IN VITRO MODELS OF CARTILAGE DEGRADATION FOLLOWING JOINT INJURY:

MECHANICAL OVERLOAD, INFLAMMATORY CYTOKINES AND THERAPEUTIC APPROACHES

by

YIHONG C.S. LU

B.S. Chemical Engineering, University of California, Los Angeles, 2004

Submitted to the Department of Biological Engineering in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY IN BIOLOGICAL ENGINEERING

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2010

© 2010 Massachusetts Institute of Technology. All rights reserved.

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
AUG 16 2010
LIBRARIES

ARCHIVES

Signature of Author _____
Department of Biological Engineering
January 28, 2010

Certified by _____
Alan J. Grodzinsky
Professor of Biological, Electrical and Mechanical Engineering
Thesis Supervisor

Accepted by _____
Peter Dedon
Professor of Toxicology and Biological Engineering
Chairman, Graduate Program Committee

Thesis Committee

Alan J. Grodzinsky.....Professor of Biological, Electrical, and Mechanical Engineering
Massachusetts Institute of Technology

Douglas A. Lauffenburger.....Professor of Biological, Chemical Engineering and Biology
Massachusetts Institute of Technology

Christopher Evans.....Professor of Orthopedic Surgery
Harvard Medical School

In Vitro Models of Cartilage Degradation Following Joint Injury: Mechanical overload, Inflammatory Cytokines and Therapeutic Approaches

By

Yihong C.S. Lu

Submitted to the Department of Biological Engineering on January 28, 2010 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biological Engineering at the Massachusetts Institute of Technology

Abstract

Osteoarthritis (OA) is the most common form of joint disorder. Individuals who have sustained an acute traumatic joint injury are at greater risk for the development of OA. The mechanisms by which injury causes cartilage degradation are not fully understood, but the elevated levels of injury-induced pro-inflammatory cytokines, such as TNFα and IL-6, have been implicated to play important roles in the pathogenesis of OA. We have used in vitro models of cartilage injury to examine the interplay between mechanical and cytokine-mediated pathways and to identify processes associated with cartilage degradation following joint injury.

The overall aims of this thesis were to characterize the combined effect of TNFα and IL-6/sIL6R on matrix degradation and chondrocyte gene expression in mechanically injured cartilage, and to investigate whether cartilage degradation could be inhibited by potential therapeutic approaches. TNFα and IL-6/sIL-6R interacted to cause aggrecanase-mediated proteoglycan degradation. Importantly, the combined catabolic effects of cytokines were highly potentiated by mechanical injury. Furthermore, cartilage degradation caused by the in vitro injury model appeared to be initiated at the transcriptional level, since the gene expression of matrix proteases, cytokines and iNOS were all highly elevated in the treatment conditions. The degradative effects of TNFα in injured cartilage was due, in part, to the action of endogenous IL-6, as proteoglycan degradation was partly reduced by an IL-6 blocking Fab fragment. Interestingly, cartilage degradation induced by the combinations of pro-inflammatory cytokines and mechanical injury was fully abrogated by short-term treatments with dexamethasone. The results of this work are significant in that they provide evidence suggesting joint injury affects cell-mediated responses as well as the transport of cytokines and proteases in extracellular matrix, making cartilage tissue more susceptible to further degradation by biochemical mediators.

Thesis Supervisor: Alan J. Grodzinsky, Professor of Biological, Electrical, and Mechanical Engineering

Acknowledgement

Many people have guided and supported me throughout my graduate study. First and foremost, I would like to express my sincere gratitude to my thesis advisor, Professor Alan Grodzinsky, for the encouragement and guidance that he has offered during my pursuit of doctoral degree. He has fostered my growth as an independent scientist, and I have learned much from his dedication to research. I also must thank my thesis committee members, Dr. Chris Evans and Dr. Doug Lauffenburger. Their helpful discussions and suggestions have been invaluable to my research.

I am grateful to all the members of the Grodzinksy group. Jenny Lee taught me the basics of working with biological materials. She also provided useful suggestions on the collaborative projects with Centocor. Han-Hwa Hung has faithfully taken care of all my needs in lab, from ordering reagents to helping me work out protocols. Rachel Miller often offered useful discussions and advices on my projects. I feel fortunate to have a lab mate who is both knowledgeable and always willing to help. I enjoyed my frequent conversations with Hsu-yi Lee about career, family and future; we shared many moments of laughter together. Sangwon Byun became a great friend during the time when I was experiencing frustration with research. He generously helped me with experiments, and was always willing to listen to my crazy ideas about research and life in general. Yunna Sinskey was a great UROP. The summer that we worked together was very fun. She made some of the painstaking experimental procedures seemed much more bearable.

I want to thank my collaborators from Centocor and Biogen Idec. They generously supplied me with reagents and expertise, which were indispensible to my research.

No words can adequately express my gratitude to my wonderful family. My parents sacrificed their own careers for my education and future. My achievement of a PhD from MIT is a fulfillment of their dreams. I am deeply indebted to their love and sacrifice for me. My husband, Allen Lu, has also been my best friend for many years. He always takes great care of me, especially during the times when I have to focus on research and thesis writing. I am forever grateful to his unconditional love.

TABLE OF CONTENTS

APPENDIX B

Preliminary Studies

LIST OF FIGURES

APPENDIX A

APPENDIX B

LIST OF TABLES

CHAPTER 1

Introduction

1.1 Cartilage Structure

Articular cartilage provides an almost frictionless articulating surface that serves as a cushion to absorb shock and withstand a variety of mechanical loadings, including compressions and tensile forces that a joint frequently experiences. Cartilage contains a single cell type, the chondrocytes, which are responsible for producing and maintaining an extensive extracellular matrix (ECM). Cartilage is avascular and alymphatic, therefore the cells rely on the diffusion of nutrients and oxygen from the synovial fluid and the underlying bones. Two major ECM macromolecular families, aggrecan and collagens, provide the necessary mechanical functions of articular cartilage. Aggrecan, a large aggregating proteoglycan, is abundant with sulfated glycosaminoglycans (GAGs), primarily chondroitin sulfate and keratan sulfate. The negatively charged GAGs give rise to a high osmotic swelling pressure that helps to maintain tissue hydration during deformation and allow cartilage to withstand compressive forces. The crosslinked collagen network enmeshes the proteoglycan aggregates and, together, resist tensile and shear forces. In cartilage ECM, the collagen network is mainly consisted of type II collagens, which form heteropolymers with type XI and IX collagens. In the pericellular matrix, the dominating collagen is type VI collagens.

Articular cartilage is a dynamic tissue that is constantly being remodeled by both anabolic and catabolic processes. The balance between synthesis and degradation of ECM is carefully maintained by the residing chondrocytes. In healthy articular cartilage, chondrocytes actively synthesize cartilage proteins and replace the old and damaged components of the matrix. In diseases, the biosynthetic activities fail to keep pace with the degenerative activities, resulting in degeneration and loss of cartilage. Once the ECM is damaged, chondrocytes have little ability to repair.

1.2 Osteoarthritis and Treatments

Osteoarthritis (OA) is the most common form of arthritis, currently affecting 27 million Americans(1). It is characterized by chronic and irreversible degeneration of articular cartilage. Symptoms include pains, swelling, movement limitation and local inflammation in the joints. OA is commonly associated with aging and is the leading cause of disability in elderly. Other risk factors include joint injury, obesity, genetics and repetitive overuse. OA commonly occurs in articulating joints such as knee, hip, hand and spine. The pathology of OA involves the entire joint, including the cartilage, the underlying bone and the surrounding synovial tissues. Cartilage is the most affected tissue and its degradation is the hallmark of joint diseases. Therefore, articular cartilage has been the focus of most OA studies.

Treatments for OA primarily focus on the management of disease symptoms, since there is no cure currently available. Commonly used pharmacologic treatments include simple analgesics such as acetaminophen and a variety of non-steroidal anti-inflammatory drugs (NSAIDS) to reduce pain and inflammation in the joint. Increasing number of OA patients

have also sought for alternative medicines, with the most common forms being the dietary supplements chondrointin and glucosamine. However, it is questionable whether these supplements, administered orally, can reach the affected joints. Other forms of treatments of OA include intra-articular injections of hyaluronan and steroids. Hyaluronan therapy appears to only help a subset of patients(2); more studies are needed to verify the efficacy of hyaluronan in OA treatment. Intra-articular injection of glucocorticoids (GCs) have demonstrated beneficial response with greater relief of pain and increased motion in the injected joints(3). The role of intra-articular GCs in OA treatment remains controversial because some studies reported such treatment may cause local tissue necrosis(4) and bone loss(5). On the other hand, it has also been noted that the deleterious side effects occur only when the GC concentration administered exceeds the normal physiological level(6). Surgical options are usually needed for late stage of joint OA, when patients experience severe pain and disability. In 2003, slightly less than half a million total knee replacements were performed in the U.S. and this number appears to be increasing (7).

Developing new drugs for the treatment of OA remains challenging due to the lack of reliable biomarkers. Joint space narrowing as determined by radiographic imaging has been used for identifying and monitoring joint disease progression. However, noticeable joint narrowing only occurs after much cartilage degradation, therefore not applicable to detect early stage OA for disease intervention.

1.3 Joint Injury and In Vitro Models

Among the many factors that could lead to the development of OA, joint injury plays an important role in the pathogenesis of joint disease. It is known young adults who sustained traumatic joint injury have high risk of developing OA (8-10). Acute traumatic injury causes immediate damage to cartilage extracellular matrix. Evidence showed increased levels of aggrecan fragments and cross-linked peptides from type II collagen in synovial fluid (11, 12). Moreover, injuries such as anterior cruciate ligament (ACL) and meniscus tear, result in an immediately surge in the synovial fluid concentrations of cytokines, including tumor necrosis factor alpha (TNFα), interleukin (IL)-1, IL-6, IL-8 and IL-10 (13). Their concentrations remain elevated for weeks and eventually decreased to levels that are similar to those found in chronic arthritis patients. It has also been shown that the levels of TNF-α, IL-1β in the synovial fluid correlate with the severity of the cartilage damage due to mechanical stress (14).

To further investigate the cartilage degeneration process after injury in vivo, biopsy samples of articular cartilage were taken from individuals with ACL injury before their ligament reconstruction surgery; the average time interval between injury and surgery was 7 weeks (15) (Figure 1.1). Surprisingly, even when the articular surfaces appeared to be intact under arthroscopic visualization, there could still be severe loss of proteoglycans and chondrocyte degeneration in the superficial zone of the cartilage. This finding indicated cartilage degradation occurs rapidly after knee injury, and raised the question whether ACL reconstruction could reduce the risk of OA(15, 16).

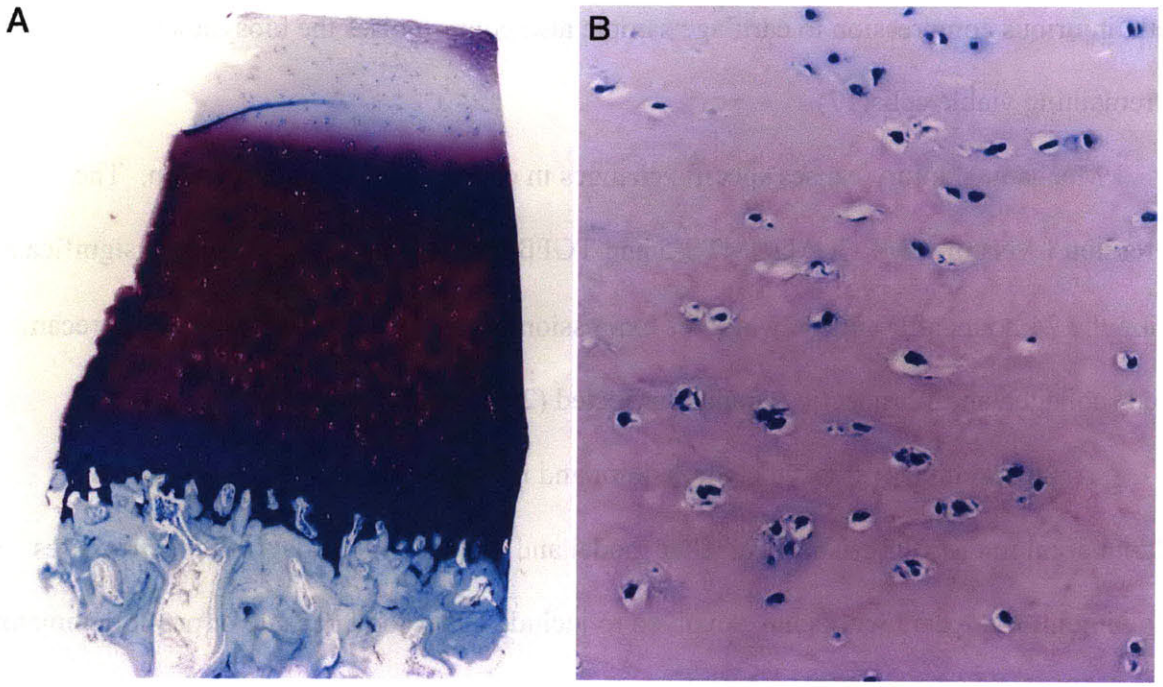


Figure 1.1 Biopsy specimen of articular cartilage from a patient with ACL injury (15). A) Articular surface is intact, but loss of toluidine blue staining in the superficial zone indicates loss of proteoglycans. B) Chondrocyte degeneration shown under higher magnification in the superficial zone of A.

In vitro injury models have helped to understand the contribution of acute damage to cartilage and degeneration commonly associated with OA. Mechanical injury causes direct damage to the cartilage extracellular matrix. Studies using single impact loading(17-20) or prolonged cyclic compression(21-23) have demonstrated loss of proteoglycans and collagen network damage(24-27), increased tissue swelling(18, 28) and reduced cartilage stiffness in compression and shear(17). Injurious compression also causes chondrocyte death (18, 24, 25, 29, 30). Necrosis has been demonstrated using high stress repetitive loading(31). Apoptosis can occur even at peak stress well below the loading required to induce significant changes in mechanical properties(30). Injurious compression associated damage to the chondrocyte-matrix interaction may be responsible for the initiation of apoptosis(32). In addition to cell

death, injurious compression to cartilage sample also compromises the biosynthetic activity in the remaining viable cells(17).

Mechanical injury causes specific changes in chondrocyte gene expression. The expression levels of MMP-3, ADAMTS-5 and TGFβ have been shown to increase significantly during the 24 hours after injury, while the expression of matrix molecules such as aggrecan, type II collagen and fibronectin remain unaffected (20).

Traumatic injury affects the whole joint and is commonly associated with local inflammation in the joint tissue. To better model and understand the degradative processes following injury, recent works have evolved to include either joint capsule or pro-inflammatory cytokines in the co-culture with injured cartilage explants. It was found mechanical injury significantly potentiated the effect of IL-1 and TNFα in inducing GAG loss in both bovine and human cartilage tissue (33), providing the first evidence that mechanical forces and biochemical factors external of cartilage can interact to cause cartilage degradation. Co-culture of injured cartilage with synovium or joint capsule results in more severe biosynthesis inhibition comparing to that measured in injured cartilage alone or co-culture alone(17, 34). Real-PCR analysis showed within 24 hours, co-culture with joint capsule increased the chondrocyte expression of MMP-13 and ADAMTS-5. Furthermore, GAG loss was most severe in the condition of co-culturing joint capsule with injured cartilage, and the degradation was generated by aggrecanases (35). These studies indicate the interactions between injured cartilage and the surrounding joint tissues may contribute to the pathogenesis of joint disease.

1.4 Pro-Inflammatory Cytokines

Osteoarthritis involves local inflammation in the joint. Injury also causes immediate increase in the synovial fluid concentrations of pro-inflammatory cytokines (13). IL-1 and TNFα are two pro-inflammatory cytokines that have prominent effects in inducing cartilage destruction. Both IL-1 and TNFα cause significant proteoglycan and collagen degradation (33, 36), which correlate with the increased expression of aggrecanases (36) and MMPs (37, 38). Both cytokines also inhibit the biosynthesis of cartilage matrix (39, 40) and cause chondrocyte apoptosis(41). In response to these pro-inflammatory molecules, chondrocytes synthesize substantial amounts of nitric oxide and inducible nitric oxide synthase (iNOS) (42, 43), which in turn exert more catabolic modulations on chondrocytes.

IL-6 is another cytokine that plays crucial roles in arthritic diseases. Upon TNFα and IL-1 stimulation, chondrocytes increase the production of IL-6. Studies have shown IL-6 knockout mice are resistant to both antigen-induced arthritis and collagen-induced arthritis (44). Moreover, IL-6 blocking antibody was effective in suppressing arthritis onset, suggesting the pro-inflammatory role of IL-6 in the early phase of arthritic disease (45). Interestingly, IL-6 may also be necessary for maintaining the normal metabolism of cartilage. It has been observed that IL-6 knockout mice showed a greater reduction in proteoglycan synthesis and more matrix breakdown in response to zymosan-induced arthritis (46).

It has been shown that cytokines are capable of acting in synergy. One study has demonstrated that the combination of TNFα and IL-6 caused significantly more GAG loss than either TNFα or IL-6 alone did (47). Another study reported oncostatin M (OSM) acts synergistically with IL-1α to induce cartilage collagen degradation and collagenase expression

(48). Therefore, incorporating more than one cytokines in the in vitro studies can help to understand and explain some of the catabolic processes observed in vivo.

1.5 Aggrecanases

Loss of aggrecan from cartilage matrix is one of the earliest events in the pathogenesis of OA. Pratta et al. illustrated using IL-1 induced cartilage that aggrecan depletion occurred prior to collagen loss(49). This observation suggested aggrecan may play a protective role in preventing collagen degradation; therefore targeting aggrecanases may be a good therapeutic strategy for OA treatment.

Before the discovery of "aggrecanases", it was thought that MMP-3 was the primary enzyme responsible for the aggrecan degradation. However, identification of the cleavage at Glu373-Ala374 site in aggrecan interglobular domain suggested that there must be another type of enzyme capable of causing aggrecan degradation(50). Aggrecanase 1 and 2 were later purified and identified as ADAMTS-4 and -5, respectively(51, 52). Both were capable of cleaving aggrecan at the interglobular domain of the core protein and generating the G1-NITEGE neo-epitope. Recent studies demonstrated ADAMTS-5, but not ADAMTS-4 knockout mice were protected from joint injury-induced OA(53). Song et al. showed siRNA suppression of ADAMTS-4 and -5 genes, either individually or together, reduced aggrecan loss in both cytokine-stimulated normal human cartilage and unstimulated OA cartilage(54), suggesting blocking either one or both aggrecanases may achieve therapeutic benefits in OA.

Aggrecanases are synthesized as latent enzymes, whose prodomains keep the enzymes from performing their biological activities. Proprotein convertase, including PC1/3, PC2, PC4,

PC5/6, PC7, PACE4 and furin, are subtilisin-like serine proteases that are capable of cleaving and activating many precursor enzymes. Among them, furin, PACE4 and PAC5/6 have been shown to be involved in the activation of aggrecanases (55-57).

1.6 Glucocorticoids

Glucocorticoids (GC) are hormones that are naturally produced in the body in response to stress and they play essential roles in maintaining metabolism in various tissues. Synthetic GCs are commonly used for the treatment of a wide variety of inflammatory and autoimmune diseases. Intra-articular injection of GC is an established procedure for both OA and rheumatoid arthritis (RA) (58, 59). Most studies and trials reported beneficial responses, including significantly greater reduction of pain and tenderness, and increased motion in the injected joints(60, 61). However, because the mechanism of GCs in cartilage function is not well understood and there have been some anecdotal reports on GC related side effects, the use of GCs in OA treatment remain controversial. It has been noted that the reports describing negative effect of GC often involved either frequent injections or high dosages(62). More careful reviews have shown the efficacy of GC is dependent on the concentration used. For example, humans daily produce on average 100nM to 700nM cortisol. In vivo administration exceeds this range often causes side effects, such as osteoporosis(6). In order to avoid complications, longer intervals between GC injections (6-12sweeks) and no more than 2 to 3 injections per year for weight-bearing joints have been recommended(7).

GCs exert their effect by binding to intracellular glucocorticoid receptors (GRs). GRs are nuclear hormone receptors, once activated they serve as transcription factors. Without

ligands, GRs are sequestered in the cytoplasm by a chaperone complex, which is composed of heat shock protein 90 and other inhibitory proteins. Upon activation, GRs translocate into the nucleus, dimerize and bind to DNA at glucocorticoid response elements (GRE). The binding of GR to GRE domain may either positively or negatively regulate gene transcriptions. GR may also interact directly with other transcription factors, such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1), to physically block them from initiating transcription. It has been shown that the association of GR and the transcription factors can inhibit the expression of a variety of inflammatory genes, including IL-1,-6, -8, TNFα, iNOS and COX-2.

Dexamethasone (DEX) is a potent synthetic GC. It has much higher receptor binding affinity than many other steroids (63). DEX has been commonly used in cartilage tissue engineering since studies have demonstrated DEX is required for progenitor cells to undergo chondrogenic differentiation and to stimulate chondrocytes to synthesize proteoglycans (64-66). However, the effects of DEX on cartilage matrix degradations and repair, particularly those following joint injury, remain to be elucidated.

1.7 Thesis Objectives

Injury-induced cartilage damage and cytokine release contribute to the pathogenesis of OA. The goal of this thesis was to characterize the combined effects of pro-inflammatory cytokines and mechanical injury on matrix degradation and chondrocyte gene expression, and to investigate whether cartilage degradation could be inhibited by potential therapeutic approaches.

The objectives of Chapter 2 were to examine the combined effect of TNFα, IL-6/sIL-6R

and mechanical injury on cartilage matrix degradation and biosynthesis, in both bovine and human cartilage tissues. Additionally, we examined the role of endogenous IL-6 in proteoglycans degradation by using an IL-6-blocking antibody.

In Chapter 3, we measured the effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression.

In Chapter 4, we tested the hypothesis that short-term glucocorticoid treatment could abolish cartilage degradation induced by the in vitro models of cartilage injury. We examined the effect of dexamethasone in both cartilage matrix degradation and chondrocyte gene expression.

Chapter 5 summarizes the major findings and conclusions from each of the studies.

1.8 References

1. Bitton R. The economic burden of osteoarthritis. Am J Manag Care 2009;15(8 Suppl):S230-5.
2. Altman RD, Moskowitz R. Intraarticular sodium hyaluronate (Hyalgan) in the treatment of patients with osteoarthritis of the knee: a randomized clinical trial. Hyalgan Study Group. J Rheumatol 1998;25(11):2203-12.
3. Hepper CT, Halvorson JJ, Duncan ST, Gregory AJ, Dunn WR, Spindler KP. The efficacy and duration of intra-articular corticosteroid injection for knee osteoarthritis: a systematic review of level I studies. J Am Acad Orthop Surg 2009;17(10):638-46.
4. Kontovazenitis PI, Starantzis KA, Soucacos PN. Major complication following minor outpatient procedure: osteonecrosis of the knee after intraarticular injection of cortisone for treatment of knee arthritis. J Surg Orthop Adv 2009;18(1):42-4.
5. Lukert BP, Raisz LG. Glucocorticoid-induced osteoporosis: pathogenesis and management. Ann Intern Med 1990;112(5):352-64.
6. Ishida Y, Heersche JN. Glucocorticoid-induced osteoporosis: both in vivo and in vitro concentrations of glucocorticoids higher than physiological levels attenuate osteoblast differentiation. J Bone Miner Res 1998;13(12):1822-6.
7. Moskowitz R, Altman, R., Hochberg, MC., Buckwalter, JA., Goldberg, VM. Osteoarthritis, Diagnosis and Medical/Surgical Management. Fourth Edition. Philadelphia, U.S.A: Lippincott Williams & Wilkins.
8. Davis MA, Ettinger WH, Neuhaus JM, Cho SA, Hauck WW. The association of knee injury and obesity with unilateral and bilateral osteoarthritis of the knee. Am J Epidemiol 1989;130(2):278-88.
9. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, et al. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000;133(8):635-46.
10. Roos H, Adalberth T, Dahlberg L, Lohmander LS. Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthritis Cartilage 1995;3(4):261-7.
11. Lohmander LS, Atley LM, Pietka TA, Eyre DR. The release of crosslinked peptides from type II collagen into human synovial fluid is increased soon after joint injury and in osteoarthritis. Arthritis Rheum 2003;48(11):3130-9.
12. Lohmander LS, Hoerrner LA, Lark MW. Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. Arthritis Rheum 1993;36(2):181-9.
13. Irie K, Uchiyama E, Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. Knee 2003;10(1):93-6.
14. Marks PH, Donaldson ML. Inflammatory cytokine profiles associated with chondral damage in the anterior cruciate ligament-deficient knee. Arthroscopy 2005;21(11):1342-7.
15. Johnson DL, Urban WP, Jr., Caborn DN, Vanarthos WJ, Carlson CS. Articular cartilage changes seen with magnetic resonance imaging-detected bone bruises associated with acute anterior cruciate ligament rupture. Am J Sports Med 1998;26(3):409-14.

16. Lohmander LS, Roos H. Knee ligament injury, surgery and osteoarthrosis. Truth or consequences? Acta Orthop Scand 1994;65(6):605-9.
17. Kurz B, Jin M, Patwari P, Cheng DM, Lark MW, Grodzinsky AJ. Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orthop Res 2001;19(6):1140-6.
18. Torzilli PA, Grigiene R, Borrelli J, Jr., Helfet DL. Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. J Biomech Eng 1999;121(5):433-41.
19. DiMicco MA, Patwari P, Siparsky PN, Kumar S, Pratta MA, Lark MW, et al. Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury. Arthritis Rheum 2004;50(3):840-8.
20. Lee JH, Fitzgerald JB, Dimicco MA, Grodzinsky AJ. Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. Arthritis Rheum 2005;52(8):2386-95.
21. Blain EJ, Gilbert SJ, Wardale RJ, Capper SJ, Mason DJ, Duance VC. Up-regulation of matrix metalloproteinase expression and activation following cyclical compressive loading of articular cartilage in vitro. Arch Biochem Biophys 2001;396(1):49-55.
22. Steinmeyer J, Knue S. The proteoglycan metabolism of mature bovine articular cartilage explants superimposed to continuously applied cyclic mechanical loading. Biochem Biophys Res Commun 1997;240(1):216-21.
23. Chen CT, Burton-Wurster N, Lust G, Bank RA, Tekoppele JM. Compositional and metabolic changes in damaged cartilage are peak-stress, stress-rate, and loading-duration dependent. J Orthop Res 1999;17(6):870-9.
24. Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. The extent of matrix damage and chondrocyte death in mechanically traumatized articular cartilage explants depends on rate of loading. J Orthop Res 2001;19(5):779-84.
25. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Jr., Lotz MK. Impact of mechanical trauma on matrix and cells. Clin Orthop Relat Res 2001(391 Suppl):S90-9.
26. Quinn TM, Grodzinsky AJ, Hunziker EB, Sandy JD. Effects of injurious compression on matrix turnover around individual cells in calf articular cartilage explants. J Orthop Res 1998;16(4):490-9.
27. Thibault M, Poole AR, Buschmann MD. Cyclic compression of cartilage/bone explants in vitro leads to physical weakening, mechanical breakdown of collagen and release of matrix fragments. J Orthop Res 2002;20(6):1265-73.
28. Farquhar T, Xia Y, Mann K, Bertram J, Burton-Wurster N, Jelinski L, et al. Swelling and fibronectin accumulation in articular cartilage explants after cyclical impact. J Orthop Res 1996;14(3):417-23.
29. Chen CT, Burton-Wurster N, Borden C, Hueffer K, Bloom SE, Lust G. Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. J Orthop Res 2001;19(4):703-11.
30. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, et al. Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 2000;381(2):205-12.
31. Lucchinetti E, Adams CS, Horton WE, Jr., Torzilli PA. Cartilage viability after repetitive loading: a preliminary report. Osteoarthritis Cartilage 2002;10(1):71-81.

32. Patwari P, Gao G, Lee JH, Grodzinsky AJ, Sandy JD. Analysis of ADAMTS4 and MT4-MMP indicates that both are involved in aggrecanolysis in interleukin-1-treated bovine cartilage. Osteoarthritis Cartilage 2005;13(4):269-77.
33. Patwari P, Cook MN, DiMicco MA, Blake SM, James IE, Kumar S, et al. Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum 2003;48(5):1292-301.
34. Patwari P, Fay J, Cook MN, Badger AM, Kerin AJ, Lark MW, et al. In vitro models for investigation of the effects of acute mechanical injury on cartilage. Clin Orthop Relat Res 2001(391 Suppl):S61-71.
35. Lee JH, Fitzgerald JB, DiMicco MA, Cheng DM, Flannery CR, Sandy JD, et al. Co-culture of mechanically injured cartilage with joint capsule tissue alters chondrocyte expression patterns and increases ADAMTS5 production. Arch Biochem Biophys 2009;489(1-2):118-26.
36. Arner EC, Hughes CE, Decicco CP, Caterson B, Tortorella MD. Cytokine-induced cartilage proteoglycan degradation is mediated by aggrecanase. Osteoarthritis Cartilage 1998;6(3):214-28.
37. Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44(3):585-94.
38. Reboul P, Pelletier JP, Tardif G, Cloutier JM, Martel-Pelletier J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. J Clin Invest 1996;97(9):2011-9.
39. Benton HP, Tyler JA. Inhibition of cartilage proteoglycan synthesis by interleukin I. Biochem Biophys Res Commun 1988;154(1):421-8.
40. Goldring MB, Birkhead J, Sandell LJ, Kimura T, Krane SM. Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J Clin Invest 1988;82(6):2026-37.
41. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146(1):75-85.
42. Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, et al. Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. J Immunol 1991;147(11):3915-20.
43. Palmer RM, Hickery MS, Charles IG, Moncada S, Bayliss MT. Induction of nitric oxide synthase in human chondrocytes. Biochem Biophys Res Commun 1993;193(1):398-405.
44. Boe A, Baiocchi M, Carbonatto M, Papoian R, Serlupi-Crescenzi O. Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis. Cytokine 1999;11(12):1057-64.
45. Lipsky PE. Interleukin-6 and rheumatic diseases. Arthritis Res Ther 2006;8 Suppl 2:S4.
46. van de Loo FA, Kuiper S, van Enckevort FH, Arntz OJ, van den Berg WB. Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. Am J Pathol 1997;151(1):177-91.
47. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. Matrix Biol 2000;19(6):549-53.
48. Cawston TE, Ellis AJ, Humm G, Lean E, Ward D, Curry V. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture. Biochem Biophys Res Commun 1995;215(1):377-85.

49. Pratta MA, Yao W, Decicco C, Tortorella MD, Liu RQ, Copeland RA, et al. Aggrecan protects cartilage collagen from proteolytic cleavage. J Biol Chem 2003;278(46):45539-45.
50. Sandy JD, Neame PJ, Boynton RE, Flannery CR. Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J Biol Chem 1991;266(14):8683-5.
51. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, et al. Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 1999;284(5420):1664-6.
52. Hurskainen TL, Hirohata S, Seldin MF, Apte SS. ADAM-TS5, ADAM-TS6, and ADAM-TS7, novel members of a new family of zinc metalloproteases. General features and genomic distribution of the ADAM-TS family. J Biol Chem 1999;274(36):25555-63.
53. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 2005;434(7033):644-8.
54. Song RH, Tortorella MD, Malfait AM, Alston JT, Yang Z, Arner EC, et al. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum 2007;56(2):575-85.
55. Wang P, Tortorella M, England K, Malfait AM, Thomas G, Arner EC, et al. Proprotein convertase furin interacts with and cleaves pro-ADAMTS4 (Aggrecanase-1) in the trans-Golgi network. J Biol Chem 2004;279(15):15434-40.
56. Tortorella MD, Arner EC, Hills R, Gormley J, Fok K, Pegg L, et al. ADAMTS-4 (aggrecanase-1): N-terminal activation mechanisms. Arch Biochem Biophys 2005;444(1):34-44.
57. Malfait AM, Arner EC, Song RH, Alston JT, Markosyan S, Staten N, et al. Proprotein convertase activation of aggrecanases in cartilage in situ. Arch Biochem Biophys 2008;478(1):43-51.
58. Stein A, Yassouridis A, Szopko C, Helmke K, Stein C. Intraarticular morphine versus dexamethasone in chronic arthritis. Pain 1999;83(3):525-32.
59. Creamer P. Intra-articular corticosteroid treatment in osteoarthritis. Curr Opin Rheumatol 1999;11(5):417-21.
60. Dieppe PA, Sathapatayavongs B, Jones HE, Bacon PA, Ring EF. Intra-articular steroids in osteoarthritis. Rheumatol Rehabil 1980;19(4):212-7.
61. Valtonen EJ. Clinical comparison of triamcinolonehexacetonide and betamethasone in the treatment of osteoarthrosis of the knee-joint. Scand J Rheumatol Suppl 1981;41:1-7.
62. Gibson T, Burry HC, Poswillo D, Glass J. Effect of intra-articular corticosteroid injections on primate cartilage. Ann Rheum Dis 1977;36(1):74-9.
63. Mollmann H, Balbach, S., Hochhaus, G., Barth, J., Derendorf, H. In: Handbook of pharmacokinetic/pharmacodynamic: CRC Press; 1995.
64. Kato Y, Gospodarowicz D. Stimulation by glucocorticoid of the synthesis of cartilage-matrix proteoglycans produced by rabbit costal chondrocytes in vitro. J Biol Chem 1985;260(4):2364-73.
65. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol 1988;106(6):2139-51.

66. Takano T, Takigawa M, Suzuki F. Stimulation by glucocorticoids of the differentiated phenotype of chondrocytes and the proliferation of rabbit costal chondrocytes in culture. J Biochem 1985;97(4):1093-100.

CHAPTER 2

Mechanical Injury Potentiates Proteoglycan Catabolism Induced by Interleukin-6 / Soluble Interleukin-6 Receptor and Tumor Necrosis Factor-α in Immature Bovine and Adult Human Articular Cartilage*

* This chapter has been published in Arthritis and Rheumatism, Vol 60, October 2009, pp 2985–2996.

2.1 Introduction

Osteoarthritis (OA) is a highly prevalent joint disease characterized by progressive degradation and loss of articular cartilage. Joint injury in young adults dramatically increases the risk for developing OA(1, 2). Acute knee injury, such as anterior cruciate ligament tear, can subject cartilage to high mechanical stress, and is accompanied by an increase in synovial fluid levels of matrix metalloproteinase-3 (MMP-3)(3) and multiple pro-inflammatory cytokines including tumor necrosis factor-α (TNF α) and interleukins IL-1β, IL-6, IL-8, and IL-10(4, 5). Additionally, there is increased release to the synovial fluid of aggrecan fragments and type II collagen cross-links(3, 6).

In vitro injury models have helped to understand the contribution of acute damage to cartilage and degeneration commonly associated with OA. Studies using single impact loading(7-10) or prolonged cyclic compression(11-13) have demonstrated loss of proteoglycans and collagen network damage(14-17), increased tissue swelling(8, 18) and reduced cartilage stiffness in compression and shear(7). Injurious compression also causes chondrocyte death(8, 14, 15, 19, 20), lower biosynthetic activity in the remaining viable cells(7), and increased gene expression of cartilage matrix-degrading enzymes(10). However, injury to cartilage, alone, does not appear to involve sustained cell-mediated degradation of matrix(7, 16, 19). Since the pathogenesis of OA is known to involve interactions between different tissues and cell types within a joint, we have studied in vitro models that include interactions between injured cartilage and inflammatory cytokines via direct cytokine treatment(21) or indirectly by co-incubation of injured cartilage with synovial tissues (10, 22).

TNFα is a pro-inflammatory cytokine that induces matrix degradation due partly to aggrecanase activity(23), suppression of proteoglycan synthesis(24), and synovial membrane inflammation. Previous studies showed a synergistic increase in proteoglycan loss from mechanically injured cartilage treated with TNFα in vitro(21). TNFα can also induce production of IL-6(24, 25), a pleiotropic cytokine involved in acute and chronic inflammation(25, 26) and present at high levels in joints of patients with rheumatoid arthritis(26). IL-6 with its soluble receptor (sIL-6R) can potentiate catabolic effects of TNFα in the degradation and loss of proteoglycans from immature bovine cartilage explants(27). IL-6 levels in synovial fluid dramatically increase after joint injury(5).

Given the increasing importance of the interplay between mechanical and cytokine-mediated pathways associated with cartilage degradation following joint injury, our objectives were [1] to characterize the combined effects of TNFα and IL-6/sIL-6R on proteoglycan loss and matrix biosynthesis in immature bovine knee cartilage subjected to injury in vitro; [2] to compare these effects with those observed in adult human knee and ankle articular cartilage, and [3] to test the hypothesis that endogenous IL-6 plays a role in the loss of proteoglycans caused by TNFα and injury. Our use of immature as well as adult articular cartilage was motivated by recent reports showing that acute injury of cartilage and subchondral bone, such as that caused by sports, is also prevalent in the immature knees of young children and adolescents(28, 29). We also hypothesized that adult human knee cartilage would respond to the combined effects of injury and cytokine stimulation very differently from ankle cartilage because of the known differences in the biomechanical properties of knee and ankle cartilage(30) and the previously reported differences in the sensitivity of knee and ankle chondrocytes to cytokine treatment(31).

