissue's compression and shear properties as in articular cartilage.

A heterogeneous population of cells, known as fibrochondrocytes, resides in the menisci and exhibits regional differences in morphologic and metabolic characteristics[88,210,221]. Despite having similar rates of collagen synthesis, fibrochondrocytes from the inner region of the meniscus had higher rates of proteoglycan synthesis (as assessed by $^{35}[S]O_4$ incorporation) than cells isolated from the outer region[38,221]. Studies of aggrecan gene expression and immunolocalization of aggrecan G1 in the meniscus suggest that regional variations in proteoglycan content are due to differences in aggrecan abundance[145,229,230]. Conversely, matrix metalloproteinase (MMP)-2 and MMP-3 expression were higher in the outer region of the meniscus, indicating that elevated catabolic activity may also contribute to lower proteoglycan content in the outer region[229]. Sandy and coworkers observed an abundance of 62-66kDa–sized aggrecan G1 fragments in extracts of bovine meniscal fibrocartilage and concluded that aggrecan cleavage within the interglobular domain (IGD) is a normal process in this tissue[198]. Furthermore, the size and C-terminal neoepitope (NITEGE) of those aggrecan fragments indicated that aggrecanases of the *a* *d*isintegrin *a*nd *m*etalloproteinase with *t*hrombo*s*pondin motifs (ADAMTS) family mediate normal aggrecan processing in the meniscus[198]. Abundance of the NITEGE neoepitope is

indicative of degeneration in articular cartilage, since this cleavage releases the functional sGAG-rich domains of the aggrecan core protein. The physiologic relevance of aggrecanase activity in the normal immature meniscus is unknown, and regional differences in aggrecanase activity of meniscal fibrocartilage have not yet been described.

The proinflammatory cytokines interleukin-1α and β (IL-1α,β) are linked to the onset of arthritis[183,208] and have been previously shown to initiate aggressive proteoglycan degradation followed by collagen network destruction in articular cartilage [47]. Aggrecan degradation is mediated almost exclusively by ADAMTS-4 and -5 (aggrecanase-1 and -2, respectively) in IL-1-stimulated bovine articular cartilage explants, and the resulting sGAG depletion leads to dramatic loss of material properties, such as compressive stiffness[22,197,223]. The proteases responsible for cartilage erosion are obvious targets of therapeutic intervention, and pharmacologic protease inhibitors have demonstrated utility in perturbing pathologic cartilage catabolism *in vitro* [25,175,196,224]. Aggrecanase and MMP inhibitors reduced IL-1-induced loss of cartilage material properties[22,246], and using an aggrecanase inhibitor, Pratta and coworkers showed that aggrecan protected collagen from proteolytic attack[175]. IL-1 triggers a cell-mediated catabolic cascade in articular cartilage, and collagen network damage marks the degenerative "point of no return."

Fibrochondrocytes also respond to IL-1 with elevated catabolic activity, similar to articular chondrocytes. IL-1-stimulation of explanted lapine menisci increased nitric oxide and MMP production[31], and cells isolated from fibrocartilage of the temporomandibular joint exhibited upregulated expression of MMPs in the presence of IL-1β[46]. Explants of human osteoarthritic meniscal fibrocartilage released proteoglycans

by an IL-1-induced cyclooxygenase-2 and prostaglandin E2 dependent mechanism[84], and Shin and coworkers demonstrated that IL-1-stimulated fibrocartilage from porcine meniscus also release proteoglycans[207]. Neither the enzymatic mechanisms responsible for proteoglycan release in IL-1-stimulated fibrocartilage nor the IL-1-induced changes in fibrocartilage material properties have been previously reported. Identifying the mechanisms of aggrecan catabolism in fibrocartilage and characterizing the effects of aggrecan processing on the tissue's material properties may contribute to our understanding of early degenerative changes in the meniscus.

4.2 Materials & Methods

Reagents and antibodies

High glucose Dulbecco's modified Eagle medium (DMEM), gentamicin, non-essential amino acids (NEAA), N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), trypsin-ethylenediaminetetraacetic acid, proteinase K, and phosphate buffered saline (PBS) were from Invitrogen (Carlsbad, CA). Antibodies to aggrecan G1 and the NITEGE neoepitope were prepared as previously described[200]. The antibody to collagen type I was from Abcam (Cambridge, MA). Alexafluor 488-conjugated anti-rabbit and Alexafluor 594-conjugated anti-mouse secondary antibodies were from Invitrogen. Fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody was from Chemicon (Temecula, CA). Guanidine hydrochloride, protease-free chondroitinase ABC, keratanase I, chloramine-T, para-(dimethylamino)-benzaldehyde, non-immune rabbit IgG, alkaline phosphatase-conjugated anti-rabbit secondary antibody, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and other histologic reagents were from Sigma (St. Louis, MO). Keratinase II was from Associates of Cape Cod (Falmouth, MA).

Dimethylmethylene blue (DMMB) was from Polysciences (Warrington, PA). Recombinant human IL-1α (*rh*IL-1α) was from Peprotech (Rocky Hill, NJ). Protease inhibitor cocktail containing ethylenediaminetetraacetic acid, 4-(2-aminoethyl)benzenesulfonylfluoride, leupeptin, and aprotinin was from Calbiochem (San Diego, CA). The chemifluorescent substrate ECF was from Amersham (Piscataway, NJ).

Small-molecule metalloproteinase inhibitors (RO3310769, aggrecanase-selective; RO1136222, MMP-selective; RO4002855, broad spectrum metalloproteinase) were provided by Roche – Palo Alto (Palo Alto, CA). Detailed selectivity profiles of these inhibitors have been reported previously[246]. Briefly, the aggrecanase-selective inhibitor was highly inhibitory against the aggrecanase ADAMTS-4, with a concentration of half-maximal inhibition (IC_{50}, lower values indicate greater inhibition) of 8nM, and IC_{50}s > 700nM for all MMPs tested. The MMP-selective inhibitor inhibited most MMPs tested with IC_{50}s ≤ 50nM, with the exceptions of MMP-1 and -7, but did not preventADAMTS-4 activity (IC_{50} = 7200nM). The broad spectrum metalloproteinase inhibitor potently inhibited ADAMTS-4 (IC_{50} = 1.5nM) and most MMPs (IC_{50}s ≤ 7.5nM) tested, with the exceptions of MMP-1 and -7. Stock solutions of the compounds were prepared with dimethylsulfoxide (DMSO), and final concentrations of DMSO in the culture media did not exceed 0.2%. Previous work in our lab and others' has shown that at concentrations <1%, DMSO does not substantially alter cell viability or proteoglycan metabolism in fibrocartilage or cartilage explants[209].

Histology and immunostaining

Immature (1-2 weeks, Research 87, Cambridge, MA), adolescent (8 weeks, Green Village Packing Company, Green Village, NJ), and mature (2.5 years, Green Village

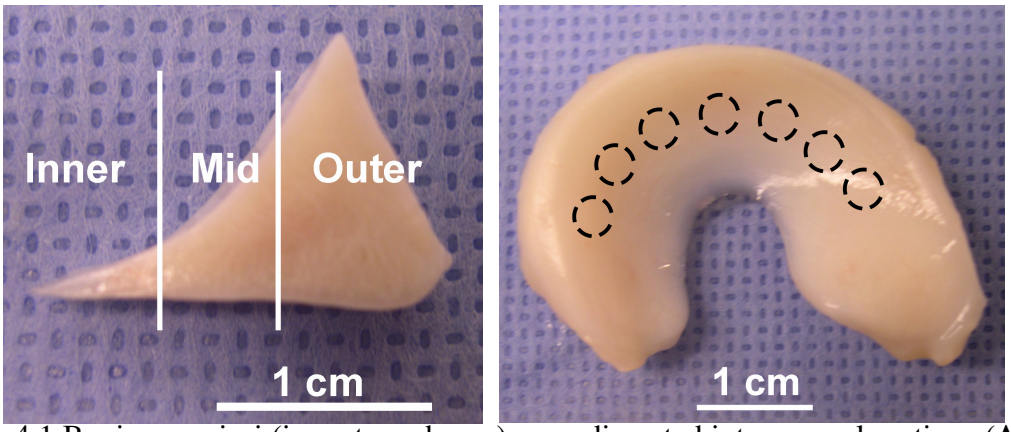

Figure 4.1 Bovine menisci (immature shown) were dissected into coronal sections (**A**) for histology or cylindrical explants from the middle region (**B**) for tissue culture experiments.

Packing Company) bovine menisci were harvested within 24h of slaughter. Coronal "slabs" from medial and lateral menisci (3-4mm thick, Figure 4.1, left) were either fixed in 10% neutral-buffered formalin (NBF) at 4°C for 48h and embedded in paraffin or fixed in 10% NBF for 4h at 4°C, incubated in 30% sucrose/PBS overnight at 4°C, embedded in OCT, and frozen in liquid-nitrogen cooled isopentane. Paraffin-embedded tissue was sectioned at 3µm and stained with safranin-O, fast green, and hemotoxylin. Frozen tissue was sectioned at 7µm and co-immunostained for the aggrecan NITEGE neoepitope and collagen type I. Sections were digested with 0.5% trypsin-EDTA in DPBS for 10min at 37°C and blocked for 1h at room temperature with blocking buffer containing 2% goat serum, 0.1% gelatin, 1% BSA, 0.1% Triton, and 0.05% Tween-20 in PBS prior to incubation with antibodies. In our experience, chondroitinase ABC treatment is not required for detection of aggrecan $G1^{230}$ or the NITEGE neoepitope in thin sections of meniscal fibrocartilage, perhaps due to the relatively low concentration of sGAG in the ECM. The NITEGE neoepitope and collagen type I were detected by indirect immunofluoresence with goat anti-rabbit Alexafluor-488 and goat anti-mouse Alexafluor-594 secondary antibodies, respectively. Following a tissue culture study

(described below), 3-4mm thick coronal slabs of meniscal fibrocartilage were fixed in 10% NBF for 48h at 4°C, embedded in paraffin, and sectioned at 3μm. The NITEGE neoepitope was detected by indirect immunofluoresence with a goat anti-rabbit FITC secondary antibody. All primary and secondary antibodies were used at 10μg/mL and antibody incubations were for 60-90min at room temperature. Cell nuclei were fluorescently stained with DAPI. Species-matched non-immune IgGs (10μg/mL) were used as negative controls. Sections were imaged on a Zeiss AxioVert 200M microscope using the Apotome optical sectioning module (Zeiss, Jena, Germany).

Tissue Culture

Menisci were harvested aseptically from immature bovine stifle joints (Research 87, Marlborough, MA). This tissue source was selected because related tissues (e.g., articular cartilage and tendon) from this age and species animal are well-characterized in a wide body of literature covering the tissues' mechanical properties, ECM composition, and response to IL-1. In an initial tissue culture study, coronal sections (Figure 4.1, left) were dissected from the menisci using two scalpels assembled in parallel and spaced 3-4mm apart. The slabs were cultured flat in 6 well plates with or without 20ng/mL *rh*IL-1α in serum-free media consisting of high glucose DMEM, 10mM NEAA, 50μg/mL gentamicin, and 50μg/mL ascorbic acid under standard conditions (5% CO_2, 95% humidity and 37ºC). Media were prepared with fresh cytokine and exchanged after 48h. After four days, the tissue slabs were fixed in formalin and processed for immunofluorescent detection of the aggrecanase-generated NITEGE neoepitope (described above).

For inhibitor studies, full-thickness cylindrical specimens (3mm diameter) were harvested from the middle region of medial and lateral menisci and cut to 2mm thickness on a custom sizing block (Figure 4.1, right). Tissue from the surface zones (superior and inferior ~500μm) was excluded. Prior to cytokine and inhibitor treatments, the explants were cultured for 72 hrs in "basal" serum-free medium consisting of high glucose DMEM, 10mM NEAA, 50ug/mL gentamicin, and 50μg/mL ascorbic acid. Explants (n = 6/group/time point) were subsequently cultured up to 12 days with or without 20ng/mL *rh*IL-1α. Some explants were additionally treated with 20μM of the aggrecanase-selective inhibitor, 5μM of the MMP-selective inhibitor, or 5μM of the broad spectrum metalloproteinase inhibitor. Media were collected, prepared with fresh cytokine and inhibitors, and replaced every 48h. Tissue culture doses of the inhibitors were determined from a previously reported dose-response study (described in chapter 3) with immature bovine articular cartilage explants[246]. Over twelve days, these inhibitor doses were found to have no significant effect on fibrochondrocyte viability as assessed by mitochondrial conversion of the tetrazolium salt WST-1 (Biovision, Mountainview, CA) to formazan (not shown). Explants harvested at days 0, 4, and 12 days of culture were stored frozen in PBS with protease inhibitors. Additional explants harvested at days 0 and 8 were lyophilized and extracted in 10 volumes of 4M guanidine hydrochloride for 48h at 4°C.

Biochemistry

Following mechanical testing (described below), explants were lyophilized, weighed dry, and digested in 0.0125 mg/mg tissue proteinase K at 60°C. Explant digests and conditioned media were spectrophotometrically assayed for sGAG content by the DMMB dye-binding method[53] and for hydroxyproline content by the chloramine-T/para-

dimethylaminobenzadehyde reaction[247]. Chondroitin sulfate AC (Sigma) standards were used for the DMMB assays. Collagen content was calculated from the hydroxyproline content assuming a collagen:hydroxyproline mass ratio of 8:1[180].

Western blotting

To assess aggrecan degradation in the inhibitor study, conditioned media and tissue extracts were analyzed by immunoblot with antibodies to the NITEGE and DLS neoepitopes and aggrecan-G1 domain, respectively. Pooled media samples from days 2 and 4, 6 and 8, or 10 and 12 of the experiment were treated with ice cold ethanol/5mM sodium acetate to precipitate proteoglycans, and precipitates were deglycosylated by sequential digestion with protease-free chondroitinase ABC and Keratanases I and II as previously described[200]. Equal volumes of media or quantities of sGAG (5μg for explant extracts) were loaded into 4-12% gradient Tris-glycine gels (Invitrogen). Following electrophoresis and transfer to nitrocellulose, aggrecan fragments were identified with primary antibodies at 1μg/mL. Blots were developed using a 1:20,000 dilution of anti-rabbit alkaline phosphatase secondary antibody followed by exposure to ECF. Bands were visualized using a Fuji FLA3000 (Fuji, Tokyo, Japan).

Mechanical Testing

Prior to testing, explants were thawed to room temperature, weighed, and the diameter and thickness of each explant were measured at three points using digital calipers. All tests were performed in the presence of PBS with protease inhibitors. Explants were compressed to 10% strain and allowed to relax for 12min between parallel plates on a CVO120 rheometer (Bohlin, East Brunskwick, NJ). Oscillatory torsion at

0.25% (0.03 rad) shear strain was applied at 0.001-0.1Hz to determine dynamic shear moduli G*. Following a 12min equilibration to the free-swelling state, the sample was moved to an unconfined compression chamber on an ELF3200 uniaxial loading frame (Enduratec, Minnetonka, MN), compressed to 10% strain at 0.1mm/s, and allowed to relax for 12min. Each explant was then tested in oscillatory unconfined compression at 0.001-0.1Hz (1.5% strain amplitude) to determine the dynamic compressive moduli E*.

Statistics

Data were analyzed by ANOVA using a general linear model in MINITAB Release 14 (MINITAB, States College, PA). Pairwise comparisons between groups were determined by Dunnett's test with IL-1-stimulated samples as controls and $p<0.05$ significant.

4.3 Results

Regional variations in sGAG content and aggrecanase activity in the bovine meniscus

Safranin-O staining for sulfated glycosaminoglycans in sections of bovine meniscus revealed regional variations in sGAG content (Figure 4.2) similar to those reported for immature porcine tissue[155] and age variations similar to those reported for ovine meniscus[145]. In all ages, the inner region, which undergoes primarily compressive and shear loading *in vivo*, was richest in sGAG. The middle region of the meniscus, which additionally bears tensile loads, appeared to have much lower sGAG than the inner region in young tissue and progressively accumulated sGAG with age. At all ages, there were "pockets" of sGAG-rich ECM in the middle region colocalized with cells and

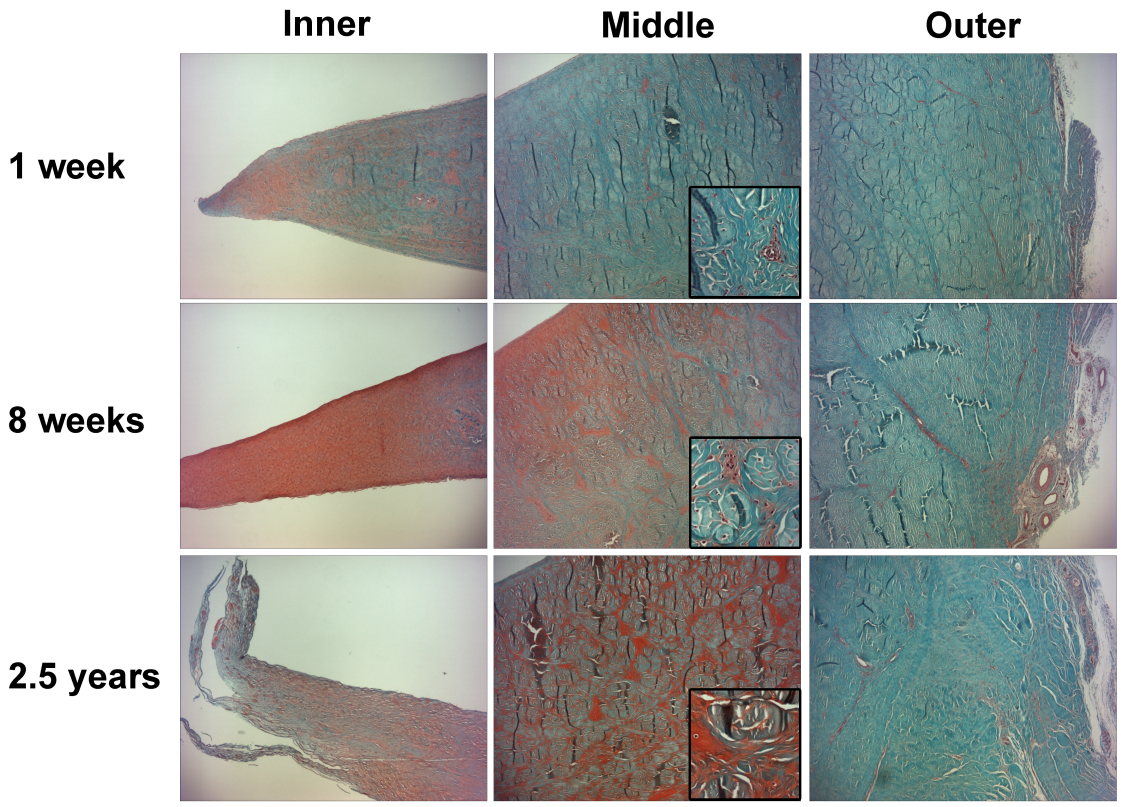

Figure 4.2 Proteoglycan distribution within the bovine meniscus depends on age. Stifle joint menisci of various ages were stained with safranin-O to localize sGAG (red) and Fast green/hemotoxylin counterstained. Original magnification = 4x (20x for insets).

situated between collagen fiber bundles. The outer region of the bovine meniscus appeared to have low sGAG content at each of the ages examined. These data were consistent with previously reported regional variations in aggrecan content, aggrecan gene expression, and proteoglycan synthesis rates in meniscal fibrocartilage[38,229,230].

Immunostaining for the NITEGE sequence, a neoepitope exposed by aggrecanase-mediated cleavage of the aggrecan IGD, indicated that aggrecanase activity also varies across regions of the meniscus (Figure 4.3, middle panels). Whereas the inner region demonstrated weak staining for NITEGE, the middle and outer regions of the meniscus stained intensely in the intra- and peri-cellular compartments. Immunoblots of tissue extracts showed that regional variations in aggrecanase activity, as indicated by

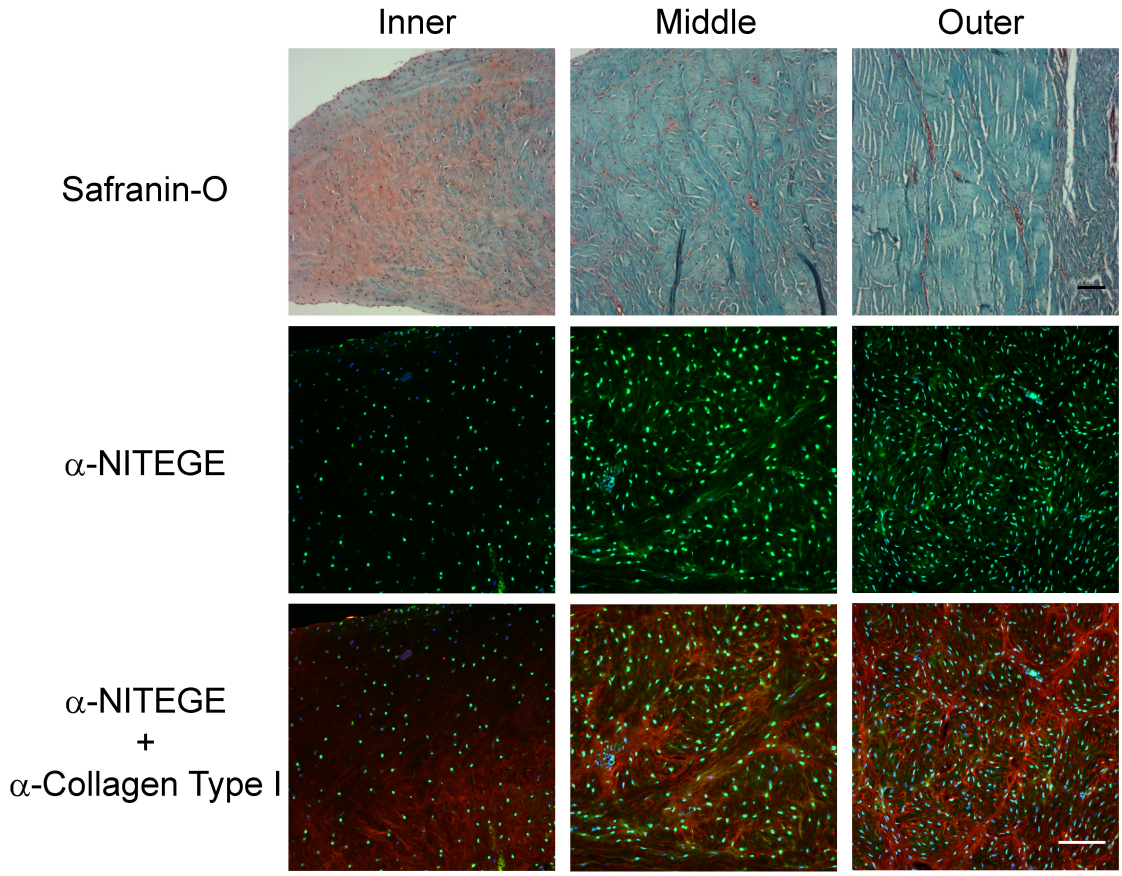

Figure 4.3 Aggrecanase activity is evident in immature bovine meniscal fibrocartilage. Coronal sections of immature bovine medial meniscus were stained for sulfated glycosaminoglycans (red) (top), or the aggrecan NITEGE neoepitope (Alexafluor-488 secondary, green) and collagen type I (Alexafluor-594 secondary, red) (middle and bottom). In immunofluorescent images, the blue indicates DAPI nuclear stain. Inner, middle, and outer refer to the radial position in the coronal plane. Scale bars = 100μm.

NITEGE-bearing aggrecan, were similar in medial and lateral menisci (Figure 4.4). The middle and outer regions exhibited staining for NITEGE in the ECM along collagen type I fibers in the plane of the section and within collagen type I fibers perpendicular to the plane of the section (Figure 4.3, lower panels). Non-immune IgG controls showed no staining (not shown). The NITEGE fragment consistently appeared in regions of weak safranin-O staining (areas low in sGAG) suggesting aggrecanase-mediated aggrecan

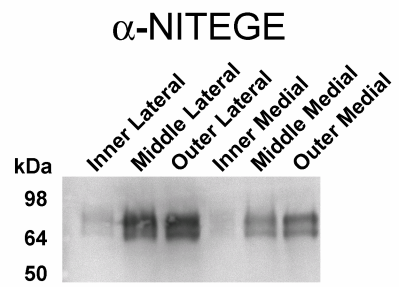

Figure 4.4 Aggrecanase activity in immature bovine medial and lateral menisci varies by region. Equal volumes of region-specific tissue extracts were separated by electrophoresis under reducing conditions and immunoblotted for the NITEGE neoepitope. Migration of globular protein standards are shown at left.

degradation precluded accumulation of sGAG in the middle and outer regions of the meniscus.