2.2 Materials And Methods

Bovine cartilage harvest and culture. Cartilage disks were harvested from the femoropatellar grooves of 1-2-week old bovine calves as described(21, 32). Briefly, cartilage-bone cylinders (9mm diameter) from 8 knee joints were cored perpendicular to the surface. After a level surface was obtained by removing the most superficial layer (~100-200 μm), 1-2 sequential 1mm-slices of middle zone cartilage were cut from each cylinder. Five disks (3mm-dia, 1mm-thick) were cored from each slice using a dermal punch. Cartilage from this middle zone in newborn calf was shown to have a reasonably homogeneous population of cells and matrix(32). All treatment groups were matched for location and depth of cartilage on the joint surface. Disks were equilibrated in serum-free medium (low-glucose DMEM (1 g/L) supplemented with 1% insulin-transferrin-selenium (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, Sigma), 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* proline, 20 μg/mL ascorbic acid, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B for 2 days prior to treatment in a 37°C, 5% CO_2 incubator.

Postmortem adult human donor tissue. Three human donor joints (61-yr-old female, modified-Collins(33) grade-1 knee and grade-0 ankle; 59-yr male, grade-1 knee; 26-yr male, grade-0 knee) were obtained from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL), approved by the Office of Research Affairs at Rush-Presbyterian-St. Luke's Medical Center and the Committee on Use of Humans as Experimental Subjects at MIT. Any fibrillated areas detected under visual inspection were excluded from study. Human cartilage harvest and culture were identical to those of bovine, except the following: [1] all human explants included the intact superficial zone and were 0.8 mm thick; [2] human knee cartilage

was obtained from both femoropatellar groove and femoral chondyles, and ankle cartilage was harvested from all surfaces of the talus; [3] culture medium for human tissues contained high-glucose (4.5 g/L) DMEM. Explants were matched for location across treatment groups.

Injurious compression. After equilibration in medium for 2 days, disks were injuriously compressed in a custom-designed incubator-housed apparatus(20, 34). Each bovine disk was subjected to radially unconfined compression to 50% final strain at 1mm/second velocity (100%/second strain rate), followed by immediate release of load at the same rate as described(21). This compression produced peak stresses of 21.5±0.2 MPa (mean±SEM, n=24 disks) directly measured by the instrument's load cell, a value within 5-7% of the peak stress reported in two previous studies using similar compression parameters and tissue source(9, 21). Inspection of cartilage immediately after injury revealed that some disks were deformed to an ellipsoidal shape (deformation score of 1 or 2 as described in (9)), but none exhibited gross fissuring.

Since adult human cartilage disks were thinner and had different biomechanical properties than immature bovine samples, we used a higher strain (60%) and strain rate (300%/second) to achieve similar peak stresses (19.7±1.1 MPa in knee and 20.4±1.3 MPa in ankle cartilage disks). Macroscopic tissue changes were similar (elliptical appearance) to those described previously using our human cartilage injury model(35). After injury, samples were immediately placed in treatment medium.

Exogenous cytokines and injury. A factorial design was used to investigate the effects of exogenous cytokines on injured cartilage using treatment conditions: (1) no-treatment-control, (2) TNFα, (3) injury, (4) [injury+TNFα], (5) [IL-6/sIL-6R], (6) [TNFα +(IL-6/sIL-6R)], (7) [injury+ (IL-6/sIL-6R)] and (8) [injury+TNFα +(IL-6/sIL6R)]. Cartilage was

either subjected to compression or left uninjured and incubated with or without cytokines (all from R&D Systems, MN). rhTNFα was used at 25 ng/mL with bovine and 100 ng/mL with human cartilage, the latter used previously with human cartilage(21, 27, 36). rhIL-6 (50 ng/mL) was used in combination with soluble IL-6 receptor (sIL-6R, 250 ng/mL) for both tissue types, since this combination was found previously to induce greater aggrecan degradation than when used separately in the presence of TNFα(27). Initial studies showed that for each treatment, human cartilage released sGAG somewhat less rapidly than bovine. Therefore, to better resolve differences between conditions for human cartilage, culture duration was extended to 8-days, in contrast to the 6-day treatment for bovine cartilage. Medium was replaced every 2 days and saved for analysis. In additional experiments, bovine disks were either uninjured or subjected to graded levels of final strain (30-60%) in the presence of TNFα (25 ng/mL) and IL-6/sIL-6R (50/250 ng/mL) to test the effect of compression-dose on sulfated glycosaminoglycan (sGAG) loss.

IL-6 blocking Fab fragment treatment. To study the role of endogenous IL-6 in matrix degradation, bovine disks were treated with an anti-IL-6 antigen binding fragment (Fab, 50 μg/mL), obtained by papain digestion of a high-affinity neutralizing anti-IL-6 monoclonal antibody. (After incubation with immobilized papain on a column, Fab was eluted and further purified by anion exchange (QSFF, GE) and size exclusion chromatography (Superdex200, GE)). Disks were pre-equilibrated with the Fab for 6-days to maximize penetration into the tissue. Using a factorial design, disks were either injuriously compressed or left intact, and immediately incubated in the presence or absence of rhTNFα (25 ng/ml) for an additional 6-days, along with continued presence of anti-IL-6 Fab. No exogenous IL-6 was added to any of these conditions.

Matrix biosynthesis. Two days before termination, bovine culture medium was supplemented with 5 μCi/ml $Na_2{}^{35}SO_4$ and 10 μCi/ml L-5-^{3}H-proline as measures of proteoglycan and total protein synthesis rates, respectively. Both concentrations were doubled for human tissue. Upon termination, disks were washed, weighed, solubilized (proteinase K, Roche), and radiolabel incorporation was measured using a liquid scintillation counter(32).

Biochemical, zymography, Western blot and ELISA analyses. Upon termination of each experiment, disks were solubilized (proteinase K, Roche) and the amount of sGAG lost to the medium and that retained in the cartilage was measured using the DMMB assay(37). Matrix metalloproteinase-3 (MMP-3) was analyzed by zymography as described(38); medium collected on days 2, 4 and 6 from the same condition were pooled and resolved on 10% casein zymogram gels. To detect aggrecanase-generated aggrecan G1 domain released to the medium, explant-conditioned medium samples containing equal amounts of sGAG were precipitated (absolute ethanol with 5 m*M* sodium acetate), deglycosylated (chondroitinase ABC, Warner; keratanase II and endobetagalactosidase, Seikagaku), and separated on SDS-PAGE (4-15% gradient gel, Biorad). Western blot analyses were performed using the monoclonal antibody AGG-C1/anti-G1-NITEGE^{373}(39) (kindly provided by C. Flannery, Wyeth) specific to the aggrecanase-generated NITEGE neoepitope in the inter-globular domain of aggrecan. To determine whether endogenous IL-6 was present in the samples treated with TNFα and/or injury, bovine disks were pulverized in liquid nitrogen and homogenized in buffer solution (20 m*M* pH7.6 Tris, 120 m*M* NaCl, 10 m*M* EDTA, 10% glyceral, 1% Nonidet P-40, and protease inhibitor cocktail, Roche). Equal amounts of protein were collected from each condition. Western blots were performed using a mouse monoclonal antibody to bovine IL-6 (GTX75226,

GeneTex). IL-6 in the culture medium of human knee and ankle cartilage was detected via ELISA (R&D Systems).

Histological analyses. Bovine and human knee samples from the various treatment groups were fixed in 2.5% glutaraldehyde (Polysciences) with 0.7% ruthenium hexaammine trichloride (Polysciences) (40). Samples were paraffin-embedded, sectioned (5 μm-thick) and stained with 0.1% toluidine blue (in deionized water, pH 7.1) to visualize sGAGs retained in the cartilage.

Statistical analyses. In studying the effects of cytokines, mechanical injury and their combinations in bovine and human cartilage, three-way analysis of variance (ANOVA) with animal or human donor as a random factor was used to analyze the data, followed by Tukey's post hoc test for comparisons. The same analyses were also used to evaluate the effect of anti-IL6 Fab in the TNFα and injury treated cartilage. The effect of compressive strain/strain rate on sGAG loss in the cytokine-treated bovine cartilage was analyzed by one-way ANOVA, as was the effect of TNFα dose on sGAG loss from human ankle tissue. All data are expressed as mean±SEM, with $p \leq 0.05$ as statistically significant. Statistical analyses were performed using SYSTAT-12 software (Richmond, CA).

2.3 Results

Combined effects of injury and cytokines on GAG loss, proteolytic activity and chondrocyte biosynthesis in bovine explants. Experiments were performed to test for interactions between injury and cytokine treatments on the resulting loss of sGAG (Figure

2.1A). Relative to control samples, which lost 7.1±0.5% of total sGAG over the 6-day period, injury alone or treatment with IL-6/sIL-6R alone did not significantly change sGAG loss (8.7± 0.7% and 7.7±0.3%, respectively), nor did the combination of injury plus IL-6/sIL-6R (8.3±0.3%). In contrast, TNFα significantly elevated sGAG loss to 18.7±1.4%, and the combination of injury plus TNFα further increased sGAG loss to 26.3±3.0%. Treatment with TNFα plus IL-6/sIL-6R induced significantly more sGAG release (32.0±2.5%) than either cytokine alone, consistent with a previous study(27). Importantly, mechanical injury markedly potentiated the combined effects of TNFα plus IL-6/sIL-6R, causing the cartilage samples to lose almost half of their total sGAG content by 6 days (45.5±3.6%). Analysis revealed significant interactions between TNFα and both IL-6/sIL-6R and injury (both $p<0.002$, three-way ANOVA).

Aggrecanases-1,2 (ADAMTS-4,5) cleave aggrecan at Glu^{373}-Ala^{374}, generating aggrecan fragments bearing the c-terminal neoepitope –NITEGE(41). For the treatment conditions of Figure 2.1A, Western blot analysis revealed NITEGE-positive fragments in all conditions involving TNFα, but primarily from TNFα plus IL-6/sIL-6R, both with and without injury (Figure 2.1B). IL-6/sIL-6R and injury, alone or together, were similar to untreated controls. Casein zymography showed that levels of pro-MMP-3 were slightly increased by injury or IL-6/sIL-6R treatments alone (Figure 2.1B). However, marked increases in pro-MMP-3 resulted from all treatments involving TNFα, most pronounced with the combination of TNFα, IL-6/sIL-6R and injury. In all conditions, only latent MMP-3 was observed by zymography.

All bovine disks of Figure 2.1A were radiolabeled to study the effects of treatments on chondrocyte biosynthesis. Compared to no-treatment controls (^{35}S-sulfate: 85.5±5.1

pmol/hr/mg-wet-weight; ^{3}H-proline: 57.0±3.1 pmol/hr/mg), TNFα alone significantly reduced proteoglycan and protein biosynthesis rates by 46% and 42%, respectively (both $p<0.001$, Figure 2.1C). TNFα in combination with injury, IL-6/sIL-6R, or injury+IL-6/sIL-6R also significantly decreased biosynthesis rates (all $p<0.01$).

In separate experiments, bovine disks were treated with TNFα plus IL-6/sIL-6R (as in Figure 2.1A) but also subjected to graded levels of strain. Compression to each different strain value utilized the same 0.5-sec ramp-time and, therefore, applied different values of strain rate (legend, Figure 2.1D). sGAG loss increased in a dose-dependent fashion with compressive load. Analysis (one-way ANOVA followed by Tukey's post hoc comparisons) showed that injury with 50% strain (corresponding to the injury conditions of Figure 2.1A) as well as 60% strain caused significantly more sGAG loss than that from non-injured controls ($p<0.02$).

Finally, we note that the experiments of Figures.2.1A,C were repeated in entirety two additional times, thereby using samples from a total of three different animals; analysis (general linear model) showed that all trends in sGAG loss and proteoglycan synthesis were independent of animal ($p=0.11$ and $p=0.51$, respectively).

Effects of injury and cytokines on GAG loss and biosynthesis in human explants. Cartilage disks having intact superficial zone, taken from the knee and ankle of the same human donor (61-yr-old female, Collins grade-1 knee and grade-0 ankle), were used in examining the combined effects of cytokines and mechanical injury in adult human cartilage. In an initial study, incubation of sets of uninjured ankle cartilage disks with 0, 0.5, 2, 10, 50, 100, and 250 ng/mL TNFα produced a dose-dependent increase in sGAG-loss to the medium by 8-days of culture (Figure 2S.9), with a significant difference between 0 and 100 ng/mL (~2-fold,

p=0.05 using one-way ANOVA followed by Tukey's test for comparisons). These results motivated the use of 100 ng/mL TNFα in subsequent experiments involving human cartilage.

The patterns for relative sGAG loss following injury and cytokine treatments of adult human knee and ankle disks (Figure 2.2A,B) were very similar to those exhibited by newborn bovine explants (Figure 2.1A). In both types of human cartilage, 8-days of combined treatment with IL-6/sIL-6R and TNFα induced more sGAG loss than treatment with TNFα alone (knee: 32.4 ± 4.8% vs. 23.6 ± 2.5% (Figure 2.2A); ankle: 19.0 ± 0.9% vs. 14.8 ± 1.2% (Figure 2.2B)). The combination of injury, TNFα and IL-6/sIL-6R caused the most severe sGAG loss compared to other treatment groups (knee: 42.1±6.6%; ankle: 22.7±2.9%). Analysis of the ankle data (Figure 2.2B) using three-way ANOVA showed a significant interaction between TNFα and IL-6/sIL-6R (p=0.003) and a main effect of mechanical injury (p=0.001). In human knee tissue, both TNFα and IL-6/sIL-6R had main effects on sGAG loss ($p \leq 0.03$); however, their interaction did not reach significance (p=0.09). The most striking difference between knee and ankle cartilage was that ankle explants showed only half the sGAG loss of knee explants following 8-days of identical treatments. The experiment of Figure 2.2A was repeated in entirety using two additional human knees (59-yr male, grade-1; 26-yr male, grade-0); the patterns of sGAG loss (Figure 2S7, 8) were similar to those of Figure 2.2A. Once again, the combination of injury, TNFα and IL-6/sIL-6R caused the most sGAG loss among all the treatment groups (26.7±5.8% and 35.2±4.5% for the 59 and 26 yr-old, respectively, compared to 45.5±3.6% sGAG loss from bovine disks).

Human knee and ankle disks of Figure 2.2A,B were radiolabeled to study the effects of treatments on chondrocyte biosynthesis. Compared to untreated controls, TNFα alone and in combination with injury, IL-6/sIL-6R, and injury+IL-6/sIL-6R decreased proteoglycan and

protein biosynthesis rates in both knee and ankle explants (Figure 2.2C,D). Three-way ANOVA analysis showed a significant interaction between TNFα and IL-6/sIL-6R in both knee and ankle ($p<0.01$).

Histology. Light microscopy of toluidine blue-stained disks was used to obtain a qualitative visualization of the spatial distribution of sGAG remaining within bovine and human knee tissue after the treatment conditions of Figs.2.1A and 2.2A. Axial cross-sections of untreated bovine control disks exhibited uniform spatial distributions of cells and toluidine blue-stained sGAG (Figure 2.3A), consistent with previous studies of this middle-zone newborn tissue(16). In contrast, cytokine and injury treatments caused highly nonuniform loss of staining, which initiated at the sample surfaces and progressed towards the center with time (Figure 2.3). Following TNFα treatment with or without injury (Figure 2.3B,D), loss of staining was noticeable at disk surfaces by day 6. Treatment with TNFα plus IL-6/sIL-6R caused additional loss of staining from the periphery (Figure 2.3C). Combined treatment with injury, TNFα and IL-6/sIL-6R caused the most severe loss of staining, seen as early as day 2 (Figure 2.3E); by day 6, about half of the tissue was free of sGAG, though the innermost region within the disks appeared intact (Figure 2.3F).

Normal adult human articular cartilage is known to have lower GAG content in the superficial zone compared to the middle zone(42), as in Figure 2.4A for control knee tissue with intact superficial zone. With treatments, loss of stain progressed mainly from the superficial region (Figs.2.4B-E). Combined treatment with injury, TNFα and IL-6/sIL-6R caused almost complete loss of stain, suggesting that the remaining GAG in this human tissue was too low to be detected by this colorimetric method. Occasional fissures caused by mechanical injury were also apparent (Figure 2.4B,E,F).

Endogenous IL-6 production. IL-6 was not detected by ELISA in the culture medium of untreated or mechanically injured human cartilage (Figure 2.5A). However, treatment with TNFα and the combination of TNFα plus injury caused release of endogenous IL-6 in both human knee and ankle cartilage. Upon normalizing the amount of IL-6 released to the medium by the volume of the explants, we estimated that the freely diffusible IL-6 content inside these human samples following TNFα± injury treatment was in the range of 50 ng/mL, within the range of that measured in human knee joint synovial fluid after joint injury in vivo(5). Western analysis of bovine tissue extracts also revealed endogenous IL-6 production following treatment with TNFα and TNFα plus injury (Figure 2.5B).

Pretreatment of bovine cartilage with Anti-IL-6 Fab fragment. To test the hypothesis that endogenous IL-6 was involved in proteoglycan catabolism induced by combined treatment with injury and TNFα, bovine disks were pretreated for 6-days with an anti-IL-6 antigen binding Fab fragment and then subjected to injury,TNFα, or combined injury plus TNFα. Pretreatment with IL-6-blocking Fab did not affect chondrocyte biosynthesis. The Fab had no effect on sGAG loss after treatment with injury or TNFα alone. However, the anti-IL-6 Fab significantly reduced sGAG loss caused by the combination of injury plus TNFα by day 2 of treatment (Figure 2.6, $p<0.001$). In similar experiments using human knee cartilage, the anti-IL-6 Fab reduced sGAG loss caused by the combination of injury plus TNFα by ~20%, though this trend did not reach statistical significance (Figure 2S.10).

2.4 Discussion

Using our model of injurious compression, we first demonstrated that mechanical injury, in a dose dependent manner, potentiated the combined effects of TNFα and IL-6/sIL-6R on GAG loss from immature bovine cartilage. Flannery et al.(27) demonstrated that aggrecanase- (and not MMP-) mediated aggrecan catabolism was caused by the combined effects of IL-6/sIL-6R and TNFα in immature bovine cartilage; our observation of NITEGE-positive aggrecan fragments is also consistent with the presence of aggrecanase activity. We then found that injury also potentiated the combined effects of TNFα and IL-6/sIL-6R on GAG loss from adult human knee and ankle cartilages with trends strikingly similar to those with bovine cartilage, though the extent and rate of loss were somewhat less with human cartilage. We also showed that blocking endogenous IL-6 activity significantly decreased the loss of GAG induced by incubation of injured bovine cartilage with TNFα. These results are significant in that they provide evidence suggesting a possible therapeutic strategy to ameliorate cartilage degradation produced by the synergistic effects of mechanical forces and interacting cytokines that originate both within (e.g., IL-6) and external (e,g., TNFα) to cartilage. In this regard, IL-6 signaling has recently been identified as a therapeutic target in rheumatic diseases(26).

Rowan et al. demonstrated that IL-6, only in the presence of its soluble receptor, synergized with IL-1α to induce chondrocyte-mediated degradation of collagen from bovine nasal cartilage(43). Flannery et al. showed that treatment of bovine cartilage with either IL-1α or TNFα in the presence of IL-6/sIL-6R caused more GAG release than treatment with IL-1α or TNFα alone(27), consistent with Figure 2.1A. Patwari et al. found that incubation of injured

bovine or human knee cartilage with IL-1α or TNFα caused a synergistic increase in the loss of GAG(21, 44), also consistent with Figures.2.1A, 2A. The present study extends these results further to examine the combined effects of injury, TNFα, and IL-6/sIL-6R. While the mechanism responsible for the synergistic action of injury and pro-inflammatory cytokines is not certain, previous studies showed the modest loss of GAG observed during the initial 24-hour period after mechanical injury, alone, was due to mechanical microdamage of the matrix and not to cell-mediated proteolysis(45), since inhibitors of aggrecanases, MMPs, and cell biosynthesis could not reduce this immediate GAG loss(9). However, injury dramatically upregulated gene expression of ADAMTS-5, and the observed release of GAG during the 2-week period following injury, alone, was associated in part with aggrecanase activity (10). In addition, mechanical microdamage to the matrix can increase tissue permeability to cytokines, which may greatly facilitate the synergistic action of these cytokines with mechanical injury.

TNFα, both with and without injury, dramatically increased chondrocyte synthesis of endogenous IL-6 (Figure 2.5); this finding is consistent with previous studies showing that IL-1 and TNFα highly increase the production of IL-6(46). To test whether endogenous IL-6 plays a role in proteoglycan degradation caused by injury plus TNFα, we treated explants with an anti-IL-6 Fab fragment, and found that loss of GAG was significantly decreased (Figure 2.6). However, the anti-IL-6 Fab did not completely reverse GAG loss, suggesting the possibility of incomplete penetration of the anti-IL-6 Fab uniformly within the explant, or that additional mechanisms may be associated with the combined action of TNFα plus injury. It is also interesting to speculate that this partial inhibition by an exogenous IL-6 blocker may exemplify situations in which an anti-ligand blocker may be less effective than an anti-receptor blocker (i.e., an IL-6 receptor blocker) in inhibiting autocrine signaling, as has been demonstrated

previously for spatially localized autocrine loops involving binding of ligands to the EGF-receptors of B82 mouse fibroblasts(47). Recent studies of Vincent et al.(48) offered further examples of the association between cutting or compressive loading of cartilage and follow-on signaling loops initiated through the interplay of mechanical and growth factor stimulation.

The potentiating effects of mechanical injury were clearly demonstrable in adult human knee and ankle cartilages. Mechanical injury combined with TNFα and IL-6/sIL-6R caused the most increase in GAG loss. This combined effect was much less for ankle compared to knee cartilage (Figure 2.2), consistent with previous observations that, a) ankle cartilage does not exhibit the same extent of mechanical damage as knee cartilage for the mechanical injury parameters used here(35), and b) chondrocyte biosynthesis in ankle cartilage does not decrease as much as that in knee cartilage when subjected to cytokines such as IL-1(31).

In the present study, cartilage samples with and without intact superficial zone exhibited certain trends that were similar (Figures 2.1, 2.2). However, other aspects of chondrocyte response to injury may be very different in superficial versus deeper layers, such as the marked up-regulation of lubricin reported in the superficial but not middle zone of similar bovine tissue (49). Additionally, the local strains in the most superficial region of human cartilage samples could be much higher than that in the middle-zone bovine samples because of the highly compliant region just below the intact surface zone (50).

In summary, we have used an in vitro model of joint injury involving the combined interactions of injurious compression of cartilage in the presence of inflammatory cytokines. IL-6/sIL-6R synergizes with TNFα to cause aggrecan degradation and their combined catabolic effects are markedly potentiated by mechanical injury. The kinetics of cartilage degradation are not solely a consequence of the activities of proteolytic enzymes, but also depend on the

transport of cytokines, proteases, and other cartilage biomolecules, all of which may be altered by overload injury, thus making the damaged cartilage more susceptible to the further insults of inflammatory mediators. This interplay between mechanical damage, macromolecular transport, and cytokine ligand-receptor interactions highlights the challenges in targeting cytokine blockade within cartilage as a means to ameliorate the consequences of injury to synovial joints.

2.5 Figures

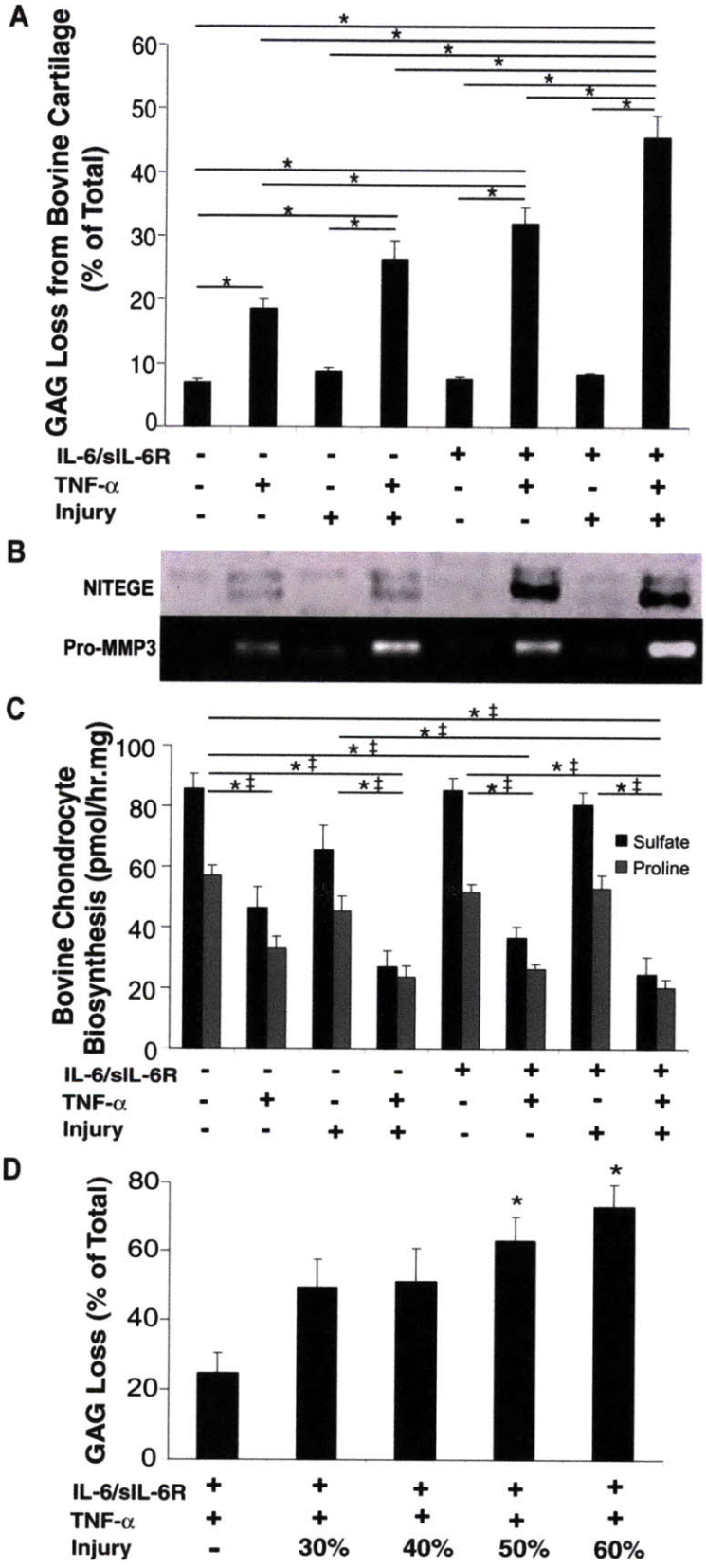

A
GAG Loss from Bovine Cartilage (% of Total)
60
50
40
30
20
10
0
IL-6/sIL-6R - - - - + + + +
TNF-α - + - + - + - +
Injury - - + + - - + +
B
NITEGE
Pro-MMP3
C
Bovine Chondrocyte Biosynthesis (pmol/hr.mg)
100
80
60
40
20
0
Sulfate
Proline
IL-6/sIL-6R - - - - + + + +
TNF-α - + - + - + - +
Injury - - + + - - + +
D
GAG Loss (% of Total)
80
60
40
20
0
IL-6/sIL-6R + + + + +
TNF-α + + + + +
Injury - 30% 40% 50% 60%

Figure 2.1 **A,** Percentage of GAG loss from bovine cartilage in response to treatments with TNFα, IL-6/sIL-6R and mechanical injury. The total GAG content was 550.1± 18.6 μg GAG/disk in the untreated control group. Mechanical injury significantly increased GAG loss induced by the combined treatment of TNFα and IL-6/sIL-6R. *$p<0.05$. **B,** Western blot for aggrecanase-generated aggrecan fragments with NITEGE neoepitope and zymography for MMP-3. **C,** Chondrocyte biosynthetic rates measured by ^{3}H-proline and ^{35}S-sulfate incorporations during days 4-6. TNFα, either alone or with additional treatment(s), significantly reduced chondrocyte biosynthetic activities. *$p<0.05$ for sulfate incorporation; ‡ $p<0.05$ for proline incorporation. **D,** Bovine samples treated with cytokines and subjected to graded levels of compressive loads. Final strain at 30%, 40%, 50% and 60% correspond to 0.6, 0.8, 1, 1.2/second strain rate, respectively. The corresponding peak stresses were 9.3 ± 1.0 MPa, 15.1 ± 1.9 MPa, 20.5 ± 3.2 MPa and 28.0 ± 1.9 MPa. Values are mean±SEM. *$p<0.05$. In **A** and **C,** n=6 cartilage samples per treatment group; in **D,** n=5.

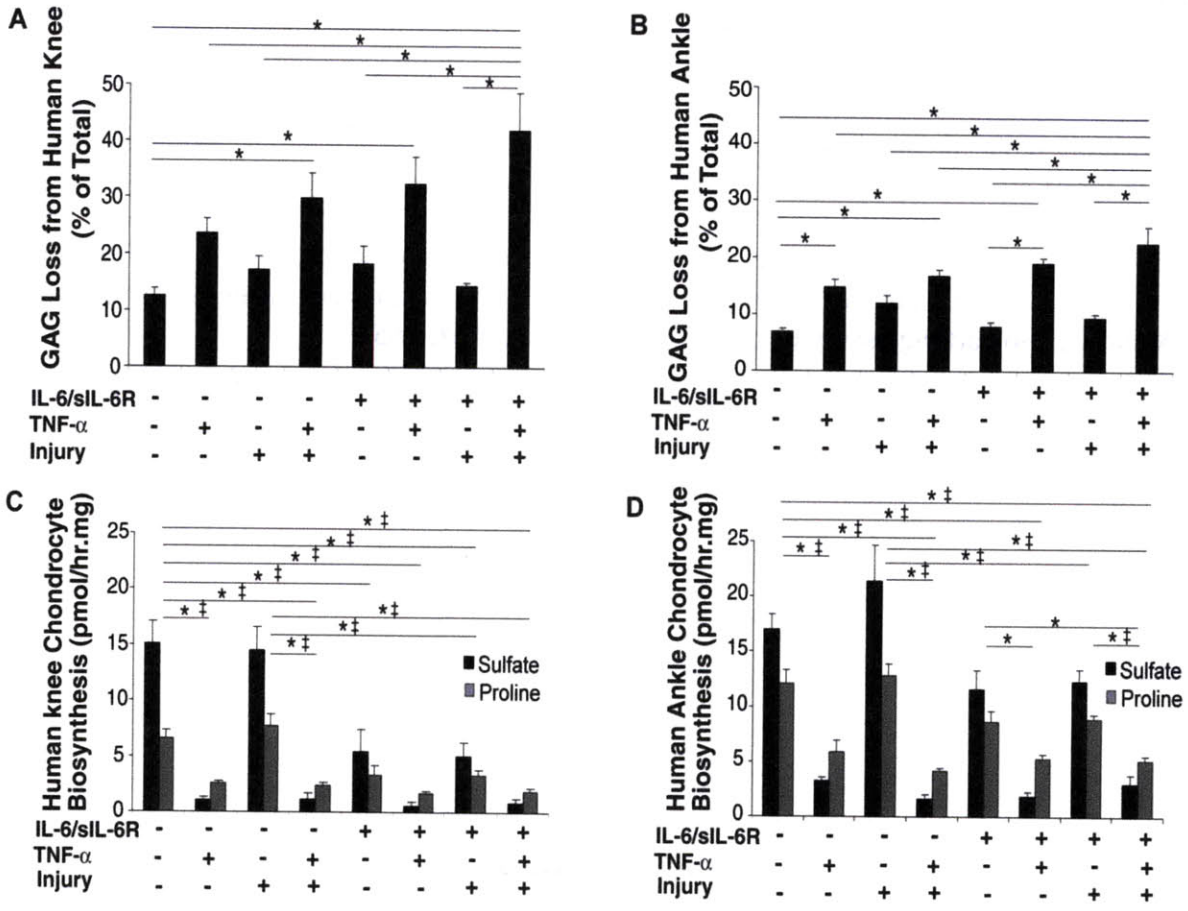


Figure 2.2 Percentage of GAG loss from **A,** human knee and **B,** human ankle cartilage in response to 8-day treatments. In the untreated control group, the total GAG content was 179.0 ± 38.1μg GAG/disk in the knee and 286.4 ± 34.6 μg GAG/disk in the ankle. *$p< 0.05$. Chondrocyte biosynthesis measured in **C,** human knee and **D,** ankle during days 6-8. *$p< 0.05$ for sulfate incorporation, and ‡$p<0.05$ for proline incorporation. Values are mean±SEM, n=6 per treatment group in **A** and **C**; n=5 in **B** and **D**.

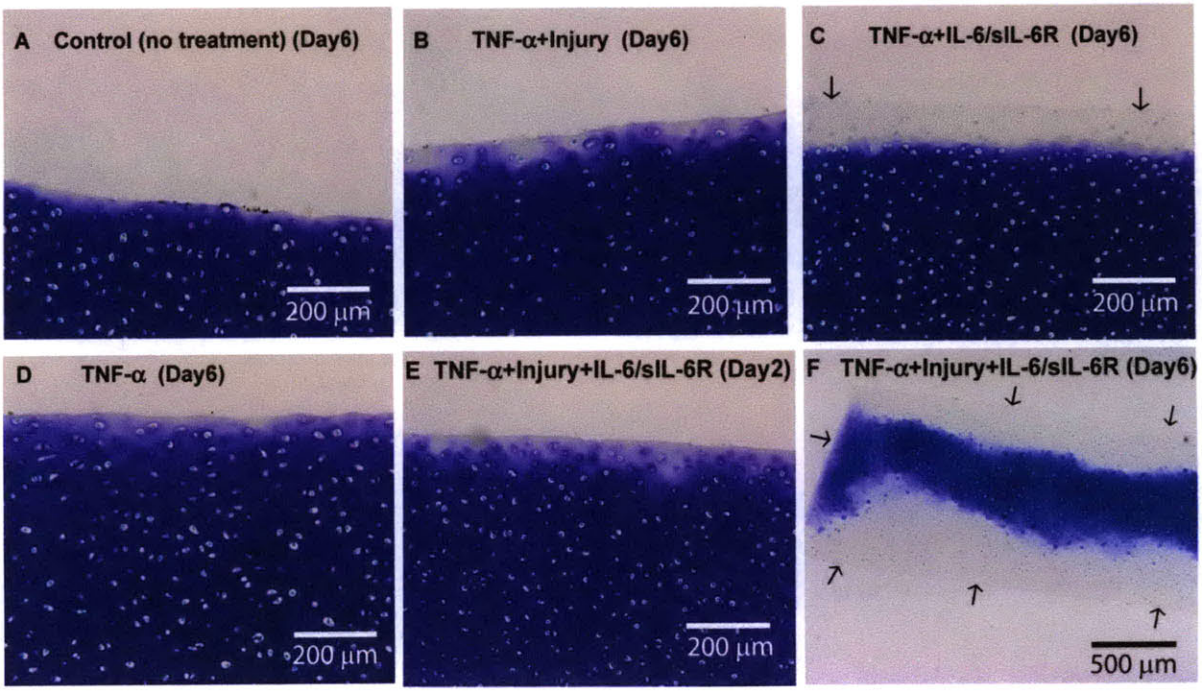


Figure 2.3 **A-F,** Histology with toluidine blue staining for sGAG in disks of middle zone bovine cartilage in response to indicated treatments. sGAG loss was initiated at the disk periphery and progressed towards the center with time. The most severe progression of sGAG loss, **F,** was due to the combination of mechanical injury, TNFα and IL-6/sIL-6R. Arrows indicate the edges of samples.

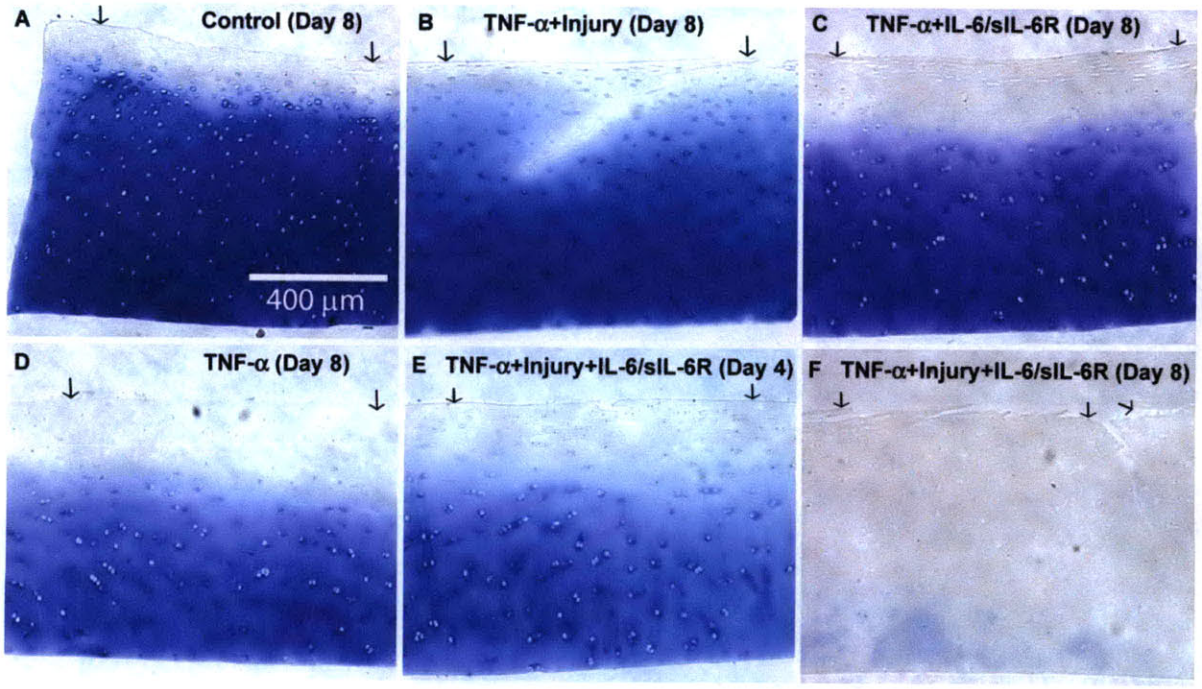


Figure 2.4 **A-F,** Histology with toluidine blue staining for sGAG in 0.8 mm disks of human knee cartilage having intact superficial zone in response to indicated treatments. The superficial zone of each cartilage explant is positioned at the top of each image. sGAG loss progressed mainly from the superficial region inward with time. Fissures caused by mechanical injury were sometimes visible (e.g., **B, E and F**). Combined treatment with injury, TNFα and IL-6/sIL-6R induced substantial sGAG loss; the remaining sGAG content was too low to be detected by histology. Arrows indicate the superficial zone surface of explants.

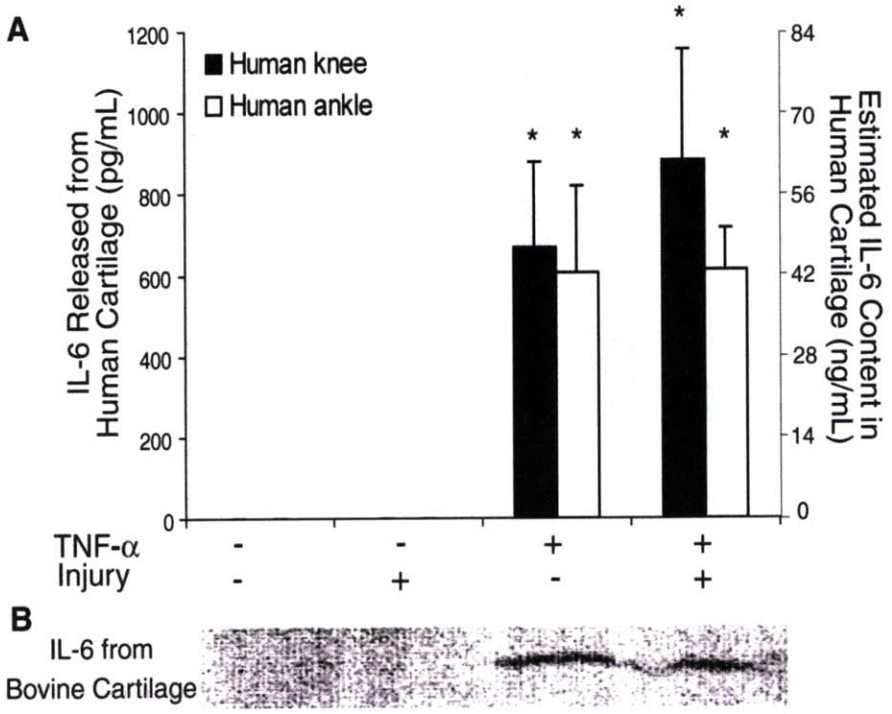


Figure 2.5 **A,** IL-6 released to the medium from human knee and ankle cartilage explants in response to TNFα and injury treatments, as measured by ELISA. Right-hand axis shows concentration of releasable IL-6 within the cartilage, based on normalization of medium concentration (left-hand axis) to cartilage explant volume. *p<0.001 vs. both no-treatment-control and injury-alone. Values are mean±SEM,n=4,5. **B,** Western blot detecting endogenous IL-6 in bovine cartilage explants. TNFα and the combined treatment with TNFα and mechanical injury induced the production of endogenous IL-6 in both human and bovine cartilages.

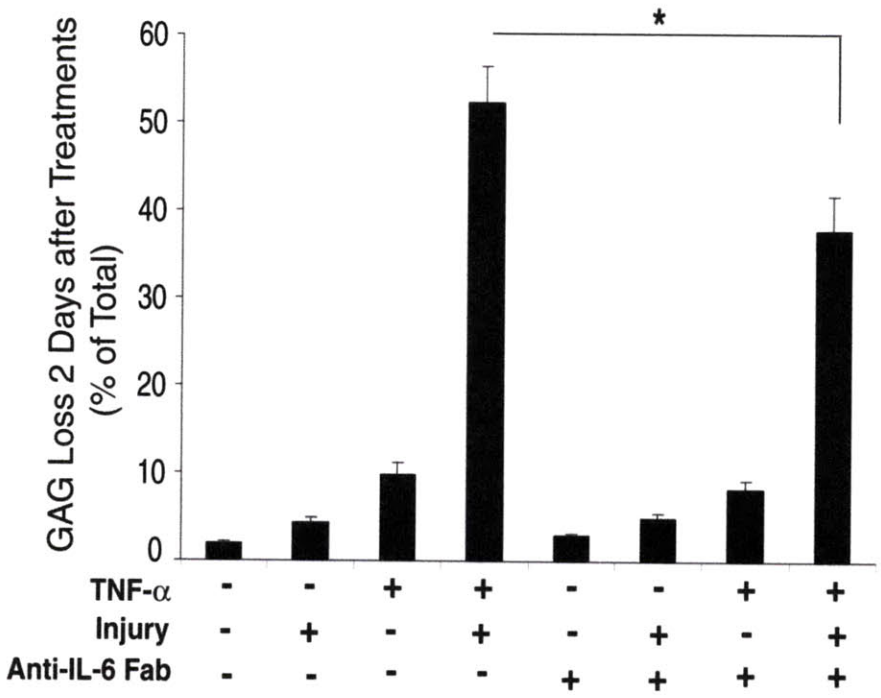


Figure 2.6 Effect of anti-IL-6 Fab on sGAG loss in response to 2-day treatment with TNFα and/or mechanical injury. Blocking endogenous IL-6 significantly decreased sGAG loss induced by the combination of TNFα and mechanical injury in bovine cartilage. Values are mean±SEM, n=4 cartilage disks per treatment group. *$p<0.001$ by Tukey's test following 3-way ANOVA (only the comparison from the selected hypothesis is shown).

2.6 References

1. Roos H, Adalberth T, Dahlberg L, Lohmander LS. Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthritis Cartilage 1995;3(4):261-7.
2. Gelber AC, Hochberg MC, Mead LA, Wang NY, Wigley FM, Klag MJ. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. Ann Intern Med 2000;133(5):321-8.
3. Lohmander LS, Roos H, Dahlberg L, Hoerrner LA, Lark MW. Temporal patterns of stromelysin-1, tissue inhibitor, and proteoglycan fragments in human knee joint fluid after injury to the cruciate ligament or meniscus. J Orthop Res 1994;12(1):21-8.
4. Higuchi H, Shirakura K, Kimura M, Terauchi M, Shinozaki T, Watanabe H, et al. Changes in biochemical parameters after anterior cruciate ligament injury. Int Orthop 2006;30(1):43-7.
5. Irie K, Uchiyama E, Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. Knee 2003;10(1):93-6.
6. Lohmander LS, Atley LM, Pietka TA, Eyre DR. The release of crosslinked peptides from type II collagen into human synovial fluid is increased soon after joint injury and in osteoarthritis. Arthritis Rheum 2003;48(11):3130-9.
7. Kurz B, Jin M, Patwari P, Cheng DM, Lark MW, Grodzinsky AJ. Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orthop Res 2001;19(6):1140-6.
8. Torzilli PA, Grigiene R, Borrelli J, Jr., Helfet DL. Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. J Biomech Eng 1999;121(5):433-41.
9. DiMicco MA, Patwari P, Siparsky PN, Kumar S, Pratta MA, Lark MW, et al. Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury. Arthritis Rheum 2004;50(3):840-8.
10. Lee JH, Fitzgerald JB, Dimicco MA, Grodzinsky AJ. Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. Arthritis Rheum 2005;52(8):2386-95.
11. Blain EJ, Gilbert SJ, Wardale RJ, Capper SJ, Mason DJ, Duance VC. Up-regulation of matrix metalloproteinase expression and activation following cyclical compressive loading of articular cartilage in vitro. Arch Biochem Biophys 2001;396(1):49-55.
12. Steinmeyer J, Knue S. The proteoglycan metabolism of mature bovine articular cartilage explants superimposed to continuously applied cyclic mechanical loading. Biochem Biophys Res Commun 1997;240(1):216-21.
13. Chen CT, Burton-Wurster N, Lust G, Bank RA, Tekoppele JM. Compositional and metabolic changes in damaged cartilage are peak-stress, stress-rate, and loading-duration dependent. J Orthop Res 1999;17(6):870-9.
14. Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. The extent of matrix damage and chondrocyte death in mechanically traumatized articular cartilage explants depends on rate of loading. J Orthop Res 2001;19(5):779-84.

15. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Jr., Lotz MK. Impact of mechanical trauma on matrix and cells. Clin Orthop Relat Res 2001(391 Suppl):S90-9.
16. Quinn TM, Grodzinsky AJ, Hunziker EB, Sandy JD. Effects of injurious compression on matrix turnover around individual cells in calf articular cartilage explants. J Orthop Res 1998;16(4):490-9.
17. Thibault M, Poole AR, Buschmann MD. Cyclic compression of cartilage/bone explants in vitro leads to physical weakening, mechanical breakdown of collagen and release of matrix fragments. J Orthop Res 2002;20(6):1265-73.
18. Farquhar T, Xia Y, Mann K, Bertram J, Burton-Wurster N, Jelinski L, et al. Swelling and fibronectin accumulation in articular cartilage explants after cyclical impact. J Orthop Res 1996;14(3):417-23.
19. Chen CT, Burton-Wurster N, Borden C, Hueffer K, Bloom SE, Lust G. Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. J Orthop Res 2001;19(4):703-11.
20. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, et al. Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 2000;381(2):205-12.
21. Patwari P, Cook MN, DiMicco MA, Blake SM, James IE, Kumar S, et al. Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum 2003;48(5):1292-301.
22. Vankemmelbeke MN, Ilic MZ, Handley CJ, Knight CG, Buttle DJ. Coincubation of bovine synovial or capsular tissue with cartilage generates a soluble "Aggrecanase" activity. Biochem Biophys Res Commun 1999;255(3):686-91.
23. Buttle DJ, Fowles A, Ilic MZ, Handley CJ. "Aggrecanase" activity is implicated in tumour necrosis factor alpha mediated cartilage aggrecan breakdown but is not detected by an in vitro assay. Mol Pathol 1997;50(3):153-9.
24. Wilbrink B, Nietfeld JJ, den Otter W, van Roy JL, Bijlsma JW, Huber-Bruning O. Role of TNF alpha, in relation to IL-1 and IL-6 in the proteoglycan turnover of human articular cartilage. Br J Rheumatol 1991;30(4):265-71.
25. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther 2006;8 Suppl 2:S3.
26. Lipsky PE. Interleukin-6 and rheumatic diseases. Arthritis Res Ther 2006;8 Suppl 2:S4.
27. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. Matrix Biol 2000;19(6):549-53.
28. Oeppen RS, Connolly SA, Bencardino JT, Jaramillo D. Acute injury of the articular cartilage and subchondral bone: a common but unrecognized lesion in the immature knee. AJR Am J Roentgenol 2004;182(1):111-7.
29. Kingma J, Ten Duis HJ. Injuries due to school sports accidents in 4 to 13-yr.-old children. Percept Mot Skills 2000;90(1):319-25.
30. Treppo S, Koepp H, Quan EC, Cole AA, Kuettner KE, Grodzinsky AJ. Comparison of biomechanical and biochemical properties of cartilage from human knee and ankle pairs. J Orthop Res 2000;18(5):739-48.
31. Eger W, Schumacher BL, Mollenhauer J, Kuettner KE, Cole AA. Human knee and ankle cartilage explants: catabolic differences. J Orthop Res 2002;20(3):526-34.
32. Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989;7(5):619-36.

33. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE. Prevalence of degenerative morphological changes in the joints of the lower extremity. Osteoarthritis Cartilage 1997;5(1):23-37.
34. Frank EH, Jin M, Loening AM, Levenston ME, Grodzinsky AJ. A versatile shear and compression apparatus for mechanical stimulation of tissue culture explants. J Biomech 2000;33(11):1523-7.
35. Patwari P, Cheng DM, Cole AA, Kuettner KE, Grodzinsky AJ. Analysis of the relationship between peak stress and proteoglycan loss following injurious compression of human post-mortem knee and ankle cartilage. Biomech Model Mechanobiol 2007;6(1-2):83-9.
36. Stevens AL, Wheeler CA, Tannenbaum SR, Grodzinsky AJ. Nitric oxide enhances aggrecan degradation by aggrecanase in response to TNF-alpha but not IL-1beta treatment at a post-transcriptional level in bovine cartilage explants. Osteoarthritis Cartilage 2007.
37. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883(2):173-7.
38. Clark IM. Matrix Metalloproteinase Protocols. Humana Press, Clifton, NJ 2001.
39. Chockalingam PS, Zeng W, Morris EA, Flannery CR. Release of hyaluronan and hyaladherins (aggrecan G1 domain and link proteins) from articular cartilage exposed to ADAMTS-4 (aggrecanase 1) or ADAMTS-5 (aggrecanase 2). Arthritis Rheum 2004;50(9):2839-48.
40. Hunziker EB, Herrmann W, Schenk RK. Improved cartilage fixation by ruthenium hexammine trichloride (RHT). A prerequisite for morphometry in growth cartilage. J Ultrastruct Res 1982;81(1):1-12.
41. Sandy JD, Flannery CR, Neame PJ, Lohmander LS. The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel proteinase which cleaves the Glu 373-Ala 374 bond of the interglobular domain. J Clin Invest 1992;89(5):1512-6.
42. Stockwell RA, Scott JE. Distribution of acid glycosaminoglycans in human articular cartilage. Nature 1967;215(5108):1376-8.
43. Rowan AD, Koshy PJ, Shingleton WD, Degnan BA, Heath JK, Vernallis AB, et al. Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. Arthritis Rheum 2001;44(7):1620-32.
44. Ng L, Grodzinsky AJ, Patwari P, Sandy J, Plaas A, Ortiz C. Individual cartilage aggrecan macromolecules and their constituent glycosaminoglycans visualized via atomic force microscopy. J Struct Biol 2003;143(3):242-57.
45. Quinn TM, Maung AA, Grodzinsky AJ, Hunziker EB, Sandy JD. Physical and biological regulation of proteoglycan turnover around chondrocytes in cartilage explants. Implications for tissue degradation and repair. Ann N Y Acad Sci 1999;878:420-41.
46. Henrotin YE, De Groote DD, Labasse AH, Gaspar SE, Zheng SX, Geenen VG, et al. Effects of exogenous IL-1 beta, TNF alpha, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes. Osteoarthritis Cartilage 1996;4(3):163-73.
47. Lauffenburger DA, Oehrtman GT, Walker L, Wiley HS. Real-time quantitative measurement of autocrine ligand binding indicates that autocrine loops are spatially localized. Proc Natl Acad Sci U S A 1998;95(26):15368-73.

48. Vincent TL, McLean CJ, Full LE, Peston D, Saklatvala J. FGF-2 is bound to perlecan in the pericellular matrix of articular cartilage, where it acts as a chondrocyte mechanotransducer. Osteoarthritis Cartilage 2007;15(7):752-63.
49. Jones AR, Chen S, Chai DH, Stevens AL, Gleghorn JP, Bonassar LP, et al. Modulation of lubricin biosynthesis and tissue surface properties following cartilage mechanical injury. Arthritis Rheum 2009;60(1):133-42.
50. Buckley MR, Gleghorn JP, Bonassar LJ, Cohen I. Mapping the depth dependence of shear properties in articular cartilage. J Biomech 2008;41(11):2430-7.

2.7 Supplementary Data

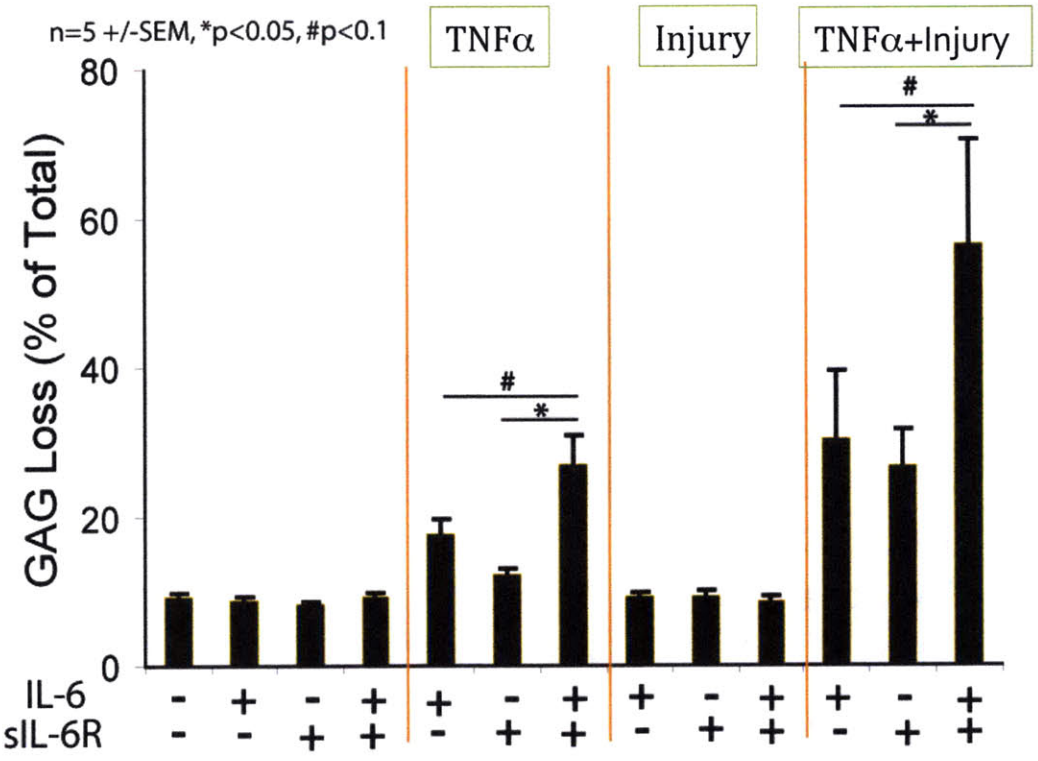


Figure 2S.1 Percentage GAG loss in response to 6-day treatments with TNFα (25ng/ml), mechanical injury and TNFα+injury, in the presence of IL-6 alone (50ng/ml), IL-6 soluble receptor alone (250ng/ml), or both together.

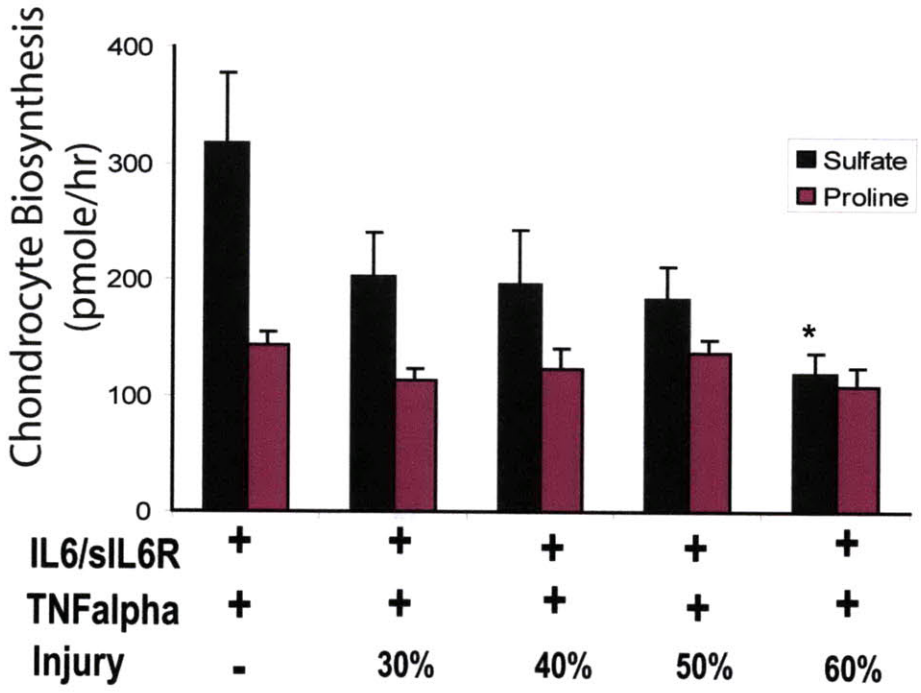


Figure 2S.2 Chondrocyte biosynthesis in response to graded levels of compressive load (final strain at 30%, 40%, 50%, and 60% corresponds to 0.6, 0.8, 1 and 1.2/second strain rate, respectively). The mean±SEM corresponding peak stresses were 9.3 ± 1.0Mpa, 15.1± 1.9MPa, 20.5±3.2MPa, 28.0±1.9 MPa. Chondrocyte biosynthetic rates were measured by ^{35}S-sulfate and ^{3}H-proline incorporation during days 4-6. TNFα was used at 25ng/ml; IL-6 and sIL-6R were used at 50ng/ml and 250ng/ml, respectively. For each condition, n=5 cartilage disks. *=p<0.05 vs. no loading control.

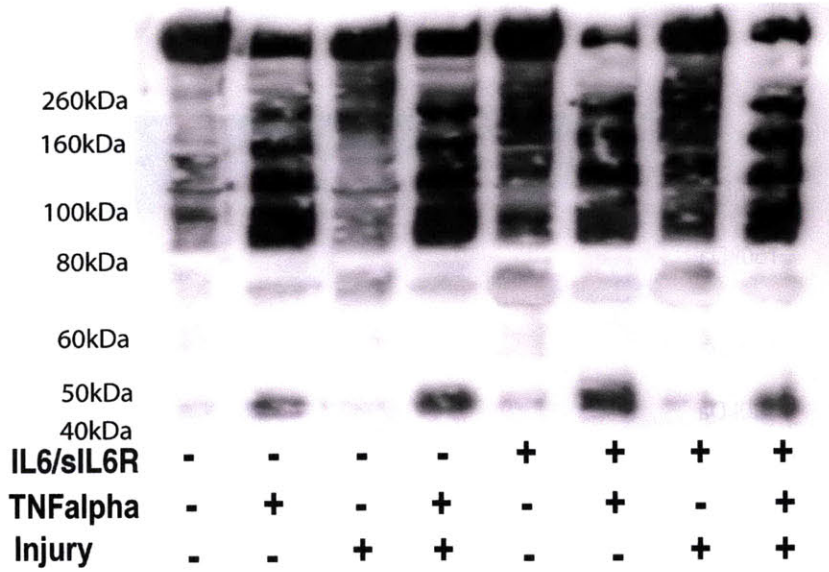


Figure 2S.3 Western analysis for aggrecan fragments with intact G3 domain following treatments with TNFα, IL-6/sIL-6R and mechanical injury. Western blot was performed using an anti-G3 antibody (J.Sandy) on medium pooled from days 2, 4 and 6 from each condition.

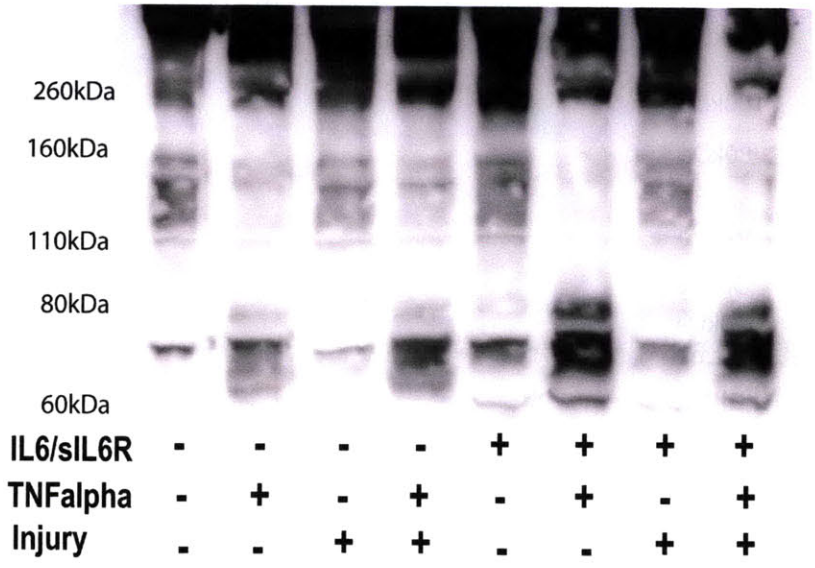


Figure 2S.4 Western analysis for aggrecan fragments with intact G1 domain following treatments with TNFα, IL-6/sIL-6R and mechanical injury. Western blot was performed using an anti-G1 antibody (J.Sandy) on medium pooled from days 2, 4 and 6 from each condition.

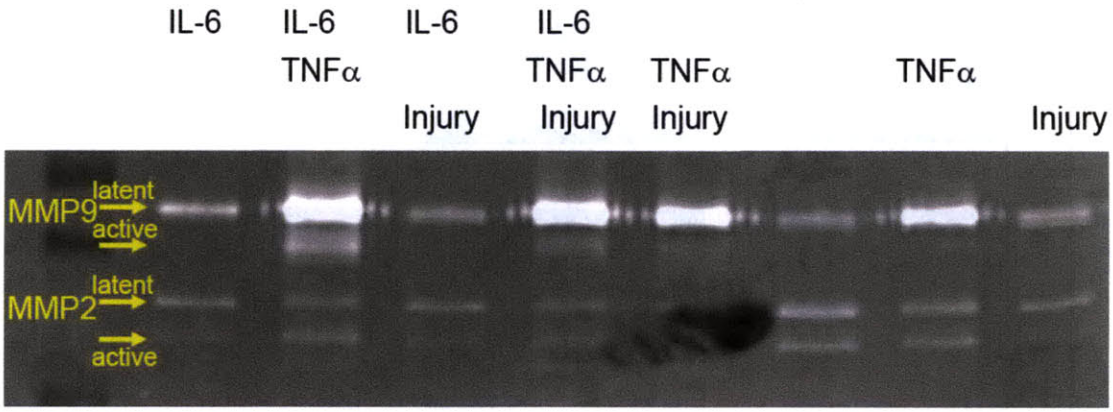


Figure 2S.5 Proteolytic activities of MMP-2 and MMP-9 analyzed by zymography. Medium was pooled from days 2, 4 and 6 within each condition, and resolved on 10% gelatin zymogram gel. Cartilage samples treated with IL-6 (50ng/ml) was also co-stimulated with sIL-6R (250ng/ml). TNFα was used at 25ng/ml. Injury protocol was consisted of radially unconfined compression to 50% final strain at velocity of 1mm/second (strain rate 100%/second), followed by immediate release of the load.

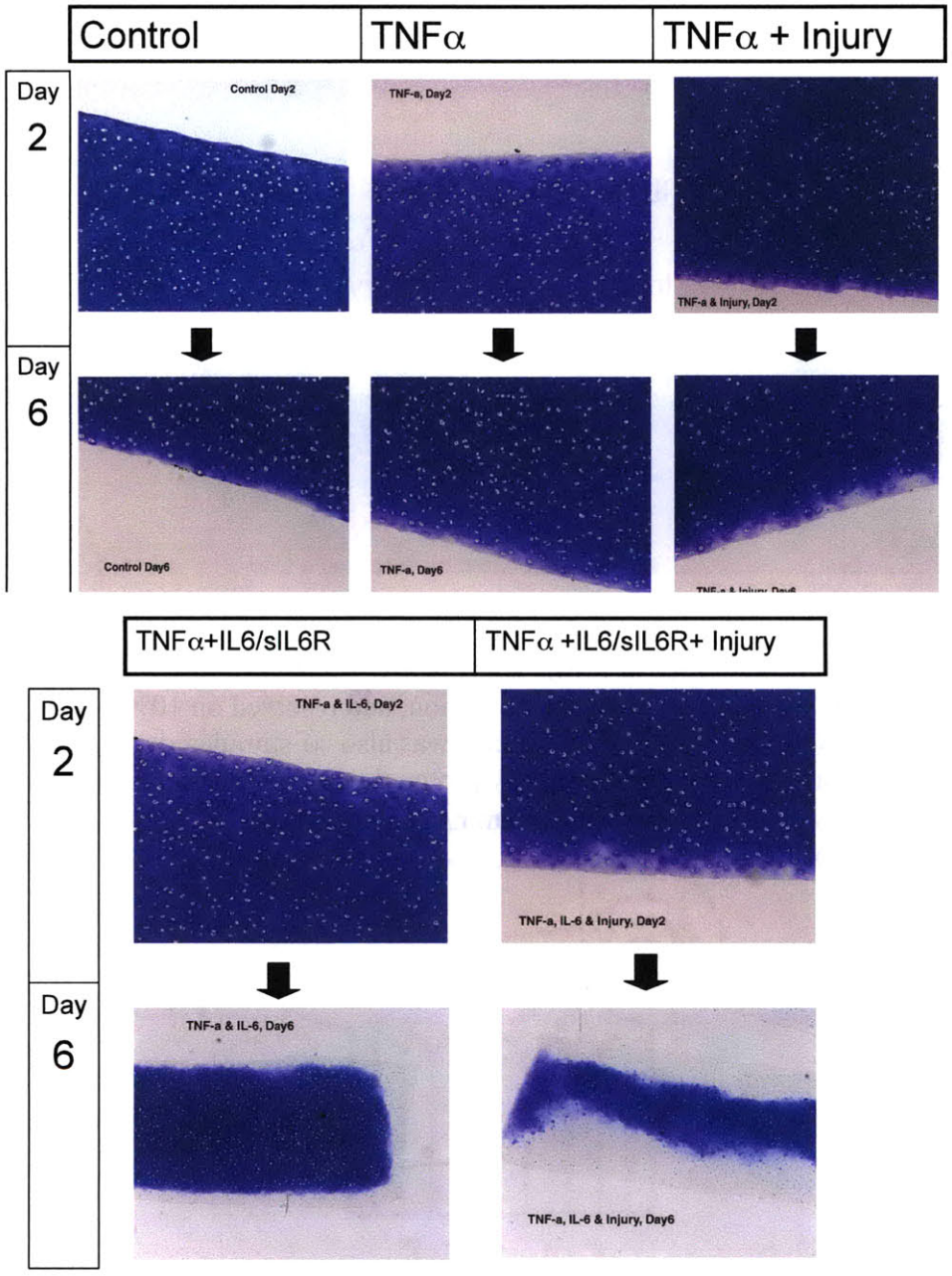


Figure 2S.6 Histologic findings of middle-zone bovine cartilage disks in treatments with TNFα, TNFα+injury, TNFα+IL-6/sIL-6R and TNFα+IL-6/sIL-6R+injury on day 2 and day 6. Toluidine blue stained for sulfated GAG. TNFα was used at 25ng/ml; IL-6 and sIL-6R were used at 50ng/ml and 250ng/ml, respectively. The loss of sGAG originated at the disk periphery and progressed toward the center with time. The most severe progression of sGAG loss was due to TNFα+IL-6/sIL-6R+injury.

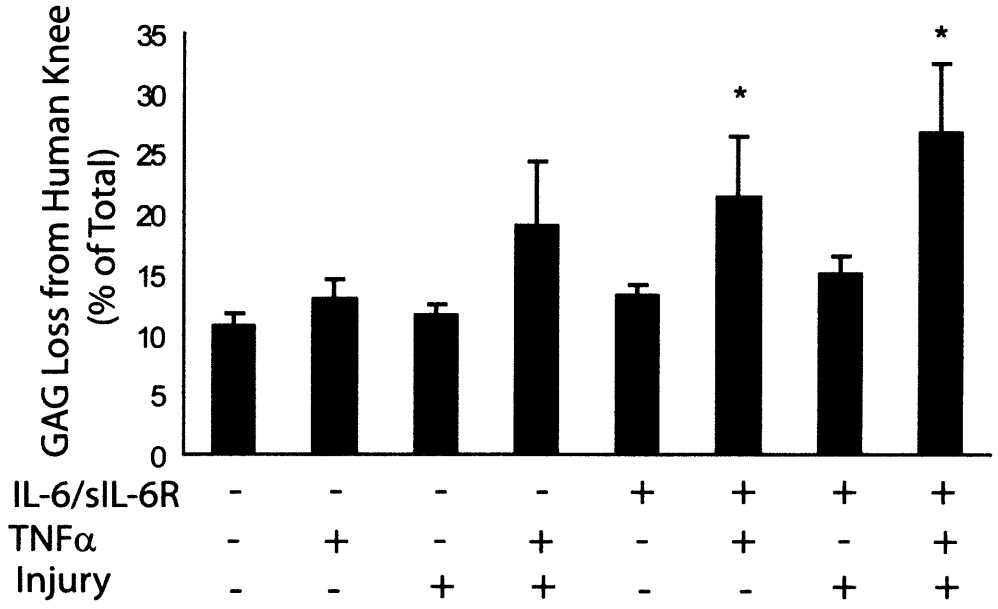


Figure 2S.7 Percentage of GAG loss from human knee cartilage (59-yr old male, Grade 1) in response to 8-day treatments with TNFα, IL-6/sIL-6R and injury. All disks included the intact superficial zone. For each treatment group, n=6 cartilage disks. *=$p<0.05$ vs. no treatment control. TNFα was used at 100ng/ml; IL-6 and sIL-6R were used at 50ng/ml and 250ng/ml, respectively.

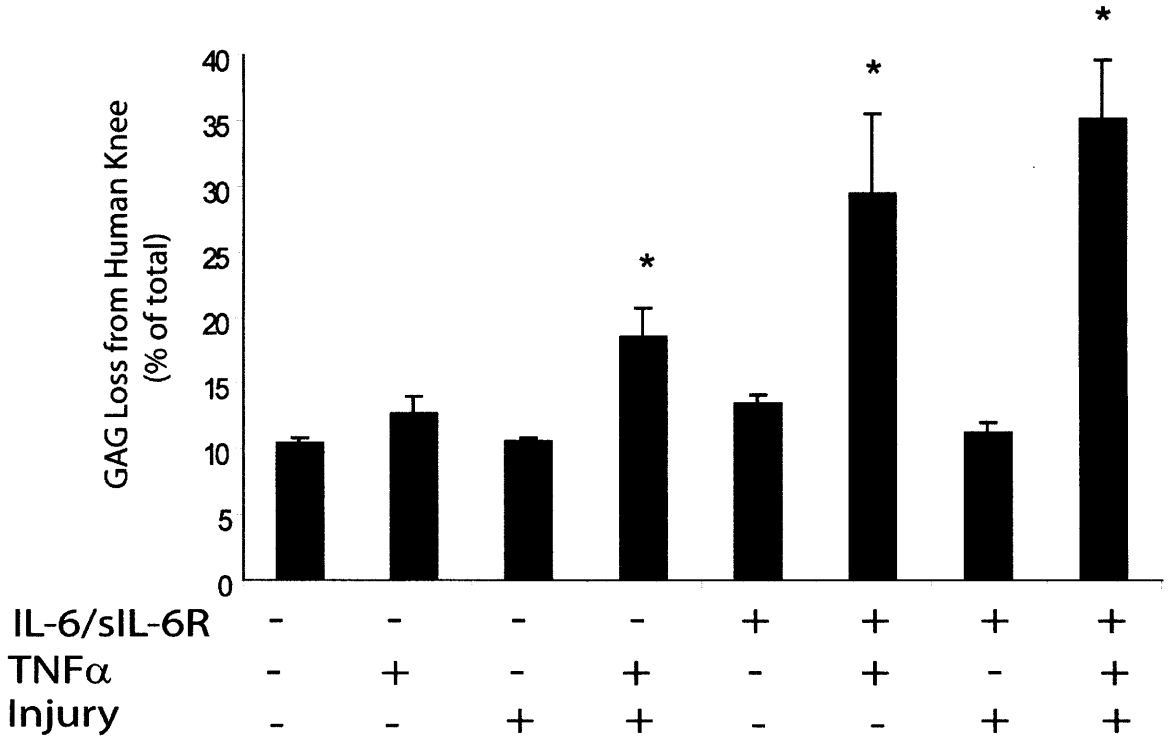


Figure 2S.8 Percentage of GAG loss from human knee cartilage (29-yr old male, Grade 0) in response to 8-day treatments with TNFα, IL-6/sIL-6R and injury. All disks included the intact superficial zone. For each treatment group, n=6 cartilage disks. *=$p<0.05$ vs. no treatment control. TNFα was used at 100ng/ml; IL-6 and sIL-6R were used at 50ng/ml and 250ng/ml, respectively.