To assess IL-1-induced changes in aggrecanase activity, 3-4mm thick coronal "slabs" of meniscal fibrocartilage were cultured for up to 4 days in the absence or presence of 20ng/mL IL-1. Similar to articular cartilage, meniscal fibrocartilage stimulated with IL-1 rapidly released sGAG to the culture media (not shown), indicating that IL-1 induced cell-mediated proteoglycan degradation in this culture system. Immunostaining sections of cultured tissue for NITEGE again revealed regional variations in aggrecanase activity (Figure 4.5). In untreated controls, the surface ECM and pericellular compartments of the inner region showed NITEGE staining. The middle and outer regions showed weak staining around fiber bundles, and the pericellular compartments stained intensely in the outer region. After 4 days of IL-1 stimulation, the inner region stained intensely for NITEGE, indicative of upregulated aggrecanase activity (Figure 4.5, lower panels). The extensive interterritorial and pericellular staining was similar to that observed of age- and species-matched articular cartilage stimulated with IL-1[246]. The middle region of IL-1-stimulated tissue also demonstrated enhanced NITEGE staining over untreated controls, and the aggrecan fragment localized to intra-

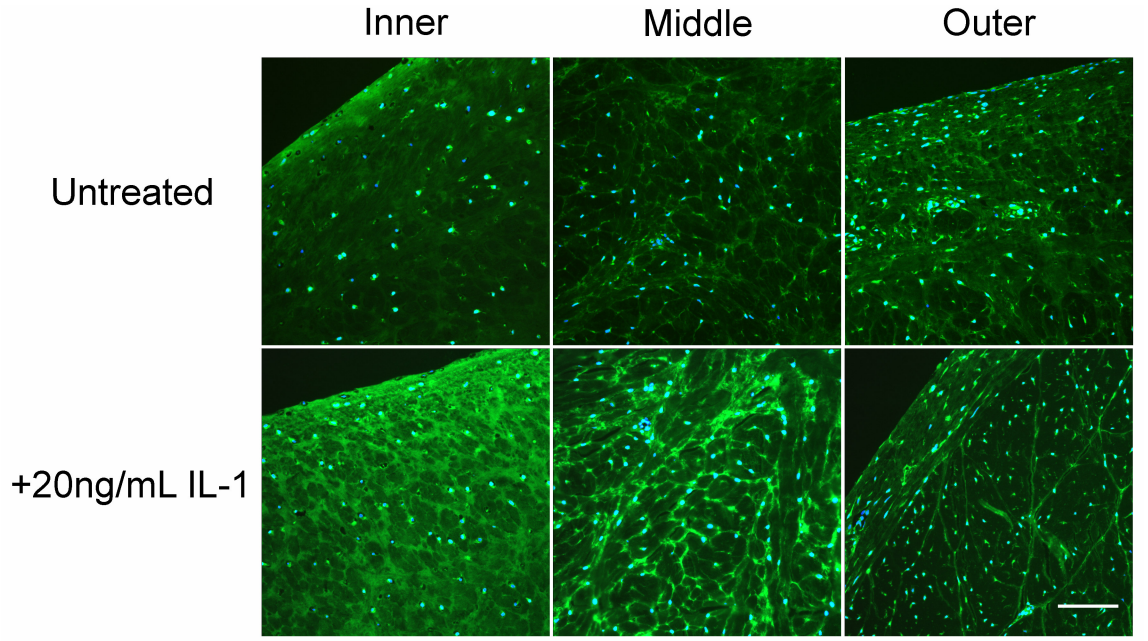

Figure 4.5 IL-1 stimulation enhances aggrecanase activity in the immature bovine meniscus. Coronal slabs of meniscal fibrocartilage were cultured for 4 days in the absence or presence of IL-1. Formalin-fixed paraffin-embedded sections were stained using an antibody to the NITEGE neoepitope (FITC secondary, green) generated by aggrecanase-mediated cleavage of the aggrecan core protein. Cell nuclei (blue) were stained with DAPI. Inner, middle, and outer refer to the radial position in the coronal plane. Scale bar = 100μm.

and peri-cellular compartments and around fiber bundles. The intensity and spatial localization of NITEGE in the outer region of IL-1-stimulated tissue was similar to that of untreated controls. These data suggest that bovine mensical fibrocartilage, like articular cartilage from this species, rapidly responded to IL-1 stimulation with aggrecanase-mediated aggrecan degradation. In addition, this response was confined to the inner and middle regions of the coronal slabs used in this study, perhaps due to the low abundance of aggrecan substrate in the outer region.

Inhibition of IL-1-induced proteoglycan depletion by selective and non-selective metalloproteinase inhibitors

To investigate mechanisms and functional consequences of IL-1-induced proteoglycan degradation in meniscal fibrocartilage, cylindrical tissue explants from the middle region of bovine menisci were cultured for up to 12 days in serum-free media. Stimulation with 20ng/mL *rh*IL-1α triggered rapid release of sGAG to the media (peak rate at day 2), whereas untreated controls demonstrated low rates of release for the duration of the experiment (Figure 4.6A). Subsets of IL-1-stimulated explants were additionally treated with inhibitors selective for aggrecanases, MMPs, or both aggrecanases and MMPs. Addition of an aggrecanase inhibitor attenuated IL-1-induced sGAG release through day 2, but led to elevated release rates after day 2 and total sGAG release over 12 days comparable to IL-1-only cultures. Addition of an MMP inhibitor had no effect on the sGAG releases through day 2, but reduced sGAG release below IL-1-only cultures at

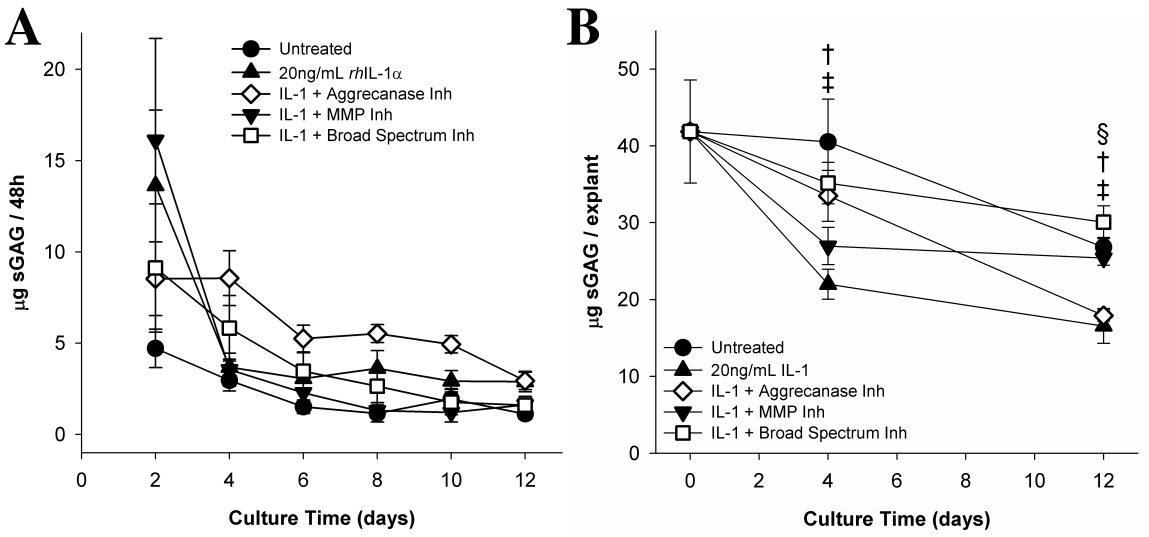

Figure 4.6 Inhibition of sGAG release to media (**A**) and loss of fibrocartilage explant sGAG (**B**) by metalloproteinase inhibitors. sGAG in conditioned media and tissue digests was quantified by the DMMB assay. Data are mean ± SEM, n = 6. §, †, and ‡ indicate $p<0.05$ vs. IL-1 for IL-1 + MMP Inh, IL-1 + P-M Inh, and untreated, respectively.

later time points. Addition of a broad spectrum metalloproteinase inhibitor capable of inhibiting both aggrecanase and MMP activity reduced IL-1-induced sGAG release at day 2, but did not substantially reduce sGAG release at later time points. These results indicate that aggrecanases are primary mediators of sGAG release early in the tissue's response to IL-1 (through day 2), and that MMPs contribute later in the catabolic cascade. In addition, these data suggest that non-metalloproteinase mechanisms of sGAG release (e.g., hyaluronidases) are active in untreated and IL-1-stimulated fibrocartilage.

Consistent with the sGAG release kinetics, measurements of explant sGAG content indicate that IL-1 stimulation triggered rapid loss of tissue sGAG (Figure 4.6B). Four days of IL-1 stimulation led to loss of 50% of the initial (day 0) sGAG content, and depletion persisted through day 12 when the sGAG contents of explants approached 40% of initial sGAG content. Untreated controls contained significantly more sGAG than IL-1-stimulated explants at days 4 and 12, but by day 12 contained only 65% of the initial sGAG, suggesting that mechanisms of proteoglycan release are active under the basal culture conditions. Addition of the aggrecanase inhibitor delayed but did not reduce IL-1-induced sGAG depletion in meniscal fibrocartilage, whereas addition of the MMP inhibitor significantly improved sGAG retention over IL-1-only cultures at day 12. Explants treated with the broad spectrum metalloproteinase inhibitor had significantly higher sGAG contents than IL-1-only controls at days 4 and 12. These data suggest that sGAG release in IL-1-stimulated fibrocartilage is initially mediated by aggrecanases and later by MMPs.

To investigate the potential contributions of aggrecanases and MMPs to proteoglycan release from unstimulated basal explants, additional groups of explants

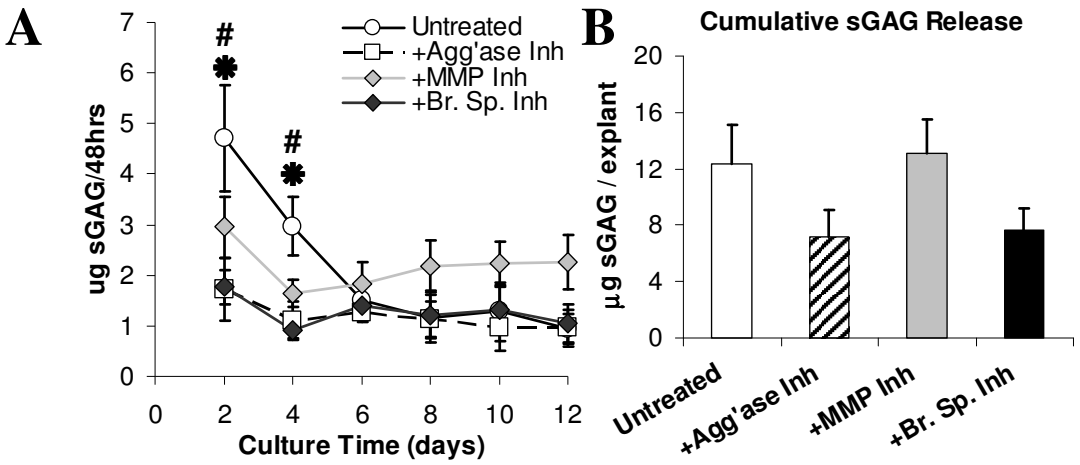

Figure 4.7 Proteoglycan release from unstimulated fibrocartilage explants was inhibited by aggrecanase-selective and broad spectrum inhibitors. Shown are kinetics (**A**) and cumulative proteoglycan release (**B**) over 12 days of serum-free culture. Conditioned media were assayed for sGAG by the DMMB dye-binding assay. Data are mean ± SEM, with n = 6. **#**,**❋** indicate $p<0.05$ for +Agg'ase Inh and +BR. Sp. Inh vs. untreated controls, respectively.

were treated with inhibitors in the absence of IL-1 stimulation. The proteoglycan release profile (Figure 4.7A&B) of untreated basal cultures demonstrates a peak in release at day 2 which decays for the duration of the experiment. Addition of the aggrecanase or broad spectrum inhibitor significantly reduced release at through day 4, and maintained release rates similar to controls thereafter. The MMP inhibitor had no significant effects on release of proteoglycans under the serum-free conditions used in these studies. These results support a role for aggrecanase-mediated aggrecan IGD cleavage during "normal" aggrecan turnover in the immature meniscus.

Inhibition of IL-1-induced aggrecan catabolism by metalloproteinase inhibitors

Mechanisms of aggrecan catabolism were investigated by probing tissue extracts for the aggrecan G1 domain and conditioned media for the NITEGE and DLS neoepitopes. Anti-G1 blots of tissue extracts indicated that a doublet migrating at ~65kDa is the dominant aggrecan species in day 0 tissue (Figure 4.8A). In separate blots for the NITEGE neoepitope (not shown), the 65kDa fragment was confirmed as the aggrecanase-generated product. In addition, there was an apparent absence of high-molecular weight aggrecan species in the explant extracts, consistent with the weak safranin-O staining and relative abundance of NITEGE observed in the middle region of freshly isolated immature bovine meniscus (Figure 4.2). Following 8 days of culture, there was a modest accumulation of this aggrecan fragment in untreated controls indicating that aggrecanase activity is maintained and aggrecanase-generated fragments are retained within the tissue under the basal culture conditions. In contrast, IL-1-stimulated tissue was exhaustively

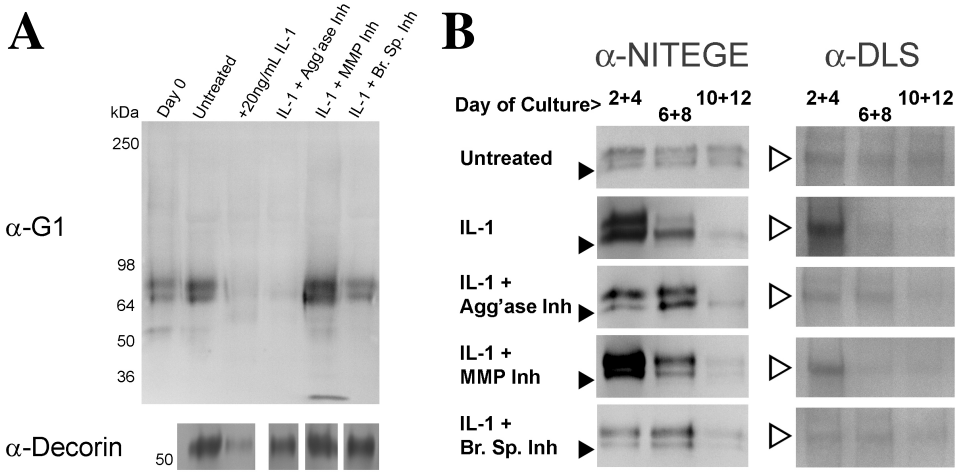

Figure 4.8 Immunodetection of aggrecan and decorin in explant extracts (**A**) and aggrecan neoepitopes in the media (**B**). In (**A**), equal portions of extract sGAG at day 0 or 8 were probed with anti-G1. Below, equal volumes of day 8 explant extracts were probed with anti-decorin. In (**B**), equal volumes of media pooled from days 2 & 4, 6 & 8, or 10 & 12 were probed for the NITEGE and DLS neoepitopes. Filled and open arrowheads indicate migration of the 64kDa and 250kDa molecular weight markers, respectively.

depleted of aggrecan G1 by day 8. Addition of the aggrecanase inhibitor had no apparent effect on this depletion, suggesting that release of this fragment is not mediated by aggrecanases. Explants treated with the MMP inhibitor showed accumulation of the G1-NITEGE fragment, demonstrating that aggrecanases are indeed active following IL-1 stimulation and suggesting that MMPs participate in release of this fragment. Treatment with the broad spectrum metalloproteinase inhibitor enhanced retention of the G1-NITEGE fragment, similar to untreated controls.

To evaluate the presence of other proteoglycans in fibrocartilage explants, extracts were also probed with anti-decorin (Figure 4.8A, lower panel). IL-1-stimulated tissue appeared to be substantially depleted of decorin in comparison to untreated controls, suggesting that some of the proteoglycans released to the conditioned media were small proteoglycans. Interestingly, additional treatment with any of the inhibitors prevented this depletion. C-terminal processing of ADAMTS-4 confers decorin-degrading activity to the aggrecanase[111], and decorin is immobilized in fibrocartilage through favorable interactions with the collagen network[27]. Thus, it is feasible that IL-1-stimulation induced aggrecanase-mediated decorin cleavage and MMP-mediated decorin release.

Neoepitope analysis of conditioned media generally reflected observations of tissue extracts (Figure 4.8B). Untreated tissue released G1-NITEGE and DLS-reactive fragments at low, steady levels through 12 days of culture. Stimulation with IL-1 led to dramatic release of both fragments over days 2-4, and treatment with the aggrecanase inhibitor attenuated and prolonged NITEGE release and reduced release of DLS for the duration of the experiment. Treatment with the MMP inhibitor did not substantially alter

NITEGE release characteristics, but reduced DLS release, and the broad spectrum metalloproteinase inhibitor reduced release of both fragments to untreated control rates.

Inhibition of IL-1-induced collagen degradation by selective and non-selective metalloproteinase inhibitors

Conditioned media and explant digests were assayed for hydroxyproline to quantify the kinetics and degree of collagen depletion. Untreated controls exhibited low basal rates of collagen release, and IL-1-stimulation led to elevated release rates from day 6 through day 12 (Figure 4.9A). Addition of the aggrecanase inhibitor had no effect on collagen release, confirming that fibrillar collagens are not substrates for aggrecanases. Treatment with the MMP or broad spectrum metalloproteinase inhibitor potently inhibited IL-1-induced collagen depletion, indicating that MMPs are primary mediators of collagen network destruction in this culture system. Assays of explant proteinase K

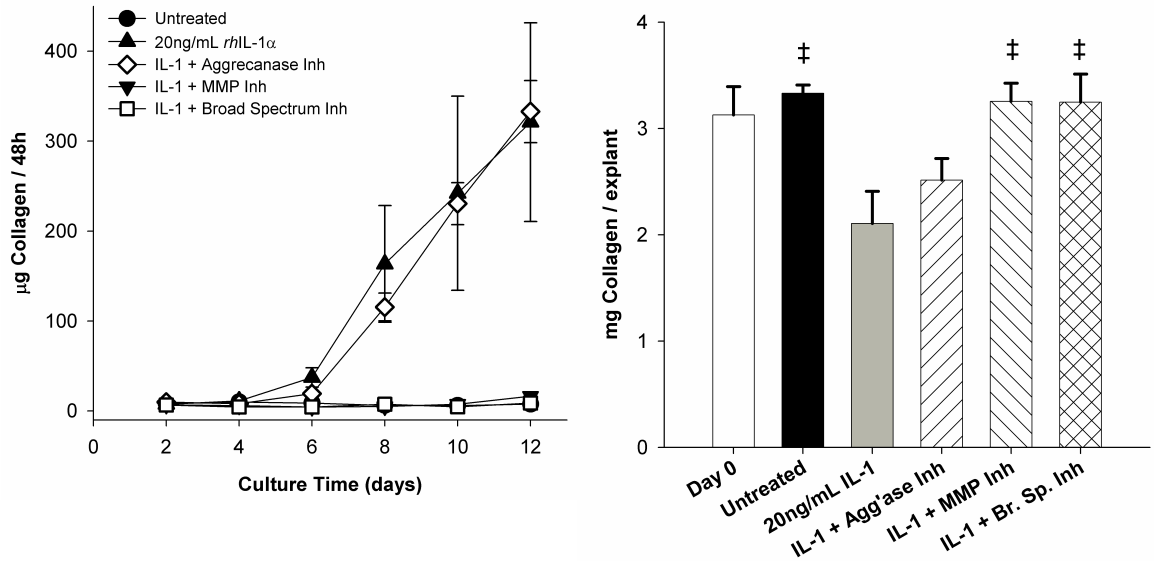

Figure 4.9 Inhibition of IL-1-induced collagen release to media (**A**) and loss from fibrocartilage explants (**B**) by metalloproteinase inhibitors. Collagen in media and explant digests (after 0 or 12 days) was quantified by the chloramine-T/p-dimethylaminobenzaldehyde reaction. Data are mean ± SEM, n = 6. ‡ indicate $p<0.05$ vs. IL-1.

digests similarly demonstrated that IL-1 stimulation triggers collagen destruction (Figure 4.9B). The aggrecanase inhibitor showed no protective effects, whereas treatment with either the MMP or broad spectrum metalloproteinase inhibitor led to significantly higher amounts of explant collagen than IL-1-stimulated tissue after 12 days in culture. This is in contrast to articular cartilage, where aggrecanase inhibition delays the onset of collagen destruction[175,246], suggesting that aggrecan does not "protect" the collagen from proteolytic attack in meniscal fibrocartilage as it appears to do in articular cartilage.

Inhibition of IL-1-induced loss of tissue mechanical properties by selective and non-selective metalloproteinase inhibitors

To investigate the effects of IL-1-induced matrix degradation on tissue material properties, explants were mechanically tested in torsion and compression. The material properties presented are useful for describing the functional implications of ECM degradation in terms of the tissue's tendency to deform under shear and compression. In both testing modes, day 0 tissue exhibited trends of increasing modulus (compression, E*, and shear, G*) with frequency, and these trends were maintained in untreated tissue following 12 days of culture (Figures 4.10A&B). Unlike the shear moduli, the compression moduli appeared to decrease in untreated tissue after 12 days in culture. Stimulation with IL-1 for 12 days led to loss of 75-80% of the day 0 tissue moduli, and treatment with the aggrecanase inhibitor did not reduce these losses. Addition of the MMP inhibitor appeared to have some beneficial effects on both material properties, indicating that preservation of the collagen network is sufficient to partially preserve both the compression and shear moduli. Tissue treated with the broad spectrum metalloproteinase inhibitor had significantly higher compression and shear moduli than

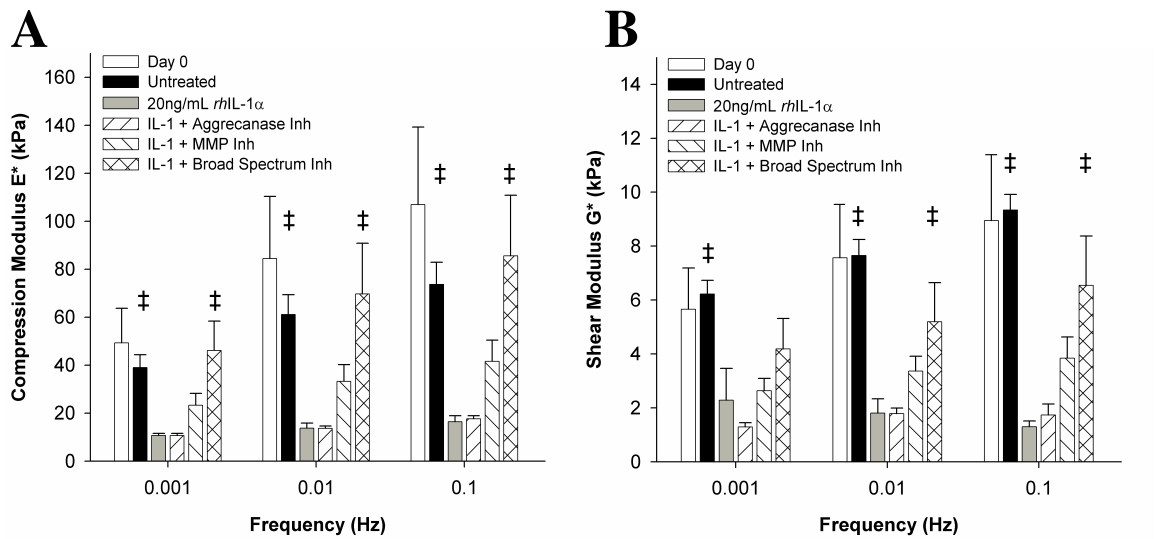

Figure 4.10 Inhibition of IL-1-induced loss of dynamic compression (**A**) and shear (**B**) moduli by metalloproteinase inhibitors. Explants were harvested after 0 or 12 days in culture and tested in oscillatory unconfined compression (1.5% amplitude) and torsion (0.25% amplitude) at a 10% compression offset. Data are mean + SEM, n = 6. ‡ indicate $p<0.05$ vs. IL-1

IL-1-only controls, and exhibited nearly complete blockade of IL-1-induced loss of tissue material properties in meniscal fibrocartilage. The efficacy of the broad spectrum inhibitor in preventing IL-1-induced fibrocartilage destruction is in marked contrast to the results presented in chapter 3 from studies with articular cartilage, and this disparity may be attributed to differences in tissue swelling characteristics. The low abundance of sGAG and structural features of the fibrocartilage ECM preclude explants from swelling as much as articular cartilage, so fibrocartilage may be less likely to undergo culture-induced loss of material properties. These results indicate that aggrecanases and MMPs are primary contributors to IL-1-induced loss of tissue material properties in meniscal fibrocartilage. In addition, culture-induced changes in explant material properties appear to be tissue-dependent.

4. 4 Discussion

The results of this study show that aggrecanases mediate aggrecan catabolism in normal fibrocartilage and both aggrecanases and MMPs mediate IL-1-induced proteoglycan release in meniscal fibrocartilage. In articular cartilage, aggrecanase-mediated cleavage of the aggrecan IGD (*i.e.*, generation of the NITEGE neoepitope) is indicative of destructive ECM catabolism and is essentially undetected in immature tissue. Yet, the G1-NITEGE fragment was readily detected in freshly isolated immature fibrocartilage. In addition, IL-1-stimulated fibrocartilage explants stained intensely for the NITEGE neoepitope in the inner and middle regions, where sGAG density was initially highest. This "pathologic" aggrecanase activity appears to be normal in the immature bovine meniscus and can be elevated in inner and middle regions with IL-1 treatment. This idea was further supported by the suppression of proteoglyan release from untreated basal cultures with the aggrecanase-selective and broad spectrum inhibitors. Cartilage explants also release proteoglycans to the culture medium under these culture conditions, but do not exhibit the aggrecanase-mediated IGD cleavage detected in the fibrocartilage-conditioned media. These data suggest that regulation of aggrecan catabolism in meniscal fibrocartilage is different from that in articular cartilage.

Spatial- and age-variations in fibrocartilage proteoglycan content appear to be controlled in part by regional differences in aggrecanase activity. Interestingly, safranin-O staining is more uniformly distributed and aggrecan gene expression is higher in menisci from mature animals than from immature tissues[138,139,155], suggesting that these regional variations in proteoglycan density and turnover are dependent on developmental stage. Age-related changes in aggrecan catabolism were previously reported for meniscal

fibrocartilage, as high molecular weight (~250kDa) aggrecan core protein and the G1-NITEGE species were abundant in immature bovine fibrocartilage, and G1-VDIPES (a product of MMP-mediated aggrecan cleavage) and a 100-130kDa fragment were additionally present in menisci from mature steer[198]. The current study extends previous work by describing regional differences in aggrecanase activity in the immature meniscus and links these differences to the normal development and region-specific functional demands of fibrocartilage. The mechanisms of aggrecan turnover may be conserved across several tissues, since aggrecan is less expressed and more extensively processed in tension-bearing regions of bovine deep flexor tendon in comparison to regions of compressed tendon[173,236]. Composition-function analysis of developing meniscal fibrocartilage may clarify the physiologic or mechanical benefits of partial aggrecan processing in different regions of the tissue. It is tempting to speculate, however, that aggrecan species of lower molecular weights may be more readily incorporated into fibrocartilage-specific structures, like the large bundles of collagen fibers. In addition, fibrochondrocytes may use partially processed aggrecan in the assembly of a pericellular matrix with composition and material properties specific to fibrocartilage. Indeed, the apparent benefits of differential aggrecan processing may be dependent on the length and time scales of the structures and processes studied.