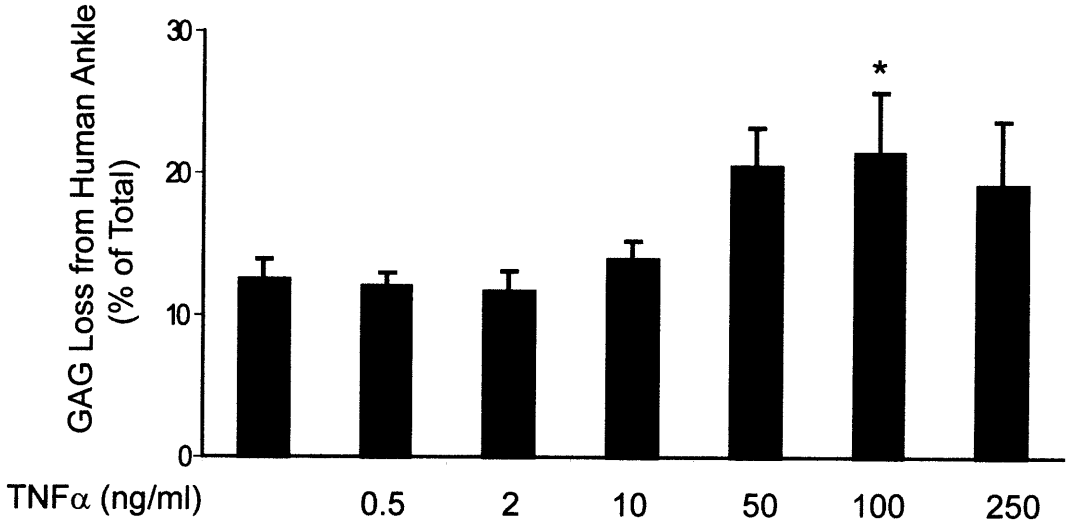


Figure 2S.9 Percentage of GAG loss from human ankle cartilage (59-yr old male, Grade 0) in response to 8-day TNFα treatments. All disks included the intact superficial zone. Values are mean and SEM, n=6 cartilage disks per condition. *=$p<0.05$ vs. no treatment control.

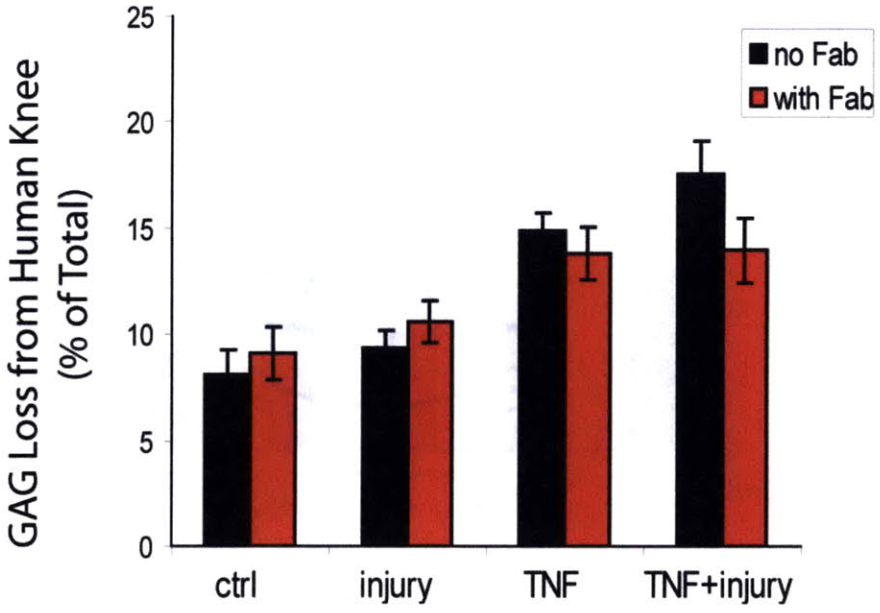


Figure 2S.10 Effect of anti-IL-6 Fab on sulfated GAG loss in response to mechanical injury and/or treatment with TNFα in human knee cartilage. Cartilage disks designed to receive anti-IL-6 Fab treatments were pre-treated with the Fab (50μg/ml) for 6 days prior to the TNFα and/or mechanical injury treatments. For these conditions, the anti-IL-6 Fab was supplied in the medium for another 6 days until the end of the experiment. All disks included the intact superficial zone. Human knee cartilage (Grade 1) was obtained from a 62-yr old female. Values are mean and SEM (n=6 cartilage disks).

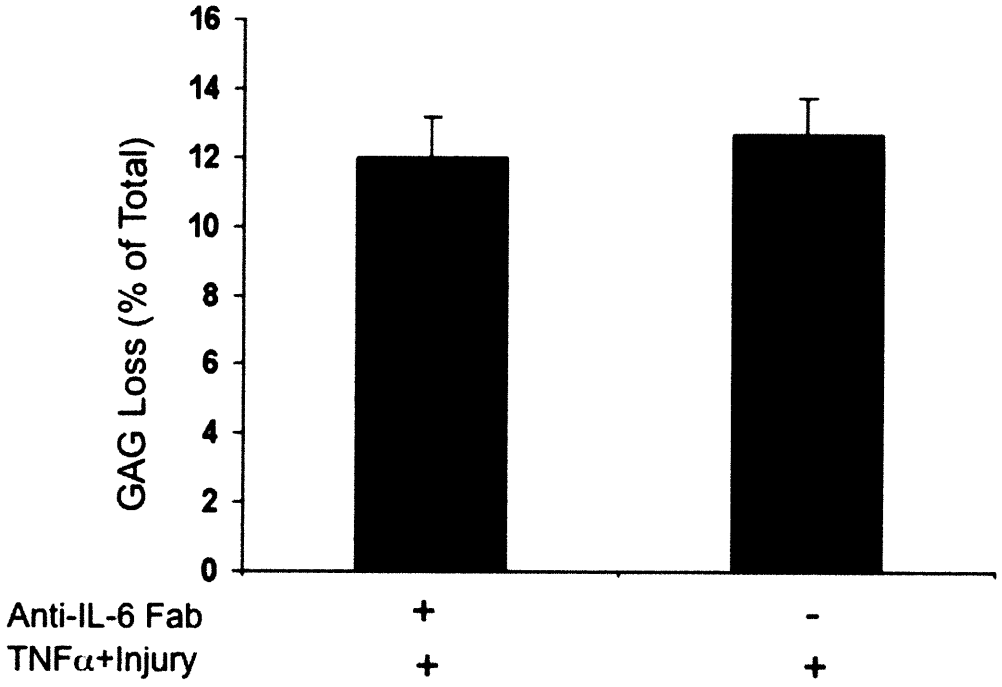


Figure 2S.11 Effect of anti-IL-6 Fab on sulfated GAG loss in response to mechanical injury and 6-day treatment with TNFα in human ankle cartilage. Cartilage disks were pre-treated with the Fab (50μg/ml) for 6 days prior to the TNFα and/or mechanical injury treatments. For these conditions, the anti-IL-6 Fab was supplied in the medium until the end of the experiment. All disks included the intact superficial zone. Human ankle cartilage (Grade 0) was obtained from a 62-yr old female. Values are mean and SEM (n=5 cartilage disks).

CHAPTER 3

Treatments with Mechanical Injury and Inflammatory Cytokines cause Specific Changes in Chondrocyte Gene Expression

3.1 Introduction

Articular cartilage provides a near frictionless and weight-bearing surface that distributes and dissipates stress in joint locomotion. Chondrocyte, the single cell type in cartilage, is responsible for the maintenance and repair of the extensive extracellular matrix (ECM). In the disease of osteoarthritis (OA), the major ECM components, aggrecan and collagen networks, are both targeted for degradation. Chondrocytes are capable of producing matrix-degrading enzymes as well as inflammatory molecules. Cartilage breakdown occurs when chondrocytes fail to balance between matrix synthesis and matrix degradation.

Acute joint trauma significantly increases the risk for development of OA in young adults (1, 2). Surgical interventions, such as reconstruction of the damaged ligament or meniscus, can restore joint stability but does not reduce the risk for development of secondary OA (3). These observations suggest the immediate biochemical and biomechanical response in the joint tissues following injury are long-term and may directly contribute to the cascades of catabolic events post-trauma. For example, increased release of aggrecan fragments and cross-linked peptides from type II collagen have been observed in the injured joints(4, 5), indicating the impact from injury causes direct damage to cartilage matrix. Moreover, immediately following knee injury, the synovial fluid concentrations of pro-inflammatory cytokines increased dramatically and remained high for weeks (6).

The pro-inflammatory cytokines released following injury can cause chondrocytes to produce nitric oxide (NO). For example, many studies have demonstrated chondrocytes respond to treatments with IL-1, and TNFα by releasing NO (7-10). Furthermore, inhibition of NO production induced by TNFα treatment partially reduced GAG loss and increased protein

synthesis in bovine cartilage, whereas blocking NO in IL-1 treated cartilage produced no effect on matrix degradation (10). Numerous studies have implicated the importance of nitric oxide in cartilage metabolism; however, its role in cartilage breakdown post-injury remains unclear.

In vitro injury models have been developed to better understand the effect of trauma on both cartilage matrix and chondrocytes. For example, in vitro acute compressive loading to articular cartilage results in the release of proteoglycans and collagen fragments (11-13), a hallmark of cartilage matrix degradation. Injurious compression also affects chondrocytes by causing apoptosis and decreased biosynthesis in the remaining viable cells(14, 15). Recently, more researchers have focused on examining whether mechanical injury can also modulate chondrocytes at the transcriptional level. For example, Lee et al. reported within 24 hours after injurious compression, the genes of ADAMTS-5 and MMP-3 were highly up-regulated, while the genes encoding matrix molecules were unaffected(16). Jones et al. demonstrated two days after mechanical injury, the expression of lubricin mRNA was increased in the superficial zone cartilage, and decreased in the middle zone cartilage(17). Together, these studies highlighted the importance of transcriptional response post-injury and its role in cartilage degradation process.

In Chapter 2 of this thesis, we have presented in vitro injury models where cartilage explants were exposed to pro-inflammatory cytokines in addition to injurious compression. We observed mechanical injury highly potentiated the combined effect of TNFα and IL-6/sIL-6R by causing severe GAG loss and biosynthesis reduction, and the resulting GAG loss was mediated by aggrecanases, not MMPs (18). To investigate whether the matrix degradation observed in this model was initiated at the transcriptional level, the current study examined the combined effects of TNFα, IL-6/sIL-6R and mechanical injury on chondrocyte gene expression

of matrix proteases, ECM molecules, natural protease inhibitors and growth factors. Additionally, we investigated the effect of mechanical injury and cytokine on NO production cartilage explants.

3.2 Materials And Methods

Bovine cartilage harvest and culture. Cartilage disks were harvested from the femoropatellar grooves of 1-2-week old bovine calves as described(19, 20). Briefly, cartilage-bone cylinders (9mm diameter) from 6 knee joints were cored perpendicular to the surface. After a level surface was obtained by removing the most superficial layer (~100-200 μm), 1-2 sequential 1mm-slices of middle zone cartilage were cut from each cylinder. Five disks (3mm-dia, 1mm-thick) were cored from each slice using a dermal punch. Cartilage from this middle zone in newborn calf was shown to have a reasonably homogeneous population of cells and matrix(20). All treatment groups were matched for location and depth of cartilage on the joint surface. Disks were equilibrated in serum-free medium (low-glucose DMEM supplemented with 1% insulin-transferrin-selenium (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, Sigma), 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* proline, 20 μg/mL ascorbic acid, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B for 2 days prior to treatment in a 37°C, 5% CO_2 incubator.

Injurious compression. After equilibration in medium for 2 days, disks were injuriously compressed in a custom-designed incubator-housed apparatus(14, 21). Each bovine disk was subjected to radially unconfined compression to 50% final strain at 1mm/second velocity (100%/second strain rate), followed by immediate release of load at the same rate as

described(19). After injury, the cartilage samples were place in culture medium containing different combinations of cytokines.

Exogenous cytokines, L-NMA and injury treatments. A factorial design was used to investigate the transcriptional effects of exogenous cytokines on injured cartilage using treatment conditions: (1) no-treatment-control, (2) TNFα, (3) injury, (4) injury+TNFα, (5) IL-6/sIL-6R, (6) TNFα+IL-6/sIL-6R, (7) injury+ IL-6/sIL-6R and (8) injury+TNFα +IL-6/sIL6R. Cartilage was either subjected to compression or left uninjured and incubated with or without cytokines (all from R&D Systems, MN). rhTNFα was used at 25 ng/mL. rhIL-6 (50 ng/mL) was used in combination with soluble IL-6 receptor (sIL-6R, 250 ng/mL), since this combination was found previously to induce greater aggrecan degradation than when used separately in the presence of TNFα(22). Cartilage samples were treated for 4 days, with medium replaced every 2 days. At the end of the experiment, cartilage disks were flash frozen in liquid nitrogen and stored at -80°C. There were 6 cartilage plugs in each condition, and all were matched across depth and location on the joint surface. The same procedure was conducted in 3-6 animals to account for animal variations.

In the experiment testing the effect of blocking NO formation in cartilage breakdown, bovine cartilage samples were treated with TNFα, TNFα+injury, TNFα+IL-6/sIL-6R, and TNFα+IL-6/sIL-6R+injury, and cultured either in the presence or absence of 1.25mM L-N-methyl-arginine (L-NMA) for 6 days.

Biochemical analysis. The amount of sGAG lost to the medium was measured using the dimethylmethylene blue (DMMB) assay. Nitrite levels in the medium were analyzed using the Griess Reagents (Invitrogen, CA).

RNA Extraction, Purification and Real-time PCR. Within each animal, six cartilage disks from the same condition were pooled and pulverized in liquid nitrogen. After homogenizing in Trizol reagent (Invitrogen, CA), the extracts were spun in Phase Gel Tubes (Eppendorf AG, Germany) with 10% v/v chloroform for 10 minutes at 4°C. RNA was isolated from the clear liquid portion of the phase gel by using the RNeasy Mini Kit (Qiagen, CA). In the RNA isolation process, DNA was digested using DNase (Qiagen, CA). The quantity and purity of the extracted RNA were measured using Nanodrop (Thermo Scientific). After determining the amount of RNA extracted from each condition, equal quantities of RNA was used for reverse transcription using the Amplitaq-Gold RT kit (Applied Biosystems, CA). Real-time PCR was performed using the ABI 7900HT (Applied Biosystems, CA). The mRNA levels for 24 genes of interests (proteaseas, protease inhibitors, matrix molecules, cytokines, growth and transcription factors) were measured. The bovine primer sequences were designed using Primer3 software (23). Measured threshold values (Ct) were converted to RNA copy number according to previously determined primer efficiencies.

Statistical Analyses. Within each condition, RNA copy numbers of each gene were normalized to that of 18S from the same condition. To examine the effects of treatments, each gene was normalized to its level in the no-treatment control group, as shown in figures. Values were represented as average of all experiments with standard errors. Statistical analysis was performed using a linear mixed-effect model with animal as a random variable, followed by Tukey's pairwise comparisons.

3.3 Results

Effects of mechanical injury on chondrocyte gene expression. Cartilage disks were subjected to radially unconfined compression to 50% final strain at 1mm/second velocity (100%/second strain rate), followed by immediate release of load at the same rate and incubation in regular medium for 4 days (Figure 3.1). None of the 23 genes measured were significantly affected by mechanical injury. However, the matrix proteases, such as MMP-3, MMP-9, MMP-13, ADAMTS-5, showed trends of increased gene expression (Figure 3.1), whereas the matrix molecules, including aggrecan, HAS, Collagen II and Collagen IX displayed trends of reduced expression.

Effects of IL-6/sIL-6R on chondrocyte gene expression. Cartilage disks were incubated with IL-6/sIL-6R (50/250 ng/ml) for 4 days before RNA extraction (Figure 3.2). Analysis showed IL-6/sIL-6R treatment significantly up-regulated MMP-13 by ~15 fold. The expression of MMP-3, MMP-9, ADAMTS-4, ADAMTS-5, iNOS and IL-6 were also elevated, though not statistically significant. The expression of Collagen I, II and IX in IL-6/sIL-6R treatment were all less comparing to those in the no-treatment control; however, no genes were significantly down-regulated by the IL-6/sIL-6R exposure.

Combined effects ofIL-6/sIL-6R and mechanical injury on chondrocyte gene expression. To investigate the transcriptional effects of the combination of IL-6/sIL-6R and mechanical injury, cartilage samples were first mechanically injured to 50% final strain at 1mm/second velocity, followed by 4-day incubation with IL-6/sIL-6R (50/250 ng/ml) (Figure 3.3). Among the 23 genes tested, the aggrecanases primarily responsible for GAG loss in cartilage were significantly up-regulated (ADAMTS-4 and ADAMTS-5 were increased by 10-

and 27-fold over control, respectively), and so were MMP-9, MMP-13, TIMP-3, iNOS, and IL-6 (expression levels were elevated by 12-, 54-, 4-, 9- and 42-fold, respectively). HAS, collagen II and IX levels were suppressed below those of the no-treatment control, though they did not reach statistical significance.

Effects of TNFα on chondrocyte gene expression. Cartilage disks were treated with TNFα (25ng/ml) for 4 days before gene analysis (Figure 3.4). TNFα treatment significantly down-regulated Collagen I and Collagen IX, while up-regulated proteins involved in matrix degradation and inflammation. For example, MMP-3 and MMP-9 expression were increased by 31-fold and 14-fold, respectively; ADAMTS-5 were increased by 30-fold; TNFα and caspase-3 were elevated by19-fold and 4-fold, respectively. The gene level of iNOS in TNFα-treated cartilage was more than 200-fold higher than that in the no-treatment control.

Combined effects of TNFα and mechanical injury on chondrocyte gene expression. To examine the combined effected of TNFα and mechanical injury, cartilage samples were injuriously compressed, then treated with TNFα for 4 additional days (Figure 3.5). TNFα plus injury treatment significantly up-regulated more genes than either treatment alone, including matrix proteases MMP-3, MMP9, MMP13, ADAMTS-4, ADAMTS-5, as well as TIMP-3, iNOS, IL-6 and Caspase-3. Furthermore, majority of these molecules were expressed at levels much higher than those induced in the condition of TNFα alone or mechanical injury alone. In contrast to these highly expressed genes, the transcriptions of matrix molecules and growth factors were either equivalent or less comparing to those in the no-treatment control.

Combined effects of TNFα and IL-6/sIL-6R on chondrocyte gene expression. TNFα treatment in combination with IL-6/sIL-6R treatment up-regulated more genes comparing to either treatment alone (Figure 3.6). In addition to MMP-3, MMP-9, ADAMTS-5, iNOS, TNFα and caspase-3, the combined treatment also significantly elevated the transcription of MMP-13, ADAMTS-4, and IL-6. Moreover, the expression levels of these molecules were all much higher in the combined treatment than those induced by TNFα alone or IL-6/sIL-6R alone. The combination of TNFα and IL-6/sIL-6R also significantly reduced the transcription of Collagen I, II and IX.

Combined effects of TNFα, IL-6/sIL-6R and mechanical injury on chondrocyte gene expression. To elucidate the combined effect of cytokines TNFα and IL-6/sIL-6R on chondrocyte gene expression in mechanically injured cartilage, samples were first injurious compressed then cultured with both TNFα and IL-6/sIL-6R for an additional 4 days (Figure 3.7). Such treatment significantly increased the expression of matrix-proteases and protease inhibitor, including MMP-3, MMP-9, MMP13, ADAMTS-4, ADAMTS-5 and TIMP-3, to levels much higher than those expressed in any other treatments. The combination of TNFα and IL-6/sIL-6R in injured cartilage also up-regulated the genes for iNOS, capsase-3 and cytokines IL-6 and TNFα, but did not significantly down-regulate any genes.

Effect of blocking nitric oxide formation in cytokine- and injury-induced proteoglycan degradation. In this study, iNOS and aggrecanases shared the same gene expression pattern, that is, their expression levels were both significantly up-regulated in the conditions of TNFα, TNFα+injury, TNFα+IL-6/sIL-6R, IL-6/sIL-6R+injury and TNFα+IL-6/sIL-6R+injury. To examine whether iNOS played a role in the aggrecanase-mediated proteoglycan degradation, cartilage samples were treated with 1.25mM of L-NMA in addition

to the combined treatments with TNFα, IL-6/sIL-6R and mechanical injury. The treatments with TNFα, TNFα+IL-6/sIL-6R, TNFα+injury and TNFα+IL-6/sIL-6R+injury all significantly increased both GAG loss and nitrite release to the medium. The addition of L-NMA significantly reduced nitrite levels in all conditions (Figure 3.8), indicating that L-NMA effectively blocked the iNOS activity. However, inhibition of NO showed no effect on proteoglycan degradation in any of the treatment conditions (Figure 3.9).

3.4 Discussion

In this study, we have examined the effect of TNFα, IL-6/sIL-6R, mechanical injury, and their combinations on chondrocyte gene expression. Cartilage subjected to mechanical injury alone showed higher expression of matrix proteases MMP-3, MMP-9, MMP-13, and ADAMTS-5 four days post-injury. Similarly, an earlier study reported both MMP-13 and ADAMTS-5 were up-regulated during the initial 24 hours post-injury (16). It is known that the GAG release from injured cartilage during the initial 24 hours was mainly due to mechanical disruption of the cartilage matrix, since inhibitors of biosynthesis and matrix enzymes could not prevent the degradation. Interestingly, from day 1 to day 7 post-injury, a general MMP inhibitor blocked the cumulative GAG loss(24). We have shown in Chapter 2 of this thesis that during the 6 days following injury, MMP-2, MMP-3 and MMP-9 released to the medium were all latent, and not proteolytic active (Figure 2.1 and 2S.3). However, it is not clear whether MMP-13 was involved in the cartilage degradation in the in vitro injury model. Interestingly, MMP-13 gene expression was significantly up-regulated by most of the treatments in this study. Studies have shown MMP-13 is capable of degrading both proteoglycans and

collagens, and is responsible for cleaving the small leucine-rich proteoglycans, which are believed to be protective of the collagens from proteolytic attacks (25). Future studies should examine whether MMP-13 was expressed at the protein level and whether it was proteolytically active in the cytokine and injury treatments.

Comparing to IL-6 or injury treatment, TNFα alone was much more potent in inducing changes in chondrocyte gene expression. For examples, TNFα significantly reduced the expression of cartilage matrix molecules while increased the levels of matrix proteases, iNOS, TNFα and caspase-3. Moreover, the addition of mechanical injury, IL-6/sIL-6R and the combination of injury with IL-6/sIL-6R treatment highly potentiated the TNFα-induced expression of matrix proteases. The up-regulation of ADAMTS-4 and ADAMTS-5 gene expression were consistent with our previous findings, which showed the treatments with TNFα, either with or without IL-6/sIL-6R, in injured or uninjured cartilage explants, were all capable of causing aggrecanase-mediated proteoglycan degradation(18). On the other hand, the MMPs were not involved in the early stage of proteoglycan degradation induced by either cytokines or combination of cytokines with injury, despite of their increased gene expression. Using a combination of OSM and TNFα treatment, researchers have discovered that aggrecanase-mediated aggrecan degradation occurs in the early (day 7) and middle stage (day 11) of the study period, whereas MMP-mediated proteoglycan and collagen degradation happened only towards the end of the study period (day 17) (26). Taken together, these findings indicate the MMPs may be subjected to modulations after transcription, and may play a more dominant role in the later stage of cartilage breakdown.

In contrast to the highly expressed catabolic molecules involved in matrix breakdown, growth factors as well as cartilage matrix molecules were either unresponsive or down-

regulated by the in vitro injury model. Collagens were particularly affected by the combined treatment with TNFα and IL-6/sIL-6R. In vivo studies have found fragments of type II collagens in injured and osteoarthritic human joints(4). It is also known that cartilage degradation becomes irreversible once the aggrecan and collagen type II were degraded by MMPs, in contrast to the reversible process of aggrecanase-mediated aggrecan degradation (27). Other researchers have also found exogenous IGF exposure could attenuate some of the MMP activities; however, our current study indicated the growth factors were not significantly up-regulated in the treatments. Together, these findings suggest cartilage exposed to cytokines and injury treatment may lose its homeostasis by experiencing increased matrix degradation and compromised matrix synthesis and capacity to repair.

IL-6 was highly expressed in all the conditions involving TNFα. It is known that TNFα, either with or without mechanical injury, can significantly induce the production of endogenous IL-6 from bovine and human ankle and knee cartilage (18). Blocking endogenous IL-6 with an anti-IL-6 Fab fragment significantly reduced GAG loss caused by the combination of TNFα and mechanical injury, suggesting endogenous IL-6 plays an important role in cartilage degradation, and therapeutics targeting IL-6 may be a beneficial strategy for OA treatment. Caspase-3 is another molecule whose gene was up-regulated in all of the conditions involving TNFα treatment. This finding was consistent with other studies. For example, in vitro experiments have shown TNFα is capable of inducing chondrocyte apoptosis(28, 29), and human osteochondral explants subjected to experimental fractures have been found to express active capspase-3(30).

From this study, we also noticed that cytokine treatments increased the expression of TIMP-3 in injured cartilage explants. TIMP-3 is a natural inhibitor for ADAMTS-4 and

ADAMTS-5(31), as well as many members of the MMP class (32). Interestingly, Wayne et al. reported the TIMP-3 activity was dependent on its interaction with aggrecan and ADAMTS-4. For examples, TIMP-3 inhibition of ADAMTS-4 enhanced multi-fold in the presence of high concentration of aggrecans, whereas its inhibitory effect was greatly compromised when the aggrecans were deglycosylated(33). From Chapter 2 of this thesis, we have already observed that cytokine treatments in injured cartilage caused significant GAG loss, as much as 50% of total GAG content in a 6-day study period. The severe degradation of aggrecan may explain why the increased expression of TIMP-3 had no effect in blocking proteoglycan degradation in the combined cytokine plus injury treatments.

Increased ADAMTS levels were consistently accompanied by elevated iNOS gene expression. We examined whether iNOS and iNOS-generated nitric oxide may be involved in the aggrecanase-mediated proteoglycan degradation. We observed that the treatments with TNFα either in the presence or absence of IL-6/sIL-6R, in both injured and uninjured cartilage explants, not only increased GAG release, but also significantly increased the level of nitrite measured in the conditioned medium, suggesting these treatments highly up-regulated the activities of both aggrecanases and iNOS, consistent with the gene expression data. The iNOS inhibitor, L-NMA, effectively suppressed the nitrite level; however, it did not block the GAG loss induced by the treatments. This observation was consistent with the findings of Voigt et al., who reported L-NMA treatment of bovine meniscal cartilage explants failed to inhibit TNFα-induced GAG loss and NITIGE production(34). In contrast, Stevens et al. found L-NMA exposure in TNFα treatment inhibited almost 50% of GAG loss and also decreased the NITEGE fragments in the medium, whereas blocking nitric oxide formation had no effect on IL-1-dependent GAG loss. In the same study, researchers also demonstrated L-NMA does not

cause significant changes in gene expressions, including those of ADAMTS-4 and ADAMTS-5. One of the major factors that may have contributed to the discrepancy between our current study and that of the Stevens et al. was the concentration of exogenous TNFα used in the treatment. The effect of L-NMA may be more prominent when there is greater GAG loss induced by TNFα stimulation. Interestingly, some studies have demonstrated that blocking NO formation in cartilage can sometimes be deleterious, implicating NO may also play a protective role in cartilage, depending on the inflammatory stimuli driving the catabolic response in chondrocytes(10).

We have shown previously that mechanical injury potentiated both proteoglycan degradation and reduced biosynthesis induced by the combined effects of TNFα and IL-6/sIL-6R. Here, we have demonstrated that the catabolic response induced by these treatments also occurs at the transcriptional level: the gene expression of MMPs, aggrecanases and iNOS were consistently up-regulated, whereas the matrix molecules and growth factors were either suppressed or not affected. Such imbalance between catabolic and anabolic processes within chondrocytes may ultimately lead to irreversible cartilage degradation following injury.

3.5 Figures

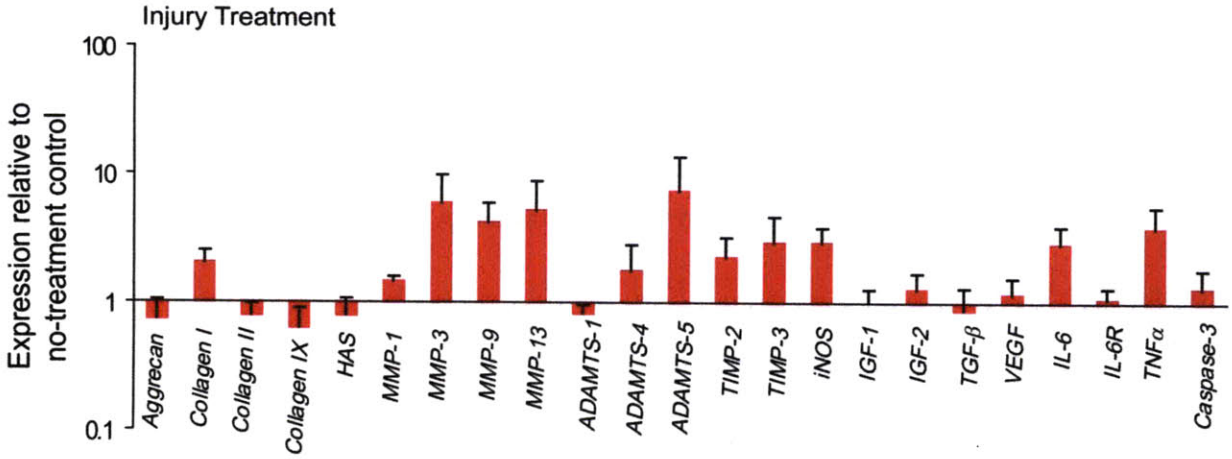


Figure 3.1 Effect of injury treatment on chondrocyte gene expression. Bovine cartilage disks were subjected to radially unconfined compression to 50% final strain at 1mm/second velocity (100%/second strain rate), followed by incubation in regular medium for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control.

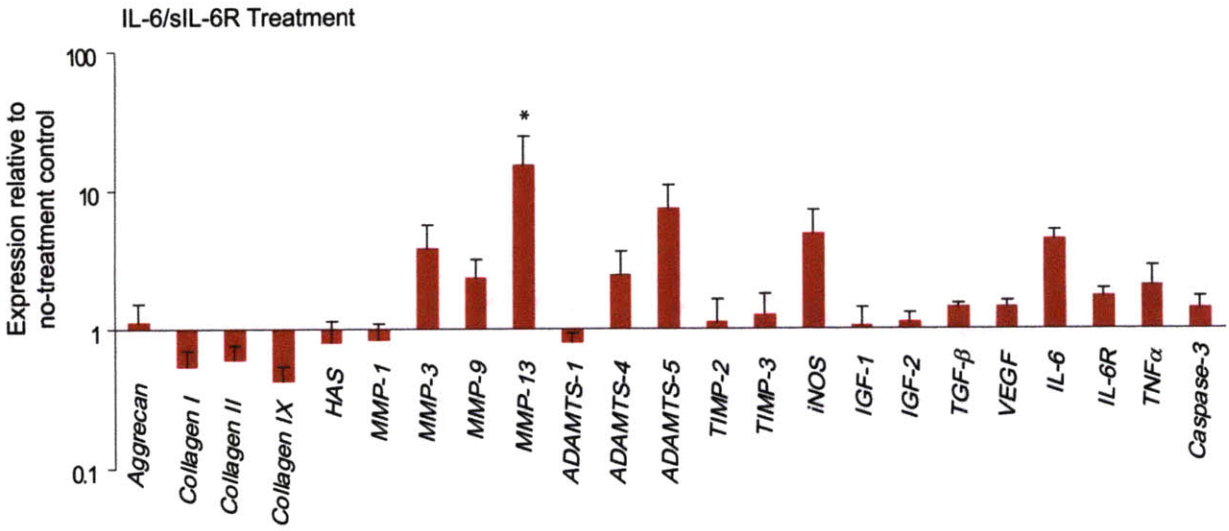


Figure 3.2 Effect of IL-6/sIL-6R treatment on chondrocyte gene expression. Cartilage disks were incubated with IL-6/sIL-6R (50/250 ng/ml) for 4 days before RNA extraction. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$ vs. no-treatment control.

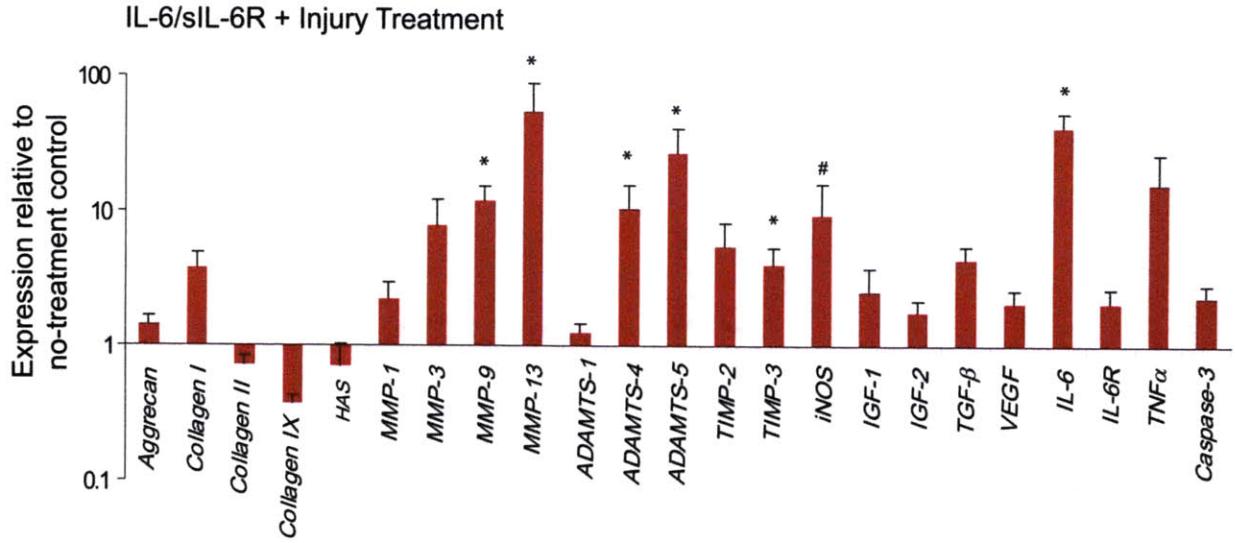


Figure 3.3 Effect of combined treatment with mechanical injury and IL-6/sIL-6R on chondrocyte gene expression. Cartilage disks were first injured and then incubated with IL-6/sIL-6R (50/250 ng/ml) for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$, #=$p<0.1$ vs. no-treatment control.

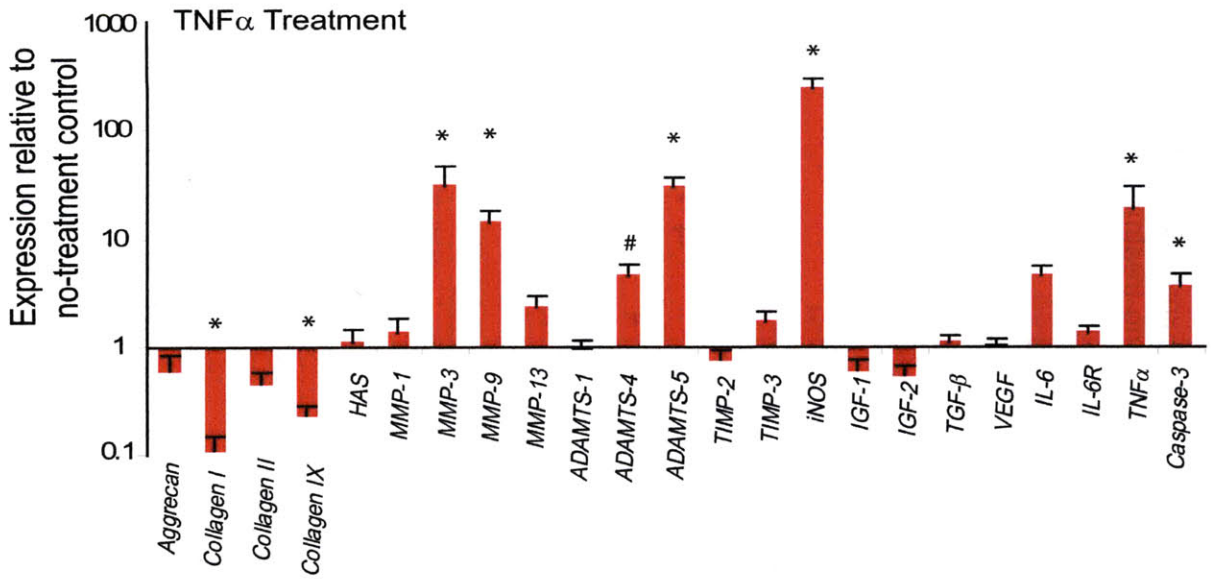


Figure 3.4 Effect of TNFα treatment on chondrocyte gene expression. Cartilage disks were incubated with TNFα (25ng/ml) for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$ vs. no-treatment control.

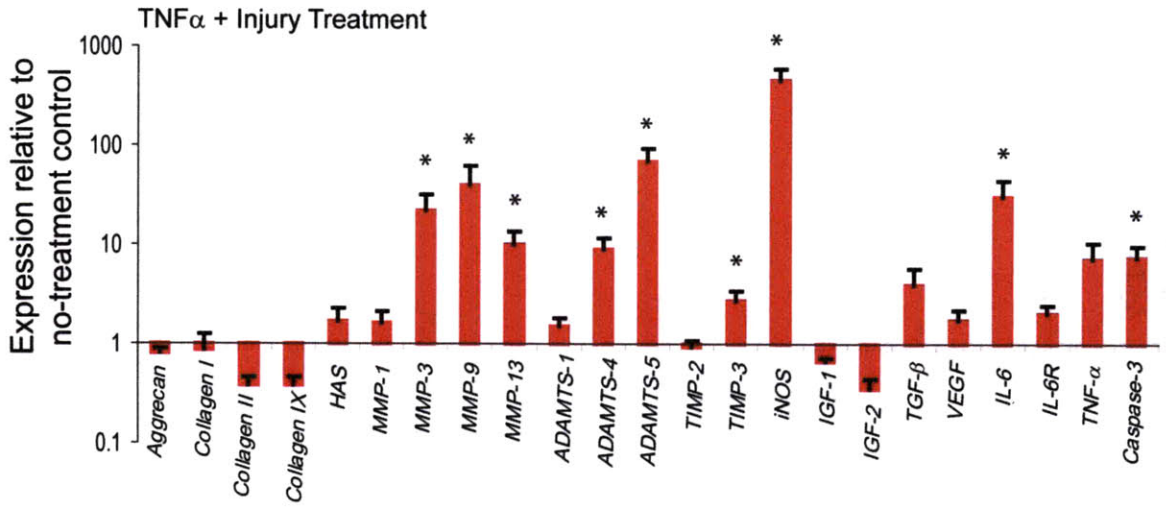


Figure 3.5 Effect of combined treatment with TNFα and mechanical injury on chondrocyte gene expression. Cartilage disks were first injuriously compressed then incubated with TNFα (25ng/ml) for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$ vs. no-treatment control.