Studies of IL-1-stimulated joint tissues have been useful in understanding the spatiotemporal dynamics of cell-mediated ECM degradation characteristic of arthritic degeneration. There is extensive evidence of IL-1-induced proteoglycan release[47,227], aggrecanase activity[7,197], and loss of material properties in articular cartilage explants [22,123]. Less is known about the catabolic response of fibrocartilage to IL-1 or its effects on

tissue material properties. Fibrochondrocytes, similar to articular chondrocytes, generated the inflammatory molecules nitric oxide and prostaglandin E2 in response to IL-1[31,84], and IL-1 abrogated the biosynthetic response of fibrochondrocytes to mechanical stimuli[207]. Surface-zone meniscal fibrocartilage explants from mature bovines were stimulated with IL-1 and exhibited increased MMP-3 expression, aggrecanase activity, and sGAG release, and lower peak compression stresses (during a ramp to 10% compression) than untreated control tissue[119]. The results of the current study are consistent with previous reports and support the hypothesis that IL-1 initiates a catabolic cascade mediated by aggrecanases and MMPs in meniscal fibrocartilage.

The catabolic response of fibrocartilage is distinct from that of articular cartilage. Most strikingly, both aggrecanases and MMPs were quantitatively important in IL-1-induced proteoglycan release. Aggrecanases were the primary mediators of aggrecan cleavage in fibrocartilage, evidenced by the abundance of G1-NITEGE and absence of MMP-generated fragments in tissue extract immunoblots. Release of sGAG, however, was significantly reduced by treatment with an MMP inhibitor, and not with an aggrecanase inhibitor. In contrast, up ~95% of the sGAG release from IL-1-stimulated articular cartilage is attributed to ADAMTS-4 and -5. In the absence of MMP-mediated aggrecan cleavage, sGAG may be released through MMP-mediated denaturation and degradation of the collagen network. In addition, diffusion of aggrecanases to substrates embedded within the fiber bundles may be limited by MMP-mediated collagen degradation. The organized bundles of radially- and circumferentially-oriented collagen fibers in meniscal fibrocartilage may demarcate a distinct metabolic pool of aggrecan for which release from the ECM is rate-limited by the reaction of MMPs with collagen. Akin

to the fascicular structures present in tendon and muscle, these structures appear to contain relatively thick collagen fiber bundles. Proteases may diffuse readily in directions parallel to the bundles, whereas diffusion into or out of these structures would be substantially slower. Presumably, MMP-mediated disruption of the fascicular structures would enhance diffusion of collagen associated aggrecan out of these structures. Thus, MMPs may participate directly or indirectly in the release of proteoglycans from fibrocartilage.

Treatment with an aggrecanase inhibitor delayed sGAG depletion in IL-1-stimulated fibrocartilage, but not did reduce the total depletion over 12 days. The selectivity of this inhibitor for aggrecanases other than ADAMTS-4 and -5 is unknown, so it is possible that other aggrecanases (*e.g.*, ADAMTS-1, -8, -9, and -15) are active later in the cascade. Alternatively, the inhibitor might be less potent against activated forms of ADAMTS-4[71,111] generated later in the culture period. Indeed, the same inhibitor was shown to delay but not block aggrecanase-mediated sGAG release in articular cartilage explants[246]. Interestingly, addition of the aggrecanase or broad spectrum inhibitor to unstimulated cultures substantially reduced sGAG release over 12 days (data not shown), suggesting that aggrecanases are primary mediators of homeostatic proteoglycan turnover under the basal culture conditions used in this study.

Degeneration of articular cartilage involves exhaustive, aggrecanase-mediated depletion of proteoglycans followed by MMP-mediated degradation of collagen type II[116]. In contrast, proteoglycan depletion in meniscal fibrocartilage was mediated by both aggrecanases and MMPs and persisted during degradation of the collagen network. It is interesting that IL-1-induced release of low molecular weight G1-NITEGE occurred in

the presence of the MMP inhibitor, whereas release of the high molecular weight DLS-positive aggrecan fragment (~250kDa) was reduced by this inhibitor treatment. These species may reside in distinct metabolic pools of aggrecan with differing susceptibility to MMP-mediated release, perhaps related to differential association with the collagen network. Understanding the distinct features (*i.e.*, kinetics and mechanisms) of IL-1-induced ECM destruction in meniscal fibrocartilage may aid in the identification of therapeutic targets and biomarkers for the treatment and diagnosis of arthritis.

The mechanical responses of meniscal fibrocartilage to shear and compression depend on the composition, integrity, and organization of its ECM[4,55,255]. Fibrocartilage explants stimulated with IL-1 for 12 days contained 44% and 67% of the day 0 tissue's sGAG and collagen, respectively. Correspondingly, the depleted tissue retained only 15-40% (depending on test frequency) of the shear stiffness and 15-22% of the compressive stiffness of the fresh tissue. Explants treated with the MMP inhibitor retained 100% of the initial collagen but only 63% of the initial sGAG. This reduction in sGAG led to a 53-57% reduction in shear stiffness and a 53-61% reduction in compressive stiffness, indicating that despite comprising a quantitatively minor ECM constituent (1-3% by dry weight)[74], proteoglycans contribute substantially to the shear and compression properties of meniscal fibrocartilage. The dominant aggrecan species (G1-NITEGE) detected in this study lacks the chondroitin sulfate binding regions of the aggrecan core molecule, so the IL-1-induced release of sGAG might be attributable to degradation of other proteoglycans. Meniscal fibrochondrocytes have been shown to express mRNA for the large aggregating proteoglycan versican in immature rabbit[89], and aggrecanase-mediated versican degradation has been observed in other tissues[96,193,201,242]. Although decorin was

cleaved by activated ADAMTS-4[111] and biglycan was shown to be a substrate for MMP-13[147], both proteoglycans were resistant to degradation in IL-1-stimulated bovine nasal cartilage[220]. Immunoblots of tissue extracts revealed that after 8 days of IL-1 stimulation, fibrocartilage explants had lost approximately 50% of the initial decorin and treatment with an aggrecanase, MMP, or broad spectrum metalloproteinase inhibitor prevented this depletion. Proteoglycan degradation clearly compromises the mechanical function of meniscal fibrocartilage, and it is likely that both small and large proteoglycans are essential for proper tissue function.

Metalloproteinase inhibitors have demonstrated utility as investigative tools for physiologic and pathophysiologic ECM turnover in orthopaedic tissues, and some inhibitors have entered clinical trials for treatment of arthritis[22,33,37,49,134,175,212,249]. The results of this study show through the use of aggrecanase- and MMP-selective inhibitors that both aggrecanases and MMPs participated in release of proteoglycan from IL-1-stimulated fibrocartilage and that aggrecanases were primary mediators of aggrecan core protein cleavage in this tissue. In addition, a broad spectrum metalloproteinase inhibitor potently inhibited IL-1-induced proteoglycan depletion and loss of the tissue's compression and shear properties. The inhibitors exhibited minimal cytotoxicity as they had no significant effects on explant viability over 12 days in culture. These results underscore the importance of metalloproteinases in normal and pathophysiologic proteoglycan turnover in the meniscus, and suggest that delivery of non-selective metalloproteinase inhibitors to the degenerative joint will have therapeutic benefit for the preservation of fibrocartilage composition and function.

These studies underscore the importance of aggrecan in fibrocartilage mechanical function. Despite the low initial abundance of aggrecan in immature meniscal fibrocartilage, loss of explant compression and shear properties aligned with loss of sGAG from the explants. On the other hand, the collagen network also played an important role in load bearing. Preservation of the collagen network appeared to partially preserve tissue material properties, which is consistent with a model describing the fibrocartilage ECM as a porous fiber-reinforced composite[55]. Cell-mediated degradation of the ECM composite and concomitant reductions in fibrocartilage stiffness would disrupt normal joint mechanics and may be early events in meniscal degeneration.

CHAPTER 5

MECHANISMS OF TGF-B-INDUCED AGGRECAN TURNOVER IN CARTILAGE AND FIBROCARTILAGE

5.1 Introduction

The healthy articular cartilage ECM is rich in aggrecan, a large aggregating proteoglycan glycosylated with up to 100 negatively charged sulfated glycosaminoglycan (sGAG) chains[83]. Aggrecan is trapped within the cartilage ECM through non-covalent binding to high molecular weight hyaluronic acid and entanglement with an extensive type II collagen network. The immobilization of charged sGAG generates an osmotic pressure that attracts water into the ECM and confers a biphasic, viscoelastic response to applied stresses that is critical to normal joint function[48]. The ECM of meniscal fibrocartilage is composed of a highly organized type I collagen network. Large collagen fibers are aligned circumferentially in the outer portions of the menisci to bear tensile hoop stresses[139], and the inner region is rich in aggrecan and collagen type II[110], consistent with its cartilage-like function of bearing primarily compressive loads. The aggrecan G1 (hyaluronan−binding) domain has been immunolocalized in meniscal fibrocartilage[230] and age-related differences in safranin-O staining suggest that aggrecan density in the middle and outer regions of the menisci increases with age[145]. Data presented in chapter 4 shows that while aggrecan G1 is present throughout the developing meniscus, substantial proteolytic processing occurs in the middle and outer regions such that sGAG-containing portions of the core molecule are released. In contrast, aggrecan from age- and species- matched articular cartilage is primarily in full-length and other high molecular weight forms.

The apparently normal, though extensive, aggrecan processing in the middle and outer regions of developing menisci is characterized by aggrecanase-mediated cleavage of the aggrecan interglobular domain (IGD) and subsequent exposure of the NITEGE^{392} neoepitope, which is typically associated with pathologic aggrecan catabolism in degenerative articular cartilage. Aggrecanolysis in articular cartilage has been studied for its role in progression of arthritis, and aggrecanase-, matrix metalloproteinase (MMP)-, and calpain-family proteases appear to be responsible for the bulk of normal and pathologic aggrecan processing in cartilage[164,194,198]. The contributions of these enzyme systems to fibrocartilage aggrecan turnover have not yet been described. In addition, the physiologic relevance or functional benefits of extensive aggrecan processing in the healthy, developing meniscus is unknown.

The transforming growth factor–β (TGF-β) family of pleiotropic cytokines has been shown to have anabolic and anti-catabolic effects on articular cartilage explants[133,148]. TGF–β1 was detected in the synovial fluid of arthritic joints[52], and appears to mediate the wound healing response[24,76]. The cartilage ECM is a reservoir of latent TGF–β1 that chondrocytes can engage through proteolytic processing of the latent-TGF–β binding complex[26,171]. The TGF–β type I receptor (TGF–βRI) is a cell-surface receptor that dimerizes with TGF–β type II receptor upon binding with TGF–β and exerts kinase activity on intracellular signaling molecules to regulate expression of genes encoding downstream effectors of ECM remodeling (*e.g.*, proteases and ECM molecules)[79]. For example, TGF–β1 has been shown to increase proteoglycan biosynthesis[133,148] and expression of tissue inhibitor of metalloproteinases (TIMP) in chondrocytes[240]. Fibrochondrocytes in explants[38,97], three-dimensional culture

scaffolds[166,251], and monolayer[221] also exhibited robust anabolic responses to TGF-β1 stimulation. Interestingly, TGF-β stimulation elevated aggrecanase activity in cartilage explants[151]. TGF-β, along with other growth factor and cytokine signaling axes[65,136,149,203,239], is a potent regulator of ECM remodeling in both cartilage and fibrocartilage.

The aims of this study were to a) compare mechanisms of aggrecan turnover in articular cartilage and meniscal fibrocartilage, and b) describe the effects of TGF–β1-induced ECM remodeling on the composition and material properties of native tissue and cell-agarose tissue constructs. Endogenous TGF-β signaling was characterized through the use of a potent and selective pharmacologic inhibitor of TGF-βRI. Pharmacologic protease inhibitors were used to evaluate the participation of aggrecanases, MMPs, and calpains in TGF–β1-stimulated ECM turnover and *de novo* assembly. Analysis of neoepitopes, the peptide sequences exposed upon cleavage of the aggrecan core protein, via antibodies raised to the C-terminal NITEGE[**392**] and DLS[**12xx-13xx**] sequences afforded detection of aggrecanase- and calpain-specific aggrecanolysis, respectively. Immunoblots of SDS-PAGE-separated samples for G1-, G2-, and G3-bearing material were also used to identify aggrecan fragments by size. The results of this study highlight tissue- and cell-type differences in the mechanisms and degree of aggrecanolysis between the tissues, and implicate all three protease systems examined in fibrochondrocyte-mediated aggrecan turnover.

5.2 Materials & Methods

Reagents and antibodies

High glucose Dulbecco's modified Eagle's medium (DMEM), antibiotic/antimycotic solution containing 100U/mL penicillin, 100μg/mL streptomycin, and 0.25μg/mL amphotericin B, 1% non-essential amino acids (NEAA), 1% N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), trypsin-ethylenediaminetetraacetic acid, proteinase K, and Dulbecco's phosphate buffered saline (DPBS) were from Invitrogen (Carlsbad, CA). Antibodies to aggrecan G1, G2, G3, and the NITEGE and DLS neoepitopes were provided by Dr. John Sandy (Shriner's Hospital for Children, Tampa, FL). The antibody LF-94 to bovine decorin was provided by Dr. Larry Fischer (NIDCR, Bethesda, MD). Guanidine hydrochloride, protease-free chondroitinase ABC, keratanase I, agarose, Hoechst 33258 dye, non-immune rabbit IgG, and alkaline phosphatase-conjugated anti-rabbit secondary antibody were from Sigma (St. Louis, MO). ITS+ Premix was from BD Biosciences (San Jose, CA). Keratinase II was from Associates of Cape Cod (Falmouth, MA). Dimethylmethylene blue (DMMB) was from Polysciences (Warrington, PA). Recombinant human TGF-β1 was from R&D Systems (Minneapolis, MN). Protease inhibitor cocktail containing ethylenediaminetetraacetic acid, 4-(2-aminoethyl)benzenesulfonylfluoride, leupeptin, E-64, and aprotinin was from Calbiochem (San Diego, CA). The chemifluorescent substrate ECF was from Amersham (Piscataway, NJ).

Small-molecule metalloproteinase inhibitors (RO3310769, aggrecanase-selective; RO1136222, MMP-selective) were provided by Roche – Palo Alto (Palo Alto, CA). Detailed selectivity profiles of these inhibitors have been reported previously[246]. Briefly,

in vitro enzyme activity assays showed that the aggrecanase-selective inhibitor was highly inhibitory against the aggrecanase ADAMTS-4, with a concentration of half-maximal inhibition (IC_{50}, lower values indicate greater inhibition) of 8nM, and IC_{50}s > 700nM for all MMPs tested. The MMP-selective inhibitor inhibited most MMPs tested with IC_{50}s ≤ 50nM, with the exceptions of MMP-1 and -7, but did not prevent ADAMTS-4 activity (IC_{50} = 7200nM). The competitive, cell-permeable calpain inhibitor carbobenzoxy-valyl-phenylalanial, also known as MDL-28170, was from Calbiochem. The manufacturer has reported an IC_{50} of 8nM against calpains I and II for this compound. Equimolar stock solutions of the protease inhibitors were prepared with dimethylsulfoxide (DMSO).

A potent and selective pharmacologic inhibitor, SB431542, of TGF-β type 1 receptor kinase was from Tocris Biosciences (Ellisville, MO). The inhibitor has been reported to have an IC_{50} of 94nM against the receptor kinase and approximately 100-fold selectivity over other kinases, such as p38[99].

Tissue Culture

Articular cartilage and meniscal fibrocartilage were harvested from immature bovine stifle joints under aseptic conditions. Cartilage explants (n = 84) were taken from the patellar groove and femoral condyles using a 4mm diameter biopsy punch, and fibrocartilage explants (n = 84) were taken from the middle regions of medial and lateral menisci. Surface zone tissue was trimmed away and explants were cut to 2mm thick using a custom cutting block. The explants were moved to 48-well plates and cultured overnight in 0.5mL basal medium consisting of high glucose DMEM, ITS+, 1% NEAA, 1% HEPES, 82µg/mL L-ascorbic acid 2-PO_4, and 100U/mL penicillin, 100µg/mL

streptomycin, and 0.25µg/mL amphotericin B. The following day (day 0), some explants were removed as day 0 samples (n = 6/tissue type) and stored at -20°C in PBS with protease inhibitors.

Explants were cultured for up to 10 days in basal medium with carrier (DMSO, 0.05%), basal medium with 2µM SB431542 (TGF-β type I receptor inhibitor, TGF−βRI inhibitor), or with 5ng/mL TGF-β1 and carrier. Groups of TGF-β1-stimulated explants were additionally treated with 5µM aggrecanase-selective inhibitor, 5µM MMP-selective inhibitor, or 5µM calpain inhibitor III. Media were collected, replaced, and prepared with fresh cytokine and inhibitors every 48h; collected media were stored at -20C for biochemical analysis. At the end of the experiment, explant viability was evaluated by the WST-1 assay. The explants were rinsed in DPBS and stored at -20C in DPBS with protease inhibitors.

For a second experiment using cell-agarose constructs, articular chondrocytes (ACs) and meniscal fibrochondrocytes (MFCs) were isolated from fresh immature bovine stifle joint tissues. Articular cartilage from the patellar groove and femoral condyles and fibrocartilage from the medial and lateral menisci were harvested aseptically. The tissues were minced to ~1mm^3, weighed, and moved to T75 flasks. Following a 30min digestion at 37C with 0.025% trypsin/EDTA in Ca^{++}/Mg^{++}-free DPBS, the tissue was digested in 0.4% collagenase/DMEM at 37C on a shaker plate. After 48h, the tissue digests were filtered through a sterile 37µm-pore size nylon mesh, and cell viability and counts were quantified using a Vi-Cell Analyzer. Cell-agarose constructs were prepared by mixing equal volumes of 45C 3% (w/v) agarose/DPBS and cells in DMEM at 20 million cells per milliliter. The molten cell-agarose suspensions were thoroughly mixed and cast

between 2 parallel glass plates approximately 2.5mm apart. The cell-agarose slab was polymerized for 20min at 4C, and cylindrical samples were cut from the slab using a 6mm diameter biopsy punch (n = 48/cell type). Cell-agarose constructs were equilibrated overnight in basal medium containing high-glucose DMEM, ITS+, NEAA, HEPES, 82μg/mL L-ascorbic acid-2-PO_4, penicillin, streptomycin, amphotericin B. ITS+ has been shown to support proteoglycan synthesis and proliferation rates comparable to serum supplementation for chondrocytes in agarose, and thus affords use of a serum-free medium [113]. Day 0 constructs (n = 6/cell type) were evaluated for cell viability by the WST-1 assay, rinsed in DPBS, moved to DPBS with protease inhibitors, and stored at 4C before undergoing mechanical tests within 72h.

Constructs were cultured for up to 16 days in basal medium with carrier (DMSO, 0.01%), or basal medium with 10ng/mL TGF-β1 with carrier. Groups of TGF-β1-stimulated constructs were additionally treated with 1μM aggrecanase inhibitor, 1μM MMP inhibitor, or 1μM calpain inhibitor. Media were collected, replaced, and prepared with fresh cytokine and inhibitor every 48h; collected media were stored at -20C. At the end of the experiment, cell-agarose constructs were either fixed in 10% NBF for 4h at 4C and embedded in paraffin for histologic analysis (n = 2/treatment/cell type) or evaluated for viability by the WST-1 assay, rinsed in PBS, and stored in DPBS with protease inhibitors at 4C prior to mechanical testing.

Mechanical Testing

Prior to mechanical testing, explants were thawed to room temperature and weighed, and explant thickness and diameter were measured at 3 points using digital calipers. All tests were performed at room temperature in DPBS and protease inhibitors.

Specimens were first tested in unconfined compression on an ELF3100 uniaxial testing frame (Enduratec, Minnetonka, MN) using a 500g button-type load cell (Sensotec, Columbus, OH). Each explant was positioned between parallel, impermeable platens and preloaded to 10-20mN to ensure contact. Following compression to 10% strain at 0.1mm/min and relaxation for 30min, each sample underwent oscillatory compression at 0.01, 0.1, and 1Hz and 1.5% strain amplitude. Samples were then equilibrated to the free-swelling state in DPBS with protease inhibitors for 30min, and positioned between roughened, impermeable, parallel plates on a CVO120 rheometer (Bohlin, East Brunswick, NJ). Following compression to 10% strain and 30min relaxation, each explant underwent oscillatory torsion at 0.5Hz and nominal 0.25% (0.017rad) shear strain amplitude. After mechanical testing, explants were frozen and lyophilized prior to biochemical analysis (described below).

Cell-agarose constructs were also tested in unconfined oscillatory compression. Prior to testing, each construct's mass, thickness, and diameter were measured. Each construct was positioned between impermeable platens on an ELF3100 and brought into contact with the platens by application of a 10mN preload. After compression to 10% strain at 0.1mm/min and relaxation for 1200s, each construct was loaded in oscillatory compression at 0.05, 0.1, 0.5, and 1Hz and 3% strain amplitude. After testing, constructs were frozen and lyophilized prior to biochemical analysis.

Total harmonic distortions were typically less than 5% for oscillatory compression and shear tests. Compressive and shear stress amplitudes at the fundamental loading frequency were determined by Fast Fourier Transform analysis of load and torque signals, respectively, and known sample geometries. Dynamic moduli were

calculated from the ratio of the measured stress amplitude to applied strain amplitude. The tangent of the phase angle was calculated by taking the ratio of loss to storage moduli.

Biochemistry

Conditioned media from both experiments were assayed for released sGAG by the DMMB assay using chondroitin sulfate standards diluted in basal medium. Following mechanical testing and lyophilization, explants and constructs were weighed and extracted in 1mL extraction buffer containing 4M guanidine hydrochloride, 10mM MES, 50mM sodium acetate, 5mM iodoacetic acid, and protease inhibitor cocktail, at pH 6.5. Extractions were performed for at least 24h at 4C on a rocker. Explant and cell-agarose extracts were assayed for sGAG by the DMMB assay, using chondroitin sulfate standards diluted in extraction buffer. The DNA content of construct extracts was also quantified by the Hoechst 33258 fluorometric assay with calf thymus DNA standards dissolved in extraction buffer [112]. After extraction, explant and construct residues were frozen, lyophilized, weighed, and digested in 500uL 300μg/mL proteinase K/100mM ammonium acetate overnight at 60C. Cell-agarose residues were then melted at 100C for 10min and further digested with 3U (15μL) agarase overnight at 43C. Explant and construct digests were assayed for sGAG content.

For both experiments, equal volumes of guanidine extracts were pooled from samples within a treatment group and added to 3 volumes of ice cold ethanol/5mM sodium acetate for overnight precipitation of sGAG at -20C. In addition, equal volumes of day 16 conditioned media from cell-agarose constructs were pooled from samples within a treatment group and ethanol precipitated. Precipitates were centrifuged at 21k x

g for 30min, the supernatants were removed, and the pellets were dried for 0.5-2h at room temperature. Pellets were resuspended in buffer containing 50mM Tris hydrochloride, 50mM sodium acetate, 10mM EDTA, and protease inhibitors, at pH 7.5 and assayed for sGAG. Precipitations typically yielded 80% recovery of sGAG from guanidine extracts and conditioned media. Samples were deglycosylated by incubation with chondroitinase ABC, keratanase I, and keratanase II overnight at 37C. Samples were frozen, lyophilized, and resuspended in sample buffer containing 3M Urea, Tris-glycine sample buffer, and dithiothreitol. Extracts of articular cartilage were resuspended in a 10-fold higher volume of sample buffer to accommodate higher sGAG concentrations.

Prior to electrophoretic separation, samples were melted at 110C for 5min and loaded into 4-12% Tris-glycine gradient gels. The samples were separated at 200V for 45min, and transferred to nitrocellulose at 50-100V for 1-2h on ice. Membranes were blocked in blocking buffer for 30min at room temperature and incubated with primary antibodies diluted 1:1000-1:5000 for 1h at room temperature or overnight at 4C. Following incubation with secondary antibody (1:10,000 dilution) for 1h at room temperature, membranes were developed in ECF for 5min and imaged on a Fuji-FLA3000. Membranes initially probed with anti-NITEGE antisera (1:1000 dilution) were subsequently stripped in 1x Re−Blot reagent (Chemicon) for 10min at room temperature and reprobed with anti-G1 antisera (1:5000). Membranes initially probed with anti-DLS antisera (1:2000) were stripped (as described above) and reprobed with anti-G2 antisera (1:4000). Membranes initially probed with the anti-G3 antisera were stripped and reprobed with the LF-94 anti-decorin antisera (1:5000).

Statistics

Data were analyzed using analysis of variance (ANOVA) and the general linear model in MINITAB Release 14. Pairwise comparisons between TGF-β1-stimulated groups were performed by Dunnett's test with TGF–β1-only as controls. Significant differences were denoted by $p<0.05$, and trends were denoted by $p<0.1$.

5.3 Results

Explant Study

Explant Mitochondrial Activity

Cell activity following 10 days of culture was assessed by the WST-1 assay. The tetrazolium salt WST-1 undergoes conversion to formazan by the mitochondria of live cells and spectrophotometric detection of the formazan product permits quantification of the differences in aggregate cell activity, which may be influenced by cell viability, metabolic activity, and cell number. Treatment with 2μM TGF-βRI inhibitor or stimulation with 5ng/mL TGF-β1 had no significant effect on the viability of articular cartilage explants (Figure 5.1). In contrast, the viability of meniscal fibrocartilage explants was reduced to approximately 60% of basal controls by treatment with the TGF-βRI inhibitor and increased by approximately 30% over basal controls with TGF-β1 stimulation. TGF- β1-stimulated articular cartilage treated with 5μM MMP-selective inhibitor showed significantly lower viability than TGF-β1-only controls, but was not lower than basal controls.

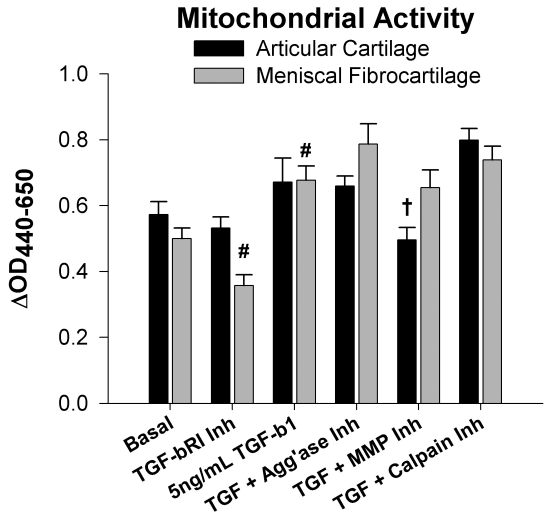

Figure 5.1 Effects of pharmacologic inhibitors and TGF-β1 stimulation on explant mitochondrial activity. Mitochondrial activity was quantified by spectrophotometric detection of the formazan product in the supernatants of explants incubated with the tetrazolium salt WST-1 for 2h at 37C. Data are mean + SEM with n=6. **#** denotes significant difference ($p<0.05$) vs. basal. **†** denotes $p<0.05$ vs. TGF-β1-only controls.

Tissue Swelling Characteristics

Explant hydration, which influences tissue sGAG concentration and material properties, was quantified by measuring explant wet mass (Figure 5.2A) and dry mass and calculating the percent water (Figure 5.2B). Articular cartilage explants cultured in basal medium for 10 days exhibited a 2-fold increase in wet mass and concomitant 13% increase in water fraction over day 0 explants. Addition of a TGF−βRI inhibitor further increased tissue swelling over basal controls. Cartilage explants stimulated with TGF−β1 had significantly lower wet masses and water fractions than basal controls, indicating that TGF−β1-driven ECM remodeling promotes maintenance of native tissue composition. TGF−β1-stimulated cartilage additionally treated with an MMP-selective inhibitor appeared to have the lowest propensity to swell, as indicated by strong trends toward lower wet mass and water fraction than TGF−β1-only controls ($p=0.057$ and 0.063, respectively). Conversely, addition of a calpain-selective inhibitor to TGF−β1-stimulated

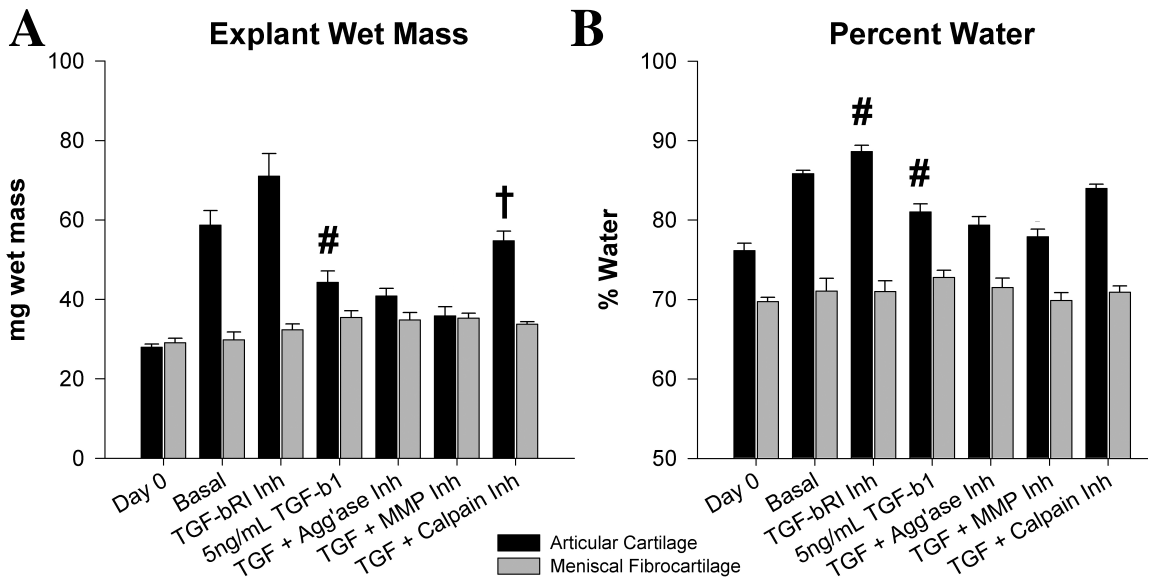

Figure 5.2 TGF-β1 stimulation reduced explant swelling in articular cartilage and meniscal fibrocartilage as indicated by wet mass (**A**) and percent water (**B**). Data are mean + SEM, and n = 6. **#** denotes $p<0.05$ vs. basal. **†** denotes $p<0.05$ vs. tissue-matched TGF-β1-only controls.

cartilage significantly increased swelling characteristics over TGF–β1-only controls. Fibrocartilage explants had lower water fractions than articular cartilage at day 0 and were much less likely to swell under any condition, although there was a strong trend for increased wet mass in TGF–β1-stimulated explants over basal controls ($p=0.055$). These results underscore important differences between cartilage and fibrocartilage swelling characteristics and implicate TGF–β, MMP, and calpain activity in the regulation of cartilage tissue hydration. In addition, explantation and serum-free culture of cartilage and fibrocartilage caused structural changes in the ECM that are likely to contribute to apparent changes in tissue composition.

Proteoglycan Content

Proteoglycan turnover was evaluated by quantifying the distribution of proteoglycans in conditioned media, explant extracts, and residue digests (Figure 5.3).

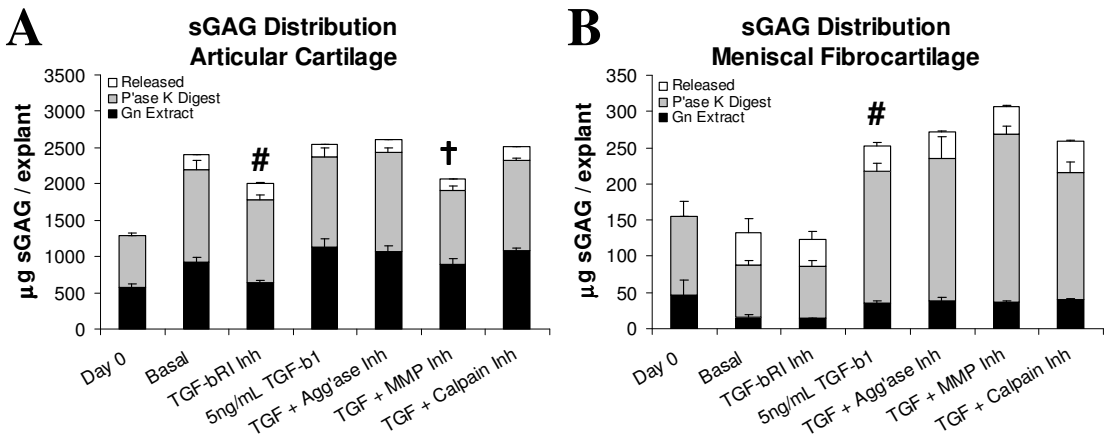

Figure 5.3 TGF-β1 stimulated sGAG accumulation in meniscal fibrocartilage. Following 0 or 10 days in culture, immature bovine cartilage and fibrocartilage explants were treated with 4M guanidine to extract intact proteoglycans and residues were digested with proteinase K. Released sGAG was the cumulative content in conditioned media. Note the 10-fold difference in scale between (**A**) and (**B**). Data are mean + SEM with n=6. **#** denotes significant difference ($p<0.05$) in total explant content (extract + digest) vs. basal. **†** denotes $p<0.05$ vs. TGF-β1-only controls.

For all experimental groups, articular cartilage explants had total explant (extract + digest) sGAG well above day 0 tissue (Figure 5.3A), indicating that chondrocyte proteoglycan synthesis is maintained under the basal culture conditions used in this study. Cartilage treated with a TGF–βRI inhibitor had significantly lower total explant content than basal controls, and TGF–β1-stimulated cartilage had modestly, but significantly, lower cumulative sGAG release than basal controls. These data suggest that endogenous TGF–β signaling is active in explanted cartilage, perhaps due to a greater abundance of TGF-β in the cartilage ECM. In sharp contrast, meniscal fibrocartilage treated with basal medium or basal medium supplemented with TGF–βRI inhibitor appeared to have very low proteoglycan synthesis (Figure 5.3B). These groups released approximately 33% of the day 0 tissue sGAG content to the media over 10 days in culture, and had a net loss of total explant sGAG. Fibrocartilage stimulated with TGF–β1, however, had significantly higher total explant sGAG than basal controls, indicating elevated proteoglycan synthesis

and retention. Based on these results, it appears that there was minimal endogenous TGF−β signaling in explanted fibrocartilage, and that fibrochondrocytes had a potent anabolic response to TGF−β1 stimulation. It is important to note that the initial sGAG content of fibrocartilage explants was approximately 10% of cartilage explants. Collectively, these data show important baseline differences in proteoglycan abundance and turnover between cartilage and fibrocartilage. Cartilage explants have an ECM rich in sGAG and relatively high proteoglycan production driven in part by endogenous TGF-β. On the other hand, fibrocartilage explants exhibit low proteoglycan production under the basal serum-free conditions used in this study, and respond to TGF-β1 stimulation with substantially increased proteoglycan production. Fibrocartilage explants also consistently released a larger fraction of sGAG to the media than cartilage explants, and structural features of the fibrocartilage ECM (*e.g.*, highly organized and oriented collagen bundles) may have facilitated diffusion of proteoglycans into the culture media.

To examine the roles of different enzyme systems in proteoglycan turnover in tissue explants, TGF−β1-stimulated tissues were additionally treated with an aggrecanase-, MMP-, or calpain-selective inhibitor. Consistent with a primarily anabolic and anti-catabolic response to TGF−β1, protease inhibitor treatments did not substantially alter proteoglycan distributions. TGF−β1-stimulated cartilage explants treated with the MMP-selective inhibitor had significantly lower total explant sGAG than TGF−β1-only controls, suggesting that MMPs were participating in the TGF−β-signaling axis. These data indicate that the aggrecanase, MMP, and calpain protease systems are not primary mediators of proteoglycan turnover (i.e., actively cleave proteoglycans) in TGF−β1-stimulated cartilage and fibrocartilage. Rather, TGF-β1 may have suppressed catabolism

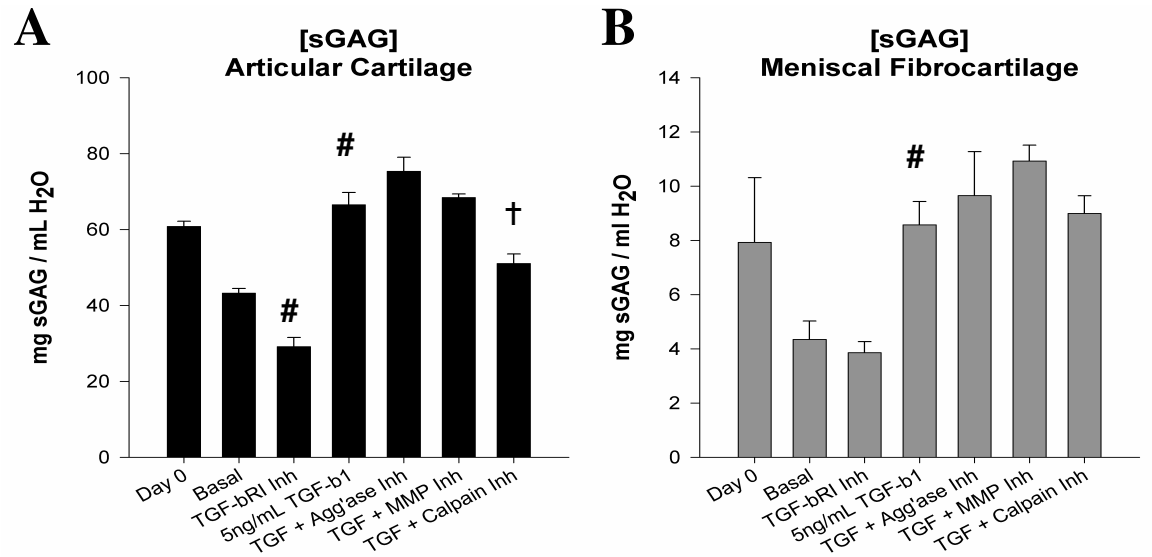

Figure 5.4 TGF-β1 stimulation promoted maintenance of proteoglycan concentration in articular cartilage (**A**) and meniscal fibrocartilage (**B**). Proteoglycan concentration was calculated from total explant sGAG content (extract + digest) and the difference between explant wet and dry weights. Note the 7-fold difference in scales between (A) and (B). Data are mean + SEM, and n=6. **#** denotes $p<0.05$ vs. basal. **†** denotes $p<0.05$ vs. TGF-β1-only controls.

under these culture conditions. It is also possible, however, that higher doses of these inhibitors would have more pronounced effects on the distribution of sGAG in these tissues.

Total explant sGAG content was normalized by explant water content as a measure of sGAG concentration and a predictor of explant mechanical properties. Cartilage explants cultured for 10 days in basal medium had a lower sGAG concentration than day 0 tissue, and addition of the TGF−βRI inhibitor led to significantly lower sGAG concentration than basal controls (Figure 5.4A). Stimulation with TGF−β1 restored sGAG concentration of cultured cartilage to that of day 0 tissue. Similarly, fibrocartilage explants cultured for 10 days in basal medium had substantially lower sGAG concentration than day 0 tissue (Figure 5.4B), consistent with the loss of total explant sGAG. In contrast to cartilage, fibrocartilage treated with the TGF−βRI inhibitor did not

show a further decrease in sGAG concentration beyond that of basal controls. As in cartilage, however, fibrocartilage stimulated with TGF−β1 had sGAG concentrations comparable to day 0 tissue.

Despite showing modest differences in absolute sGAG abundance, TGF−β1-stimulated tissue additionally treated with protease inhibitors demonstrated some differences in explant sGAG concentration from TGF−β1-only controls. In TGF−β1-stimulated articular cartilage, treatment with a calpain-selective inhibitor led to significantly lower sGAG concentration than TGF−β1-only controls. In TGF−β1-stimulated fibrocartilage explants, none of the protease inhibitor treatments led to significant differences in sGAG concentration from TGF−β1-only controls.

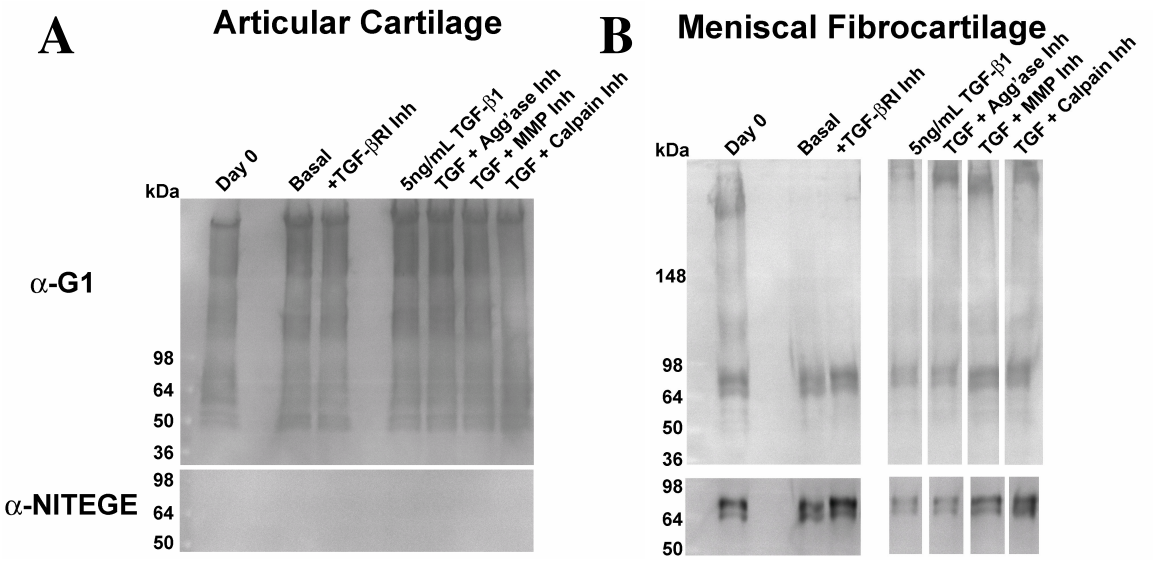

Figure 5.5 Characterization of aggrecan in fresh and cultured articular cartilage (**A**) and meniscal fibrocartilage (**B**). Explants were cultured in serum-free media for 0 or 10 days. Equal volumes of deglycosylated tissue extracts were separated by SDS-PAGE. Membranes were probed with anti-NITEGE, stripped, and reprobed with anti-G1.

Immunodetection of Proteoglycans

In order to identify aggrecan species present in cultured cartilage and fibrocartilage tissue, explant extracts were analyzed by Western blot using antibodies to domains and neoepitopes of the aggrecan core protein. Blots probed with an antibody to the G1 (HA–binding) domain show that day 0 articular cartilage explants contain an abundance of aggrecan with a wide variation in degree of processing (Figure 5.5A). Aggrecan G1 species as small as 50kDa and as large as the full length (~350kDa) were present, as well as abundant ~250kDa and ~120kDa species. After 10 days of culture in basal medium or basal medium supplemented with TGF–βRI inhibitor, the G1 profile of the explants did not change substantially. Stimulation with TGF–β1 did not dramatically alter the size distribution of G1–bearing aggrecan species in the cartilage explants, nor did addition of selective protease inhibitors. In contrast, the size of G1-positive aggrecan species in day 0 meniscal fibrocartilage extracts was more restricted to a high molecule-

weight band ~250kDa, a broad, faint 120kDa band, and a doublet at 65-70kDa. After 10 days of culture in basal medium or basal medium supplemented with TGF−βRI inhibitor, fibrocartilage explants were depleted of high molecular weight aggrecan species but retained the low molecular weight doublet. Treatment with TGF−β1 restored the presence of high-molecular weight aggrecan, but did not restore the mid-molecular weight species detected in day 0 tissue, and addition of selective protease inhibitors did not further alter the size distribution of G1 species in fibrocartilage. The 65-70kDa G1 doublet in day 0 and cultured fibrocartilage was identified as the G1-NITEGE aggrecanase-generated aggrecan fragments using an anti-NITEGE antibody, and these fragments were strikingly absent from cartilage extracts. These data highlight intrinsic differences in proteoglycan processing between articular cartilage and meniscal fibrocartilage and indicate, along with total explant sGAG data (Figure 5.2), that TGF−β1 stimulation promotes synthesis and retention of aggrecan in fibrocartilage explants.

To further characterize the proteoglycans in fresh and cultured explants, explant extracts were probed for the G3 domain of aggrecan and the small proteoglycan decorin. G3 blots of articular cartilage explants confirmed that full length aggrecan is present in fresh and cultured tissue, indicated by the high molecular weight band. In addition, a band migrating at 120kDa was observed in all tissue extracts and indicates that a subset of the aggrecan population has undergone moderate C-terminal cleavage in fresh and cultured tissue. The size of this fragment suggests that cleavage occurred at the TAQE^{1791}—^{1792}AGEG bond, a substrate for aggrecanases[200,224], but peptide sequencing or additional neoepitope immunoblots are required for positive identification. Fibrocartilage extracts exhibited a different pattern of aggrecan processing as assessed by G3 reactivity. As in articular cartilage, fresh fibrocartilage contained full-length aggrecan and a species migrating at 120kDa. After 10 days of culture in basal medium or basal

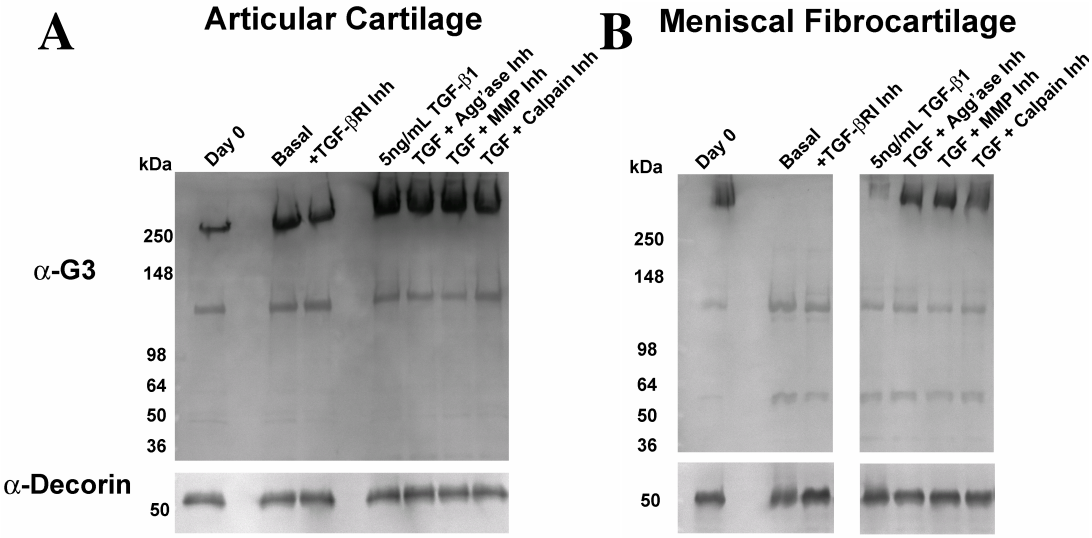

Figure 5.6 Further Characterization of proteoglycans in fresh and cultured articular cartilage (**A**) and meniscal fibrocartilage (**B**). Explants were cultured in serum-free media for 0 or 10 days. Equal volumes of deglycosylated tissue extracts were separated by SDS-PAGE. Membranes were probed with anti-G3, stripped, and reprobed with LF-94 anti-decorin. The apparent shift in migration of high molecular weight bands in lanes 1-3 of (**A**) was probably due to gel distortion during transfer to nitrocellulose.

medium with TGF−βRI inhibitor, the high molecular weight G3 species was absent and a fragment migrating at ~55kDa emerged. Stimulation with TGF−β1 restored the presence, but not the abundance, of full-length G3 positive species to the explants, and additional treatment with protease inhibitors did not substantially alter the restoration of full-length aggrecan or emergence of the low molecular weight fragment. All three protease inhibitors did appear to promote accumulation of full-length aggrecan, suggesting that aggrecanases, MMPs, and calpains are active in TGF-β1-stimulated fibrocartilage. Blots of cartilage and fibrocartilage extracts probed with the LF-94 antibody to bovine decorin indicated a single band migrating at ~55kDa. Band intensity was in similar for both tissues and all conditions, suggesting that differences in sGAG content (Figure 5.2) were not attributable to differences in decorin abundance. Collectively, these results support the concepts that aggrecan is a major proteoglycan in both cartilage and fibrocartilage and that aggrecan turnover is fundamentally different in these two tissues.

Tissue Material Properties

The effects of TGF−β-driven ECM remodeling on cartilage and fibrocartilage material properties were investigated by oscillatory torsion and oscillatory unconfined compression tests. Culture of cartilage explants for 10 days in basal medium led to a ~65% reduction in shear modulus (0.5Hz, 0.05% shear strain amplitude) from day 0 tissue, and culture in basal medium with a TGF−βRI inhibitor led to a further, significant

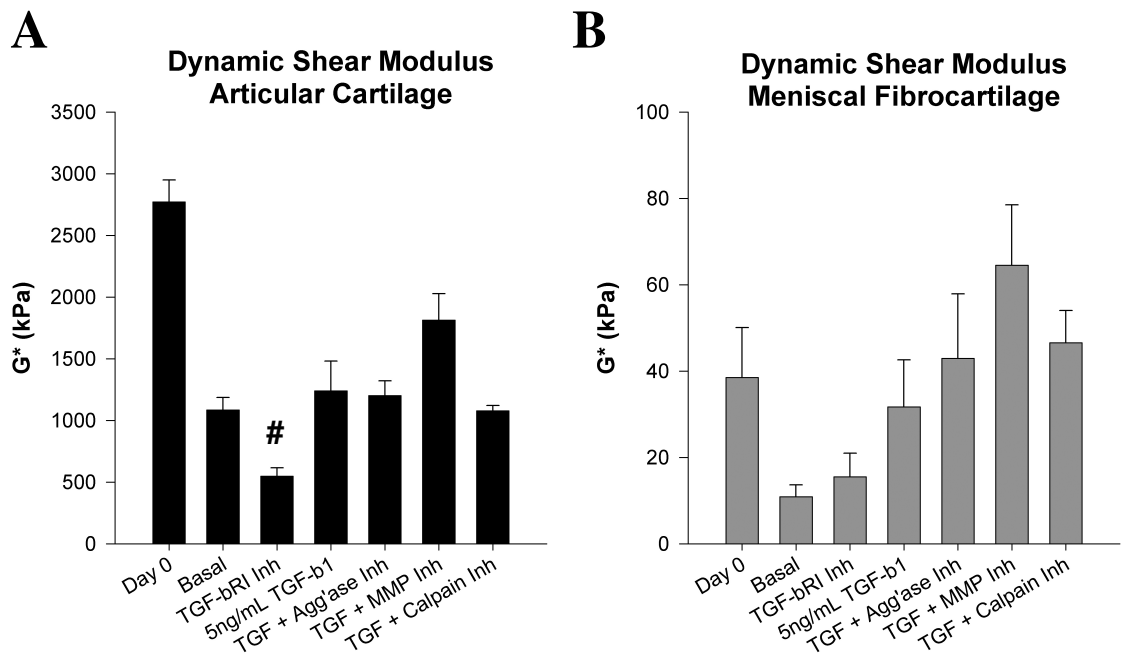

Figure 5.7 Retention of explant shear properties was enhanced by TGF-β1 stimulation in meniscal fibrocartilage (B) and by an MMP-selective inhibitor in articular cartilage (A). Dynamic shear moduli were determined by oscillatory torsion tests at 0.5Hz. Cartilage and fibrocartilage explants were tested at 0.05% and 0.25% shear amplitude, respectively. Data are mean + SEM with n=6. **#** denotes $p<0.05$ vs. basal.

decrease in shear modulus from basal controls. TGF−β1-stimulated cartilage explants exhibited similar shear properties to basal controls. Fibrocartilage explants cultured in basal medium underwent a 75% reduction in shear modulus (0.5Hz, 0.25% shear strain amplitude), but in contrast to articular cartilage, additional treatment with a TGF−βRI inhibitor did not induce further loss of this property. These results parallel explant sGAG concentration (Figure 5.4) for both tissue types, supporting the idea that explant sGAG concentration is a predictor of tissue material properties. It is important to note that TGF−β1-stimulated cartilage had sGAG concentrations similar to day 0 samples, yet exhibited substantially lower shear properties than the day 0 tissue, and in contrast, TGF−β1-stimulated fibrocartilage maintained both sGAG concentration and shear properties similar to day 0 tissue.