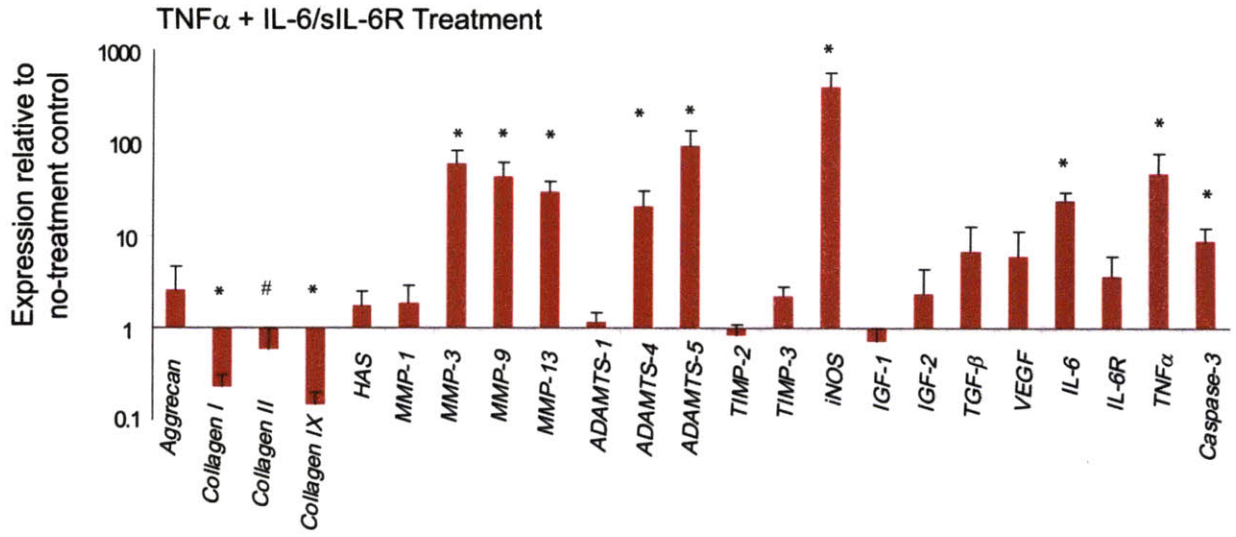


Figure 3.6 Effect of combined treatment with TNFα and IL-6/sIL-6R on chondrocyte gene expression. Cartilage disks were incubated with TNFα (25ng/ml) and IL-6/sIL-6R (50/250ng/ml) for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$, #=$p<0.1$ vs. no-treatment control.

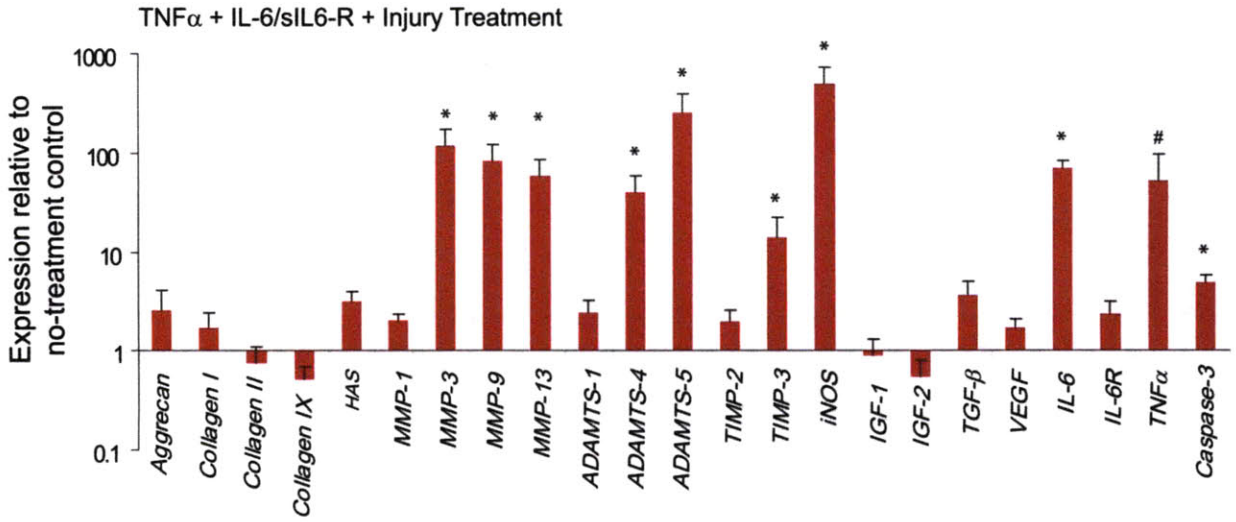


Figure 3.7 Effect of combined treatment with TNFα, IL-6/sIL-6R and mechanical injury on chondrocyte gene expression. Cartilage disks were first injuriously compressed then incubated with TNFα (25ng/ml) and IL-6/sIL-6R (50/250ng/ml) for 4 days. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control. *=$p<0.05$, #=$p<0.1$ vs. no-treatment control.

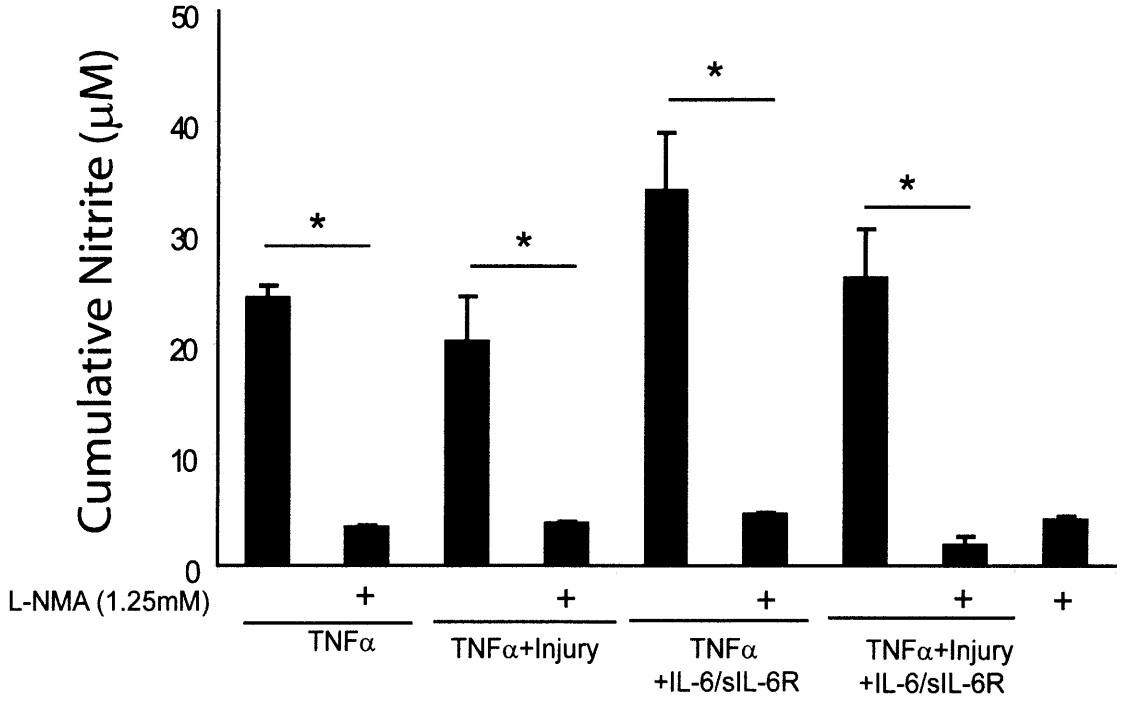


Figure 3.8 Cumulative nitrite released to medium in response to 6-day treatments with L-NMA (1.25mM, nitric oxide synthase inhibitor), TNFα(25ng/ml), IL-6/sIL-6R (50/250 ng/ml) and mechanical injury. L-NMA significantly reduced nitrite formation caused by TNFα, TNFα+injury, TNF+IL-6/sIL-6R and TNFα+injury+IL-6/sIL-6R treatments. In each condition, n=5 cartilage samples. *=$p<0.05$.

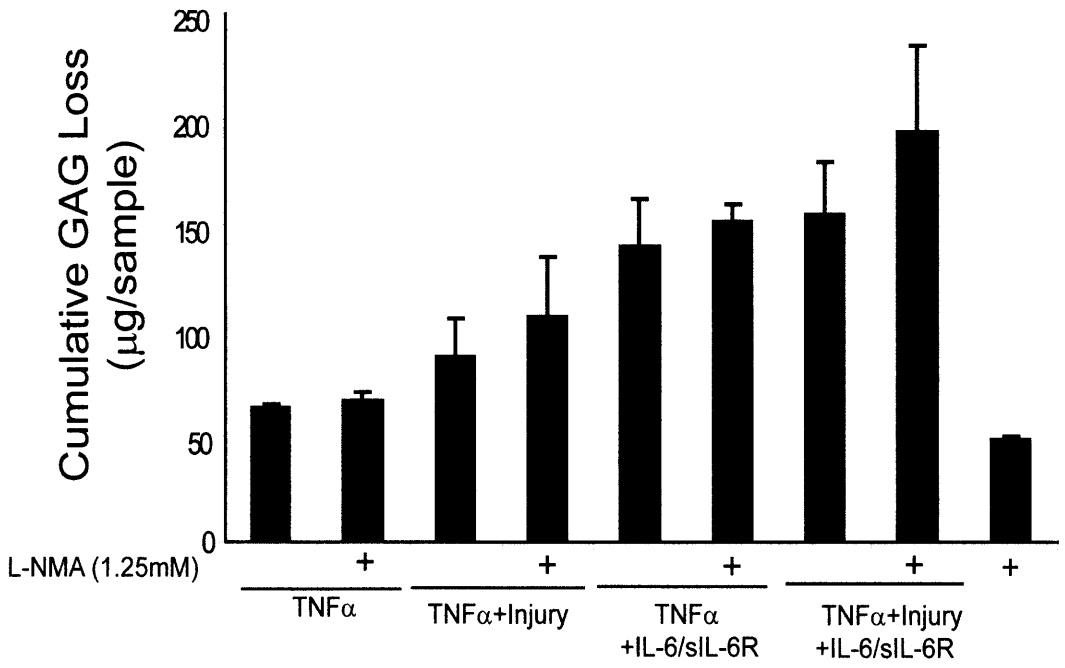


Figure 3.9 Cumulative GAG loss in response to 6-day treatments with L-NMA (1.25mM, nitric oxide synthase inhibitor), TNFα(25ng/ml), IL-6/sIL-6R (50/250 ng/ml) and mechanical injury. L-NMA showed no effect in reducing GAG loss caused by treatments. In each condition, n=5 cartilage samples.

3.6 References

1. Gelber AC, Hochberg MC, Mead LA, Wang NY, Wigley FM, Klag MJ. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. Ann Intern Med 2000;133(5):321-8.
2. Lohmander LS, Ostenberg A, Englund M, Roos H. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. Arthritis Rheum 2004;50(10):3145-52.
3. Roos H, Adalberth T, Dahlberg L, Lohmander LS. Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthritis Cartilage 1995;3(4):261-7.
4. Lohmander LS, Atley LM, Pietka TA, Eyre DR. The release of crosslinked peptides from type II collagen into human synovial fluid is increased soon after joint injury and in osteoarthritis. Arthritis Rheum 2003;48(11):3130-9.
5. Lohmander LS, Hoerrner LA, Lark MW. Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. Arthritis Rheum 1993;36(2):181-9.
6. Irie K, Uchiyama E, Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. Knee 2003;10(1):93-6.
7. Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH. The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. J Immunol 1996;156(3):1213-20.
8. Rediske JJ, Koehne CF, Zhang B, Lotz M. The inducible production of nitric oxide by articular cell types. Osteoarthritis Cartilage 1994;2(3):199-206.
9. Goodstone NJ, Hardingham TE. Tumour necrosis factor alpha stimulates nitric oxide production more potently than interleukin-1beta in porcine articular chondrocytes. Rheumatology (Oxford) 2002;41(8):883-91.
10. Stevens AL, Wheeler CA, Tannenbaum SR, Grodzinsky AJ. Nitric oxide enhances aggrecan degradation by aggrecanase in response to TNF-alpha but not IL-1beta treatment at a post-transcriptional level in bovine cartilage explants. Osteoarthritis Cartilage 2007.
11. Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. The extent of matrix damage and chondrocyte death in mechanically traumatized articular cartilage explants depends on rate of loading. J Orthop Res 2001;19(5):779-84.
12. Quinn TM, Grodzinsky AJ, Hunziker EB, Sandy JD. Effects of injurious compression on matrix turnover around individual cells in calf articular cartilage explants. J Orthop Res 1998;16(4):490-9.
13. Thibault M, Poole AR, Buschmann MD. Cyclic compression of cartilage/bone explants in vitro leads to physical weakening, mechanical breakdown of collagen and release of matrix fragments. J Orthop Res 2002;20(6):1265-73.
14. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, et al. Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 2000;381(2):205-12.

15. Kurz B, Jin M, Patwari P, Cheng DM, Lark MW, Grodzinsky AJ. Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orthop Res 2001;19(6):1140-6.
16. Lee JH, Fitzgerald JB, Dimicco MA, Grodzinsky AJ. Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. Arthritis Rheum 2005;52(8):2386-95.
17. Jones AR, Chen S, Chai DH, Stevens AL, Gleghorn JP, Bonassar LP, et al. Modulation of lubricin biosynthesis and tissue surface properties following cartilage mechanical injury. Arthritis Rheum 2009;60(1):133-42.
18. Sui Y, Lee JH, DiMicco MA, Vanderploeg EJ, Blake SM, Hung HH, et al. Mechanical injury potentiates proteoglycan catabolism induced by interleukin-6 with soluble interleukin-6 receptor and tumor necrosis factor alpha in immature bovine and adult human articular cartilage. Arthritis Rheum 2009;60(10):2985-96.
19. Patwari P, Cook MN, DiMicco MA, Blake SM, James IE, Kumar S, et al. Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum 2003;48(5):1292-301.
20. Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989;7(5):619-36.
21. Frank EH, Jin M, Loening AM, Levenston ME, Grodzinsky AJ. A versatile shear and compression apparatus for mechanical stimulation of tissue culture explants. J Biomech 2000;33(11):1523-7.
22. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. Matrix Biol 2000;19(6):549-53.
23. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365-86.
24. DiMicco MA, Patwari P, Siparsky PN, Kumar S, Pratta MA, Lark MW, et al. Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury. Arthritis Rheum 2004;50(3):840-8.
25. Monfort J, Tardif G, Reboul P, Mineau F, Roughley P, Pelletier JP, et al. Degradation of small leucine-rich repeat proteoglycans by matrix metalloprotease-13: identification of a new biglycan cleavage site. Arthritis Res Ther 2006;8(1):R26.
26. Karsdal MA, Byrjalsen I, Leeming DJ, Christiansen C. Tibolone inhibits bone resorption without secondary positive effects on cartilage degradation. BMC Musculoskelet Disord 2008;9:153.
27. Karsdal MA, Madsen SH, Christiansen C, Henriksen K, Fosang AJ, Sondergaard BC. Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. Arthritis Res Ther 2008;10(3):R63.
28. Fischer BA, Mundle S, Cole AA. Tumor necrosis factor-alpha induced DNA cleavage in human articular chondrocytes may involve multiple endonucleolytic activities during apoptosis. Microsc Res Tech 2000;50(3):236-42.
29. Aizawa T, Kon T, Einhorn TA, Gerstenfeld LC. Induction of apoptosis in chondrocytes by tumor necrosis factor-alpha. J Orthop Res 2001;19(5):785-96.
30. Dang AC, Kim HT. Chondrocyte apoptosis after simulated intraarticular fracture: a comparison of histologic detection methods. Clin Orthop Relat Res 2009;467(7):1877-84.

31. Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem 2001;276(16):12501-4.
32. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 2003;5(2):94-103.
33. Wayne GJ, Deng SJ, Amour A, Borman S, Matico R, Carter HL, et al. TIMP-3 inhibition of ADAMTS-4 (Aggrecanase-1) is modulated by interactions between aggrecan and the C-terminal domain of ADAMTS-4. J Biol Chem 2007;282(29):20991-8.
34. Voigt H, Lemke AK, Mentlein R, Schunke M, Kurz B. Tumor necrosis factor alpha-dependent aggrecan cleavage and release of glycosaminoglycans in the meniscus is mediated by nitrous oxide-independent aggrecanase activity in vitro. Arthritis Res Ther 2009;11(5):R141.

3.7 Supplementary Data

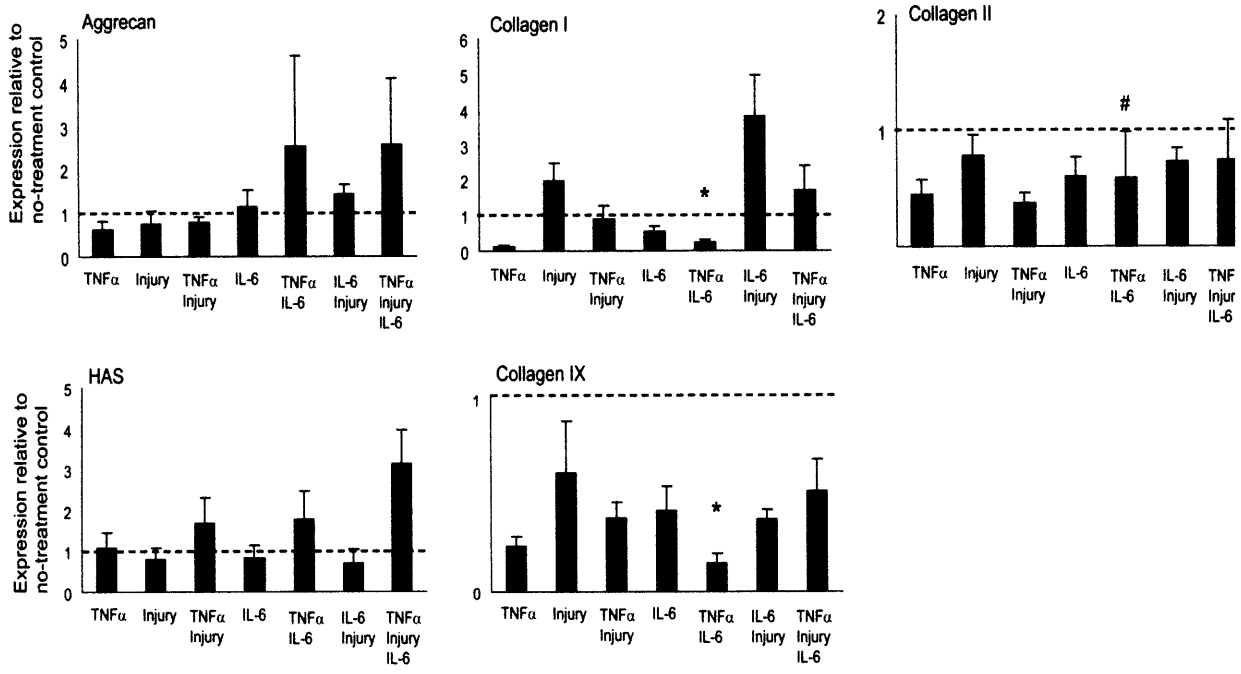


Figure 3S.1 Effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression of matrix proteins. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). *=p<0.05, #=p<0.1 vs. no-treatment control.

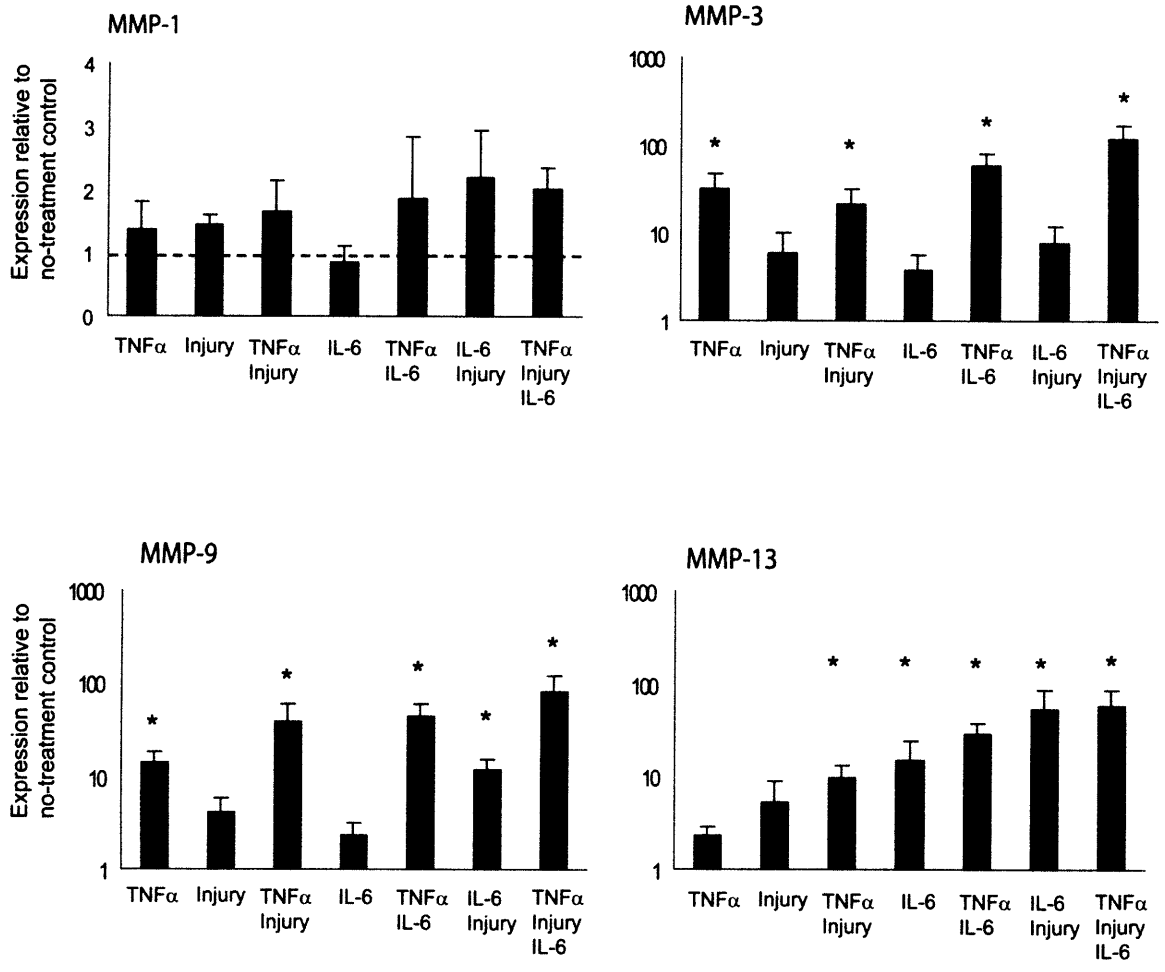


Figure 3S.2 Effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression of matrix metalloproteinases. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). *=$p<0.05$ vs. no-treatment control.

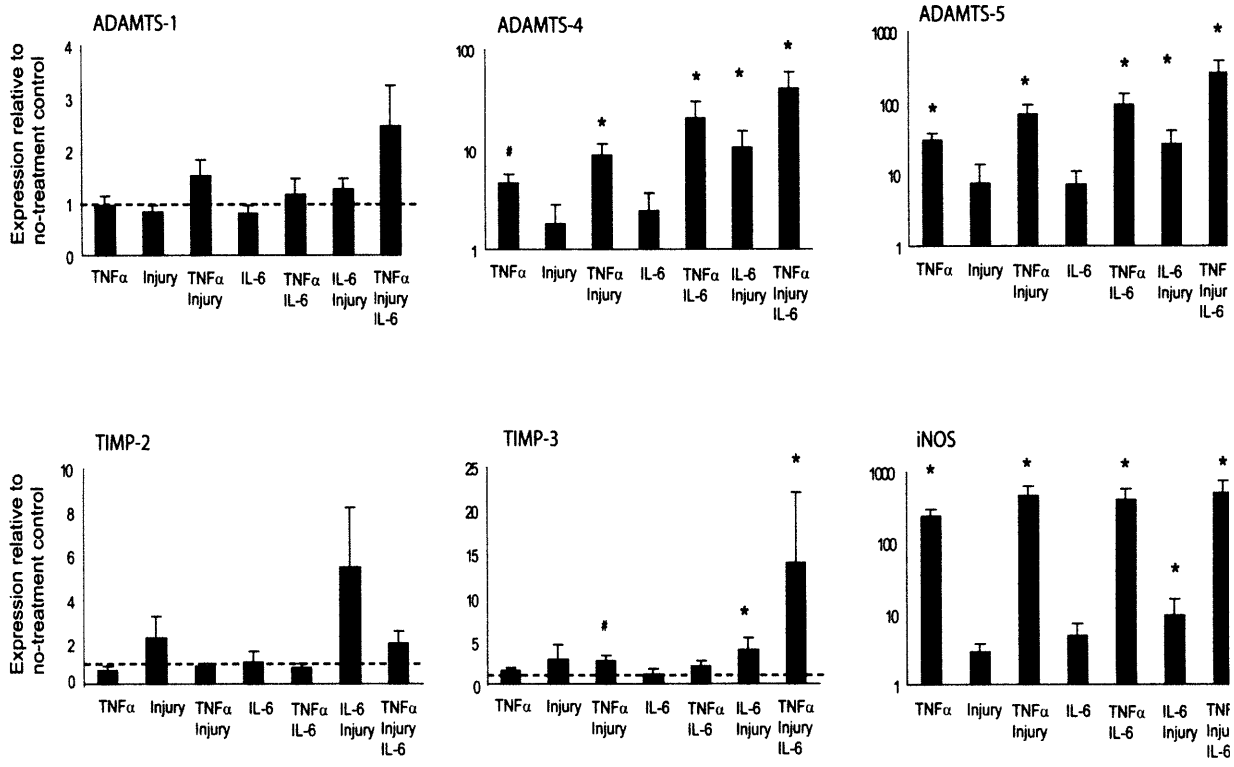


Figure 3S.3 Effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression of aggrecanases, tissue inhibitor of matrix metalloproteinases (TIMPs) and inducible nitric oxide synthase (iNOS). Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). *=$p<0.05$, #=$p<0.1$ vs. no-treatment control.

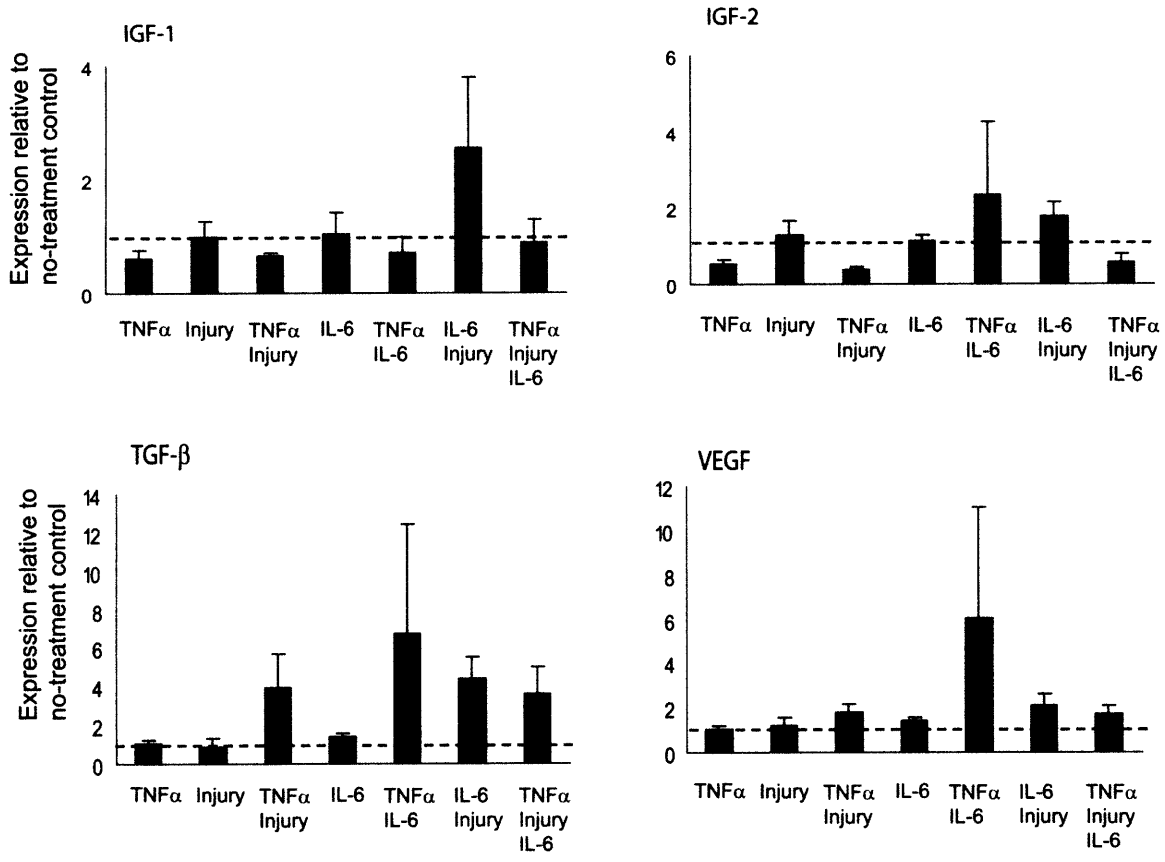


Figure 3S.4 Effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression of growth factors. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line).

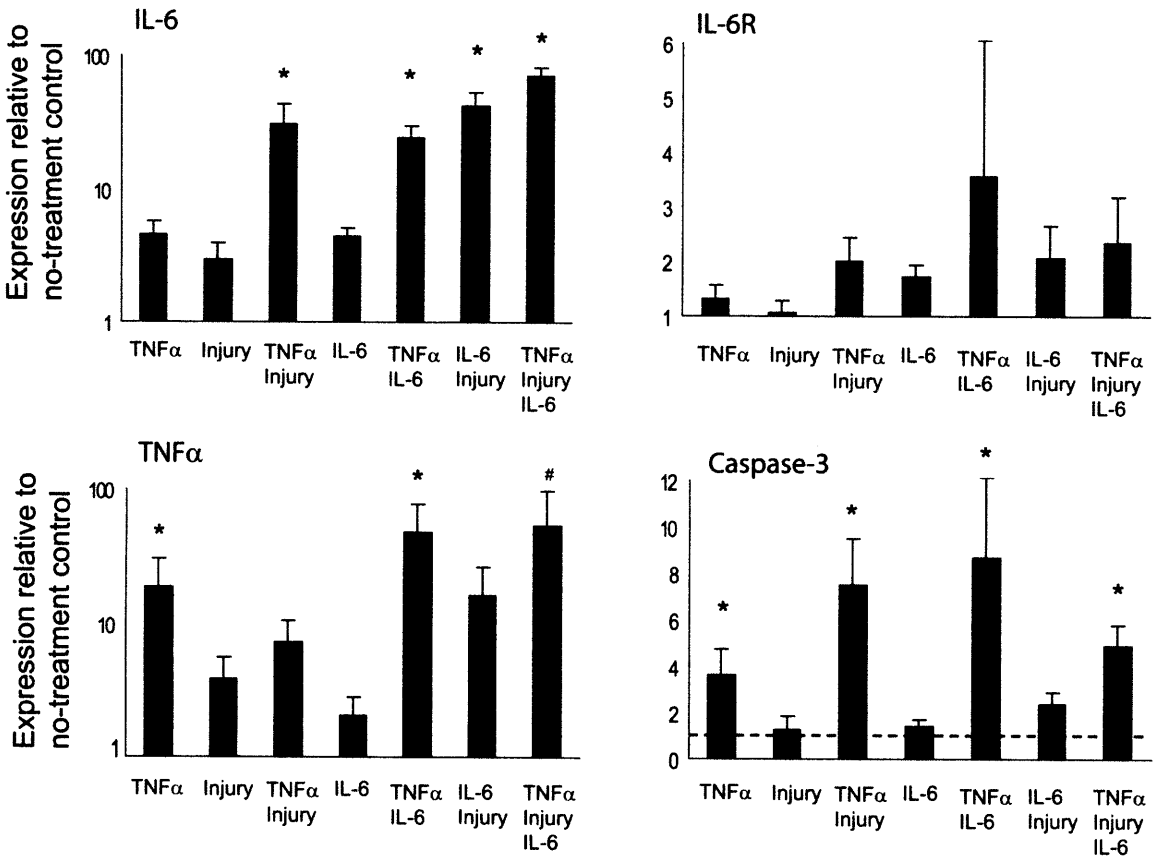


Figure 3S.5 Effect of TNFα, IL-6/sIL-6R, mechanical injury and their combinations on chondrocyte gene expression of cytokines, cytokine receptor and capspase-3. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). *=$p<0.05$, #=$p<0.1$ vs. no-treatment control.

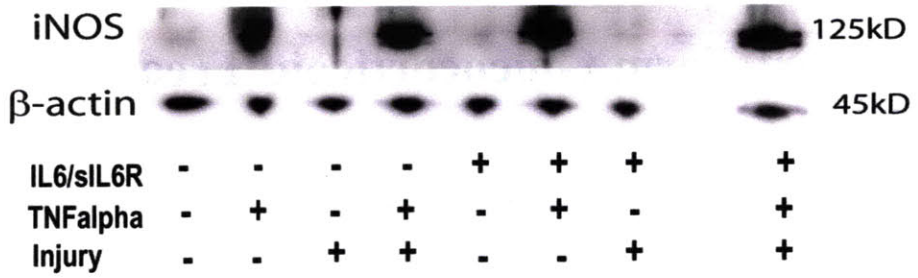


Figure 3S.6 Western blot for iNOS protein in the combined treatments with TNFα, IL-6/sIL-6R and mechanical injury. Cartilage samples were either injured or left uninjured, then treated with TNFα, IL-6/sIL-6R or their combinations for 4 additional days. Equal quantities of tissue extracts from each condition were run on a 4-15% gradient SDS-PAGE gel, transferred to PVDF membranes and immunoblotted with a primary antibody recognizing iNOS.

CHAPTER 4

Effects of Short-Term Glucocorticoid Treatment in Cartilage Degradation and Chondrocyte Gene Expression Induced by the Combined Treatments with Cytokines and Mechanical Injury*

* This chapter is a manuscript in preparation for submission

4.1 Introduction

Osteoarthritis (OA) is characterized by chronic degradation of articular cartilage. Joint injury in young adults greatly increases the risk for the development of OA (1, 2). Treatments following trauma primarily focus on reducing pain and swelling, as well as re-stabilizing the injured joint when necessary. However, these interventions do not prevent the progression of secondary OA after injury(3). Studies have shown following knee injury, high levels of aggrecan fragments and cross-linked peptides from type II collagen were found in the synovial fluid(4, 5). Moreover, joint injury results in an immediate surge in synovial concentrations of matrix metalloproteinase-3 (MMP-3) (6) and pro-inflammatory cytokines including tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6 and IL-8 (7, 8). The levels of these cytokines remained elevated for weeks and eventually became similar to those detected in chronic OA joints (8).

Intra-articular injection of glucocorticoid (GC) is an established treatment for both OA and rheumatoid arthritis (RA) (9, 10). GCs exert their effect by binding to the intracellular glucocorticoid receptors (GRs), which act as transcription factors in cells. The activated GRs either directly or indirectly regulate the transcription of target genes. For example, it has been shown GRs enhance the production of anti-inflammatory cytokines such as IL-1 receptor antagonist and IL-10 (11), while repress the expression of molecules associated with inflammatory processes, including cytokines IL-1β, IL-6, TNFα, and cyclooxygenase-2 (12-15). The mechanisms of GC in cartilage are less understood. Since human chondrocytes have been shown to express GR (16, 17), the effect of GC in treating joint disorders may be due to direct regulation of chondrocytes.

Dexamethasone (DEX) is a very potent synthetic GC due to its high receptor binding affinity(18). DEX has been commonly used in cartilage tissue engineering. Numerous studies have demonstrated that DEX is required for progenitor cells to undergo chondrogenic differentiation and to stimulate chondrocytes to synthesize proteoglycans (19-21). However, the effect of DEX on cartilage matrix degradations and repair, particularly those associated with joint injury, remain unclear.