The roles of aggrecanases, MMPs, and calpains on TGF−β1-mediated preservation of explant shear properties were investigated through the use of selective protease inhibitors. In both cartilage and fibrocartilage, the aggrecanase and calpain inhibitors had no significant effect on tissue shear modulus but treatment with an MMP-selective inhibitor appeared to enhance retention of this property over TGF−β1-only controls, though this effect was not significant. Structural damage to the collagen network during explantation may activate latent MMPs in the ECM that denature or degrade collagen during culture. Alternatively, relief of pre-stresses in the collagen network during explantation may induce MMP secretion and activation via aberrant mechanotransduction signaling. In either case, blockade of enzymatic collagen destruction with the MMP inhibitor would feasibly have beneficial effects on maintenance of tissue shear properties.

Unconfined compression tests showed that cartilage and fibrocartilage explants cultured in basal medium for 10 days also underwent substantial loss of tissue compression properties. Cultured cartilage explants exhibited a 6-fold decrease in dynamic compression modulus that was consistent across the 0.01-1Hz test frequencies. Treatment with a TGF−βRI inhibitor led to a further and significant decrease in cartilage compression modulus. Cartilage stimulated with TGF−β1 had similar compression properties to the basal group. Fibrocartilage explants cultured in basal medium, basal medium with TGF−βRI inhibitor, or TGF−β1 all exhibited similar compression properties of approximately 33% of day 0 tissue properties. Addition of an MMP-selective inhibitor to TGF−β1-stimulated explants appeared to increase compression properties over TGF−β1-only controls for both cartilage and fibrocartilage, although

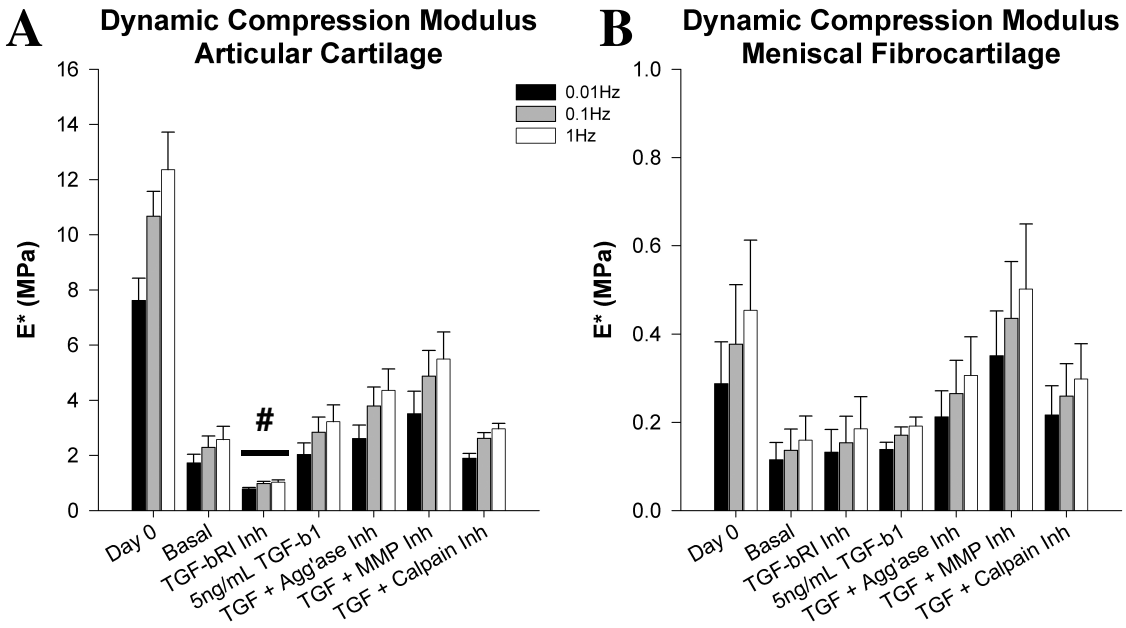

Figure 5.8 Maintenance of explant compression properties was mediated by TGF-β in cartilage and by MMPs in both cartilage (**A**) and fibrocartilage (**B**). Compression moduli were determined by oscillatory unconfined compression (1.5% compression amplitude) over the 0.01-1Hz test frequencies. Data are mean + SEM with n=6. **#** denotes $p<0.05$ vs. basal.

these increases were not statistically significant. Treatment of either tissue with an aggrecanase- or calpain-selective inhibitor yielded no significant differences in explant compression properties in comparison with TGF−β1-only controls.

Cell-Agarose Study

Construct Mitochondrial Activity and DNA Content

To further investigate mechanisms of TGF−β1-induced aggrecan deposition and remodeling in cartilage and fibrocartilage, chondrocytes and fibrochondrocytes isolated from immature bovine tissues were suspended in agarose and cultured under serum free conditions for up to16 days. Quantitative measurements of mitochondrial activity at days 0 and 16 via the WST-1 assay afforded examination of cytotoxic effects of

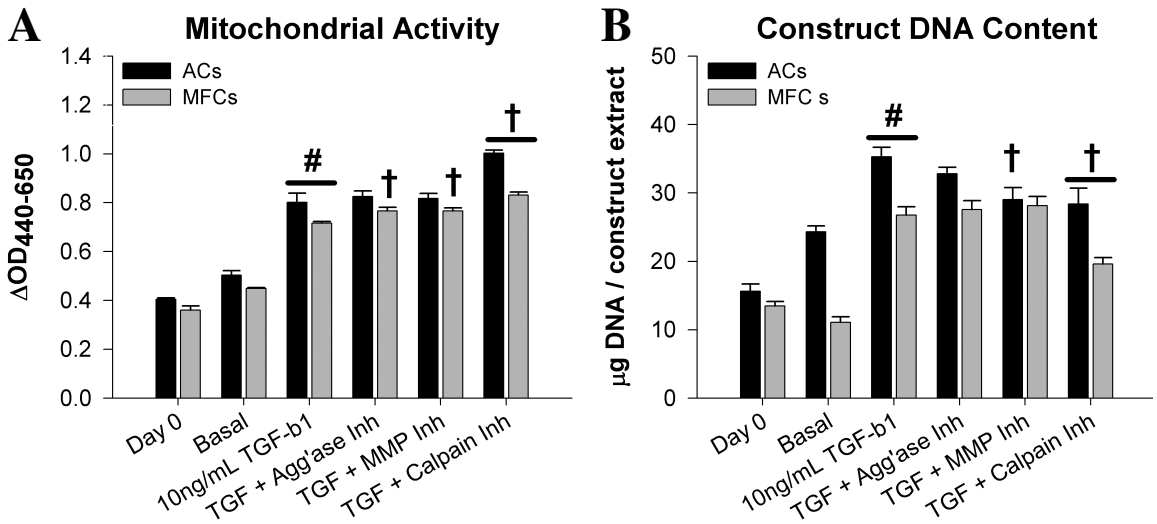

Figure 5.9 Pharmacologic protease inhibitors did not exhibit cytotoxic effects in cell-agarose constructs, as indicated by mitochondrial activity (**A**) and DNA content (**B**). Cell-agarose constructs were cultured for 0 or 16 days in serum-free media. Data are mean + SEM with n=6. **#** denotes $p<0.05$ vs. basal. **†** denotes $p<0.05$ vs. TGF-β1-only controls.

pharmacologic inhibitors and cell proliferation over the course of the experiment (Figure 5.9A). Culture for 16 days in basal or TGF–β1-supplemented media led to significant increases in both chondrocyte and fibrochondrocyte cell number over day 0 constructs. None of the inhibitors exhibited cytotoxic effects on either cell type. Rather, addition of a calpain-selective inhibitor significantly increased mitochondrial activity over TGF–β1-only controls in both cell-types. Treatment with the MMP- or aggrecanase-selective inhibitors also increased the mitochondrial activity of fibrochondrocyte-agarose constructs over TGF–β1-only controls. To examine the possibility that the per-cell mitochondrial activity was in influenced by cytokine or inhibitor treatments, the DNA content of construct extracts (Figure 5.9B) was determined by fluorometric assay. A previous report indicated that guanidine could be used for efficient, near-total extraction of DNA from cell-agarose constructs[91]. TGF-β1-stimulation of both chondrocytes and fibrochondrocytes led to higher construct DNA contents than day 0 constructs and day 16

basal controls, indicative of TGF-β1-induced proliferation and consistent with the changes in mitochondrial activity. In sharp contrast to the WST-1 data, however, the DNA contents of calpain inhibitor-treated cell-agarose constructs were significantly lower than TGF-β1-only controls for both cell types. Thus, the calpain inhibitor modifies the responses of chondrocytes and fibrochondrocytes to TGF-β1 by inhibiting proliferation and increasing the per-cell mitochondrial activity. The physiologic basis for these perturbations is unknown, but may be related to inhibition of calpain-mediated activation of latent TGF-β in the pericellular matrix[1].

These results show that these pharmacologic protease inhibitors are not cytotoxic at the dose used (1μM) and indicate that, consistent with previous reports [38,82], chondrocytes and fibrochondrocytes proliferate in response to stimulation with TGF–β1. In addition, the calpain inhibitor consistently elevated the per-cell mitochondrial activity of these cells for unknown reasons.

Construct Proteoglycan Content

Proteoglycan turnover in cell-agarose constructs was initially assessed by assaying the conditioned media for released sGAG. The cumulative sGAG released to the media was comparable between cell types and increased substantially with TGF–β1 stimulation (Figure 5.10). Cumulative sGAG release from chondrocyte-agarose constructs was significantly reduced by treatment with an aggrecanase-, MMP-, or calpain-selective inhibitor to 89%, 89%, and 78% of TGF–β1-only controls, respectively. In contrast, the aggrecanase and MMP inhibitors had no significant effects on sGAG release from fibrochondrocyte-agarose constructs, and treatment with the calpain-selective inhibitor increased sGAG release by 16%. These results indicate that

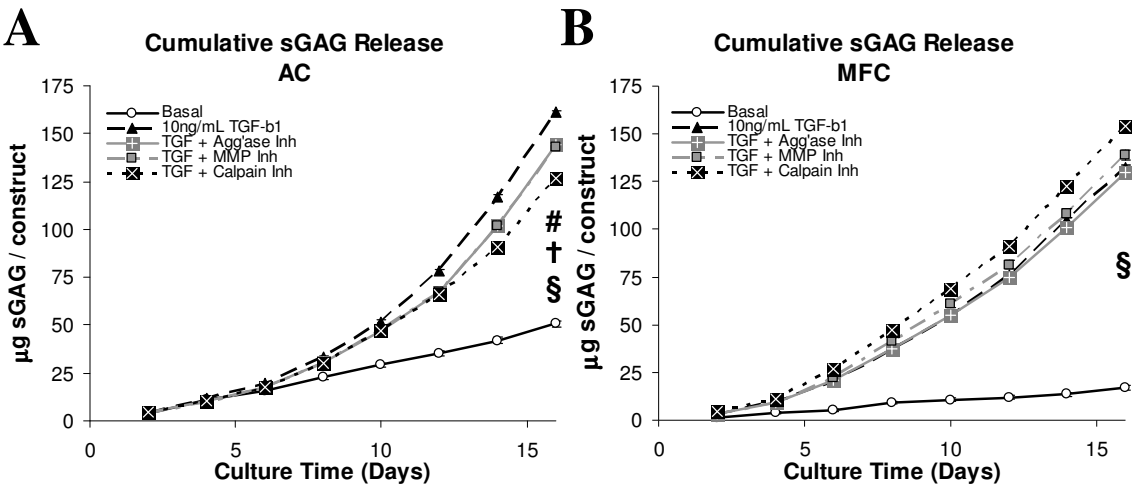

Figure 5.10 Cumulative sGAG release in articular chondrocyte (AC, **A**)- and fibrochondrocyte (MFC, **B**)-agarose constructs increased with TGF-β1 stimulation and was differentially modulated by selective protease inhibitors. Data are mean ± SEM with n=8. **#**,**†**, **§** denote $p<0.05$ between TGF-β1-only and TGF + Agg'ase Inh, TGF + MMP Inh, and TGF + Calpain Inh groups, respectively.

mechanisms of proteoglycan turnover vary by cell type.

Proteoglycan turnover in cell-agarose constructs was further characterized by the distribution of sGAG between the construct and media compartments (Figure 5.11). TGF−β1-stimulated constructs of both cell types exhibited significantly higher total construct sGAG (extract + digest) than basal controls, consistent with the expected anabolic responses to TGF−β. Additional treatment of chondrocytes with the calpain inhibitor led to significantly higher construct sGAG content than TGF−β1-only controls. Fibrochondrocyte constructs additionally treated with an aggrecanase-, MMP-, or calpain-selective inhibitor exhibited significantly higher total construct sGAG than TGF−β1-only controls. Perhaps the most striking feature of these data is that the proportion of sGAG released to the media was substantially higher for fibrochondrocyte (~50%) than chondrocyte constructs (~15%). These results add further evidence of intrinsic differences in ECM assembly and turnover between chondrocytes and meniscal

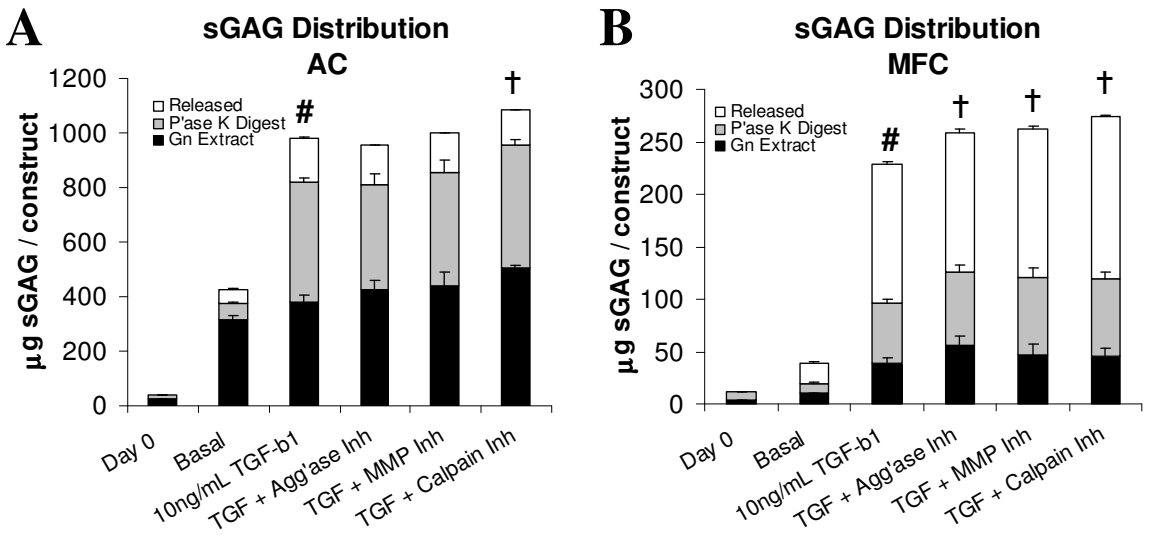

Figure 5.11 sGAG accumulation in cell-agarose constructs was dependent on cell type and can be modulated by treatment with pharmacologic protease inhibitors. Following 0 or 16 days in culture, articular chondrocyte (AC, **A**)- and fibrochondrocyte (MFC, **B**)-agarose constructs underwent extraction of intact proteoglycans in 4M guanidine and residues were digested in proteinase K and agarase. Note the 4-fold difference in scale between **A** and **B**. Data are mean + SEM, with n=6. **#** denotes significant difference ($p<0.05$) in total construct content (extract + digest) vs. basal. **†** denotes $p<0.05$ vs. TGF-β1-only controls.

fibrochondrocytes, and indicate that structural differences between cartilage and fibrocartilage are not enough to explain the tissue-specific proteoglycan distributions observed in explants.

Immunodetection of Aggrecan

Aggrecan processing in cell-agarose constructs was examined by Western blot of construct extracts. G3 (Fig 5.12, upper left panel), G2 (middle left), and G1 blots (data not shown) indicated the presence of full length aggrecan in chondrocyte-agarose constructs cultured for 16 days in basal serum-free medium. In addition, the presence of a G3-positive band migrating at 120kDa indicates that aggrecan underwent some aggrecanase-mediated C-terminal trimming in these constructs. Stimulation of chondrocytes with TGF−β1 increased the intensity of the 120kDa G3 band, and

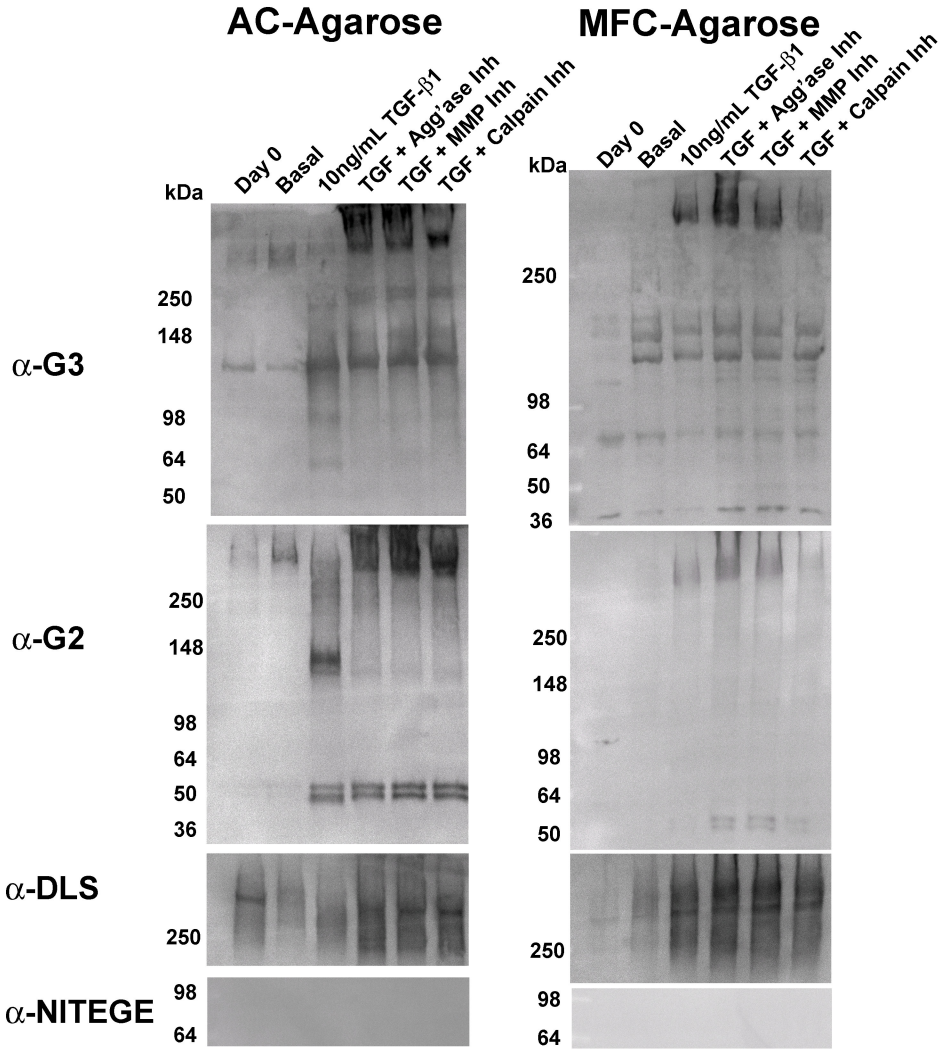

Figure 5.12 Characterization of aggrecan extracted from chondrocyte-agarose (left) and fibrochondrocyte-agarose (right) constructs. Equal volumes of extracts from day 0 or day 16 constructs were deglycosylated and separated by SDS-PAGE on 4-12% gradient gels. Anti-G2 blots are reblots of membranes initially probed with anti-DLS.

substantially reduced intensity of the high molecular weight band observed in basal constructs, suggesting that TGF−β1 stimulation promoted aggrecan processing. Aggrecan G2 blots confirmed this finding, showing a broad, faint band migrating at ≥250kDa, an intense band at ~140kDa, and a doublet at 50-55kDa in TGF−β1-stimulated samples. The ~140kDa band was later identified as an m-calpain-generated aggrecan fragment bearing the GVA^{791} C-terminal neoepitope (not shown). Fibrochondrocyte-agarose constructs demonstrated different patterns of G3- and G2-positive aggrecan species. In contrast to the chondrocyte-agarose constructs, fibrochondrocyte-agarose constructs cultured in basal serum-free medium contained very little full length aggrecan as indicated by weak staining in the high molecular weight regions of G3 (Figure 5.12 upper right), G2 (Figure 5.12 middle right), and G1 (data not shown) blots. Consistent with enhanced C-terminal trimming in fibrochondrocyte constructs, G3 blots also revealed the presence of several aggrecan fragments, including a 120kDa band similar to that observed in chondrocyte constructs and bands at 140kDa, 65kDa, and 40kDa. The 140kDa fragment may also be aggrecanase-generated [224]. Fibrochondrocytes stimulated with TGF−β1 accumulated high molecular weight aggrecan species positive for the G1, G2, and G3 domains, but G3 blots showed that variable C-terminal trimming persisted in TGF−β1-stimulated constructs. More specifically, G3-reactive bands migrating at ~40kDa, 70kDa, and 120kDa were present in fibrochondrocyte constructs and absent in chondrocyte constructs. Surprisingly, the aggrecan G1-NITEGE product was not detected in extracts of fibrochondrocyte cultures (Figure 5.12, bottom panels) as it was in tissue extracts. This suggests that agarose culture inhibits or prevents the aggrecanase-mediated IGD cleavage characteristic of fibrochondrocytes in their native ECM. These results show that aggrecan

is processed in different ways by chondrocytes and fibrochondrocytes under the basal conditions used in this study, and demonstrate that TGF–β1-induced changes in aggrecan processing are cell-type specific. In addition, agarose culture does not appear to support some mechanisms of fibrochondrocyte-mediated aggrecan turnover detected in native tissue.

Western blots of constructs extracts for the DLS neoepitope also revealed differences in aggrecan processing between cell type and with culture condition (Figure 5.12 lower panels). Day 0 chondrocyte-agarose constructs contained DLS-reactive material migrating at ~250kDa, suggesting that this product is intracellular or tightly bound in the pericellular compartment. Culture in basal medium for 16 days appeared to reduce the abundance of this fragment, and TGF–β1 stimulation yielded a similar result to basal constructs. In contrast, meniscal fibrochondrocyte constructs exhibited no DLS reactivity at day 0 but accumulated this product upon culture for 16 days in basal medium or with TGF–β1-stimulation (Figure 5.12, lower right). These results indicate that the abundance and accumulation of calpain-generated aggrecan fragments in cell-agarose constructs was cell-type dependent.

Treatment with selective pharmacologic protease inhibitors perturbed TGF–β1-driven aggrecan processing in both chondrocyte- and fibrochondrocyte-agarose constructs. In chondrocyte-agarose constructs, treatment with any of the protease inhibitors enhanced retention of high-molecular weight G1 (data not shown), G2, and G3 reactive material (Figure 5.12, upper and middle left, lanes 4-6), and these effects were similar in fibrochondrocyte-agarose constructs (Figure 5.12, upper and middle right, lanes 4-6). In addition, accumulation of DLS-reactive aggrecan species was also

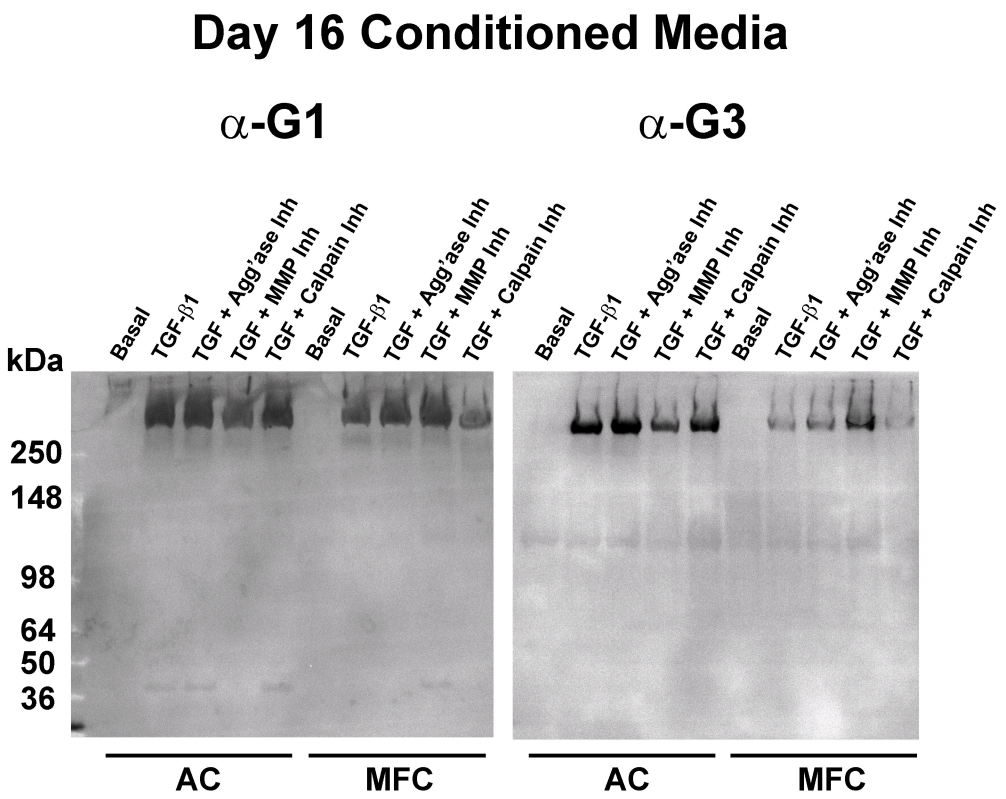

Figure 5.13 Characterization of aggrecan released to media from cell-agarose constructs. Equal volumes of conditioned media from day 16 of the experiment were probed with antibodies to the G1 (left) and G3 (right) domains of the aggrecan core molecule. Chondrocyte- and fibrochondrocyte-agarose samples were loaded in lanes 1-5 and 6-10, respectively.

increased with inhibitor treatments for both cell types. Consistent with the sGAG accumulation data in Figure 5.11, these results show that inhibition of aggrecanases, MMPs, or the calpains enhanced retention of full length and other high molecular weight aggrecan species. Most strikingly, these inhibitor treatments appeared to reduce TGF−β1-induced aggrecan processing in articular chondrocyte-agarose constructs.

Aggrecan species released from cell-agarose constructs were characterized by G1 and G3 immunoblots of conditioned media from the final 48h of the experiment. TGF−β1-stimulated chondrocyte and fibrochondrocyte constructs released high molecular weight G1 and G3 reactive species, whereas media from basal cultures showed

no reactive material (Figure 5.13). G3 blots from media conditioned by either cell type exhibited a weak band at 120kDa, indicating that these fragments of C-terminally trimmed aggrecan freely diffuse to the media compartment. Interestingly, other mid- and low-molecular weight G3-reactive species identified in construct extracts (Figure 5.12 upper panels) were not detected in day 16 media. The aggrecan G3 domain contains lectin domains known to associate with tenascin-C, a glycoprotein found in developing cartilage. Perhaps *de novo* ECM assembly by chondrocytes and fibrochondrocytes involves some aspects of development, including tenascin-C deposition, and subsequent retention of G3 fragments. Treatment with pharmacologic protease inhibitors had no dramatic effects on the size of aggrecan species released to media from constructs of either cell type.

Construct Material Properties

The functional implications of proteoglycan accumulation and aggrecan processing in cell-agarose constructs were examined by testing the constructs in oscillatory unconfined compression. Constructs cultured in basal medium for 16 days exhibited higher dynamic compression moduli and a lower tangent of the phase angle [tan δ, defined as the ratio of viscous (loss) modulus to elastic (storage) modulus] than day 0 constructs (Figure 5.14), indicating that the encapsulated cells assembled a load–bearing extracellular matrix. TGF–β1-stimulation significantly increased the dynamic compression moduli of chondrocyte and fibrochondrocyte constructs above basal controls by 89% and 7%, respectively, and also decreased tan δ below basal controls for both cell types. Consistent with the increase in construct sGAG content (Figure 5.11), treatment with a calpain-selective inhibitor significantly increased the

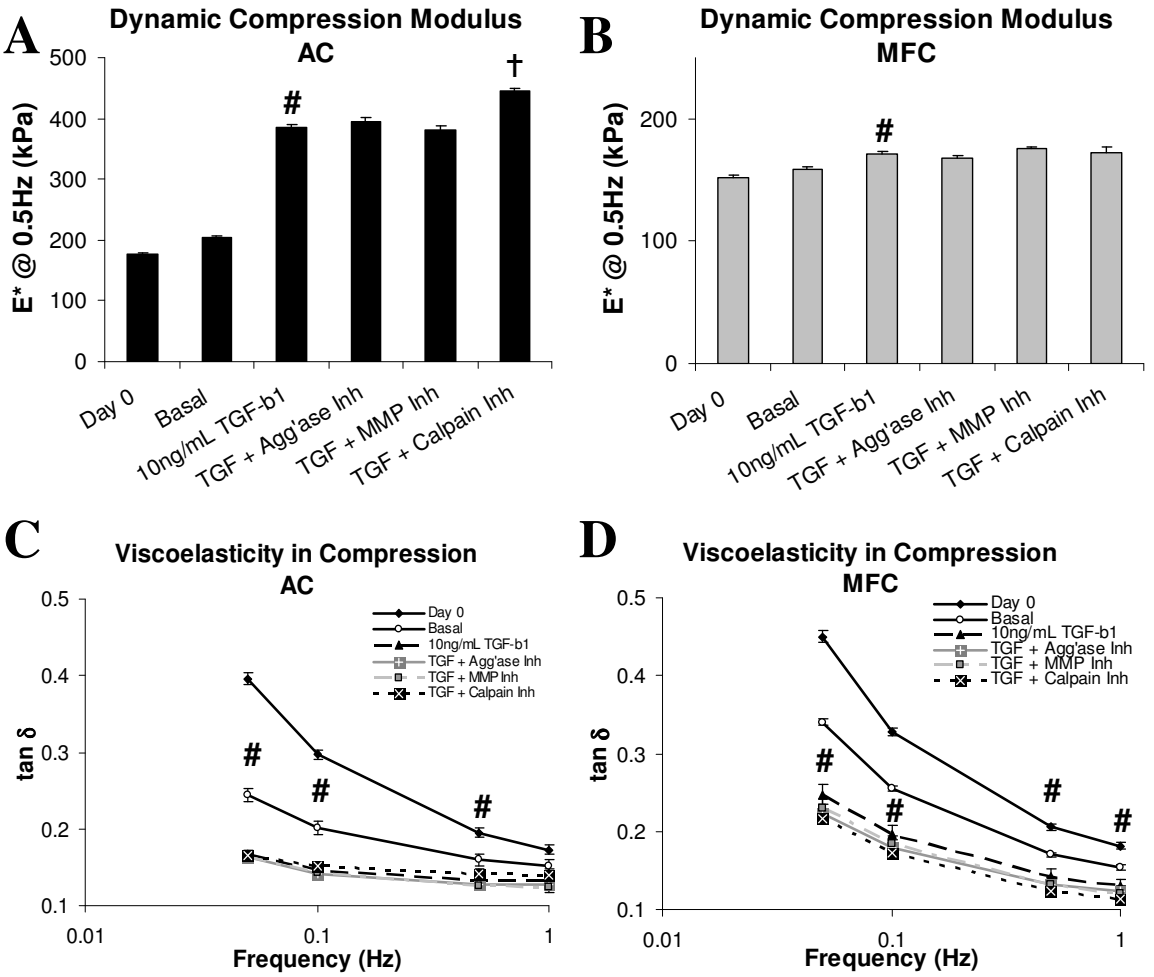

Figure 5.14 TGF-β1-stimulation increases material properties of cell-agarose constructs. The dynamic compression moduli (0.5Hz, **A**,**B**) and tangents of the phase angles, (0.05-1Hz, tan δ) (**C**,**D**) of chondrocyte-(**A**,**C**) and fibrochondrocyte-seeded constructs (**B**,**D**) were measured after 0 or 16 days of culture. Note the 2-fold difference in scale between (**A**) and (**B**). Data are mean ± SEM, with n=6. **#** denotes significant difference $p<0.05$ between TGF-β1-stimulated and basal. † denotes $p<0.05$ vs. TGF-β1-only controls.

dynamic compression modulus of TGF–β1-stimulated chondrocyte constructs above TGF–β1-only controls (Figure 5.14A). Inhibitor-treated fibrochondrocyte constructs had compression moduli similar to TGF–β1-only controls, and the aggrecanase- and MMP-selective inhibitors had no effects on TGF–β1-stimulated chondrocyte constructs. Taken together, these results indicate that TGF–β1 stimulates chondrocytes and fibrochondrocytes to assemble a loading-bearing extracellular matrix and suggest that

calpains participate in this response in chondrocytes.

5.4 Discussion

The current study was designed to identify mechanisms of aggrecan turnover in articular cartilage and meniscal fibrocartilage and to characterize tissue-level changes in ECM function (*i.e.*, material properties) under anabolic conditions. *In vitro* culture of tissue explants and cell-agarose constructs were used to evaluate TGF-β-induced aggrecan turnover and *de novo* ECM assembly, respectively, and pharmacologic protease inhibitors were used to identify active proteases. Describing the tissue-dependent differences in aggrecan processing between meniscal fibrocartilage and articular cartilage may be useful in identifying tissue-specific therapeutic targets for the treatment of arthritis and engineering grafts with tissue-specific aggrecan compositions for joint repair.

TGF−β signaling promotes aggrecan turnover in both cartilage and fibrocartilage explants in tissue-specific ways. Cartilage treated with a TGF−βRI inhibitor (in basal medium, without exogenous TGF-β) had lower proteoglycan content and material properties than basal controls, suggesting that endogenous anabolic TGF−β signaling was active in this tissue. Fibrocartilage exhibited apparently low endogenous TGF-β signaling activity, as explants treated with the TGF−βRI inhibitor had similar proteoglycan content and material properties to untreated basal controls. There may be more TGF-β available for endogenous TGF-β-signaling in the cartilage ECM than in fibrocartilage. Inhibitor treatment significantly reduced the mitochondrial activity of fibrocartilage explants below basal controls, however, suggesting that some endogenous TGF−β signaling

provided a survival or proliferative signal for fibrochondrocytes. Although the inhibitor may be specifically cytotoxic to fibrochondrocytes at this dose, several cell types have tolerated this inhibitor at doses up to 30μM[90,99]. Cartilage explants stimulated with exogenous TGF−β1 swelled significantly less than basal controls and exhibited proteoglycan synthesis similar to basal. Indeed, TGF−β1-stimulated maintenance of proteoglycan concentration was due primarily to maintenance of tissue water content. Cartilage swelling is highly dependent on the collagen network, and TGF−β1-stimulation may have induced anti-catabolic activity (e.g., downregulated MMP expression or upregulated TIMP expression) that reduced denaturation or degradation of the collagen. In contrast to cartilage and consistent with previous reports[38], fibrocartilage explants responded to exogenous TGF−β1 with significantly higher proteoglycan synthesis than basal controls, and cytokine treatment had no effect on tissue swelling. It is interesting to note that both tissues responded to exogenous TGF−β1 by maintaining proteoglycan *concentrations*, rather than absolute abundance, comparable to day 0 tissues. This suggests a point of convergence in two otherwise disparate mechanisms of TGF−β1-induced aggrecan turnover. Alternatively, this may have simply been an artifact of the tissue explant culture system and changes in swelling related to the collagen network.

TGF−β1 stimulation promotes maintenance of fibrocartilage shear properties, but is not sufficient to prevent loss of cartilage or fibrocartilage compression properties. Fibrocartilage cultured for 10 days in the presence of TGF−β1 had shear moduli comparable to day 0, whereas identically treated cartilage explants retained <50% of the day 0 moduli. Both tissues underwent substantial loss (~67%) of compression moduli after 10 days in culture, suggesting that the abundance and/or integrity of non-

proteoglycan ECM constituents were altered during the experiment. Changes in the collagen network were not characterized in this study, but likely contributed substantially to the culture-induced loss of compression and shear properties. Indeed, TGF–β1-stimulated explants (of both tissue types) treated with an MMP-selective inhibitor exhibited the greatest retention of compression and shear moduli. Thus, MMP-mediated collagen degradation may have precluded complete preservation of tissue function. Preparation of the explants involved separation from surrounding structures that would support the maintenance and development of tissue material properties. Thus, the contributions of non-proteoglycan constituents and the tissue culture model to changes in tissue material properties cannot be completely decoupled from the effects of aggrecan turnover in this study.

MMPs regulate TGF–β1-stimulated ECM remodeling in both cartilage and fibrocartilage. Treatment of TGF–β1-stimulated cartilage with a potent MMP-selective inhibitor led to significantly lower mitochondrial activity than TGF–β1-only controls (but not lower than basal cultures), suggesting that MMPs regulate survival or proliferation signals in cartilage. MMP-3 can activate latent TGF–β1 in the cartilage ECM[131,132], and treatment with an MMP inhibitor would thus be expected to interfere with the activity of endogenous TGF–β1 and progression of autocrine TGF–β signaling[72,186]. The significantly lower sGAG content of MMP-inhibitor-treated tissue may be the indirect result of reducing TGF–β-stimulated cell survival or proliferation. MMPs also regulated tissue swelling in cartilage explants, as MMP-inhibitor treated cartilage had the lowest wet weight and water fraction of all culture conditions. In contrast to cartilage, the MMP inhibitor had no effect on fibrocartilage viability. TGF–β1-stimulated fibrocartilage

treated with the MMP-inhibitor had higher sGAG content and concentration and a lower fraction of sGAG released to the media than TGF−β1-only controls, which suggests that MMP activity potentiated the TGF−β1-induced increase in proteoglycan synthesis and contributed to loss of sGAG from the fibrocartilage ECM. It is unclear how MMPs might potentiate the TGF−β1 stimulus, but MMPs may mediate sGAG release by cleaving link protein and interfering with proteoglycan aggregation. Despite the disparate tissue-dependent effects of MMP inhibition on explant viability and proteoglycan synthesis, the MMP inhibitor treatments revealed another point of convergence in ECM remodeling: MMP inhibition with TGF−β1-stimulation conferred the greatest preservation of explant material properties. Thus, MMPs are likely participants not only in proteolytic disruption of each tissue's collagen network, but also as regulators of the TGF−β signaling axis and retention of sGAG in articular cartilage and meniscal fibrocartilage.

The balance of proteoglycan synthesis and processing in meniscal fibrochondrocytes is different from that of articular chondrocytes. TGF−β1-stimulated explants and constructs derived from both tissues had increased proteoglycan biosynthetic activity than basal controls. However, the rate of proteoglycan synthesis in fibrochondrocytes (as indicated by accumulation of sGAG in the cultures) was approximately 10% of that in articular chondrocytes. This result is consistent with previous reports and work in our lab using $^{35}SO_4$ incorporation as an index of proteoglycan synthesis [38,97,101,231]. Interestingly, proteoglycans synthesized by fibrochondrocytes were less likely to be retained within the explant or construct than were proteoglycans synthesized by chondrocytes. Over 10 days, approximately 14-34% (depending on culture condition) of the total sGAG in fibrocartilage cultures was found

in the media, whereas analogous cartilage cultures released 7-11%. Differences between the collagen networks of these tissues might explain differences in sGAG retention, as proteoglycans may diffuse more readily along the more oriented collagen fibrils in fibrocartilage. However, cell-agarose constructs with immature collagen networks exhibited similar differences in sGAG retention: fibrochondrocyte constructs released 48-58% of the total sGAG when chondrocyte constructs only released 11-17%. Differences in the abundance, molecular weight, and/or localization of hyaluronan in fibrochondrocyte cultures may preclude retention of newly synthesized proteoglycan. Indeed, fibrocartilage is known to have a restricted localization of hyaluronan, often appearing exclusively between collagen fibrils and lamellar structures[98,235]. For the same anabolic stimulus, fibrochondrocytes have lower proteoglycan synthesis and retain a lower fraction of newly synthesized proteoglycans than articular chondrocytes.

Fibrochondrocytes process aggrecan more extensively than chondrocytes. Despite the anabolic culture conditions used in this study, fibrocartilage explants exhibited proteolysis of the aggrecan IGD, indicated by the presence of G1-NITEGE, and G3 blots of explant and construct extracts showed additional sites of C-terminal aggrecan trimming by fibrochondrocytes that were not detected in chondrocytes cultures. Cell-type differences in aggrecan catabolism appear to be intrinsic, since they were observed in day 0 tissues, basal cultures, and TGF–β1-stimulated cultures. Fibrochondrocyte cultured in agarose did not exhibit aggrecanase-mediated IGD cleavage, as indicated by the lack of aggrecan G1-NITEGE in the extracts (Figure 5.12, bottom right panel) and BC-3-reactive material in the conditioned media (not shown). These results suggest that suspending these cells in agarose reduces their catabolic activity. Proteoglycans from the inner region

of immature menisci contain substantially less aggrecan G1-NITEGE than material extracted from the outer region, and cells from the inner meniscus are more phenotypically similar to articular chondrocytes (*e.g.*, exhibit rounded morphology and express more aggrecan and collagen type II)[88,156,204,221]. Thus, agarose suspension may induce fibrochondrocytes to exhibit a less aggrecan-destructive phenotype characteristic of cells isolated from the inner meniscus, and there is no doubt that native ECM is a potent regulator of cell metabolism as well. Regardless of culture system, however, fibrochondrocytes consistently demonstrated more extensive aggrecan catabolism (evidenced by IGD cleavage and/or more extensive C-terminal trimming) than articular chondrocytes and these differences in aggrecan processing may explain the propensity for fibrochondrocytes to release a larger fraction of sGAG to the media.

The calpain protease system regulates the response of fibrochondrocytes and chondrocytes to TGF–β1. Calpain is a primarily cytosolic protease that regulates a variety of intracellular signaling events [144,202]. In addition, m-calpain (calpain-2, active in the presence of mM-range Ca^{++} concentrations) is secreted to the extracellular space in osteoarthritic tissues[66,213,217,218,248] and efficiently cleaves aggrecan[164,214]. Antibodies to the calpain-generated neoepitopes (bearing the C-terminal sequence $DLS^{\mathbf{12xx\text{-}13xx}}$) were used to identify calpain-modified aggrecan in these studies. Abundant DLS reactive material was detected in cartilage and fibrocartilage explants (data not shown), as well as in chondrocyte and fibrochondrocyte-agarose construct extracts. In the explant study, calpain-inhibitor-treated tissue was generally similar to TGF–β1-only controls, although cartilage explants treated with the calpain inhibitor had higher wet weights ($p<0.05$) and water fractions ($p<0.1$) than TGF–β1-only controls. Thus, calpain regulated cartilage

swelling, but the mechanism is unclear and does not appear to be due to differences in the population of aggrecan species. In cell-agarose constructs, calpain inhibitor treatment led to significantly higher mitochondrial activity and sGAG content than TGF–β1-only controls for both fibrochondrocytes and chondrocytes. Calpain activity also had different effects on proteoglycan release and construct material properties in fibrochondrocyte and chondrocyte cultures. Whereas calpain inhibitor-treated chondrocyte constructs had significantly less sGAG release and higher compression moduli than TGF–β1-only controls, calpain inhibitor-treated fibrochondrocyte constructs exhibited significantly higher sGAG release than controls and no differences in construct material properties. These differences were not readily explained by differences in aggrecan processing; even the generation of DLS-reactive material in conditioned media (data not shown) and construct extracts was unchanged with calpain inhibitor-treatment. Rather, calpain appears to regulate proteoglycan turnover by an upstream mechanism, perhaps related to cell metabolism and proliferation, as evidenced by increased mitochondrial activity and decreased DNA content in constructs treated with the calpain inhibitor. Calpain can regulate cell proliferation[6], and the cell adhesion molecules focal adhesion kinase and talin are substrates for calpain[18,64]. It is feasible that the calpain inhibitor perturbed basic cell functions such as division, migration, or adhesion, and these perturbations led to differences in cell metabolism. Further work, perhaps with inhibitors that can selectively target calpain's aggrecanolytic activity, will be necessary to elucidate the functional implications of calpain-mediated aggrecan processing in cartilage and fibrocartilage.

Collectively, the results of these experiments indicate that fibrochondrocytes exhibit a pattern of anabolic aggrecan turnover that is distinguished from chondrocytes by

lower overall synthesis rates and more extensive processing. The material properties of TGF−β1-stimulation generally promoted maintenance of tissue-specific aggrecan populations for up to 10 days in tissue explants and 16 days in cell-agarose constructs, and perturbation of TGF−β1-driven remodeling with selective protease inhibitors resulted in differences in tissue-level material properties for both tissue types. Injury- or age-related divergence from tissue-specific ECM remodeling pathways may be an early event in onset of cartilage or fibrocartilage degeneration.

CHAPTER 6

CONCLUSIONS

6.1 Summary

Arthritis refers to several conditions causing joint pain and degeneration and is the nation's leading cause of disability. As the population ages, the number of arthritis diagnoses is expected to increase to nearly 65 million patients by the year 2030. The pathogenesis of arthritis is largely unknown, but advanced age and joint injury are highly prognostic for the onset of osteoarthritis. Traumatic injury triggers an aggressive inflammatory response and disrupts normal joint biomechanics, and these factors potentiate each other as joint degeneration progresses. Increasing evidence suggests that arthritis with advanced age, in contrast, is due to more subtle processes in which joint tissues undergo degenerative shifts in material properties. The cell-mediated mechanisms of ECM remodeling underlying injury- and age-related degeneration are not well understood, but aggrecan, a large aggregating proteoglycan abundant in knee joint tissues, may be one of the earliest targets of cell-mediated ECM degradation in the degenerative joint. The work in this dissertation investigated mechanisms by which cells of articular cartilage and meniscal fibrocartilage process aggrecan and the effects of aberrant aggrecan turnover on tissue material properties.

The studies presented in Chapter 3 describe the kinetics, mechanisms, and functional implications of IL-1-stimulated articular cartilage destruction. In this model of aggressive degradation, cartilage explants are completely resorbed over a period of weeks, whereas *in vivo* this process takes years to decades. Aggrecanase-selective, MMP-selective, and broad spectrum metalloproteinase inhibitors demonstrated differential

perturbation of IL-1-induced ECM destruction. Consistent with previous reports[73,134,175], proteoglycans were released first from the tissue by aggrecanases and subsequent degradation of the collagen network was MMP-mediated. The aggrecanase inhibitor treatment also inhibited collagen degradation, and this finding supports the hypothesis that aggrecan protects collagen from proteolytic attack in articular cartilage[175]. Hence, the function of aggrecan in cartilage is more than just mechanical, and clinical interventions that maintain intact aggrecan within articular cartilage may have the additional benefit of preventing collagen degradation. Indeed, the pharmacologic inhibitors used in this work were designed for therapeutic use with that rationale. Metalloproteinase inhibitors, however, ultimately failed to prevent proteoglycan depletion and loss of tissue function. Conditioned media from aggrecanase inhibitor-treated cultures contained aggrecanase-generated aggrecan at later time points (not shown) and immunostaining showed that intra- or pericellular aggrecanase activity persisted with aggrecanase inhibitor treatment. Thus, aggrecanases were present in stoichiometric excess of the inhibitor, other aggrecanases not inhibited by the inhibitors were active, or aggrecanases were somehow sequestered from exposure to the inhibitors. ADAMTS-9 is a candidate molecule that may be protected from exposure to the inhibitors, since it does not readily diffuse through the ECM. This aggrecanase is localized to the cell-surface and upregulated at the mRNA and protein levels by IL-1 in chondrocytes[45,95] These studies demonstrated that articular cartilage responds to IL-1 with high levels of aggrecanase activity which ultimately led to exhaustive proteoglycan depletion and loss of tissue compression and shear properties.

Chapter 4 summarizes studies of aggrecan catabolism and IL-1-induced ECM degradation in meniscal fibrocartilage. Aggrecanase-mediated cleavage of the aggrecan

IGD activity, typical of IL-1-stimulated articular cartilage, was detected in freshly isolated tissue from the middle and outer regions of the menisci. IL-1 stimulation triggered formation of the NITEGE neoepitope in the inner and middle regions. NITEGE positive material was consistently localized along radial fibers and along and within circumferential collagen fibers in the meniscus, indicating that aggrecan is present and processed in these compartments of the fibrocartilage ECM. IL-1-stimulated fibrocartilage exhibited aggrecanase-mediated sGAG release and, in contrast to articular cartilage, MMP-mediated sGAG release. Aggrecanases triggered the initial "burst" of sGAG release, whereas MMP activity later in the experiment maintained a lower, persistent rate of sGAG release. Whereas the bulk of IL-1-induced aggrecan catabolism occurs within the first 2-4 days, treatment with an aggrecanase-selective inhibitor prolonged release of NITEGE-positive fragments through days 6 or 8 of the experiment, when collagen degradation began. Treatment with the MMP-inhibitor promoted accumulation of NITEGE-positive aggrecan in the tissue and reduced release of DLS-positive aggrecan, perhaps by completely blocking collagen degradation. In addition, collagen degradation in IL-1-stimulated fibrocartilage began prior to exhaustive proteoglycan depletion. These results indicated that disruption of the collagen network is required for release of a substantial fraction of the tissue sGAG. In this way, notably opposite from articular cartilage, the collagen network protected the aggrecan in fibrocartilage (from release, but not necessarily from proteolytic attack). Treatment with the aggrecanase inhibitor did not influence the extent or kinetics of collagen degradation as it did in IL-1-stimulated articular cartilage, which suggests that the abundance and/or molecular species of aggrecan in the fibrocartilage matrix are insufficient to protect

collagen from proteolytic attack. Although aggrecanase-selective and MMP-selective inhibitor treatments did not significantly reduce IL-1-induced loss of material properties, treatment with a broad spectrum inhibitor of both aggrecanases and MMPs completely blocked degradation. Broad spectrum inhibitor-treated samples had compression and shear properties similar to day 0 tissue after 12 days of stimulation with IL-1. It is clear from these results that proteoglycan, putatively aggrecan, in vulnerable and collagen-protected compartments contributes to the mechanical competence of meniscal fibrocartilage. In addition, these studies revealed mechanisms of normal and pathologic ECM remodeling that distinguish fibrocartilage from articular cartilage.