Our previous study has highlighted the importance of the interplay between mechanical and cytokine-mediated pathways associated with cartilage degradation following joint injury (22). We used an in vitro model, which involves injurious compression of cartilage explants and direct cytokines treatments. TNFα and mechanical injury synergistically increased proteoglycan degradation in cartilage. TNFα also induced the production of IL-6, which combined with its soluble receptor (sIL-6R) synergistically increased the catabolic effects of TNFα in cartilage. Moreover, in both bovine and human cartilage, injury potentiated the catabolic effects of TNFα combined with IL-6/sIL-6R exposure, causing the most severe glycosaminoglycan (GAG) loss among all the treatment conditions, and the proteoglycan degradation was aggrecanase generated.

ADAMTS-4 and -5 are the primary aggrecanases responsible for the pathological process of aggrecan degradation in human OA. Aggrecanases are synthesized as latent, inactive enzymes whose pro-domains have to be removed by proprotein convertases (PCs) in order to express their catalytic function. Studies have shown increased activity of PCs in both osteoarthritic and cytokine-stimulated cartilage, and inhibiting PC activity significantly reduced cytokine-induced aggrecan degradation (23). Among all the PCs, furin, PACE4 and PC5/6

have been proved to be capable of removing the prodomain of ADAMTS-4 (24), while furin and PC7 have been shown to process pro-ADAMTS-5 (25).

In this study, the objectives were 1) to test the hypothesis that short-term treatment with GCs, such as DEX, could abolish the cartilage degradation induced by mechanical injury, TNFα and IL-6/sIL-6R in both bovine and human cartilage, 2) to investigate whether DEX regulates this catabolic response at the transcriptional level in chondrocytes, and 3) to examined the role of DEX in modulating the proteolytic activities of aggrecanases.

4.2 Materials And Methods

Bovine cartilage harvest and culture. Cartilage disks were harvested from the femoropatellar grooves of 1-2-week old bovine calves as described(26). Briefly, cartilage-bone cylinders (9mm diameter) from knee joints were cored perpendicular to the surface. After a level surface was obtained by removing the most superficial layer (~100-200 μm), 1-2 sequential 1mm-slices of middle zone cartilage were cut from each cylinder. Five disks (3mm-diameter, 1mm-thick) were cored from each slice using a dermal punch. Cartilage from this middle zone in newborn calf was shown to have a reasonably homogeneous population of cells and matrix(27). All treatment groups were matched for location and depth of cartilage on the joint surface. Disks were equilibrated in serum-free medium (low-glucose DMEM supplemented with 1% insulin-transferrin-selenium (10 μg/ml, 5.5 μg/ml and 5 ng/ml, respectively) (Sigma, St. Louis, MO), 10 m*M* HEPES buffer, 0.1 m*M* nonessential amino acids, 0.4 m*M* proline, 20 μg/mL ascorbic acid, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B for 2 days prior to treatment in a 37°C, 5% CO_2 incubator.

Postmortem adult human donor tissue. Human donor joints (49-yr-old female, modified-Collins (28) grade-1 knee; 64-yr-old male, grade-1 ankles) were obtained from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL), approved by the Office of Research Affairs at Rush-Presbyterian-St. Luke's Medical Center and the Committee on Use of Humans as Experimental Subjects at MIT. Any fibrillated areas detected under visual inspection were excluded from study. Human cartilage harvest and culture were identical to those of bovine, but included the intact superficial zone and each disk was 0.8 mm thick. Human knee cartilage was obtained from both femoropatellar groove and femoral condyles, and ankle cartilage was harvested from all surfaces of the talus.

Injurious compression. After equilibration in medium for 3 days, disks were injuriously compressed in a custom-designed incubator-housed apparatus(29, 30). Each bovine disk was subjected to radially unconfined compression to 50% final strain at 1mm/second velocity (100%/second strain rate), followed by immediate release of load at the same rate as described(26). Immediately after injury, some disks were deformed to an ellipsoidal shape (deformation score of 1 or 2 as described in (31)), but none exhibited gross fissuring. Adult human cartilage disks were thinner and had different biomechanical properties than immature bovine samples, therefore we used a higher strain (60%) and strain rate (300%/second). Macroscopic tissue changes were similar (elliptical appearance) to those described previously using our human cartilage injury model(32). After injury, samples were immediately placed in treatment medium.

Exogenous cytokines, injury and DEX treatment. Cartilage samples were either subjected to compression or left uninjured, incubated with or without cytokines (all from R&D Systems, MN), and with or without DEX (Sigma, St. Louis, MO). In the DEX dose-response

study, bovine cartilage samples were treated either with or without rhTNFα, and incubated with increasing concentrations of DEX, from 0.1nM to 100μM, for 6 days. Previously, we have observed the treatments with TNFα, TNFα+injury, TNFα+IL-6/sIL-6R, and TNFα+injury+IL-6/sIL-6R caused significant release of GAGs from both bovine and human cartilage explants. In this study, we examined the effects of DEX in cartilage explants treated with these conditions. For bovine cartilage, DEX and rhTNFα was used at 10nM and 25 ng/mL, respectively. For human cartilage, DEX and rhTNFα was used at 100nM and 100ng/mL, respectively. rhIL-6 (50 ng/mL) was always used in combination with soluble IL-6 receptor (sIL-6R, 250 ng/mL), since this combination was found previously to induce greater aggrecan degradation than when used separately in the presence of TNFα(33). Bovine cartilage disks were cultured in various conditions for 6 days. Earlier studies showed that for each treatment, human cartilage released sGAG somewhat less rapidly than bovine(22). Therefore, the culture duration for human explants was extended, 10 day for human knee and 8 day for human ankle study. Medium was replaced every 2 days and saved for analysis.

A parallel study investigated whether pre-exposing cartilage with DEX could prevent the matrix degradation induced by cytokines. Cartilage samples were either pre-treated with DEX for 2 days or incubated in medium alone. Afterwards, both groups were incubated in medium containing TNFα for additional 4 days. To test whether DEX could still produce its effects in samples where catabolic process has already started, cartilage explants were pre-treated with TNFα for 2 days, and then DEX was added in addition to the TNFα treatment for another 4 days.

Another study examined the role of GR in chondrocyte response to DEX. Bovine cartilage samples were treated with the GR antagonist, RU486 (1μM, Sigma,St. Louis, MO), in the presence of TNFα and TNFα+DEX for 6 days.

To examine the effects of DEX, injury and TNFα treatment on chondrocyte gene expression, bovine cartilage were cultured in the conditions of 1) no-treatment control, 2) DEX, 3) injury, 4) DEX+injury, 5) TNFα, 6) TNFα+DeX, 7) TNFα+injury, and 8) TNFα+injury+DEX for 4 days. RNA was extracted for the determination of the expression levels of 24 genes associated with cartilage metabolism. The same experiment was repeated in total of 6 animals.

Finally, the role of proprotein convertases in matrix degradation was tested in bovine cartilage cultured in different combinations of TNFα, IL-6/sIL-6R and mechanical injury. Each of these conditions was also added with 10μM PC inhibitor, decanoyl-RVKR-CMK (Calbiochem, La Jolla, CA) and cultured for 6 days.

Matrix biosynthesis and Biochemical analysis. Two days before termination, bovine culture medium was supplemented with 5 μCi/ml $Na_2{}^{35}SO_4$ and 10 μCi/ml L-5-^{3}H-proline as measures of proteoglycan and total protein synthesis rates, respectively. The amount of radiolabeled sulfate and proline were doubled in human studies. Upon termination, disks were washed, weighed and solubilized (proteinase K, Roche). The rate of radiolabel incorporation was measured using a liquid scintillation counter(27). The amount of sGAG lost to the medium and that retained in the cartilage was measured using the DMMB assay(34).

RNA extraction and real-time PCR. After incubating cartilage samples in treatment conditions for four days, 6 cartilage disks from each condition were pooled together for RNA extraction. RNA extraction, reverse transcription and quantification were performed as

previously described (35). Bovine primers were designed for genes involved in cartilage homeostasis, including matrix molecules (aggrecan, collagen II and IX), cytokines (IL-1β, IL-6, TNFα), proteases and protease inhibitors (ADAMTS-4,-5, MMP-3, TIMP-3), inducible nitric oxide synthase (iNOS) and house keeping gene (18S). The bovine primer sequences all have been used in previous studies(36, 37). Real-time PCR was performed using Applied Biosystems ABI 7900HT instrument and SYBR Green Master Mix (Applied Biosystems, CA). Measured threshold values (Ct) were converted to RNA copy number according to previously determined primer efficiencies. Within each condition, the RNA copy numbers for each gene were normalized to that of 18S from the same condition. To examine the effects of treatments, each gene was then normalized to its level in the no-treatment control group, as plotted in Figure 4.6.

Statistical analyses. In studying the dose-response of DEX on GAG loss and chondrocyte biosynthesis, a general linear model was used to analyze the data, followed by Tukey's post hoc test for pairwise comparisons. The same analysis was also used to evaluate the effect of DEX on cytokines- and mechanical injury-treated bovine and human cartilage, as well as the effect of RU486 in reversing the action of DEX. In the study of pre-treating cartilage with either DEX or TNFα, student's t-test was used to evaluate the difference between conditions at each time point. For the gene expression study, log-transformed expression data were analyzed using a linear mixed-effect model with animal as a random variable, followed by Tukey's pairwise comparison. All values were expressed as mean±SEM, with $p \leq 0.05$ as statistically significant. Statistical analyses were performed using SYSTAT-12 software (Richmond, CA).

4.3 Results

DEX dose-dependently inhibited both GAG loss and biosynthesis reduction in TNFα-treated bovine cartilage explants. Experiments were performed to test the effect of DEX (0.1nM to 100μM) on both TNFα-treated and regular medium cultured cartilage explants and to measure the resulting loss of sGAG (Figure 4.1). Compare with control samples (mean±SEM total sGAG loss 8.5±0.2%, over a 6-day period), TNFα treatment significantly increased GAG loss (16.2±0.5%), a finding that was consistent with previous studies (22). Interestingly, 1nM or higher concentrations of DEX treatment significantly inhibited GAG loss induced by TNFα treatment, all to levels comparable to control samples. At higher concentrations (i.e.100nM and 1μM), DEX treatment alone resulted in even less GAG loss comparing to the no-treatment group.

All cartilage samples from Figure 4.1A were also radiolabeled with ^{35}S-sulfate and ^{3}H-proline to measure the rates of proteoglycan and protein biosynthesis in response to treatment conditions (Figure 4.1B). Compared with controls (^{35}S-sulfate 51.6±2.1pmol/hour/mg wet weight), TNFα treatment significantly reduced the biosynthetic rate of proteoglycan (25.3±2.3 pmol/hour/mg) and protein (data not shown). In contrast, treatments with DEX at concentrations 1nM and higher abolished the biosynthesis reduction caused by the TNFα treatment. Moreover, 1μM and 100μM of DEX treatment alone resulted in significantly higher sulfate incorporations (75.9±3.5 and 73.0±1.0 pmol/hour/mg, respectively) than no-treatment control.

DEX inhibited GAG loss and biosynthesis reduction from bovine cartilage treated with combinations of mechanical injury, TNFα and IL-6/sIL6R. Consistent with our

previous findings, TNFα treatment, either alone or together with mechanical injury and IL-6/sIL-6R treatment, significantly increased sGAG release from bovine cartilage (Figure 4.2) (22). The combined treatment of TNFα, IL-6/sIL-6R and injurious compression caused the most severe GAG loss (53.6±9.8%) in 6 days. Strikingly, the addition of 10nM DEX treatment significantly reduced proteoglycan degradation from all of the above conditions (i.e. 7.7±0.9% and13.8±1.5% GAG loss from [TNFα+DEX] and [TNFα+IL-6/sIL-6R+injury+DEX] treatments, respectively), to levels comparable to that of the normal control samples (7.3±0.2%).

TNFα treatment, either alone or together with mechanical injury and IL-6/sIL-6R treatment, also significantly decreased the incorporation rate of both sulfate (Figure 4.2B) and proline (data not shown), agreeing with our previous findings (22). DEX alone resulted in increased proteoglycan synthesis from bovine cartilage samples. More importantly, it also abolished the biosynthesis reduction caused by TNFα and the combined treatments with TNFα, IL-6/sIL-6R and mechanical injury. For example, the treatment with [TNFα+IL-6/sIL-6R+injury] reduced sulfate incorporation to 26.2±7.2 pmol/hour/mg, whereas the addition of DEX to the same condition significantly increased the incorporation rate to 96.2±13.44 pmol/hour/mg.

DEX treatment reduced GAG loss in human cartilage explants. TNFα treatment and [TNFα+injury+IL-6/sIL-6R] treatment significantly increased GAG loss from human knee cartilage (23.5±2.3% and 36.0±4%, respectively. Figure 4.3), consistent with what we reported previously (22). In these conditions, the addition of 100nM DEX significantly lowered the GAG loss (16.2±1.4% and 20.5±1.5%, respectively), but showed no effect on sulfate incorporation (data not shown). Similarly, DEX treatment markedly reduced the GAG loss

induced by TNFα+IL-6/sIL-6R+mechanical injury treatment in human ankle cartilage, while imposing no effect on proteoglycan biosynthesis (data not shown).

Pre-incubation with DEX lowered GAG loss and increased sulfate incorporation with TNFα treatment. Bovine cartilage samples were pre-incubated with DEX for two days, and then cultured in medium supplemented with TNFα, without DEX, for additional four days. Two-day pre-treatment with DEX significantly reduced TNFα-induced GAG loss at both day 2 and day 4 (Figure 4.4A). Cartilage samples pre-treated with DEX also showed significantly higher sulfate incorporation rate comparing to the ones without DEX treatment (Figure 4.4B).

DEX reduced GAG loss and increased sulfate incorporation in TNFα-pre-treated cartilage samples. We next examined whether DEX would exert anti-catabolic effect in cartilage samples where matrix degradation has already been induced by cytokine stimulation. All cartilage samples were pre-incubated with TNFα for two days. Afterwards, one group of samples was cultured in medium with TNFα+ DEX, while the other condition only had treatment with TNFα. After the pre-incubation with TNFα, both conditions lost approximately 6% of total GAGs. The addition of DEX significantly attenuated GAG loss and increased proteoglycan biosyntheis (Figure 4.4 C,D).

The anti-catabolic effects of DEX were glucocorticoid receptor (GR) mediated. To assess whether the inhibition on GAG loss and the increase in chondrocyte biosynthesis in DEX-treated cartilage were GR mediated, samples were treated with GR antagonist RU486 in addition to TNFα ± DEX treatment. As shown in Figure 4.5, DEX inhibited the release of GAG induced by TNFα and increased the sulfate incorporation rate. The effect of DEX is reversed in the presence of RU486. RU486 alone has no effect on both normal controls and TNFα-treated samples.

Effects of DEX, TNFα and mechanical injury on chondrocyte gene expression.

Real-time PCR was performed to determine chondrocyte gene response to 4-day treatments with DEX, TNFα and mechanical injury as well as their combinations (Figure 4.6). From all the genes tested, none showed significant response to DEX treatment alone, mechanical injury lone and the combined treatment with DEX+injury, while majority of the genes responded to either TNFα treatment or the combination of TNFα and injury.

Matrix molecules collagen II and IX responded to both TNFα and TNFα+injury treatments with a significant decrease in expression levels. Both molecules also showed elevated expression in the combination of DEX plus TNFα. However, DEX has no effect on their expression levels in the treatment with TNFα+injury. Aggrecan showed trends of decrease in response to TNFα and TNFα+injury treatment, and its expression in response to TNFα+DEX was near control level. However, none of the treatments significantly affected the expression of aggrecan. Both TNFα and IL-1β gene showed increased expression with TNFα and TNFα+injury treatment and decreased expression in response to TNFα+DEX treatment, although the trends were not statistically significant. IL-6 responded to all treatments involving TNFα with significantly elevated expression levels, irrespective of the presence of DEX and mechanical injury treatment. Similarly, iNOS responded to treatments with TNFα and TNFα plus injury with significantly increased expression, regardless of DEX treatment. Interestingly, nitrite formation measured in conditioned media was markedly reduced when DEX was added to the TNFα and TNFα+injury treatment.

Analysis also showed that TNFα significantly upregulated the expression of ADAMTS-4 and MMP-3. More genes related to protease and protease inhibitor were up-regulated in response to the TNFα+injury treatment, including ADAMTS-4,-5, TIMP3 and MMP-3.

Among the matrix proteases, only MMP-3 showed reduced expression in response to DEX+TNFα and DEX+TNFα+injury treatment, whereas the aggrecanases were not down-regulated in the presence of DEX.

Proprotein Convertase (PC) Inhibitor decreased GAG loss induced by cytokines and mechanical injury treatment. To assess the role of PC in cartilage degradation, a general PC inhibitor, decanoyl-RVKR-CMK, was added to the condition of TNFα,TNFα+IL-6/sIL-6R and TNFα+IL-6/sIL-6R+injury. Treatments with TNFα,TNFα+IL-6/sIL-6R and TNFα+IL-6/sIL-6R+injury all significantly increased GAG loss comparing to control samples, whereas 10μM CMK significantly decreased GAG release induced by TNFα+IL-6/sIL-6R and TNFα+IL-6/sIL-6R+injury (Figure 4.7).

4.4 Discussion

The objective of this study was to determine the effect of DEX in cartilage degradation induced by combined treatments with mechanical injury and pro-inflammatory cytokine stimulation. We previously reported co-stimulation of cartilage with TNFα and IL-6/sIL-6R caused significantly more GAG release than either cytokine exposure alone, in both immature bovine knee and adult human knee and ankle cartilage. Moreover, mechanical injury substantially potentiated the combined catabolic effect of TNFα and IL-6/sIL-6R by inducing severe matrix degradation. In this study, we first demonstrated that DEX, over a wide range of concentrations (1nM to 100μM), completely blocked TNFα-induced GAG loss and biosynthesis reduction in bovine cartilage. Even in the absence of cytokine stimulations, cartilage disks exposed to higher concentrations (i.e.1μM) of DEX released less GAG and showed increased sulfate incorporation than samples incubated in medium without DEX.

More importantly, DEX (10nM) also recovered chondrocyte biosynthesis and inhibited GAG loss caused by the treatments with TNFα+IL-6/sIL-6R, TNFα+injury, and TNFα+IL-6/sIL-6R+injury. The proteoglycan fragments from these conditions were previously found to be generated by aggrecanases, not MMPs (22), suggesting the inhibitory effect of DEX in matrix degradation may involve modulating the proteolytic activities of aggrecanases. Recently, Malfait et al. demonstrated that DEX blocked the aggrecanase activity in an in vivo model of cartilage degradation. They showed intra-articular injection of TNFα in rats resulted in aggrecanase-generated proteoglycan degradation, which could be inhibited by either an aggrecanase inhibitor or DEX, but not a non-steroidal anti-inflammatory drug (38).

Surprisingly, DEX did not regulate aggrecanases at the transcriptional level. We observed the gene expression of ADAMTS-4 and -5 significantly increased with TNFα and TNFα+injury treatment, but their levels remained elevated even in the presence of DEX. Similarly, DEX did not down-regulate the gene expression of iNOS in treatment conditions, whereas we and others have observed that DEX could reduce the level of iNOS protein as well as nitric oxide production, in both cytokine-stimulated and cytokine plus injury-treated cartilage(39). Therefore, DEX may not regulate chondrocytes at the transcriptional level alone. For aggrecanases, there are multiple levels that could be modulated, including altered protein expression, pro-enzyme activation and binding to aggrecan from the C-terminal thrombospondin motif. In this study, we hypothesized that DEX may block the aggrecanase activity by inhibiting the activation of latent pro-ADAMTS-4 and -5. We have shown blocking proprotein convertases activity significantly reduced GAG loss in the cytokine and cytokine plus injury treatments, suggesting proprotein convertases play important roles in proteoglycan degradation. Others have made similar observations with TNFα-treated cartilage (23).

Ongoing studies are investigating how DEX modulates proprotein convertases activities as well as other possible mechanisms involved in DEX-induced inhibition on proteoglycan degradation.

We demonstrated that treating cartilage with DEX either before or after TNFα stimulation significantly reduced GAG loss and increased proteoglycan biosynthesis. This observation suggested the effect of DEX was long lasting and preventative to future cytokine assaults. Furthermore, even when the catabolic process has already occurred in cartilage, DEX treatment could still suppress GAG loss and increase biosynthesis.

In this study, we also observed that DEX (100nM) significantly reduced GAG loss in human cartilage tissues, though having no stimulatory effects on proteoglycan biosynthesis. This finding was consistent with previous studies. For example, Hardy et al. observed DEX blocked IL-1 stimulated proteoglycan degradation in OA cartilage cultured with synovial membrane (40). Guerne et al. reported DEX inhibited the down-regulating effect of IL-1 and IL-6/sIL-6R on proteoglycan synthesis, enhanced matrix synthesis in normal human chondrocytes, and to a lesser extent in osteoarthritic chondrocytes (39). Together these reports indicate DEX may produce favorable response in human cartilage.

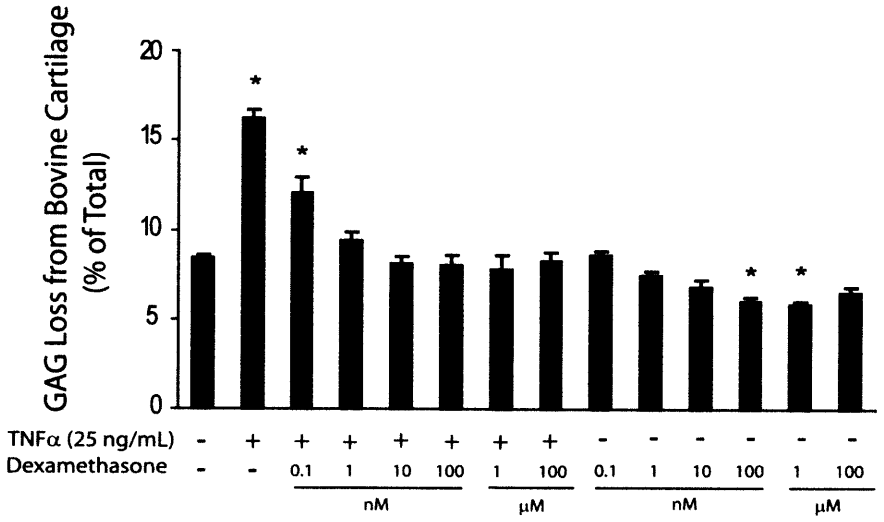
GCs have been widely used in the treatment of joint diseases (9, 10). Most studies and trials reported beneficial responses, including significantly greater reduction of pain and tenderness, and increased motion in the injected joint(41, 42). However, because the mechanism of GCs in cartilage function was not well understood and there have been some anecdotal reports on GC related side effects in treating joint diseases, the use of GCs in OA treatment remain controversial. It has been noted that the reports describing negative effect of GCs often involved either frequent injections or high dosage(43). More careful reviews have

shown that the efficacy of GC is dependent on the concentration used (44). In order to avoid complications, longer intervals between GCs injections for the weight-bearing joints have been recommended(45). There have not been any reports on the long-term effects of GC treatments in joint injury. However, the current study suggests immediate treatment of DEX in injured knee may greatly retard the progression of cartilage degradation.

In summary, it has been established that acute knee injury initiates cascades of catabolic events in joint tissues, including mechanical disruption of cartilage matrix and increasing synovial concentrations of pro-inflammatory cytokines. Glucocorticoid treatment in cartilage tissue can effectively abolish the matrix degradation induced by the combination of pro-inflammatory cytokines and injury. We speculate that dexamethasone may protect cartilage matrix from post-traumatic catabolism by modulating the aggrecanase proteolytic activities, therefore may be a better therapeutic strategy in preventing cartilage degeneration in individuals with joint injury.

4.5 Figures

A.



B.

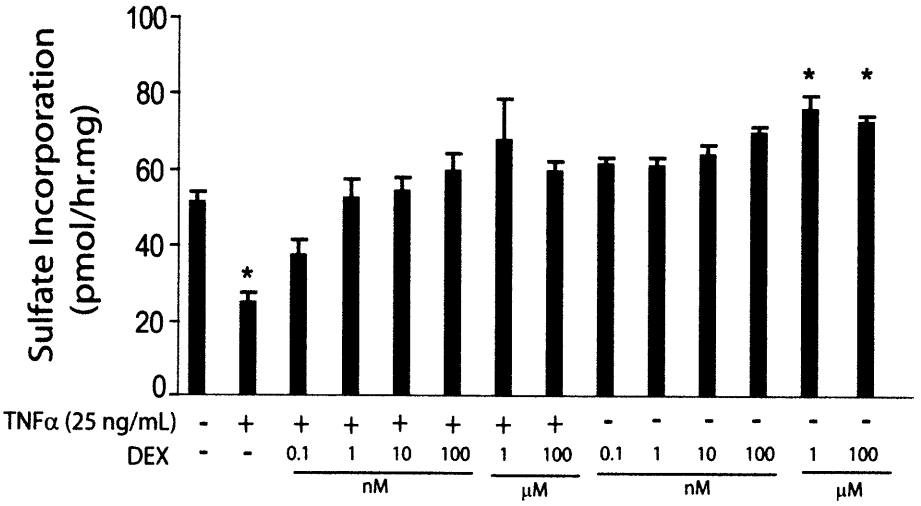
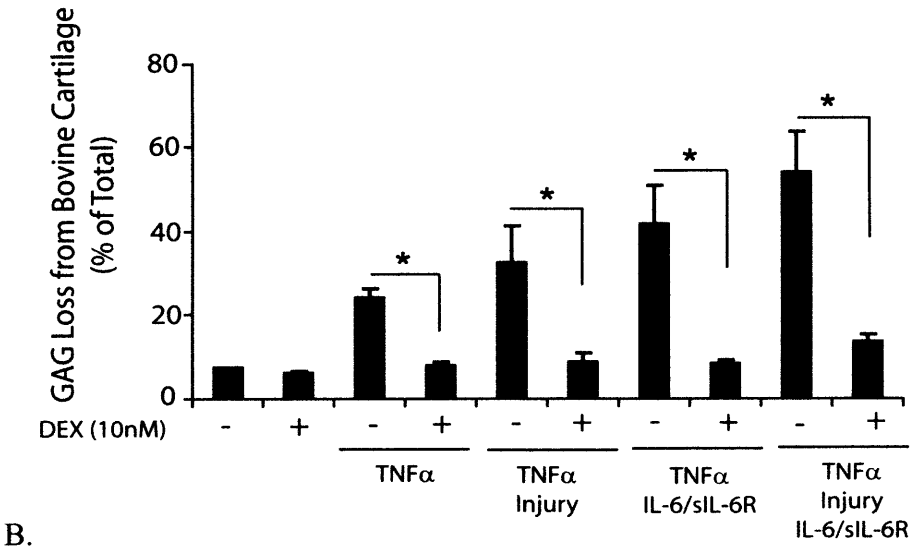


Figure 4.1 **A,** Effect of DEX on TNFα-stimulated GAG loss in bovine cartilage explants. Cartilage tissues were cultured in DEX (0.1nM-100μM)-supplemented media, with or without TNFα (25ng/ml) for 6 days. The mean±SEM total GAG content was 465.6±23.1μg GAG/disk in the untreated control group. DEX, at 1nM and higher, significantly inhibited GAG loss induced by TNFα treatment. **B,** Effect of DEX on chondrocyte biosynthetic rates as measured by ^{35}S-sulfate incorporation during days 4-6. TNFα resulted in significantly lowered biosynthesis of proteoglycan, which is recovered by DEX at concentrations 1nM or higher. Values in **A** and **B** are presented as mean±SEM. For each condition, n=5 cartilage samples. *= $p<0.05$ vs. no-treatment control.

A.



B.

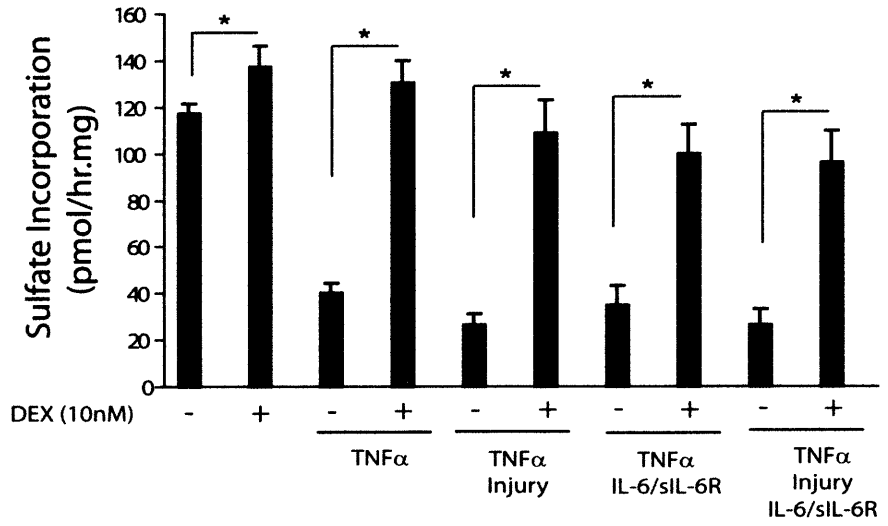


Figure 4.2 **A**, Percentage of GAG loss in bovine cartilage in response to 6-day treatments. The mean±SEM total GAG content was 466.3±21.5μg GAG/disk in the untreated control group. 10nM DEX significantly reduced GAG loss from conditions involving TNFα treatment and its combination with IL-6/sIL-6R and mechanical injury treatment. **B,** Chondrocyte biosynthetic rates measured by ^{35}S-sulfate incorporation during days 4-6. TNFα, either with or without IL-6/sIL-6R and mechanical injury, significantly lowered biosynthesis of proteoglycan, while the addition of DEX to these conditions blocked the biosynthesis reductions. Values in **A** and **B** are presented as mean±SEM. In each condition, n=5 cartilage samples. *=$p<0.05$ (only comparisons from selected hypothesis are shown).

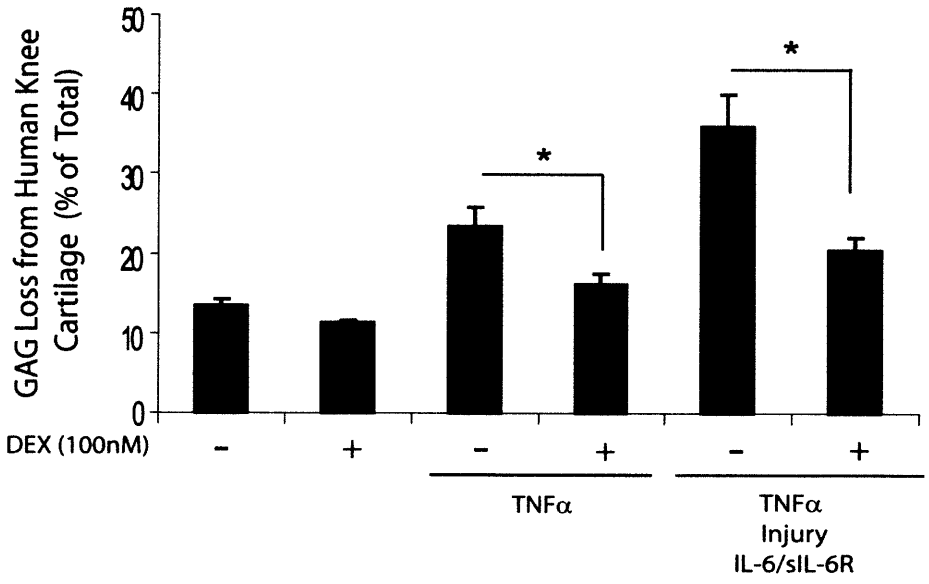


Figure 4.3 Effect of DEX on human knee cartilage treated with TNFα, and TNFα in combination with injury and IL-6/sIL-6R. Percentage of GAG loss was measured from 8-day treatments. All cartilage samples included superficial surface. The total GAG content was 168.9±17.1μg GAG/disk in the untreated control group. 100nM DEX significantly reduced GAG release induced by treatments with TNFα and the combined treatment with TNFα, IL-6/sIL-6R and mechanical injury. In each condition, n=6 cartilage samples. *=$p<0.05$ (only comparisons from selected hypothesis are shown).

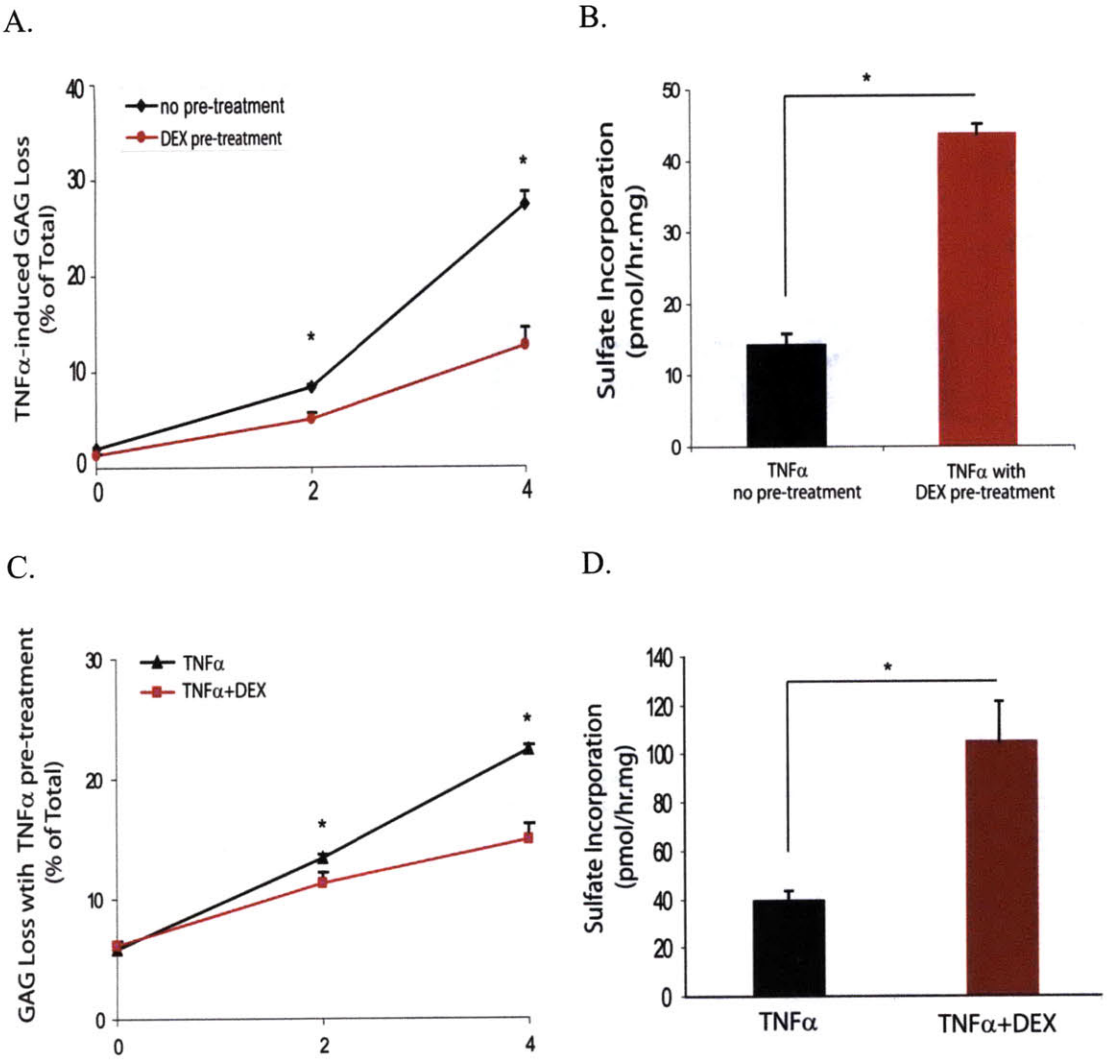
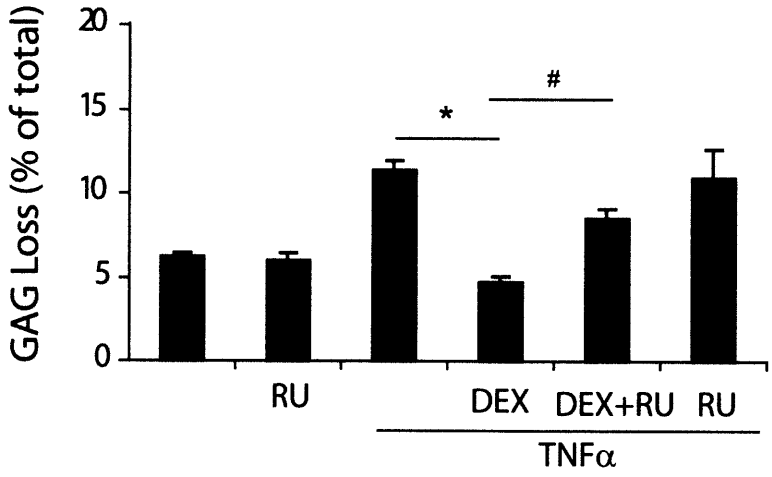


Figure 4.4 **A,** Cumulative GAG loss and **B**, sulfate incorporation (measured in the last 2 days) from bovine cartilage samples pre-treated with 10nM DEX for 2 days prior to a 4-day TNFα treatment. Cartilage samples pre-incubated with DEX released significantly less GAG, and showed significantly higher proteoglycan synthesis in the TNFα treatment comparing to samples without DEX pre-treatment. **C,** Cumulative GAG loss and **D**, sulfate incorporation (measured in the last 2 days) in bovine cartilage samples treated with TNFα, in the presence or absence of 10nM DEX, with a 2-day pre-exposure to TNFα. DEX treatment introduced after the TNFα pre-treatment significantly reduced GAG loss and increased sulfate incorporation. In each condition, n=5 cartilage disks. *=$p<0.05$.