The complementary set of experiments in Chapter 5 explored the role of aggrecan turnover under anabolic conditions. Based on the literature[198] and results of earlier experiments, the aggrecan population in native meniscal fibrocartilage appeared to be more extensively processed than that of articular cartilage and these experiments aimed to resolve the functional consequences, and potential benefits, of such processing. Oscillatory mechanical testing of fresh tissue explants showed that cartilage had substantially higher shear (~50x) and compression (~25x) moduli than fibrocartilage, and biochemical analysis of these tissues showed that cartilage has a 10-fold higher concentration of sGAG than fibrocartilage. Western analysis of tissue extracts revealed similarities and differences between the native aggrecan populations of cartilage and fibrocartilage. High molecular weight G3-bearing aggrecan (presumably full length) and 250kDa DLS-bearing aggrecan were present in both tissues. As described in Chapter 4, however, G1-NITEGE fragments were localized exclusively in fibrocartilage. Explants and cell-agarose constructs served as models of aggrecan turnover and *de novo* matrix

assembly, respectively. In the presence of TGF-β1, cartilage and suspended chondrocytes maintained and assembled a matrix rich in full length aggrecan. TGF-β1-stimulation promoted retention of initial value material properties and swelling characteristics in cartilage explants over 10 days in culture, with minimal release of sGAG to the medium. TGF-β1 stimulation also promoted maintenance of tissue material properties, sGAG content, and fibrocartilage-specific aggrecan species (*e.g.*, G1-NITEGE) in fibrocartilage. TGF-β1-stimulated fibrochondrocytes in both explants and agarose gel cultures retained a lower fraction of sGAG in the ECM than chondrocytes, suggesting that there are fundamental differences in the ways these cells assemble and deposit proteoglycans. Perturbation of TGF-β1-driven ECM turnover with pharmacologic protease inhibitors had no dramatic effects on aggrecan processing. Rather, these inhibitors appeared to have more pronounced effects on other aspects of cell metabolism and ECM remodeling. Treatment with the calpain inhibitor consistently enhanced mitochondrial activity and MMP inhibitor-treated cartilage demonstrated less tissue swelling and proteoglycan production than TGF-β1-only controls. Although TGF-β1 has been shown to upregulate ADAMTS-4 expression at the mRNA and protein levels in chondrocytes[95,151], supplementation of TGF-β1-stimulated cultures (of both tissue and cell types) with a potent and selective aggrecanase inhibitor had only subtle effects on aggrecan processing and construct material properties. Chondrocyte-agarose constructs cultured for 16 days had substantially higher moduli and a lower tendency to dissipate stress (evidenced by lower tan δ) than day 0 cell-agarose constructs, whereas fibrochondrocyte constructs assembled a matrix with only a minimal increase in construct stiffness. Thus, these two cell types exhibit distinct biosynthetic and catabolic activities with respect to the

aggrecan component of their ECMs, and the material properties of tissue explants and cell-agarose constructs are related to the tissue-specific ECM remodeling activities.

6.2 Conclusions

The work in this dissertation shows that chondrocytes and fibrochondrocytes exhibit distinct kinetics and mechanisms of aggrecan turnover in response to catabolic and anabolic stimuli. Correlation studies[181,244] and microstructural models[29,48] have previously indicated that soft tissue compression properties increase with aggrecan concentration, and mechanistic descriptions of this relationship cite the electrochemical properties of the sGAG chains attached to aggrecan[80]. Cartilage is loaded primarily in compression and the abundance of aggrecan is substantially higher in cartilage than fibrocartilage, and regions of the menisci that undergo primarily compressive loading (*e.g.*, the inner region) were richer in sGAG and exhibited higher rates of proteoglycan production[38,101,221]. The middle and outer regions of the menisci, which bear higher tensile loads, contain extensively processed aggrecan, low sGAG contents, and low rates of aggrecan expression and production[38,229,230]. Similar relationships between sGAG abundance and loading environment have been observed in tendon and even the developing heart valve[75,236,252]. Thus, production and accumulation of sGAG-bearing aggrecan appears to be a conserved mechanism in soft tissues exposed to compressive loads.

Aggrecanase-mediated cleavage of the aggrecan IGD is normal in developing meniscal fibrocartilage. Lower quantities of sGAG in the middle and outer regions of calf fibrocartilage coincide with increased abundance of aggrecan G1-NITEGE, and such extensive aggrecan catabolism may be necessary for the proper assembly and

organization of large collagen fibers in these regions. It remains unclear what signal(s) regulate this spatial variation in aggrecan processing, although region-specific mechanical signals may be predominant regulators of aggrecan anabolism and catabolism. In this scenario, sGAG is localized to regions of compressive loading through elevated proteoglycan synthesis rates and suppressed protease expression. In regions where the ECM undergoes less compression and more tensile loading, sGAG accumulation is reduced by lower rates of aggrecan production and increased aggrecan degradation. There is precedent for such a mechanism in tendon[51,75]. Age-related increases in compressive loading and sGAG content of the middle and outer regions might be due to increases in animal weight and/or activity. The proximity of fibrochondrocytes to a vascular supply may also regulate aggrecan turnover, as the peripheral one third of the menisci is vascularized, whereas the inner one third is avascular and sustained by the synovial fluid. Thus, regional variations in access to soluble factors such as cytokines, growth factors, and oxygen in the blood may be an important regulator of aggrecan turnover in the meniscus.

IL-1 stimulation induces cell-mediated ECM degradation in cartilage and fibrocartilage explants. IL-1-stimulated chondrocytes and fibrochondrocytes use similar downstream signaling effectors, including nitric oxide and prostaglandin E2, in the downregulation of proteoglycan production and upregulation of protease expression[87,124,160,207]. In addition, IL-1 triggers aggrecanase and MMP activity in both cartilage and fibrocartilage. IL-1 disrupts ECM homeostasis in arthritic joints and elicits potent autocrine signaling in chondrocytes and fibrochondrocytes from the intervetebral disc [105,163,226]. IL-1-induced disruption of ECM turnover is also modulated by mechanical

stimulation in both tissue types[36,97,192,207,228] . Thus, the physical proximity, shared exposure to cytokines in synovial fluid, and similar responses to IL-1 underscore the potential convergence in the upstream regulation of proteoglycan turnover in cartilage and fibrocartilage.

The IL-1-induced catabolic cascade is different for cartilage and fibrocartilage. Most strikingly, the concomitant proteoglycan release and collagen degradation in fibrocartilage is markedly different from the apparent requirement in articular cartilage for exhaustive proteoglycan depletion prior to collagen degradation. The lower abundance of proteoglycans or different interactions between proteoglycans and collagen in fibrocartilage may favor diffusion of collagen-degrading proteases for reaction with their substrates. In addition, since the collagen network is degraded relatively early in the fibrocartilage catabolic cascade, the menisci may be more susceptible to degenerative tears than articular cartilage during the progression of joint disease. On the other hand, metalloproteinase inhibitors are more effective at preventing IL-1-induced degradation in fibrocartilage than cartilage. Pharmacologic inhibition of aggrecanases and MMPs abolishes IL-1-induced loss of fibrocartilage composition and material properties over 12 days, but is substantially less effective at preserving the composition and properties of IL-1-stimulated cartilage over the same time period. These results suggest that the ECM of immature meniscal fibrocartilage is more vulnerable to cell-mediated destruction, but that fibrochondrocytes exhibit a less robust catabolic response to IL-1 than articular chondrocytes. Future studies will need to examine the relevance of these findings to mature human tissues and the progression of joint degeneration.

Inhibiting ADAMTS and MMP family proteases may not be sufficient for the clinical management of degenerative or damaged cartilages. Potent ADAMTS and MMP inhibitors delayed, but did not block, the destruction of IL-1-stimulated articular cartilage, suggesting that redundant or compensatory degradation mechanisms were active in this model. More upstream targets in the catabolic cascade, such as the cytokines and/or receptors that enhance protease production and activation, may have more therapeutic potential. IL-1-receptor antagonist (Anakinra©) and anti-TNF-α (Infliximab©) are biologics that were recently approved for clinical use. They appear to prevent the proinflammatory cytokines IL-1 and TNF-α, respectively, from binding cell-surface receptors and can reduce degenerative joint damage in some rheumatoid and osteoarthritis patients[15,69,103,114,243,257]. Indeed, the compounds used to block ADAMTS and MMP activity in this dissertation research were initially developed for therapeutic use, but were ultimately abandoned for compounds targeting more upstream cell signaling events.

In contrast to articular cartilage, meniscal fibrocartilage explants are depleted of proteoglycans and lose tissue material properties in serum-free culture conditions. The basal (unstimulated) culture conditions used in these studies do not support maintenance of fibrochondrocyte ECM biosynthesis, and explanted fibrocartilage from the midsubstance of bovine menisci undergoes cell-mediated and cell-independent loss of sGAG. This loss occured in the absence or presence of ITS+ supplementation, and addition of an aggrecanase-selective inhibitor significantly reduces this loss, indicating that aggrecanases are primary mediators of aggrecan catabolism in basal fibrocartilage cultures. Cell-independent proteoglycan loss, amounting to approximately 15% of the

initial sGAG content over 12 days, is presumably due to diffusion of proteoglycans out of the tissue. The explants' cut surfaces and highly oriented collagen fibers in fibrocartilage would facilitate this process. Also in contrast to articular cartilage, meniscal fibrocartilage treated with a TGF-β type I receptor inhibitor has proteoglycan synthesis and rates of release similar to untreated controls, so endogenous TGF-β-driven ECM synthesis in fibrocartilage appears to be much lower than in articular cartilage; this could be due to a low abundance of latent TGF-β in the fibrocartilage in the ECM. Stimulation with exogenous TGF-β1, however, induces sufficient proteoglycan production to maintain fibrocartilage sGAG concentrations similar to day 0 tissue. Serum-supplemented media has also been shown to support proteoglycan synthesis and retention in intact menisci through two weeks of culture[232], and other anabolic stimuli, such as insulin-life growth factor and platelet-derived growth factor, may also promote matrix homeostasis in fibrocartilage[97,126,166].

Chondrocytes and fibrochondrocytes assemble functionally distinct ECMs. Native fibrocartilage has lower compression and shear moduli and lower hydraulic permeability than articular cartilage[56,176]. Mechanical testing data from the studies in this thesis are generally consistent with previously published reports. Fibrocartilage explants exhibit much lower moduli and higher tangents of the phase angle (tendency to dissipate vs. store energy) than articular cartilage. Accordingly, fibrochondrocytes in agarose gels synthesize a *de novo* ECM with substantially lower compressive stiffness than chondrocytes in agarose. These cells inherently produce different ECMs, and the agarose gel culture system appears to preserve some aspects of tissue-specific behavior.

The role of aggrecan in meniscal fibrocartilage mechanics appears to be different from that in articular cartilage. The apparent sGAG density in fibrocartilage is quite low, on the order of 6-10mg/mL. In contrast, the sGAG density of age-matched articular cartilage is 60-80mg/mL. Microstructural models of proteoglycan solutions indicate that electrostatic effects contribute to tissue stiffness at sGAG densities of ≥40mg/mL[29,44]. The studies in this dissertation demonstrate that aggrecan confers compressive stiffness to meniscal fibrocartilage, despite the tissue's low bulk sGAG density. This may be reconciled by the possibility that localized regions of the ECM in the pericellular space, at the junctions of collagen fibers, or surrounding collagen fibers contain high concentrations of aggrecan. Consistent with this idea, safranin-O staining show pockets of sGAG-rich ECM in the middle region of the meniscus. Aggrecan also contributes to fibrocartilage shear properties, although its function in bearing shear loads is considerably less clear. In contrast to compression, shear deformations do not introduce volumetric deformations, and time-dependent effects observed in the shear stress-strain response are attributed to inherent viscoelasticity of the ECM solid fraction (*i.e.*, independent of water extrusion). It is possible that aggrecan localized to the surfaces of collagen fibers in some way alters the viscoelasticity of the collagen network, perhaps by altering the frictional coefficient between adjacent fibers. Indeed, aggrecan has been localized in the plane of the circumferentially-oriented collagen fibers found in the outer region of the menisci. The sGAG in fibrocartilage is less readily extracted than aggrecan in articular cartilage, which suggests that aggrecan in fibrocartilage is more tightly associated with the collagen network.

The physiologic benefits of differential aggrecan processing in cartilage and fibrocartilage remain unclear. Aggrecan contributes to the compression and shear properties of both tissues, yet there are marked differences in the abundance and degree of proteolytic processing between the two tissues. Aggrecan has been shown to inhibit nerve outgrowth in part due to the presence of sGAG, and perineuronal nets are composed of aggrecan[107,137]. It is possible that fibrochondrocytes, particularly in the middle and outer regions of the meniscus, would not attach to the ECM or retain a stellate morphology in the presence of abundant full-length aggrecan. Indeed, there is a direct relationship between rounded cell morphology and sGAG abundance in articular cartilage and the developing meniscus. Maintenance of the stellate morphology may be essential for fibrocartilage—specific mechanotransduction pathways, which in turn regulate fibrochondrocyte-specific ECM remodeling responses. Aggrecan may also exhibit differential associations with other ECM molecules in cartilage and fibrocartilage. The relative abundance of G3-bearing aggrecan fragments in fibrocartilage extracts suggests that these molecules are functional. The G3 domain of aggrecan contains repeated lectin domains that can bind the glycoproteins tenascin C and fibulin[11,43,130], which have been immunolocalized in developing meniscal fibrocartilage and cartilage, respectively[191,253]. In addition, the sizes of aggrecan G3 fragments detected in tissue extracts indicate that they contain sGAG chains from the chondroitin sulfate-rich 2 domain. Thus, aggrecan fragments resulting from extensive proteolytic processing may still be functional in fibrocartilage.

Tissue-specific patterns of aggrecan turnover appear to be the result of intrinsic differences in cell activity. Clarifying the function of different aggrecan processing

events and aggrecan species in the development of a mechanically competent tissue will be useful in the design and evaluation of engineered tissue grafts. In addition, identification of aggrecan cleavage products indicative of pathologic fibrochondrocyte ECM remodeling may be useful in the early detection of arthritis. On this point, however, it is important to note that while aggrecan G1-NITEGE is indicative of pathologic remodeling in articular cartilage, it is indicative of normal developmental remodeling in meniscal fibrocartilage. Identification of alternative biomarkers, such as the recently-characterized fibronectin neoepitope in arthritic cartilage[250], may be necessary to distinguish tissue development from tissue degeneration within the joint.

6.3 Future Directions

The work in this thesis raises many more questions than it answers. The tissue source used in most of the studies presented here was the immature bovine stifle joint. This tissue source was abundant, received disease- and injury-free, and well-characterized by decades of research on the material properties, chondrocyte metabolism, and aggrecan turnover of the cartilage and fibrocartilage. An important extension of this work, however, will be investigation of chondrocyte- and fibrochondrocyte-mediated ECM remodeling in human tissues of varying ages. Species-dependent variations in meniscus composition and material properties have been reported[2,109,215], and chondrocytes exhibit diminished sensitivity to growth factor stimulation with age[81,135]. Thus, elucidation of human-specific ECM remodeling in pathologic, developmental, and homeostatic states will be important.

Differences in injury-induced mechanisms of proteoglycan remodeling in cartilage and fibrocartilage will also be worth investigating. IL-1 stimulation of explanted

tissue loosely approximates a traumatic tissue tear and ensuing inflammatory cascade. However, in the absence of mechanical overload, this model fails to capture the roles of collagen network fissures and apoptosis in injury-induced ECM catabolism. Bovine and human articular cartilage release proteoglycans following compressive overload, and addition of IL-1 to injured cultures synergizes with the injury to enhance proteoglycan release[168,177]. Examination of loading mode will also be relevant for study of injury-induced cell-mediated ECM remodeling in meniscal fibrocartilage.

The loading environment is known to have profound effects on chondrocyte and fibrochondrocyte metabolism, and future studies would benefit from the superposition of physiologic loading on cytokine-stimulated explants. The cytokine treatments used in this dissertation provided aggressive stimuli for perturbing cell metabolism. Nevertheless, the effects of the cytokine treatments cannot be fully decoupled from the effects of being removed from an actively loaded environment. Dynamic compression enhanced IL-1-induced proteoglycan release from fibrocartilage explants[207], and static compression suppressed proteoglycan synthesis in fibrocartilage explants[97]. Adaptation of the current studies to *in vivo* models of fibrocartilage development and degeneration would be helpful in maintaining physiologic mechanical signals.

The role of inter-tissue paracrine signaling in regulating ECM turnover during development and degeneration would be another interesting extension of this work. Chondrocytes exhibit autocrine IL-1 and TGF-β signaling[163,240], and release of these and other signaling molecules to the synovial fluid may play a significant role in the pathogenesis of arthritis. The co-culture of explanted tissues or isolated cells under catabolic and anabolic conditions may reveal important kinetic and mechanistic features

of arthritic changes in one tissue compartment resulting from injury or inflammatory insult to the other.

The use of pharmacologic inhibitors is ultimately limited by the specificity of the inhibitors for their target. Non-specific effects of inhibitor treatments can be manifested in obvious cell death and aberrant behavior, or in more subtle shifts in cell metabolism that are difficult or impossible to detect. The inhibitors and range of doses used in this dissertation work demonstrated low or no adverse effects on the mitochondrial activity of tissue explants and cell-agarose constructs. Evidence of specific enzyme activity in aggrecan processing events, however, should be substantiated by alternative methods of protease blockade. Aggrecanase-1 or -2 knockout animals[77,78,211] would be helpful in characterizing the functional implications of aggrecanase-mediated aggrecan cleavage in the developing meniscus. Specific inhibition of MMP- and calpain- mediated aggrecanolysis is problematic because they are so multifunctional, but cleavage site-directed mutagenesis of the aggrecan core protein is a promising approach[129].

Further investigation of the signals regulating ECM molecule abundance, structure, and localization in cell-scaffold constructs may aid in the development of engineered tissue replacements and model tissues. TGF-β1-stimulated explant cultures maintained sGAG concentration, rather than absolute sGAG quantity, suggesting that homeostatic proteoglycan turnover is regulated by sGAG concentration. A model describing the kinetics of chondrocyte-mediated *de novo* tissue formation postulates that the rate of ECM molecule deposition is related to a "target" abundance of that ECM component[245]. Chondrocytes and fibrochondrocytes may exhibit cell type-specific target ECM compositions that depend on the culture conditions, and these responses may be

governed by a common mechanism. In addition, the chondrogenic or fibrochondogenic potential of viable tissue engineering cell sources, such as bone-marrow-derived stromal cells (BMSCs), may be characterized by the kinetics and mechanisms of aggrecan processing. Pilot experiments with BMSCs cultured in agarose and chondrogenic media demonstrated increases in construct material properties in the presence of an aggrecanase-selective inhibitor, suggesting that BMSCs expressed aggrecanases and exhibited some aspects of fibrochondrocytic ECM turnover. Engineered tissue constructs can thus serve as useful model systems for examining ECM remodeling, tissue development, and ECM composition-function relationships.

APPENDIX A

MATERIALS & SUPPLIES

Product	Vendor	Location
Anti-collagen I antibody	Abcam	Cambridge, MA
ECF substrate	Amersham	Piscataway, NJ
Keratanase II	Associates of Cape Cod	E. Falmouth, MA
ITS+ media supplement	Becton Dickinson	San Jose, CA
Gel/Mount	Biomeda	Foster City, CA
WST-1 reagent	BioVision	Mountain View, CA
Calpain inhibitor III (MDL 28170)	Calbiochem	La Jolla, CA
Protease inhibitor cocktail set I	Calbiochem	La Jolla, CA
Re-blot plus stripping buffer	Chemicon	Temecula, CA
0.11μm Polyethersulfone filter	Corning	Corning, NY
ELF3200	Enduratec	Minnetonka, MN
ELF3100	Enduratec	Minnetonka, MN
Proteinase K	EMD Chemicals	Gibbstown, NJ
24-Well tissue culture plates	Falcon	Franklin Lakes, CA
48-Well tissue culture plates	Falcon	Franklin Lakes, CA
Dimethyl sulfoxide	Fisher Scientific	Pittsburg, PA
Sodium dodecyl sulfate	Fisher Scientific	Pittsburg, PA
Triton X-100	Fisher Scientific	Pittsburg, PA
FLA-3000 phosphorimager	FujiFilm Lifescience	Stamford, CT
SMT1 5.6lbf load cell	Interface	Scottsdale, Arizona
SMT1 1.1lbf load cell	Interface	Scottsdale, Arizona
2x Sample loading buffer	Invitrogen	Carlsbad, CA
4',6-Diamidino-2-phenylindole	Invitrogen	Carlsbad, CA
4-12% Tris glycine PAGE gels	Invitrogen	Carlsbad, CA
AlexaFluor 488 goat anti-rabbit	Invitrogen	Carlsbad, CA

AlexaFluor 594 goat anti-mouse	Invitrogen	Carlsbad, CA
Antibiotic/antimycotic	Invitrogen	Carlsbad, CA
Collagenase Type II	Invitrogen	Carlsbad, CA
Fungizone (Amphotericin B)	Invitrogen	Carlsbad, CA
Gentamicin	Invitrogen	Carlsbad, CA
HEPES buffer	Invitrogen	Carlsbad, CA
High glucose Dulbecco's Modified Eagle's Medium	Invitrogen	Carlsbad, CA
Non-essential amino acids	Invitrogen	Carlsbad, CA
Phosphate buffered saline	Invitrogen	Carlsbad, CA
SeeBlue Plus MW markers	Invitrogen	Carlsbad, CA
Trypsin-EDTA	Invitrogen	Carlsbad, CA
p-dimethylaminobenzaldehyde	JT Baker	Phillipsburg, NJ
Urea	JT Baker	Phillipsburg, NJ
anti-aggrecan G1 antibody	John Sandy, PhD	Tampa, FL
anti-aggrecan G2 antibody	John Sandy, PhD	Tampa, FL
anti-aggrecan G3 antibody	John Sandy, PhD	Tampa, FL
anti-DLS antibody	John Sandy, PhD	Tampa, FL
anti-NITEGE antibody	John Sandy, PhD	Tampa, FL
anti-VDIPEN antibody	John Sandy, PhD	Tampa, FL
anti-decorin antibody (LF-94)	Larry Fisher, PhD	Bethesda, MD
Chloramine-T	Mallinckrodt	Paris, KY
Bohlin CVO 120 rheometer	Malvern	Worcestershire, UK
4mm Biopsy punch	Miltex	York, PA
6mm Biopsy punch	Miltex	York, PA
*rh*IL-1α	R & D Systems	Minneapolis, MN
*rh*TGF-β1	R & D Systems	Minneapolis, MN
Bovine calf stifle joints	Research 87	Marlborough, MA
Broad Spectrum inhibitor	Roche-Palo Alto	Palo Alto, CA
Aggrecanase inhibitor	Roche-Palo Alto	Palo Alto, CA
MMP inhibitor	Roche-Palo Alto	Palo Alto, CA
Model 31 250g load cell	Sensotec	Columbus, OH
Guanidine-HCl	Shelton Scientific	Shelton, CT
Agarase	Sigma	St. Louis, MO

Alkaline Phos. anti-rabbit antibody	Sigma	St. Louis, MO
Ammonium acetate	Sigma	St. Louis, MO
1,9-Dimethylmethylene Blue	Sigma	St. Louis, MO
Bovine serum albumin	Sigma	St. Louis, MO
Calf thymus DNA	Sigma	St. Louis, MO
Chondroitin sulfate	Sigma	St. Louis, MO
Chondroitinase ABC	Sigma	St. Louis, MO
Dithiothreitol	Sigma	St. Louis, MO
Goat serum	Sigma	St. Louis, MO
Hoechst 33258	Sigma	St. Louis, MO
Hydroxyproline	Sigma	St. Louis, MO
Hydrochloric acid	Sigma	St. Louis, MO
Iodoacetic acid	Sigma	St. Louis, MO
Keratanase I	Sigma	St. Louis, MO
L-ascorbic acid	Sigma	St. Louis, MO
L-ascorbic acid 2-PO_4	Sigma	St. Louis, MO
MES	Sigma	St. Louis, MO
Non-immune rabbit IgG	Sigma	St. Louis, MO
Rabbit serum	Sigma	St. Louis, MO
Safranin O	Sigma	St. Louis, MO
Sodium acetate	Sigma	St. Louis, MO
Sodium chloride	Sigma	St. Louis, MO
Tris base	Sigma	St. Louis, MO
TWEEN-20	Sigma	St. Louis, MO
Sequenza staining racks/chambers	Thermo Electron	Waltham, MA
SB431542	Tocris	Ellisville, MO
#12 Razor blades	VWR Scientific	West Chester, PA
#22 Scalpel blades	VWR Scientific	West Chester, PA
Filter paper	VWR Scientific	West Chester, PA
Nitrocellulose	VWR Scientific	West Chester, PA
Axiovert 200M w/ Apotome	Zeiss North America	Thornwood, NY

APPENDIX B

PROTOCOLS

B.1 Western Blotting

B.1.1 Sample Preparation

1. Proteins were precipitated from conditioned media or tissue/construct extracts in 3 volumes of ice cold ethanol/5mM sodium acetate overnight at -20C. Lyophilized and minced explants or constructs were first extracted in 1mL 4M Guanidine·HCl/10mM MES/50mM sodium acetate/5mM EDTA/pH 6.5 with supplemented with protease inhibitor cocktail I (Calbiochem). Extractions were typically performed 24-48h with gentle agitation at 4C. Volumes of media or extract for precipitation should contain no more than ~100μg sGAG, as determined by DMMB dye-binding assay.
2. Precipitates were spun at max speed at 4C for 30 minutes, and the supernatant removed. Pellets were allowed to dry 15-30min on the bench (with tube tops open).
3. Pellets were resuspended in 100-200μL chondroitinase buffer (50mM Tris·HCl/50mM sodium acetate/10mM EDTA supplemented with protease inhibitor cocktail I) and assayed for sGAG to check recovery. Stubborn pellets were warmed to 37C for 15-30min and triturated with a micropipet. Recovery should be approximately 80% of the sGAG in the initial volume of media or extract.

4. Samples were deglycosylated with protease-free chondroitinase ABC (5μL of stock at 4U/mL), Keratanase I (1μL of stock at 60U/mL), and Keratanase II (3μL of stock at 0.1U/mL) overnight at 37C. It was helpful to periodically examine the deglycosylation efficiency by assay the digest for sGAG. Effective deglycosylation yields at least an 80% reduction in measureable sGAG.
5. Samples were frozen and lyophilized, then resuspended in sample loading buffer consisting of 2X Tris-glycine sample buffer, 6M urea, and DTT (stock composed of 200μL sample buffer, 200μL 6M urea, and 12mg DTT). Samples were diluted in sample loading buffer to approximately 1mg sGAG/mL.