A.



B.

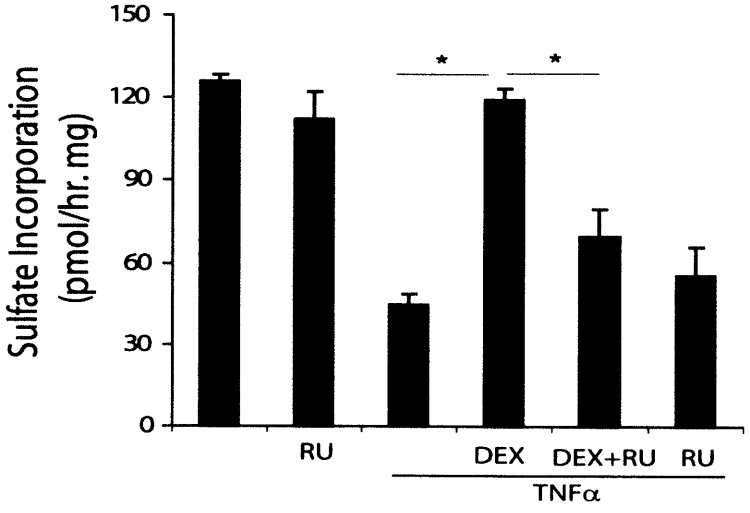


Figure 4.5 **A,** Percentage of GAG loss in 6 days and **B**, proteoglycan biosynthesis measured from days 4-6 in bovine cartilage in response to TNFα, DEX and glucocorticoid receptor antagonist, RU 486. RU reversed the effect of DEX in both GAG loss and sulfate incorporation. In each condition, n=5 cartilage explants. *= $p<0.05$, #=$p<0.06$ (only comparisons from selected hypothesis are shown).

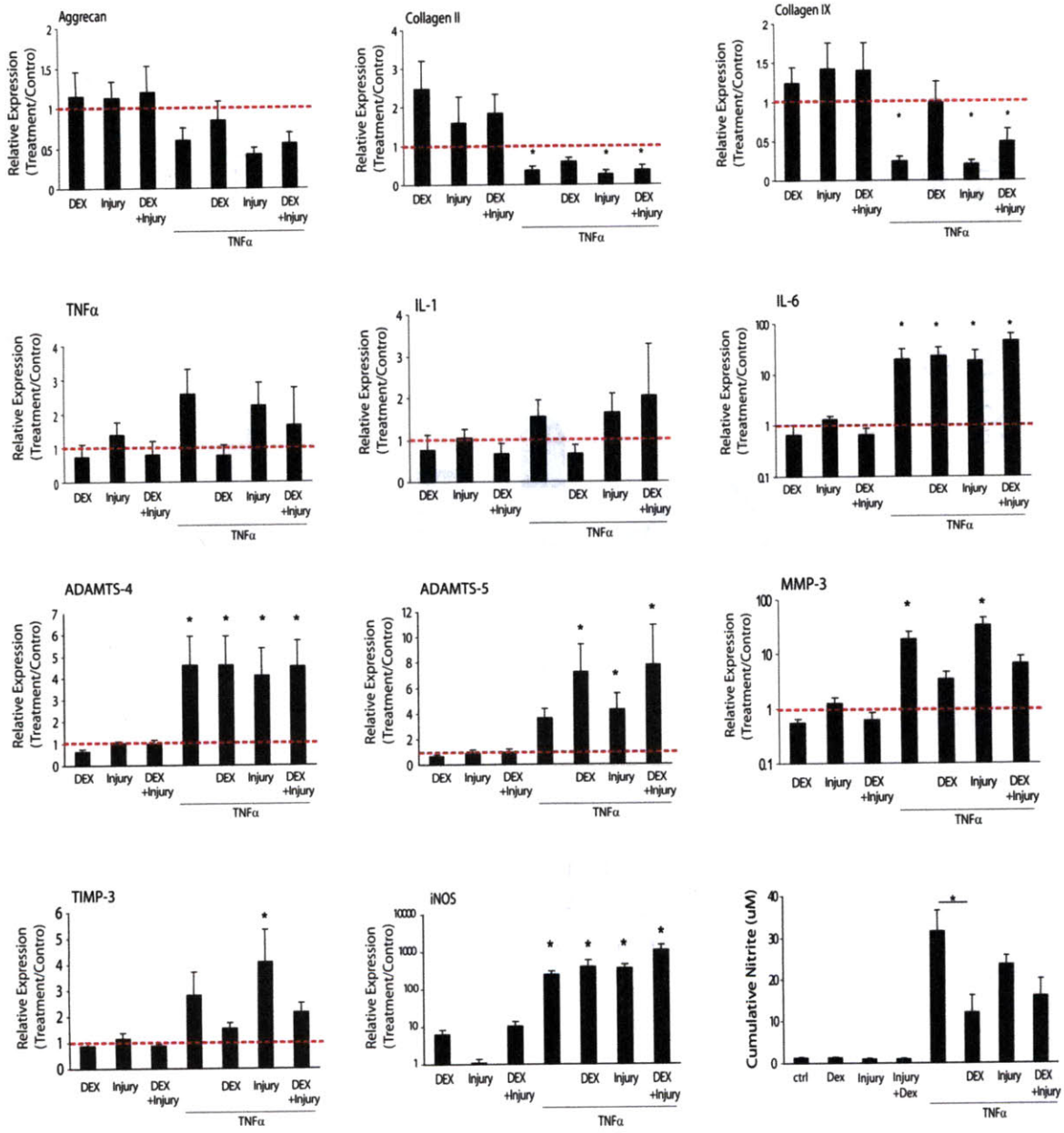


Figure 4.6 Changes in chondrocyte gene expression after 4-day treatments with DEX, TNFα, injury and their combinations. Values at the y-axis represent the expression level of each gene normalized to that of the no-treatment control (y=1). Values are mean±SEM, n=6 animals. *=$p<0.05$ vs. no-treatment control.

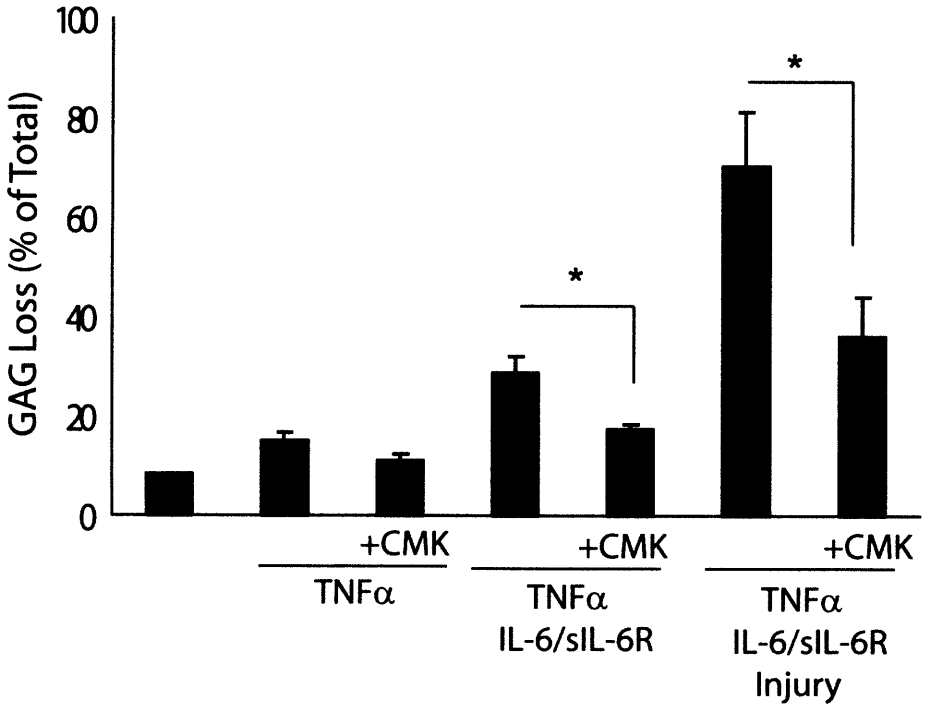


Figure 4.7 Percentage of GAG loss from bovine cartilage in response to treatments with TNFα, IL-6/sIL-6R, mechanical injury and 10μM proprotein convertase inhibitor, decanoyl-RVKR-CMK. CMK significantly reduced GAG loss induced by TNFα+IL-6, with or without mechanical injury. Values are mean±SEM, n=5 cartilage disks. *=$p<0.05$ (only comparisons from selected hypothesis are shown).

4.6 References

1. Roos H, Adalberth T, Dahlberg L, Lohmander LS. Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthritis Cartilage 1995;3(4):261-7.
2. Gelber AC, Hochberg MC, Mead LA, Wang NY, Wigley FM, Klag MJ. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. Ann Intern Med 2000;133(5):321-8.
3. Lohmander LS, Roos H. Knee ligament injury, surgery and osteoarthrosis. Truth or consequences? Acta Orthop Scand 1994;65(6):605-9.
4. Lohmander LS, Atley LM, Pietka TA, Eyre DR. The release of crosslinked peptides from type II collagen into human synovial fluid is increased soon after joint injury and in osteoarthritis. Arthritis Rheum 2003;48(11):3130-9.
5. Lohmander LS, Hoerrner LA, Dahlberg L, Roos H, Bjornsson S, Lark MW. Stromelysin, tissue inhibitor of metalloproteinases and proteoglycan fragments in human knee joint fluid after injury. J Rheumatol 1993;20(8):1362-8.
6. Lohmander LS, Hoerrner LA, Lark MW. Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. Arthritis Rheum 1993;36(2):181-9.
7. Higuchi H, Shirakura K, Kimura M, Terauchi M, Shinozaki T, Watanabe H, et al. Changes in biochemical parameters after anterior cruciate ligament injury. Int Orthop 2006;30(1):43-7.
8. Irie K, Uchiyama E, Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. Knee 2003;10(1):93-6.
9. Stein A, Yassouridis A, Szopko C, Helmke K, Stein C. Intraarticular morphine versus dexamethasone in chronic arthritis. Pain 1999;83(3):525-32.
10. Creamer P. Intra-articular corticosteroid treatment in osteoarthritis. Curr Opin Rheumatol 1999;11(5):417-21.
11. Asadullah K, Schacke H, Cato AC. Dichotomy of glucocorticoid action in the immune system. Trends Immunol 2002;23(3):120-2.
12. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, Haegeman G. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. Biochem Pharmacol 2000;60(8):1185-95.
13. Grewe M, Gausling R, Gyufko K, Hoffmann R, Decker K. Regulation of the mRNA expression for tumor necrosis factor-alpha in rat liver macrophages. J Hepatol 1994;20(6):811-8.
14. Bendrups A, Hilton A, Meager A, Hamilton JA. Reduction of tumor necrosis factor alpha and interleukin-1 beta levels in human synovial tissue by interleukin-4 and glucocorticoid. Rheumatol Int 1993;12(6):217-20.
15. Lukiw WJ, Pelaez RP, Martinez J, Bazan NG. Budesonide epimer R or dexamethasone selectively inhibit platelet-activating factor-induced or interleukin 1beta-induced DNA binding activity of cis-acting transcription factors and cyclooxygenase-2 gene expression in human epidermal keratinocytes. Proc Natl Acad Sci U S A 1998;95(7):3914-9.

16. DiBattista JA, Martel-Pelletier J, Wosu LO, Sandor T, Antakly T, Pelletier JP. Glucocorticoid receptor mediated inhibition of interleukin-1 stimulated neutral metalloprotease synthesis in normal human chondrocytes. J Clin Endocrinol Metab 1991;72(2):316-26.
17. DiBattista JA, Martel-Pelletier J, Cloutier JM, Pelletier JP. Modulation of glucocorticoid receptor expression in human articular chondrocytes by cAMP and prostaglandins. J Rheumatol Suppl 1991;27:102-5.
18. Mollmann H, Balbach, S., Hochhaus, G., Barth, J., Derendorf, H. In: Handbook of pharmacokinetic/pharmacodynamic: CRC Press; 1995.
19. Kato Y, Gospodarowicz D. Stimulation by glucocorticoid of the synthesis of cartilage-matrix proteoglycans produced by rabbit costal chondrocytes in vitro. J Biol Chem 1985;260(4):2364-73.
20. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol 1988;106(6):2139-51.
21. Takano T, Takigawa M, Suzuki F. Stimulation by glucocorticoids of the differentiated phenotype of chondrocytes and the proliferation of rabbit costal chondrocytes in culture. J Biochem 1985;97(4):1093-100.
22. Sui Y, Lee JH, DiMicco MA, Vanderploeg EJ, Blake SM, Hung HH, et al. Mechanical injury potentiates proteoglycan catabolism induced by interleukin-6 with soluble interleukin-6 receptor and tumor necrosis factor alpha in immature bovine and adult human articular cartilage. Arthritis Rheum 2009;60(10):2985-96.
23. Malfait AM, Arner EC, Song RH, Alston JT, Markosyan S, Staten N, et al. Proprotein convertase activation of aggrecanases in cartilage in situ. Arch Biochem Biophys 2008;478(1):43-51.
24. Tortorella MD, Arner EC, Hills R, Gormley J, Fok K, Pegg L, et al. ADAMTS-4 (aggrecanase-1): N-terminal activation mechanisms. Arch Biochem Biophys 2005;444(1):34-44.
25. Longpre JM, McCulloch DR, Koo BH, Alexander JP, Apte SS, Leduc R. Characterization of proADAMTS5 processing by proprotein convertases. Int J Biochem Cell Biol 2009;41(5):1116-26.
26. Patwari P, Cook MN, DiMicco MA, Blake SM, James IE, Kumar S, et al. Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: interaction with exogenous cytokines. Arthritis Rheum 2003;48(5):1292-301.
27. Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989;7(5):619-36.
28. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE. Prevalence of degenerative morphological changes in the joints of the lower extremity. Osteoarthritis Cartilage 1997;5(1):23-37.
29. Frank EH, Jin M, Loening AM, Levenston ME, Grodzinsky AJ. A versatile shear and compression apparatus for mechanical stimulation of tissue culture explants. J Biomech 2000;33(11):1523-7.
30. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, et al. Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 2000;381(2):205-12.

31. DiMicco MA, Patwari P, Siparsky PN, Kumar S, Pratta MA, Lark MW, et al. Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury. Arthritis Rheum 2004;50(3):840-8.
32. Patwari P, Cheng DM, Cole AA, Kuettner KE, Grodzinsky AJ. Analysis of the relationship between peak stress and proteoglycan loss following injurious compression of human post-mortem knee and ankle cartilage. Biomech Model Mechanobiol 2007;6(1-2):83-9.
33. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. Matrix Biol 2000;19(6):549-53.
34. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883(2):173-7.
35. Lee JH, Fitzgerald JB, DiMicco MA, Cheng DM, Flannery CR, Sandy JD, et al. Co-culture of mechanically injured cartilage with joint capsule tissue alters chondrocyte expression patterns and increases ADAMTS5 production. Arch Biochem Biophys 2009;489(1-2):118-26.
36. Fitzgerald JB, Jin M, Dean D, Wood DJ, Zheng MH, Grodzinsky AJ. Mechanical compression of cartilage explants induces multiple time-dependent gene expression patterns and involves intracellular calcium and cyclic AMP. J Biol Chem 2004;279(19):19502-11.
37. Fitzgerald JB, Jin M, Grodzinsky AJ. Shear and compression differentially regulate clusters of functionally related temporal transcription patterns in cartilage tissue. J Biol Chem 2006;281(34):24095-103.
38. Malfait AM, Tortorella M, Thompson J, Hills R, Meyer DM, Jaffee BD, et al. Intra-articular injection of tumor necrosis factor-alpha in the rat: an acute and reversible in vivo model of cartilage proteoglycan degradation. Osteoarthritis Cartilage 2009;17(5):627-35.
39. Guerne PA, Desgeorges A, Jaspar JM, Relic B, Peter R, Hoffmeyer P, et al. Effects of IL-6 and its soluble receptor on proteoglycan synthesis and NO release by human articular chondrocytes: comparison with IL-1. Modulation by dexamethasone. Matrix Biol 1999;18(3):253-60.
40. Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, et al. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum 2002;46(7):1789-803.
41. Dieppe PA, Sathapatayavongs B, Jones HE, Bacon PA, Ring EF. Intra-articular steroids in osteoarthritis. Rheumatol Rehabil 1980;19(4):212-7.
42. Valtonen EJ. Clinical comparison of triamcinolonehexacetonide and betamethasone in the treatment of osteoarthrosis of the knee-joint. Scand J Rheumatol Suppl 1981;41:1-7.
43. Gibson T, Burry HC, Poswillo D, Glass J. Effect of intra-articular corticosteroid injections on primate cartilage. Ann Rheum Dis 1977;36(1):74-9.
44. Ishida Y, Heersche JN. Glucocorticoid-induced osteoporosis: both in vivo and in vitro concentrations of glucocorticoids higher than physiological levels attenuate osteoblast differentiation. J Bone Miner Res 1998;13(12):1822-6.
45. Moskowitz R, Altman, R., Hochberg, MC., Buckwalter, JA., Goldberg, VM. Osteoarthritis, Diagnosis and Medical/Surgical Management. Fourth Edition. Philadelphia, U.S.A: Lippincott Williams & Wilkins.

4.7 Supplementary Data

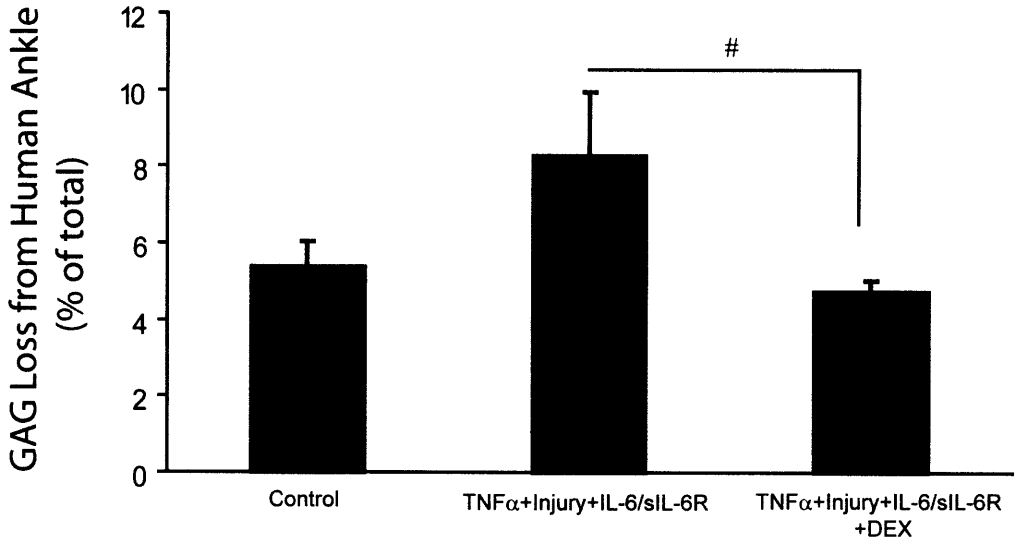


Figure 4S.1 Percentage of GAG loss from human ankle cartilage in response to 8-day treatments with TNFα(100ng/ml), IL-6/sIL-6R(50/250ng/ml) and mechanical injury (60% strain, 3/second strain rate), in the presence or absence of DEX (100nM). Human ankle cartilage (grade 1) was obtained from a 64-yr old male. Values are mean and SEM, n=5 cartilage samples in each condition. # p=0.08.

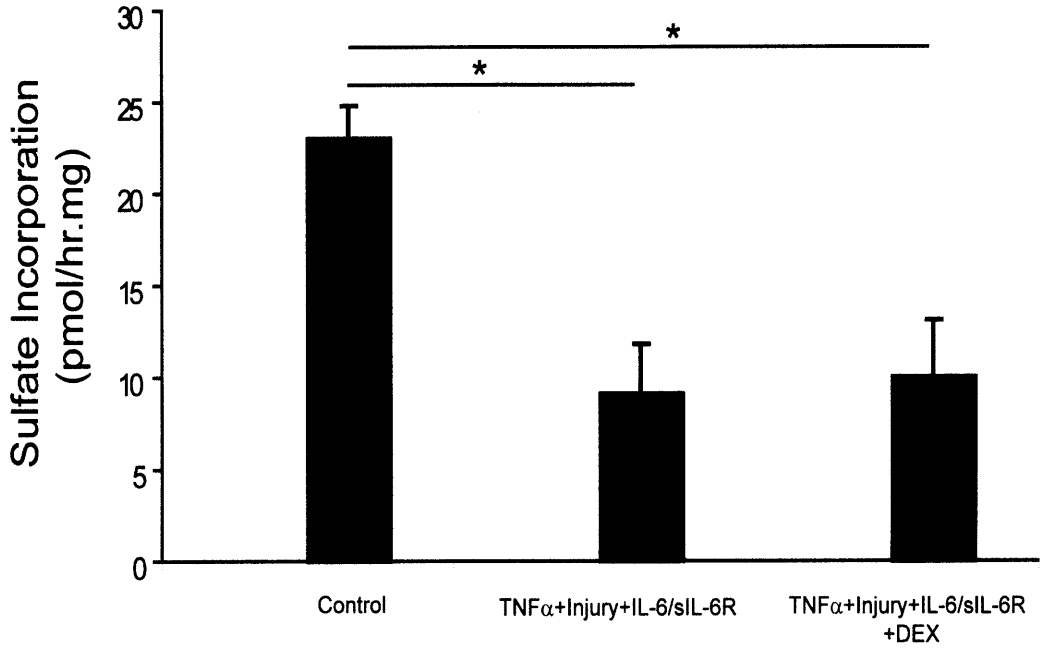


Figure 4S.2 Sulfate incorporation from human ankle chondrocytes measured from days 6-8 in response to treatments with TNFα(100ng/ml), IL-6/sIL-6R(50/250ng/ml) and mechanical injury (60% strain, 3/second strain rate), in the presence or absence of DEX (100nM). Human ankle cartilage (grade 1) was obtained from a 64-yr old male. Values are mean and SEM, n=5 cartilage samples in each condition. *=$p<0.05$.

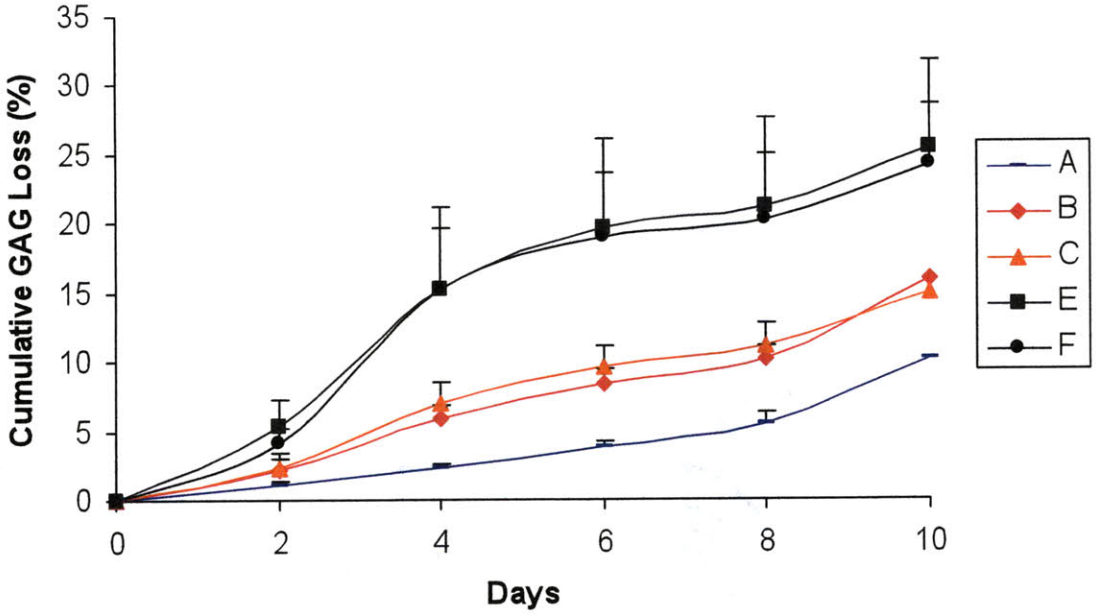


Figure 4S.3 Cumulative GAG loss from cartilage samples pre-incubated with TNFα (25ng/ml), then treated with DEX (10nM). A) Cartilage incubated in regular medium for 10 days. B) Cartilage samples pre-treated with TNFα for 4 days followed with DEX only treatment. C) Cartilage samples were incubated in the presence of TNFα for the entire duration of the study. E). Cartilage samples were injurious compressed on day 0, then incubated in the presence of TNFα for the remainder of the study. F) Cartilage disks were injurious compressed on day 0, followed by 4 days of TNFα treatment; afterwards samples were treated with DEX, in the absence of TNFα, from days 4-10. Values are mean and SEM, n=5 cartilage disks.

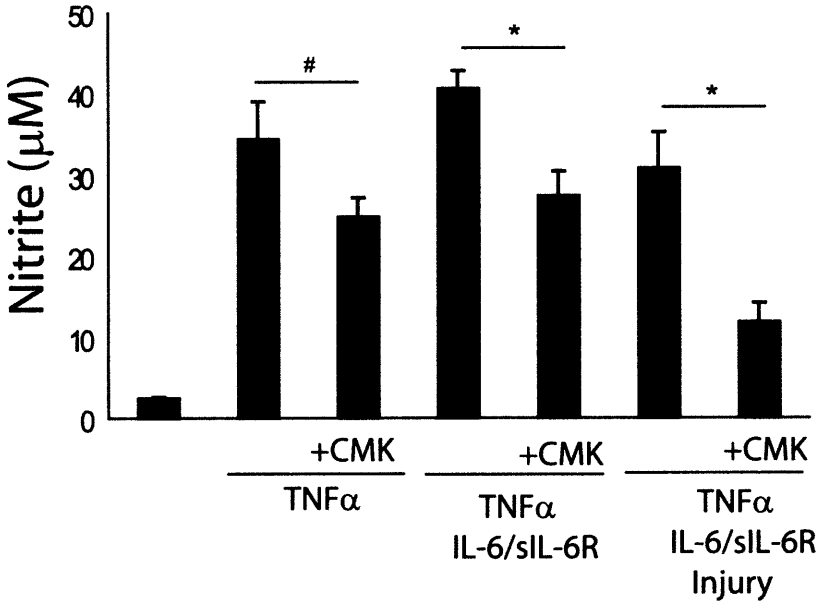


Figure 4S.4 Nitrite levels measured in the medium following 6-day treatments of TNFα (25ng/ml), IL-6/sIL-6R(50/250 ng/ml) and mechanical injury, in the presence or absence of decanoyl-RVKR-CMK (10mM). CMK, a general inhibitor for proprotein convertases, significantly reduced the nitrite levels induced by treatments with TNFα, TNFα+IL-6/sIL-6R, and TNFα+IL-6/IL-6R+injury. In each condition, n=5 bovine cartilage explants. *$p<0.05$, #$p<0.1$.

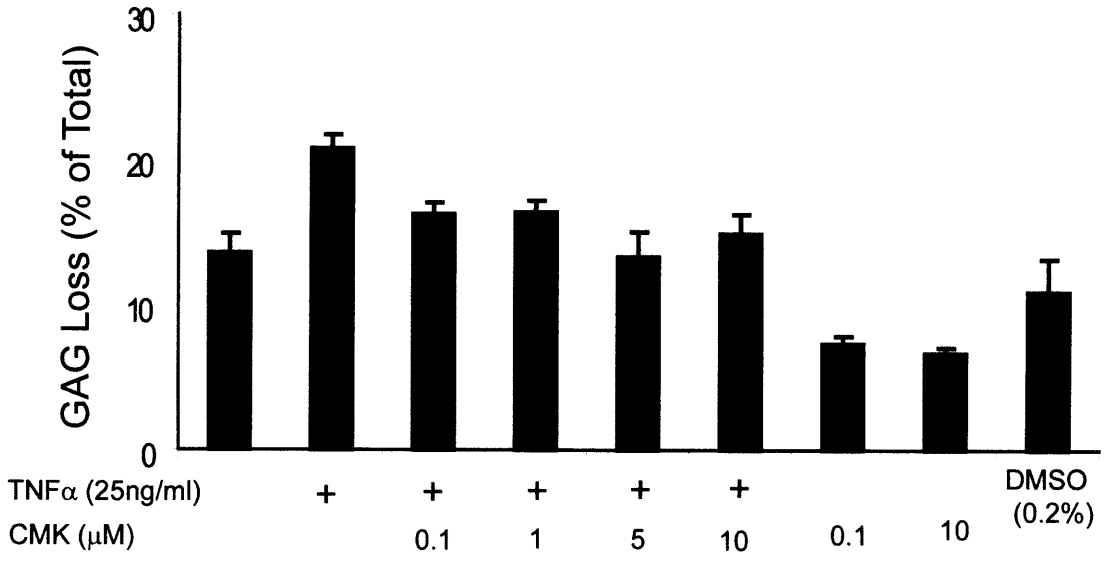


Figure 4S.5 Effect of CMK on cartilage GAG loss induced by TNFα. Bovine cartilage samples were incubated in presence of TNFα (25ng/ml) and different concentrations of CMK. CMK was dissolved in 0.2% DMSO, which alone has no effect on GAG loss. Values are mean and SEM, n=5.

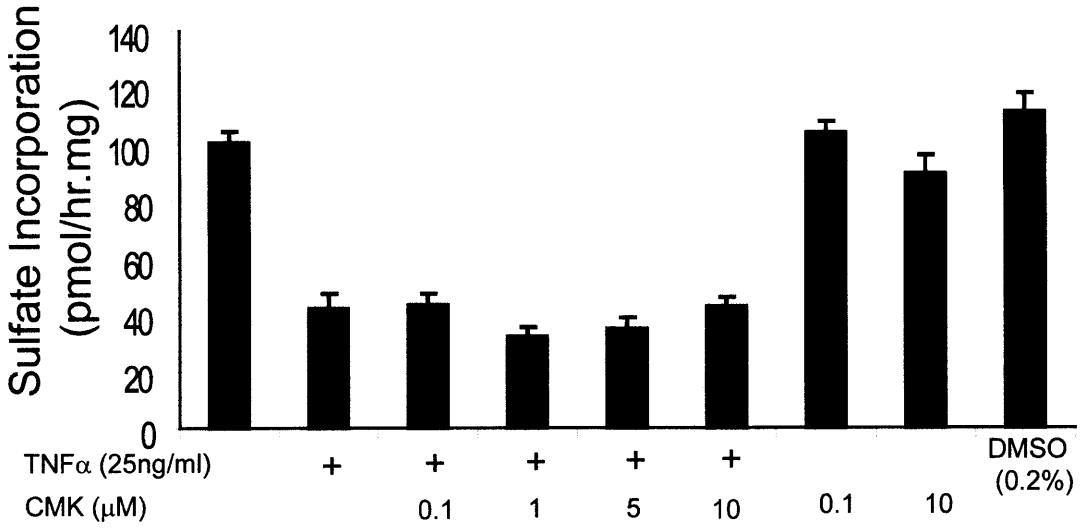


Figure 4S.6 Effect of CMK on chondrocyte proteoglycan synthesis as measured by ^{35}sulfate incorporation during days4-6. Bovine cartilage samples were incubated in presence of TNFα (25ng/ml) and different concentrations of CMK. CMK showed no effect on proteoglycan biosynthesis. CMK was dissolved in 0.2% DMSO, which alone has no effect on sulfate incorporation. Values are mean and SEM, n=5.

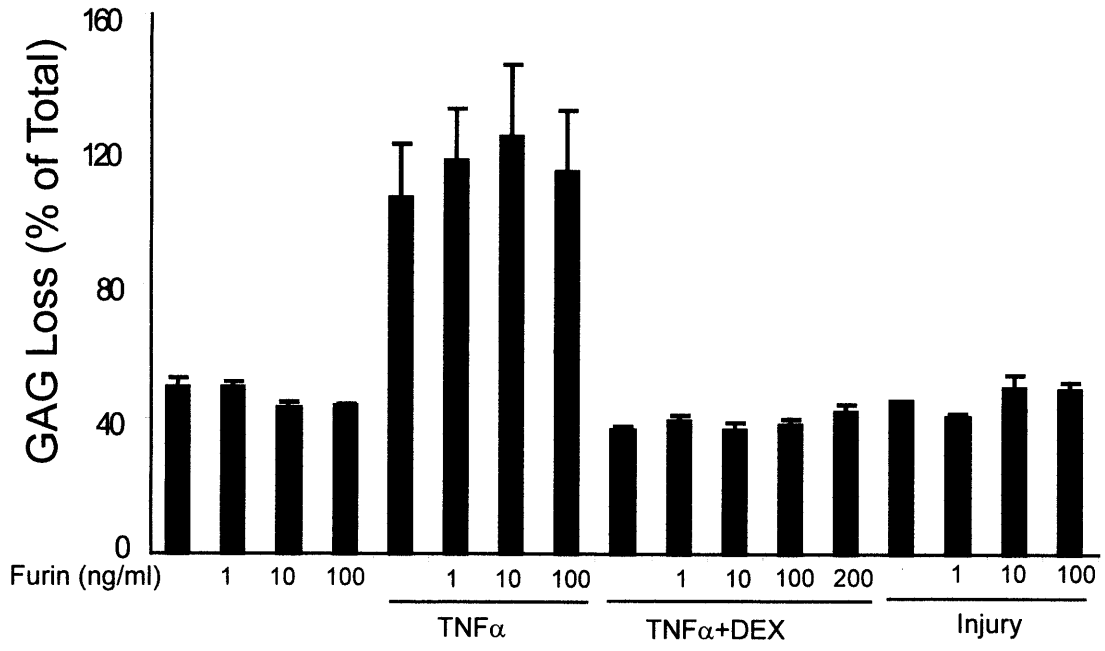


Figure 4S.7 Effect of Furin on GAG loss from bovine cartilage treated with TNFα, TNFα+DEX, and injury. Cartilage samples were either treated with TNFα (25ng/ml), TNFα+DEX(1nM) or mechanical injury, in the presence of 1, 10, 100 and 200 ng/ml of furin (R&D systems). Values are mean and SEM, n=5 cartilage disks.

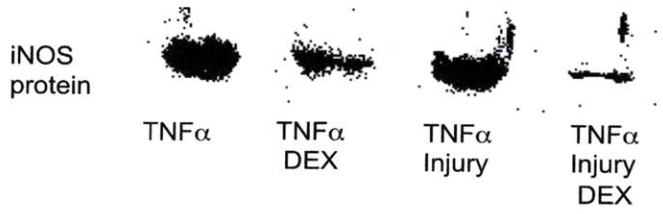


Figure 4S.8 Western blot for iNOS protein from bovine cartilage treated with 4-days of TNFα (25ng/ml), with or without mechanical injury, in the presence or absence of DEX (10nM).

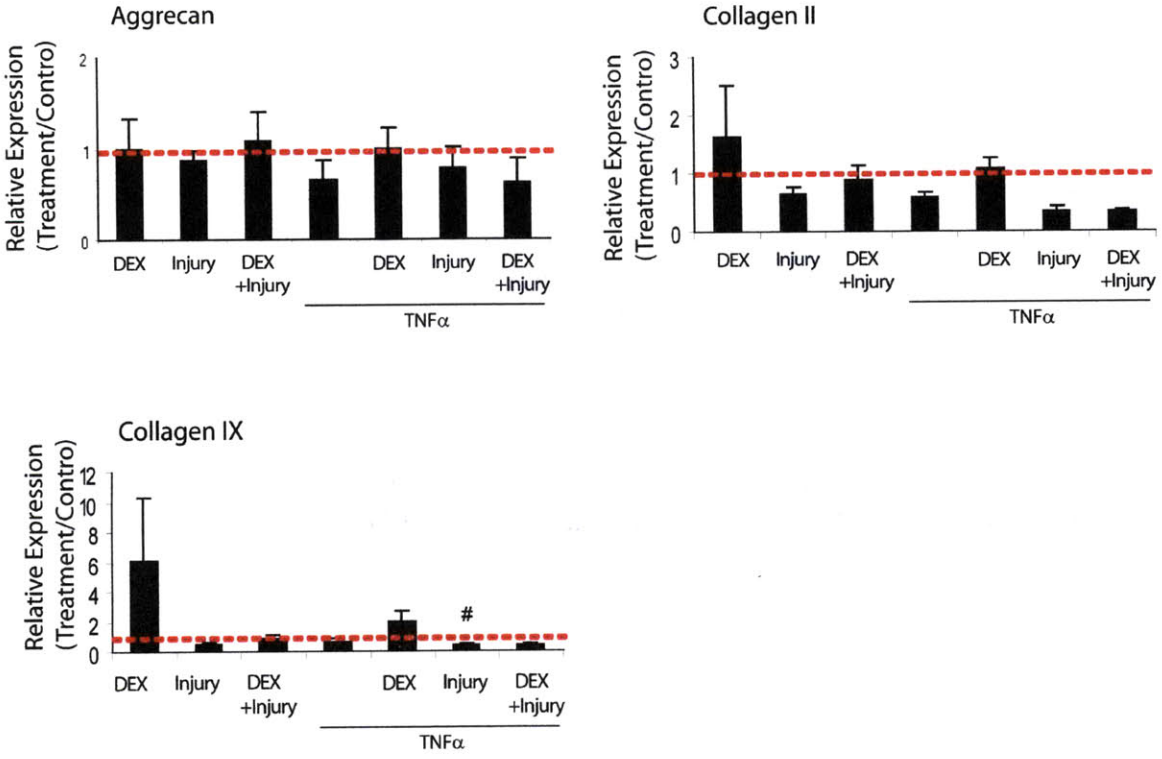


Figure 4S.9 Effects of 1-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of matrix molecules. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=5 animals. #=$p<0.01$ vs. no-treatment control.