B.1.2 Immunoblotting

1. Samples in sample loading buffer were boiled at 100C for 5 min. Screw-top or lockable lids worked best.
2. 10-15μL of molecular weight standards or experimental samples were loaded into 12- or 15-lane 4-12% Tris-glycine gradient gels.
3. Proteins were separated at 200V for 45-50min at room temperature. Running ("electrode") buffer was prepared from the following recipe: [6g Tris base (50mM) + 28.8g Glycine (384mM) + 2g SDS + 1L deionized water].
4. Gels were moved to immunoblot transfer modules with nitrocellulose membranes. Transfer buffer was prepared from the following recipe: [3.03g Tris base (25mM) + 14.4g Glycine (192mM) + 800mL deionized water + 200mL molecular biology grade methanol]. The best transfers were achieved at 70V for 2-3h, with the transfer boxes packed with ice. Transfer also appeared complete following 30V overnight at 4C.

5. Following transfer, membranes were rinsed in Tris buffered saline with TWEEN (TBS-T) in an empty micropipet tip box. A 10X stock of TBS was prepared form the following recipe: [24.2g Tris base (200mM) + 80g sodium chloride (1.37M) + 1L deionized water & pH'd to 7.6]. TBS-T was prepared by adding 1mL TWEEN-20 to 1L 1X TBS.
6. Membranes were blocked 30-60min with gentle agitation at room temperature in blocking buffer. Blocking buffer was prepared by adding 800mg non-fat dry milk to 80mL TBS-T. Occasionally, membranes were blocked overnight at 4C.
7. Membranes were incubated with primary antibody diluted (typically 1:1000~1:5000) in Blotto with TWEEN. Blotto with TWEEN was prepared by adding 4g non-fat dry milk to 80mL TBS-T. Incubations with primary antibody were either 1h at room temperature or overnight at 4C, with gentle agitation.
8. Membranes were then washed with TBS-T 3 x 5min.
9. Membranes were then incubated with secondary antibody diluted (typically 1:10,000~1:30,000) in Blotto with TWEEN. Incubations with secondary antibody were either 1h at room temp or overnight at 4C with gentle agitation.
10. Membranes were washed with TBS-T 3 x 10min.
11. Membranes were developed by exposure to 1mL of Amersham's ECF substrate for 5min at room temperature, on a transparency (protein-side of membrane faced "down" into pool of ECF).
12. Developed membranes were imaged on an FLA-3000 FujiFilm phosphorimager.
13. Membranes that underwent re-blotting were immediately stripped in Re-Blot Plus solution (Chemicon) for 10min at room temperature with gentle agitation.

14. Stripped membranes were rinsed 3 x 5min in TBS-T and subject to primary, secondary, and development steps as described in steps 7-12.

B.2 Immunostaining

1. Paraffin-embedded sections of tissue explants or gel constructs cut at 4-7μm and placed on Superfrost+ slides were first deparaffinized in a Leica autostainer.
2. Slides were then loaded into Sequenza staining cassettes and racks with ~300μL PBS.
3. The sections typically underwent enzymatic antigen retrieval. With sections of cartilage, in particular, detection of many epitopes required deglycosylation. Sections were typically incubated with 150μL 0.1U/mL Ch'ase ABC at 37C for 30-60min. Detection of collagen (types I and II, in particular) could be enhanced by brief incubation with trypsin (0.5x, 37C, 10-30min) or pepsin.
4. Following antigen retrieval, sections were rinsed with PBS 3 x 500μL.
5. Sections were blocked for 1h at RT with a blocking buffer prepared from the following recipe: [3.4mL PBS + 1mL 5% BSA (dissolved in PBS, supplemented with 0.1% azide to prevent mold growth) + 25μL 10% TWEEN-20 (in PBS) + 100μL normal goat serum + 500μL 1% gelatin (in PBS)].
6. Sections were incubated with primary antibody diluted (typically 1:100~1:200) in primary antibody buffer consisting of: [3.5mL PBS + 1mL 5% BSA (as above) + 500μL 1% gelatin (as above)]. Incubations were either for 1h at room temperature or overnight at 4C. For some experiments, a second primary antibody, raised in a different species (mouse vs. rabbit), was also added during this step.
7. Sections were rinsed 3 x 500μL PBS.

8. Sections were incubated with secondary antibody diluted (typically 1:200~1:500) in PBS for 1h at room temperature. The fluorescent nuclear stains Hoetsch 33258 or DAPI were added at this step as well. Again, dual stained sections were additionally treated with a secondary to the second primary (anti-mouse vs. anti-rabbit).
9. Sections were rinsed 3 x 500μL PBS, removed from the racks, mounted in Gelmount, coverslipped, and allowed to set overnight at 4C.
10. Following mounting, coverslips were fixed to the slides with a bead of clear nail polish.

- In some follow-up experiments not included in the dissertation, an alternative approach to dual labeling was developed. The approach was designed to accommodate use of two primary antibodies raised in the same species. It required the use of a biotinylated primary antibody, an avidin- or streptavidin-fluorophore, and sequential use of the antibodies. Following blocking, the sections were incubated *only* with the non-biotinylated primary and then with the corresponding secondary antibody. After this step, the sections were blocked for endogenous biotin with a commercially available kit (Vector Labs), and subsequently incubated with the biotinylated primary antibody. After rinsing, the sections were then incubated with avidin-AlexaFluor 594 and DAPI for 1h at room temperature. Sections were then mounted, coverslipped, and sealed as described above.

B.3 Mechanical Testing

- Samples were stored in an isotonic solution (e.g., PBS) supplemented with protease inhibitor cocktail I to ensure that sample material properties were not substantially influenced by the activity of endogenous proteases. Tissue explants were stored for long periods (weeks to months) at -20C prior to testing, whereas the much softer gel constructs were stored at 4C for less than 1wk prior to testing.
- Samples were typically tested in torsional shear prior to testing in compression.
- A typical testing protocol is outline below.

1. Sample was thawed or warmed at room temperature for 30min.
2. Sample was patted dry on a laboratory napkin, and the wet mass was measured on a fine balance.
3. Sample diameter and thickness was measured in 3 separate sample locations (for each dimension) using digital calipers.
4. Sample was placed between two parallel, impermeable plates (6mm Ø, with 600 grit water proof sandpaper glued to the surfaces). The "gap" was defined in the Bohlin software to be 90% of the sample thickness (in μm), and the upper plate was then moved to the gap setting to compress the sample to 10% strain. The sample was enveloped with a bead of room temperature PBS with protease inhibitors, and the humidity chamber put in place to prevent dehydration.
5. Sample was allowed to equilibrate to the new stress state for 15-30min (depending on sample type).
6. Sample was loaded in oscillatory torsion over a) a range of frequencies or b) a range of peak strains. Frequency sweeps were performed at ±0.25% strain from

0.001-1Hz. Amplitude sweeps were performed at 0.1Hz from ±0.1-2.5% strain. Samples were cycled for 30s prior to measurements of stress and strain.

7. Bohlin software automatically calculated complex, storage, and loss moduli, and phase angles, from these tests. All data was either manually transcribed or copied from the Bohlin software window into a Microsoft Excel© spreadsheet for further analysis.
8. Following shear testing, the sample was returned to free swelling conditions in PBS with protease inhibitors for 30min at room temperature. Compression test parameters were defined in the Enduratec Wintest software. The "Block" waveform was used to define and collect data from stress relaxation steps. Oscillatory compression tests were programmed using either the Block waveform (as in chapter 4) or the Dynamic Mechanical Analysis package (as in chapters 5 & 6).
9. Sample was placed into an unconfined compression chamber in either the ELF3100 or ELF3200 uniaxial loading frame. Sample was immersed in 3-5mL PBS with protease inhibitors. The main actuator with attached 12mm Ø indenter was moved to the zero displacement position and the load channel was tared. The compression chamber was then moved into close proximity of the indenter using the motorized stroke extender (on the ELF3200) or manually adjustable lower platen (on the ELF3100).
10. The sample was then manually preloaded to 10-20mN with the stroke extender (ELF3200) or the main actuator (ELF3100).

11. All compression tests involved ramping to 10% compressive strain (at a rate of 100μm/min), a period of stress relaxation (20-30min, depending on sample type), and oscillatory loading over a range of frequencies at ±1.5% strain. Oscillatory loading tests were performed at frequencies ranging from 0.001Hz-5Hz. Some experiments (presented in chapter 4) included 4 steps of stress relaxation (5% each up to 20% strain) for measurement of an equilibrium modulus.
12. The Dynamic Mechanical Analysis package was used to collect data from the oscillatory loading tests performed in chapters 5 and 6. For the experiments described in chapter 4, a custom MATLAB code was written to extract material properties from the oscillatory loading test data. More specifically, the code imported raw time-displacement and time-load waves, applied a fast fourier transform to these waves to determine the magnitude of the fundamental sinusoidal components of the waves, and outputted a complex modulus and phase angle for each frequency tested.
13. Following testing, the sample was moved to an empty screw-top 2mL tube, frozen, and lyophilized for biochemical analysis.

The code for the MATLAB routine is given below:

```
infile = char(textdata(1,1));
[p, name] = fileparts(infile);
outfile = [name ' fft.txt'];

prompt = {'Enter Sample Radius (mm):', 'Enter Sample Thickness (mm)'};
dlg_title = 'Your input is Requested!';
num_lines= 1;
def2 = {'2.0', '2.0'};
dim = inputdlg(prompt,dlg_title,num_lines,def2);
radius = str2num(char(dim(1,1)));
```

```
thickness = str2num(char(dim(2,1)));

LoadWave1 = data(2403:2602, 5);
LW1Freq = 0.001;
LoadWave2 = data(2604:3003, 5);
LW2Freq = 0.01;
LoadWave3 = data(3005:4004, 5);
LW3Freq = 0.1;
LoadWave4 = data(4006:5005, 5);
LW4Freq = 1;
LWFFT1 = fft(LoadWave1);
LWFFT2 = fft(LoadWave2);
LWFFT3 = fft(LoadWave3);
LWFFT4 = fft(LoadWave4);
lm1 = abs(LWFFT1);
lm2 = abs(LWFFT2);
lm3 = abs(LWFFT3);
lm4 = abs(LWFFT4);
lm1(1,1) = 0;
lm2(1,1) = 0;
lm3(1,1) = 0;
lm4(1,1) = 0;
lm1(102:200) = [];
lm2(202:400) = [];
lm3(503:1000) = [];
lm4(504:1000) = [];

DispWave1 = data(2403:2602, 4);
DW1Freq = 0.001;
DispWave2 = data(2604:3003, 4);
DW2Freq = 0.01;
DispWave3 = data(3005:4004, 4);
DW3Freq = 0.1;
DispWave4 = data(4006:5005, 4);
DW4Freq = 1;
DWFFT1 = fft(DispWave1);
DWFFT2 = fft(DispWave2);
DWFFT3 = fft(DispWave3);
DWFFT4 = fft(DispWave4);
dm1 = abs(DWFFT1);
dm2 = abs(DWFFT2);
dm3 = abs(DWFFT3);
dm4 = abs(DWFFT4);
dm1(1,1) = 0;
dm2(1,1) = 0;
dm3(1,1) = 0;
```

```
dm4(1,1) = 0;
dm1(102:200) = [];
dm2(202:400) = [];
dm3(503:1000) = [];
dm4(504:1000) = [];

f1 = 0.2*(1:100)/200;
f2 = 2*(1:200)/400;
f3 = 20*(1:500)/1000;
f4 = 200*(1:500)/1000;

[a,flpos1] = max(lm1);
[a,flpos2] = max(lm2);
[a,flpos3] = max(lm3);
[a,flpos4] = max(lm4);

[a,fdpos1] = max(dm1);
[a,fdpos2] = max(dm2);
[a,fdpos3] = max(dm3);
[a,fdpos4] = max(dm4);

langle1 = (angle(LWFFT1));
langle2 = (angle(LWFFT2));
langle3 = (angle(LWFFT3));
langle4 = (angle(LWFFT4));

dangle1 = (angle(DWFFT1));
dangle2 = (angle(DWFFT2));
dangle3 = (angle(DWFFT3));
dangle4 = (angle(DWFFT4));

phanglediff1 = (180/(pi))*(dangle1(fdpos1)-langle1(flpos1));
phanglediff2 = (180/(pi))*(dangle2(fdpos2)-langle2(flpos2));
phanglediff3 = (180/(pi))*(dangle3(fdpos3)-langle3(flpos3));
phanglediff4 = (180/(pi))*(dangle4(fdpos4)-langle4(flpos4));

freq1_dyn_mod = (max(lm1)/((pi)*(radius)))/(max(dm1)/(thickness));
freq2_dyn_mod = (max(lm2)/((pi)*(radius)))/(max(dm2)/(thickness));
freq3_dyn_mod = (max(lm3)/((pi)*(radius)))/(max(dm3)/(thickness));
freq4_dyn_mod = (max(lm4)/((pi)*(radius)))/(max(dm4)/(thickness));

freq = [0.001, 0.01, 0.1, 1];
dyn_mod = [freq1_dyn_mod, freq2_dyn_mod, freq3_dyn_mod, freq4_dyn_mod];
phase_angle = [phanglediff1, phanglediff2, phanglediff3, phanglediff4];

sumd = zeros(1,4);
```

```
THDd = zeros(1,4);
suml = zeros(1,4);
THDl = zeros(1,4);
   for i = 2:1:25;
      sumd(1) = ((dm1(i*fdpos1))^2) + sumd(1);
      sumd(2) = ((dm2(i*fdpos2))^2) + sumd(2);
      sumd(3) = ((dm3(i*fdpos3))^2) + sumd(3);
      sumd(4) = ((dm4(i*fdpos4))^2) + sumd(4);

      THDd(1) = 100*((sumd(1))^.5)/max(dm1);
      THDd(2) = 100*((sumd(2))^.5)/max(dm2);
      THDd(3) = 100*((sumd(3))^.5)/max(dm3);
      THDd(4) = 100*((sumd(4))^.5)/max(dm4);

      suml(1) = ((lm1(i*flpos1))^2) + suml(1);
      suml(2) = ((lm2(i*flpos2))^2) + suml(2);
      suml(3) = ((lm3(i*flpos3))^2) + suml(3);
      suml(4) = ((lm4(i*flpos4))^2) + suml(4);

      THDl(1) = 100*((suml(1))^.5)/max(lm1);
      THDl(2) = 100*((suml(2))^.5)/max(lm2);
      THDl(3) = 100*((suml(3))^.5)/max(lm3);
      THDl(4) = 100*((suml(4))^.5)/max(lm4);
   end;


subplot(2,1,1); semilogx (freq, dyn_mod, '-or','LineWidth', 2), grid on;
xlabel('Frequency (Hz)');
ylabel('MPa');
title('Dynamic Modulus');
subplot(2,1,2); semilogx (freq, phase_angle, '-ob', 'LineWidth', 2), grid on;
xlabel('Frequency (Hz)');
ylabel('Degrees');
title('Phase angle');

output = [freq; dyn_mod; phase_angle; THDd; THDl];

fid = fopen(outfile,'w');
fprintf(fid,'Frequency(Hz), Dynamic Modulus(MPa), Phase(degrees), dispTHD(%%),
loadTHD(%%) \r');
fprintf(fid,'radius = %1.3f\r', radius);
fprintf(fid,'thickness = %1.3f\r', thickness);
fprintf(fid,'%1.5f, %1.5f, %1.5f, %1.5f, %1.5f\r', output);
fclose(fid);
```

APPENDIX C

RETINOIC ACID STIMULATES AGGRECAN PRODUCTION AND PROCESSING IN MENISCAL FIBROCARTILAGE

C.1 Introduction

The vitamin A derivative all-trans retinoic acid (RA) is a pleiotrophic cytokine with demonstrated roles in skeletal patterning and chondrocyte differentiation. In explanted articular cartilage, RA induces aggrecanase-mediated release of sulfated glycosaminoglycans (sGAG) leading to loss of material properties, and RA upregulates MMP-2 in growth plate chondrocytes. Interestingly, RA dramatically upregulates expression of the small proteoglycan decorin in isolated articular chondrocytes, and RA has anti-inflammatory effects in a collagen-induced model of arthritis. It is unknown, however, what roles RA may play in fibrocartilage development. The objective of this study was to examine the effects of RA on proteoglycan turnover and material properties of explanted meniscal fibrocartilage.

C.2 Methods

Fibrocartilage explants (n=24, 4mm dia, 2mm thick) were harvested aseptically from the middle zone midsubstance of menisci from a 1-2 week old calf. Explants were cultured in serum free high-glucose DMEM in the absence or presence of 1 or 10□M RA. Media were changed every 2 days. Explants were removed after 10 days and stored at –20C with protease inhibitors for subsequent analysis. Explants were tested at room temperature in torsional shear and in unconfined compression. Complex shear moduli G* were determined via oscillatory shear testing with 0.1 - 5% shear strain at 0.1Hz and a

10% compressive offset. Dynamic compression moduli E* were determined via sinusoidal compression with a 1.5% strain amplitude at 0.01 – 3.16 Hz and a 10% compressive offset. Following mechanical testing, explants were lyophilized and extracted in 4M Gn•HCl with protease inhibitors. Residual explant tissue was digested in proteinase K. Extracts, digests, and conditioned media were then assayed for sGAG content via the DMMB assay. Extracts and media samples were ethanol precipitated, deglycosylated, separated by SDS-PAGE and immunoblotted for aggrecan G1 and decorin.

C.3 Results & Discussion

Untreated fibrocartilage demonstrated a low basal release of sGAG (Fig. C1A) which led to a significant decrease in explant sGAG (extract+digest) (Fig. C1B). Treatment with RA induced a dose-dependent increase in sGAG release that peaked at day 4. Total sGAG (extract+digest+media) in RA-treated tissue was higher than in untreated controls, suggesting that sGAG release is due to both anabolic and catabolic activity. Western blots of conditioned media indicate that basal aggrecanolysis occurs via the aggrecanases, denoted by a 65-70kDa species corresponding to the G1-NITEGE fragment; this aggrecanase activity was not attenuated by RA treatment (Fig.C2A). Interestingly, RA treatment induced release of full-length aggrecan in a dose-dependent manner, and G1-blots of tissue extracts show that RA-treatment led to accumulation of high molecular weight aggrecan species essentially absent in untreated tissue (Fig. C2B). Untreated tissue released decorin to the media, and RA treatment appeared to reduce release (Fig. C2C). Decorin was detected in tissue extracts from all groups. Oscillatory compression and shear tests indicate that RA treatment produced dose-dependent

increases in tissue material properties over untreated controls (Fig. C3A&B). The unconfined compression modulus exhibited characteristic increases with frequency, whereas the shear modulus showed characteristic strain softening for all groups. RA stimulation was shown to maintain proteoglycan turnover and enhance material properties in explanted immature bovine meniscus. RA treatment sustained aggrecanase activity of untreated controls, and in contrast to its effects on articular cartilage from immature bovines, RA appears to stimulate proteoglycan synthesis as evidenced by accumulation of full length aggrecan in the tissue. RA-treated tissue contains an aggrecan population similar to that of day 0 tissue, including full-length and near full length species, suggesting that RA is driving homeostasis in this model. Interestingly, the increases in compressive and shear properties were not associated with elevated sGAG content in tissue. RA-induced increases in the mechanical properties may, then, be due to changes in proteoglycan distribution, or collagen content, cross-linking, or organization.

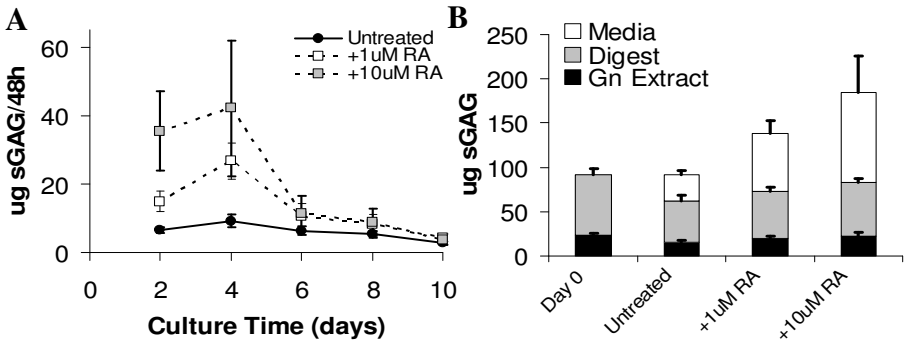

Figure C.1 sGAG release to media (A) and sGAG content (B) of fibrocartilage explants after 10 days. Data are mean ± SEM, n = 5-6.

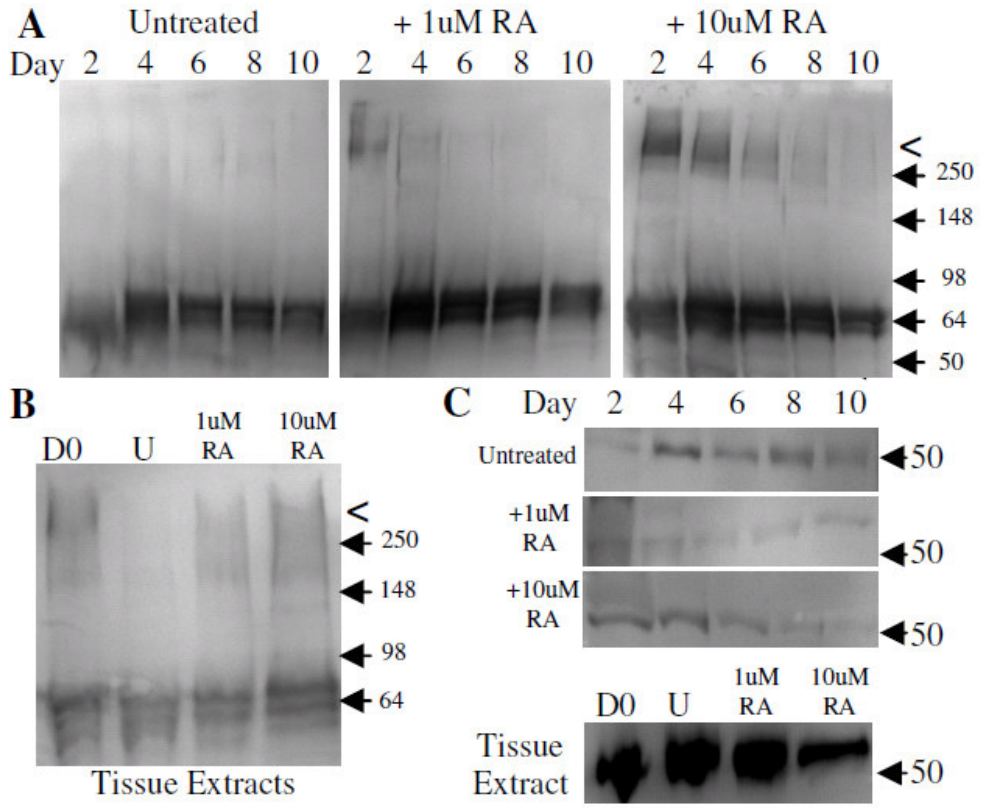

Figure C.2 Aggrecan G1 (A&B) and decorin (C) blots of media (A,C) and tissue extracts after 10 days. (B,C). Equal volumes of media or quantities of sGAG (extracts) were loaded per lane. D0 = Day 0, U = Untreated. In A & B, < denotes migration of full-length aggrecan.

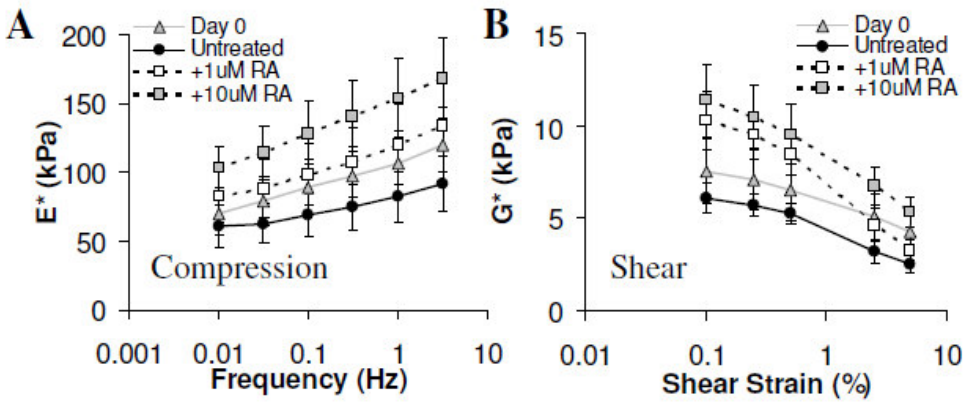

Figure C.3 Dynamic compression (A) and shear (at 0.1Hz) (B) properties of fibrocartilage explants after 10 days. Data are mean ± SEM. n = 5-6

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VITA

Christopher Garrison Wilson was born in Lowell, MA in September of ’78 to George and Susan Wilson. Chris was immediately surrounded by an energetic and supportive extended family led by 4 important role models, his grandparents Mary and Ted Settanny and Eileen and George Wilson. After graduating from Nashua Senior High School in 1996, he attended Worcester Polytechnic Institute in Worcester, MA and earned Bachelor’s (’00) and Master’s (’02) degrees in Biomedical Engineering. It was as a young graduate student that Chris’s eyes opened to the allure of two-wheeled travel and the power of Halloween. Also during this time, Chris interned at biomedical device companies, found inspiration and mentorship in several people, and decided to pursue doctoral studies. Chris moved south to join the white and gold ramblin’ wreck of Georgia Tech in Atlanta, GA, where he grew into a new “family” of fellow graduate students, mentors, and coworkers. In addition to his studies in the Orthopaedic Bioengineering Lab at the Institute for Bioengineering and Biosciences, Chris devoted time to the B^2UGS student organization, was an active patron and bar captain of the ABC brewhouse, raced with the Georgia Tech Cycling Team, and did not freak out with the East Atlanta Optimists’ Club. Following graduation, Chris plans to pursue post-doctoral work in the field of developmental biology, with the vision of attaining a bioengineering faculty position.