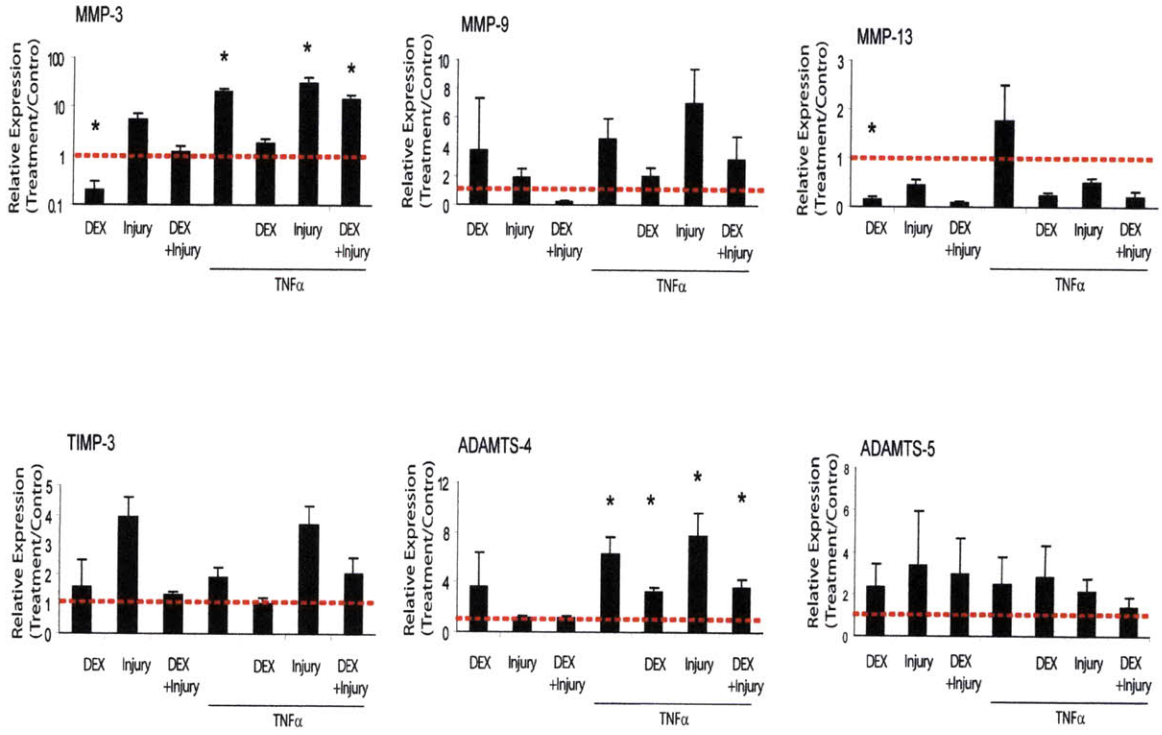


Figure 4S.10 Effects of 1-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of matrix proteases and protease inhibitor. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=5 animals. *=$p<0.05$ vs. no-treatment control.

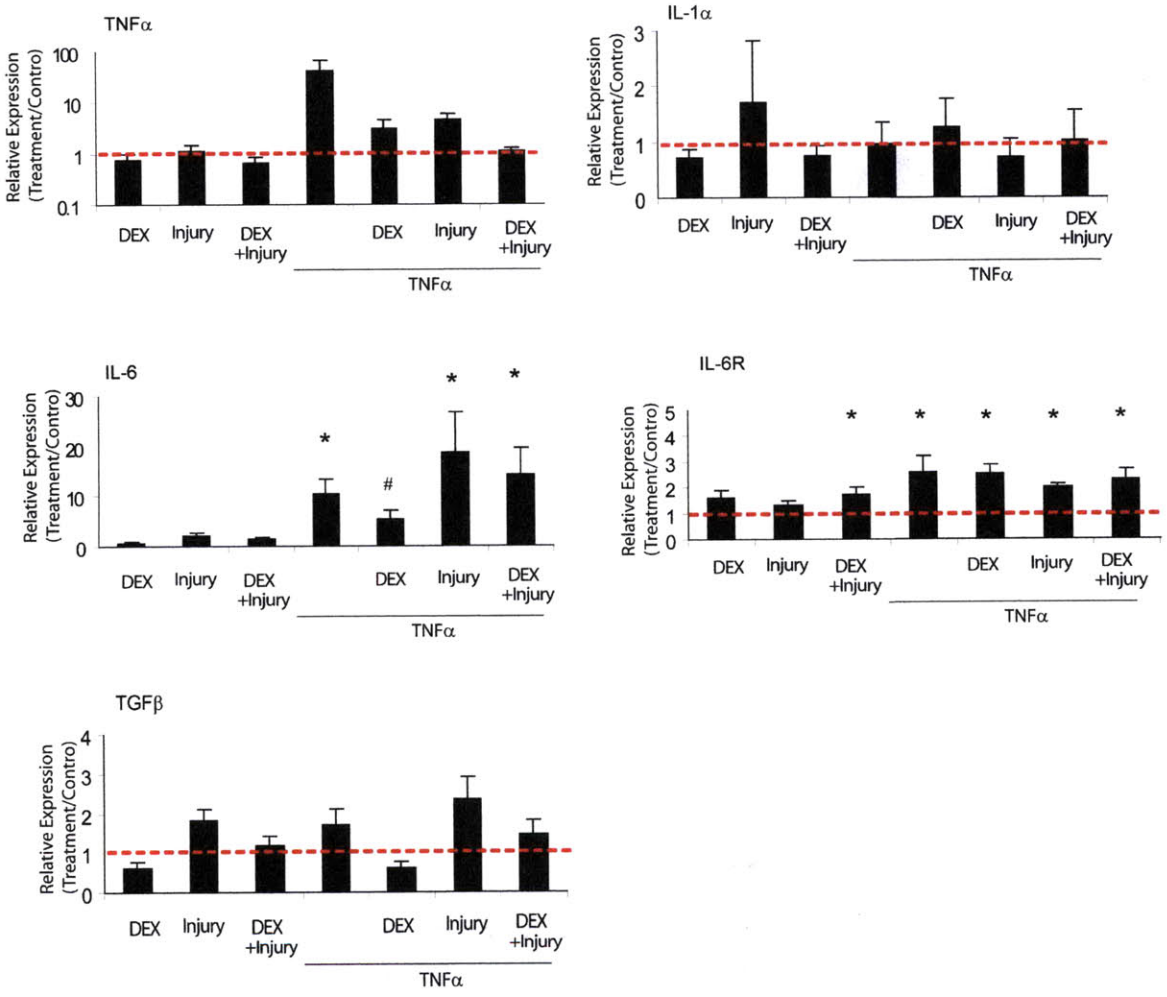


Figure 4S.11 Effects of 1-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of cytokines and growth factor. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=5 animals. *=$p<0.05$ vs. no-treatment control.

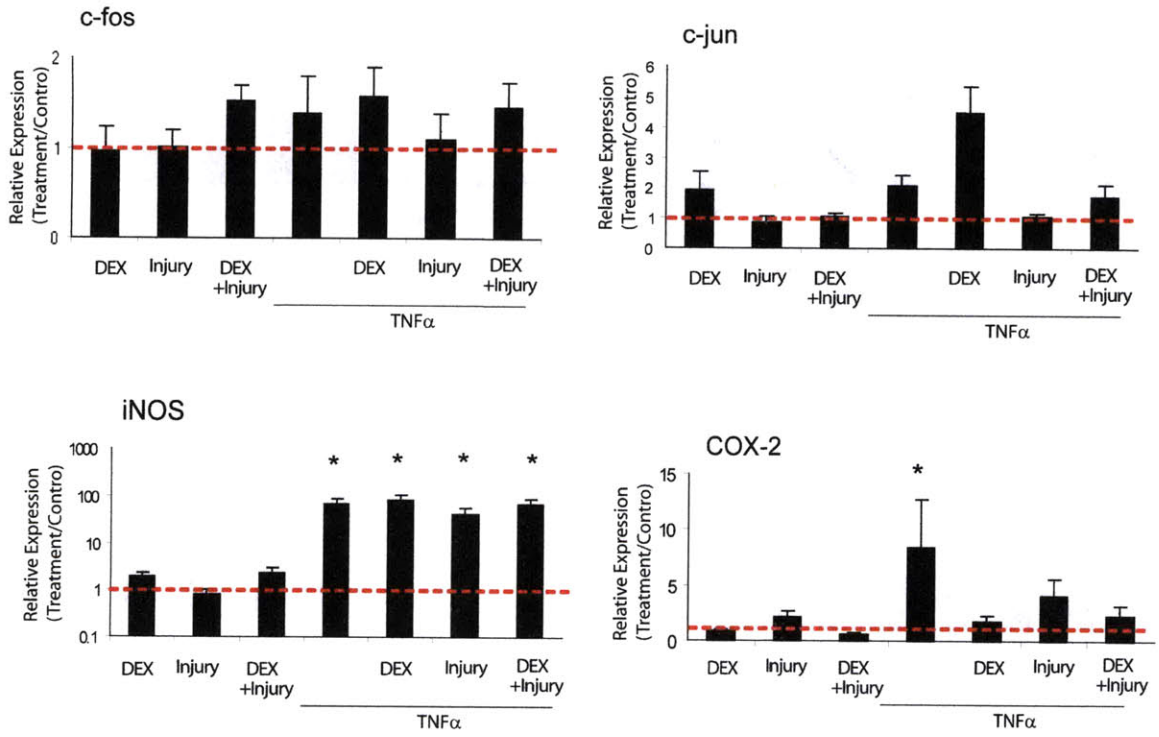


Figure 4S.12 Effects of 1-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of c-fos, c-jun, iNOS and COX-2. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=5 animals. *=$p<0.05$ vs. no-treatment control.

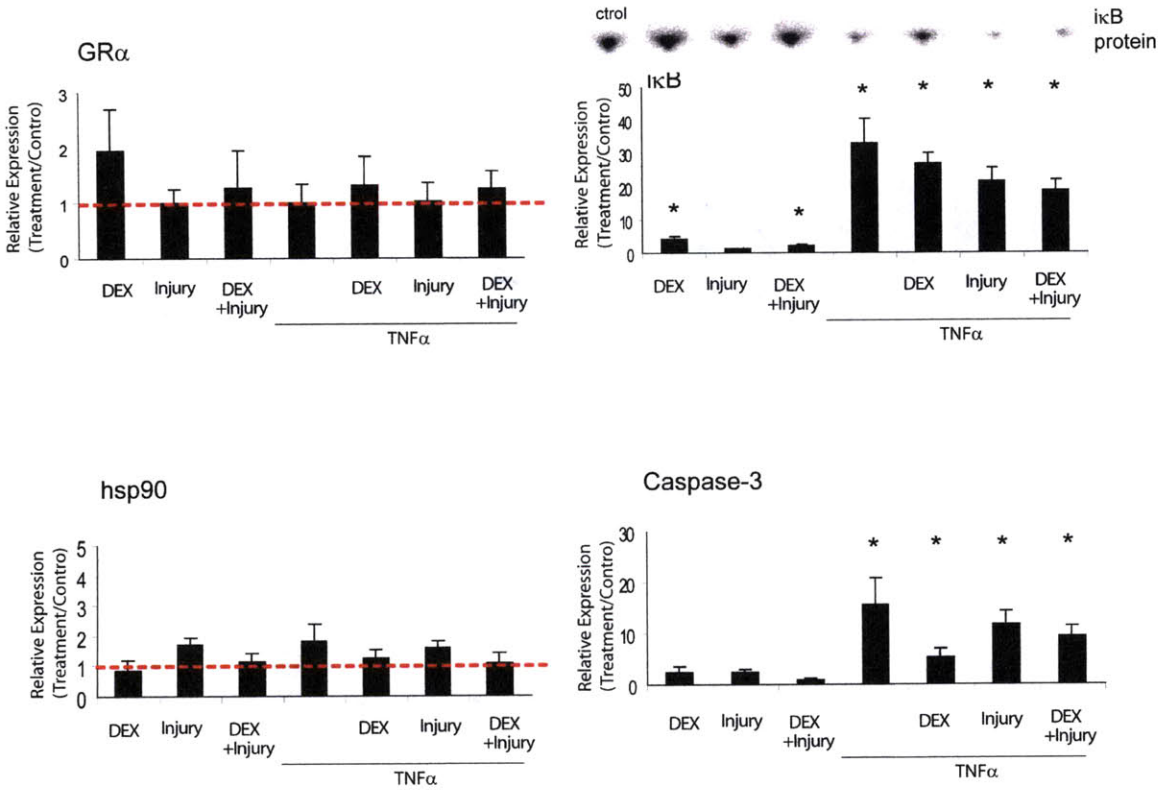


Figure 4S.13 Effects of 1-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of glucocoticoid receptor, ikB, hsp90 and caspase-3. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=5 animals. *=$p<0.05$ vs. no-treatment control. Western blot detects ikB protein from bovine cartilage in treatments.

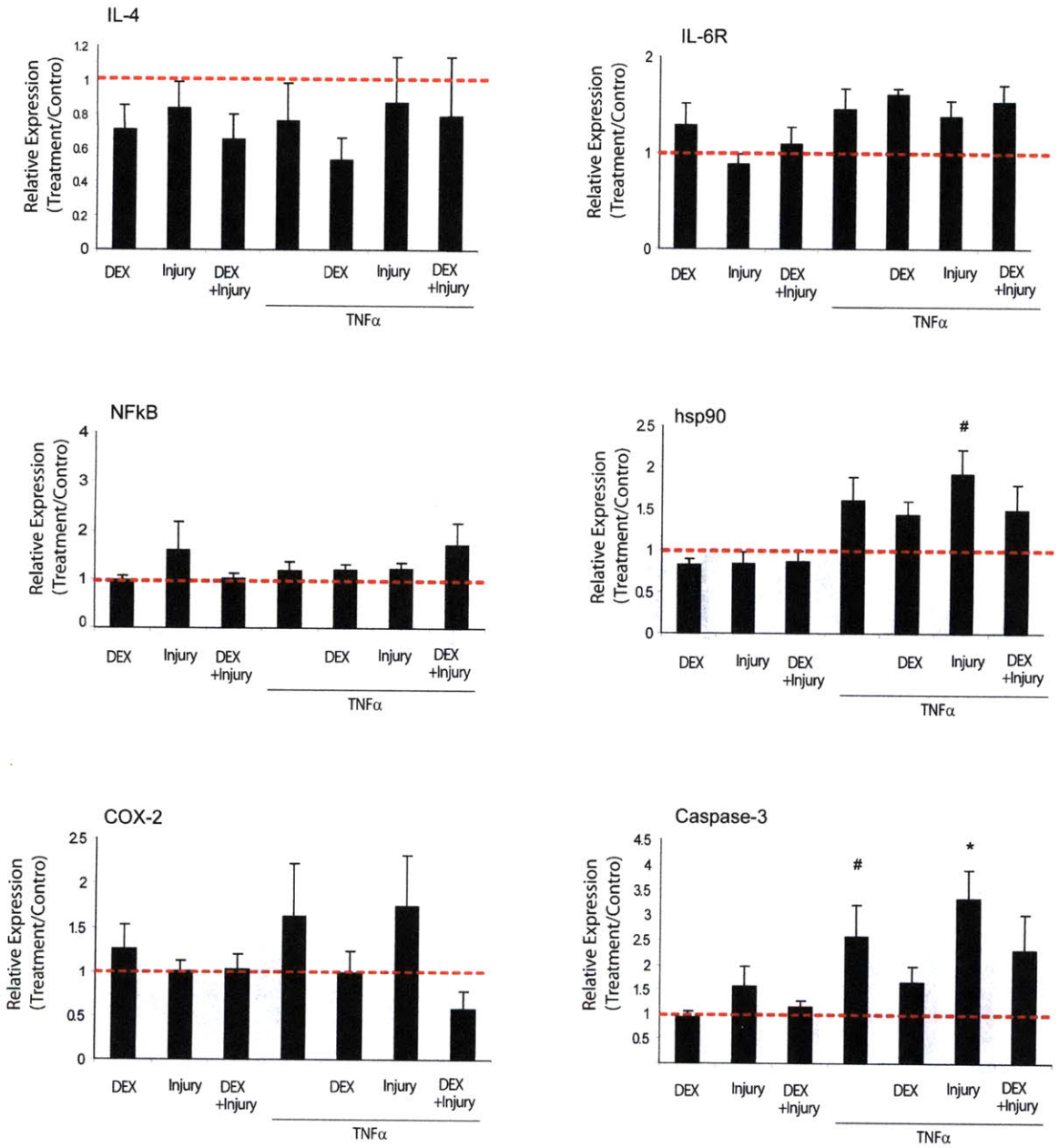


Figure 4S.14 Effects of 4-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of IL-4, IL-6 receptor, NFkB, hsp90, cox-2 and caspase-3. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=6 animals. *=$p<0.05$, #=$p<0.01$ vs. no-treatment control.

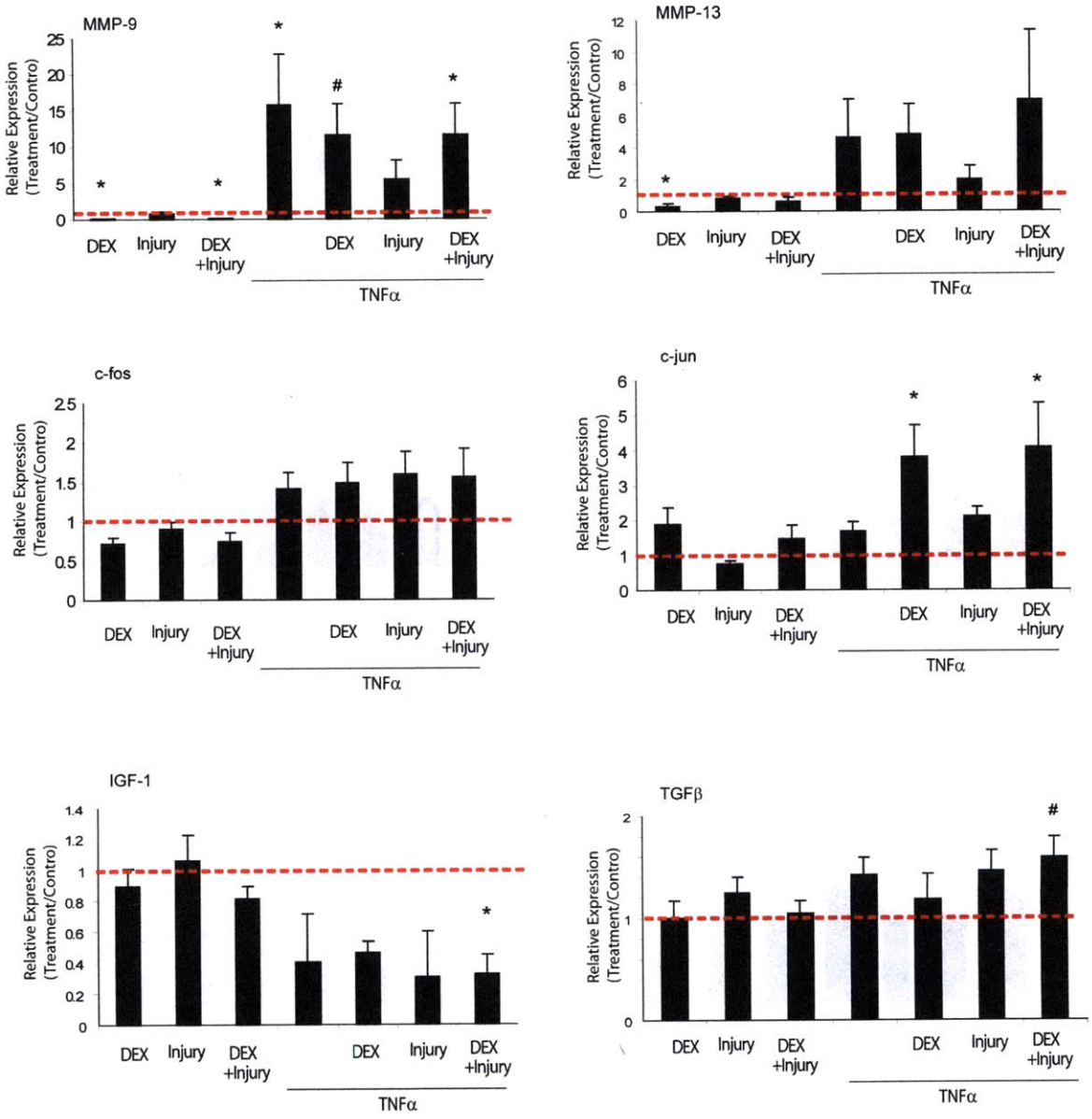


Figure 4S.15 Effects of 4-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of MMP-9, -13, c-fos, c-jun, IGF-1 and TGFβ. Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=6 animals. *=$p<0.05$, #=$p<0.01$ vs. no-treatment control.

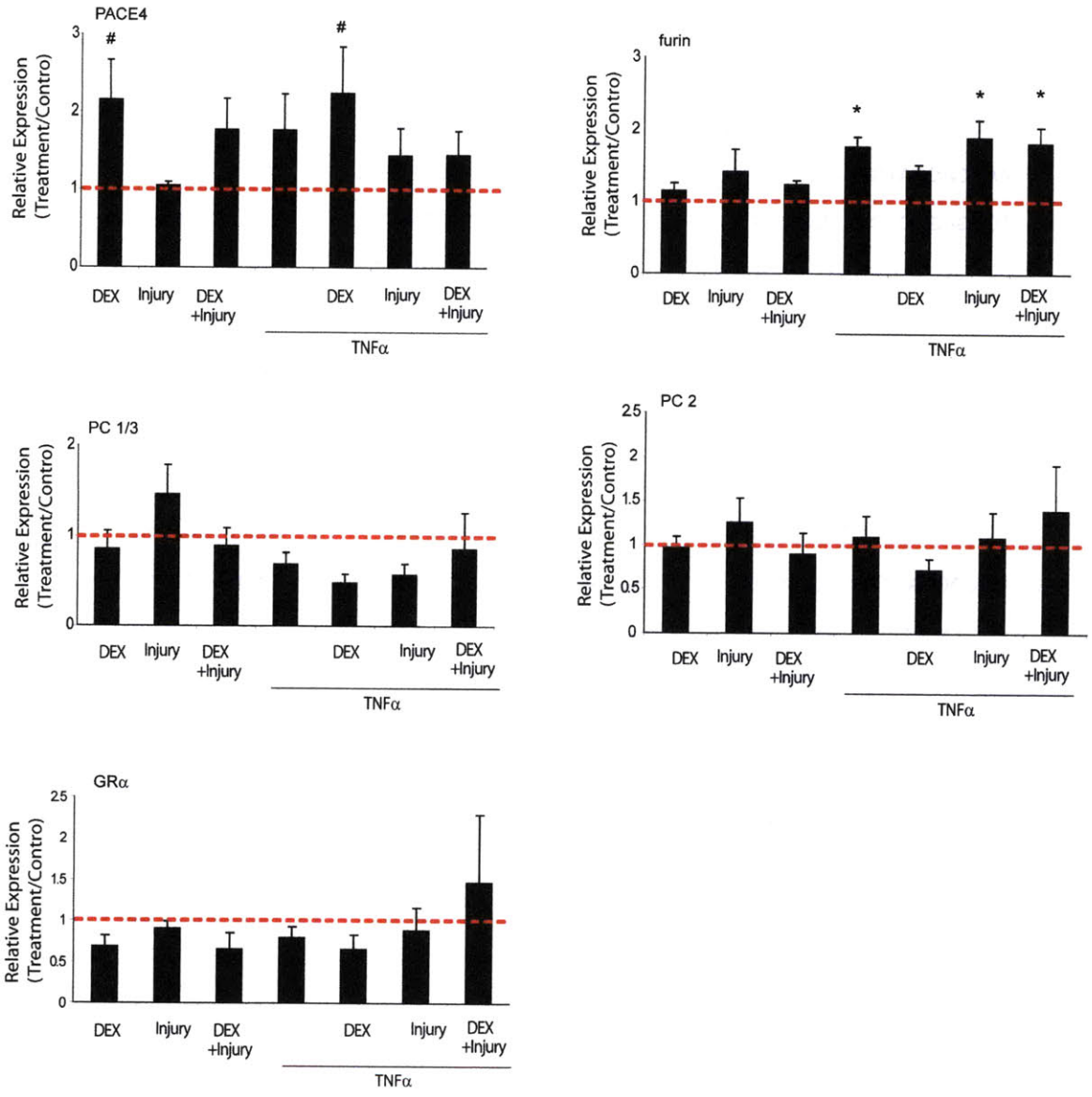


Figure 4S.16 Effects of 4-day treatments with TNFα, mechanical injury, DEX and their combinations on bovine chondrocyte gene expression of PACE4, furin, PC1/3, PC2, and GRα (glucocorticoid receptor α). Values on the y-axis represent expression levels relative to the no-treatment control (y=1, dotted line). Values are mean±SEM, n=6 animals. *=$p<0.05$, #=$p<0.01$ vs. no-treatment control.

Table 4S.1 Primer Sequences

Gene	Forward Sequence	Reverse Sequence	Slope	Offset
IL-6	TGGAGGAAAAGGACGGATGCT	TGGCTGGAGTGGTTATTAGAT	-0.8544	24.502
TNFα	CTCAAGCCTCAAGTAACAAGC	GCAATGATCCCAAAGTAGACC	-0.9585	30.333
IL-1	AAGGAGAATGTGGTGATGGTG	CAGAAGAAGAGGAGGTTGGTC	-0.9638	27.292
iNOS	TCAGAGCCACGATCCTCTTT	GTTGAAGGCACAGCTGAACA	-0.9235	24.859
iKB	CTACACCTTGCCTGTGAGCA	GACACGTGTGGCCATTGTAG	-1.0648	26.995
Fn14*	AAGGTGTCTGACTGCCCTGT	CAAATGCTGCGGTTCCTTAG	-1.0271	27.055
Furin	CTTCATGACGACCCATTCCT	ACGAGGGTGAACTTGGTCAG	-0.8864	21.428
PC2	ACCCTCTTTTCACGAAGCAA	TCTGCCACATTCAAGTCGAG	-0.7149	25.399
PACE4	CCACCATCAGGAGGTGAACT	CTTACAGCTTGGGTGGCATT	-0.7068	19.34
PC1/3	TGGAAGCAAATCCCAATCTC	CTCCGTTCTTTTTCCATCCA	-0.9285	23.788
ADAMTS5	GGGACCATATGCTCTCCTGA	GCCAAGCAGATGTCCAATTT	-0.8644	21.011
GRα	CACAACTCACCCCAACACTG	GAGTCTGGAACCGAGCTGTC	-0.9435	27.876

* TNFRSF12A (based on predicted bovine sequence)

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/), and were based on bovine genome sequences.

CHAPTER 5

Summary and Conclusions

Cartilage degradation following joint injury involves a complex interplay between mechanical and cytokine-mediated pathways. The objectives of this thesis were to characterize the catabolic responses of cartilage in the in vitro models of joint injury, and to investigate whether cartilage degradation could be inhibited by potential therapeutic agents.

In Chapter 2, our objectives were to characterize the combined effects of TNFα and IL-6/sIL-6R on proteoglycan degradation in mechanically injured cartilage and to determine the role of endogenous IL-6 in cartilage catabolism. We found that the combined treatment with TNF-α and IL-6/sIL-6R induced significantly more GAG loss than either cytokine alone and this catabolic response was associated with aggrecanase activity. Additionally, the catabolic effect of TNFα, and the combined effect of TNFα and IL-6/sIL-6R were both highly potentiated by mechanical injury. Human knee and ankle cartilages showed strikingly similar trends as bovine tissues, though GAG loss was less severe for ankle tissue. The degradative effects of injury plus TNFα appear to be due, in part, to the action of endogenous IL-6, as sGAG loss was partly abrogated by the IL-6 blocking Fab fragment. Histology observations suggest the kinetics of cartilage degradation is not merely a consequence of the activities of proteolytic enzymes, but it also depends strongly on the transport of cytokines, proteases and other cartilage biomolecules, which may be altered by overload injury.

In Chapter 3, we investigated whether the matrix degradation induced by the combinations of TNFα, IL-6/sIL-6R and mechanical injury was initiated at the transcriptional

level. Real-time PCR was used to determine the expression levels of matrix proteases, ECM molecules, protease inhibitors and growth factors. The combined treatment with TNFα and IL-6/sIL-6R significantly increased the gene expression of MMPs, aggrecanases, cytokines, iNOS, and caspase-3, to levels much higher than those induced by either cytokine alone. The addition of mechanical injury to the combined cytokine treatment caused the most dramatic increase in the expression of matrix proteases and protease inhibitor. Aggrecanases and iNOS shared the same gene expression pattern, that is, their expression levels were both significantly up-regulated in the conditions involving TNFα, as well as the combined treatment with IL-6/sIL-6R and mechanical injury. However, blocking iNOS activity did not abrogate proteoglycan degradation. Together these findings suggest cytokines exposure to injured cartilage can cause specific changes in chondrocyte gene expression. The molecules that are affected at the transcriptional level may contribute to the initial phase of cartilage degradation following joint injury.

The objectives of Chapter 4 were to study the effect of glucocorticoid treatment, such as dexamethasone, in cartilage degradation caused by the in vitro models of joint injury and to investigate whether dexamethasone would regulate the catabolic response at the transcriptional level. We have found that dexamethasone could effectively abolish both proteoglycan degradation and biosynthesis reduction caused by the pro-inflammatory cytokines and mechanical injury treatments in bovine cartilage. Dexamethasone also showed similar inhibitory effects on GAG loss in human cartilage. Surprisingly, real-time PCR analyses revealed dexamethasone did not down-regulated the expression of aggrecanases, which are primarily responsible for the matrix degradation induced by the in vitro injury models. Similarly, dexamethasone produced no effect on iNOS gene expression, despite of its ability to

lower the levels of both iNOS protein and nitrite. These findings suggest that dexamethasone may exert its anti-catabolic response at levels other than transcription (i.e., post-transcription, translation, enzyme activation). We also showed that proprotein convertases played important roles in proteoglycan degradation since blocking the proprotein convertases activity significantly reduced the GAG loss caused by the cytokine and injury treatments. It is possible that dexamethasone protects proteoglycans from aggrecanase attacks by inhibiting the proprotein convertases from activating pro-ADAMTS-4 and -5. Further studies are necessary to determine the mechanisms of dexamethasone in cartilage degradation process, particularly its role in modulating aggrecanase activity.

In summary, our study shows that mechanical injury could potentiate the combined effects of pro-inflammatory cytokines: altering chondrocyte gene expression and inducing matrix degradation. The mechanobiological responses to overload, as well as the transport of cytokines and proteases in the matrix, may both be affected by joint injury, making the damaged cartilage tissue more susceptible to further degradation by biochemical mediators. Therapeutics targeting either a specific cytokine or multiple inflammatory pathways may be useful for the treatment of cartilage degeneration following joint injury.

APPENDIX A

Collaborative Projects with Centocor

A.1 A preliminary study of the interaction between TNFα, IL-6/sIL-6R and mechanical injury in bovine cartilage

A.1.1 Objective

To determine whether the inclusion of IL-6/sIL-6R affects the TNFα and injury interaction in bovine cartilage explants.

A.1.2 Methods

9mm diameter osteochondral cores were harvested from the patellofemoral groove of an immature (1-2 week old) bovine joint under aseptic conditions [1]. After removal of the articular surface, two sequential 1 mm slices were collected from the middle-deep zone of the tissue. From these, five 3mm diameter cartilage specimens were prepared and each was distributed to separate groups to create two sets of five location-matched groups (A-E and F-J; 5 disks in each group), in order to reduce variability arising from tissue location within the joint.

Recombinant human IL-6 (206-IL-010), recombinant human IL-6 soluble receptor (227-SR-025), and recombinant human TNF-α (210-TA) in lyophilized form were obtained from R&D Systems (Minneapolis, MN). The doses of both TNF- α and IL-6/sIL-6R were

equivalent to those used in the Flannery paper [2]. TNFα, IL-6 and sIL-6R were used at 100, 50 and 250ng/ml, respectively.

Cartilage specimens were cultured in separate wells of a 96-well plate, each containing 300 μL of medium (low-glucose DMEM with 1% ITS, 20 μg/mL ascorbate, 0.1 mM non-essential amino acids, 0.4 mM additional L-proline, 10mM HEPES, 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.25 μg/mL amphotericin B), in a 37C humidified environment. The cartilage samples were cultured in the medium for 2 days before the experimental treatment begin. Samples were arranged into 10 groups, with five samples in each group. Samples in Group C, D, E, and H were injuriously compressed to 50% of the measured thickness, at 100%/second, and followed with immediate release of load. Medium was collected and changed every two days. On Day 4, fresh medium contained 5 μCi/mL [^{3}H] proline and 10 μCi/mL [^{35}S] sulfate, to measure protein and GAG biosynthesis, respectively. On Day 6, cartilage disks were digested with 1mL proteinase K solution. Then both medium and tissue digests were analyzed for sulfated GAG content, and the tissue digest was also measured for incorporated radiolabel.

A.1.3 Results

Samples were distributed into two location-matched experiments (A-E and F-J). Group A and I, and Group B and J received the same treatment. They were used to determine whether the two experiments could produce the same results under the same condition. It was found that there was no significant difference in the percentage of GAG loss between Group A and I ($p=0.074$), or between Group B and J ($p=0.523$). The differences in biosynthesis of Group A

and I, and those of Group B and J were not significant either (all $p>0.35$). Therefore the two experiments were grossly similar and could be compared.

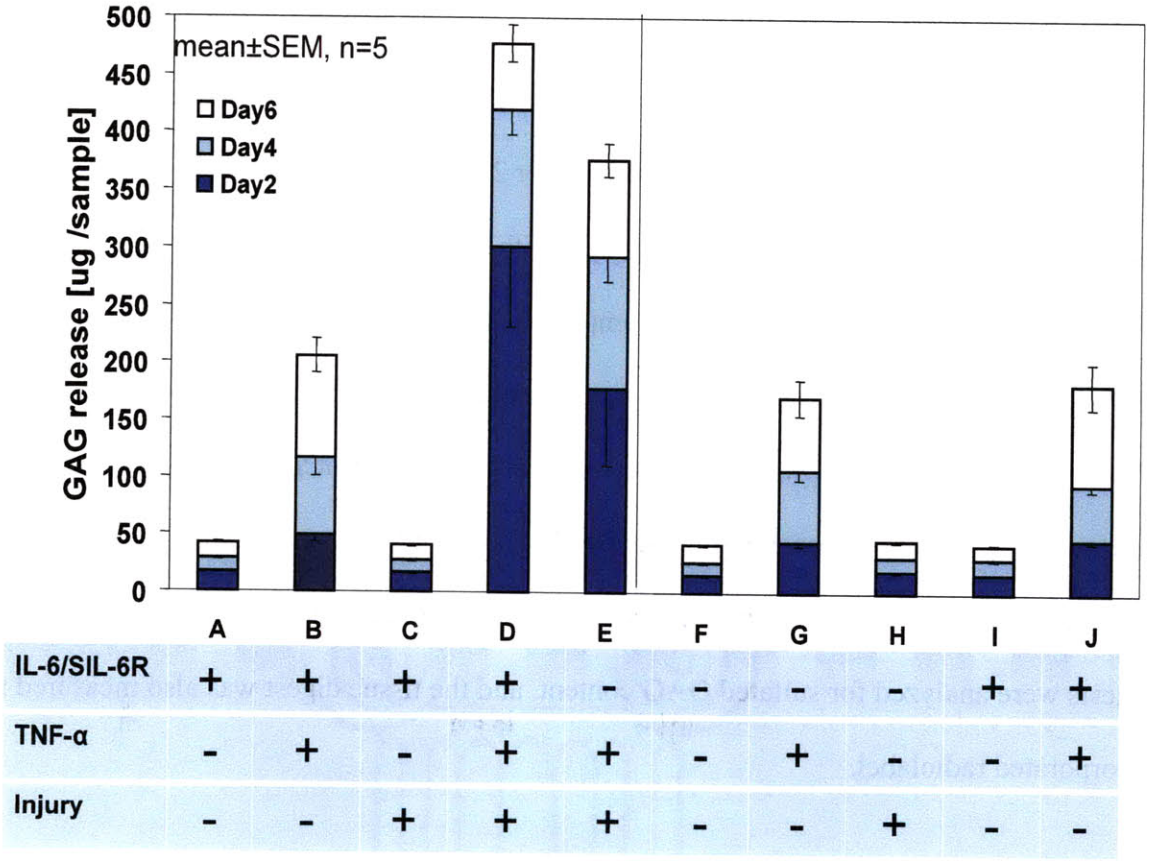


Figure A.1 Cumulative GAG loss in response to treatments with combinations of IL-6/sIL-6R (50/250 ng/mL), TNFα (100 ng/mL), and mechanical injury.

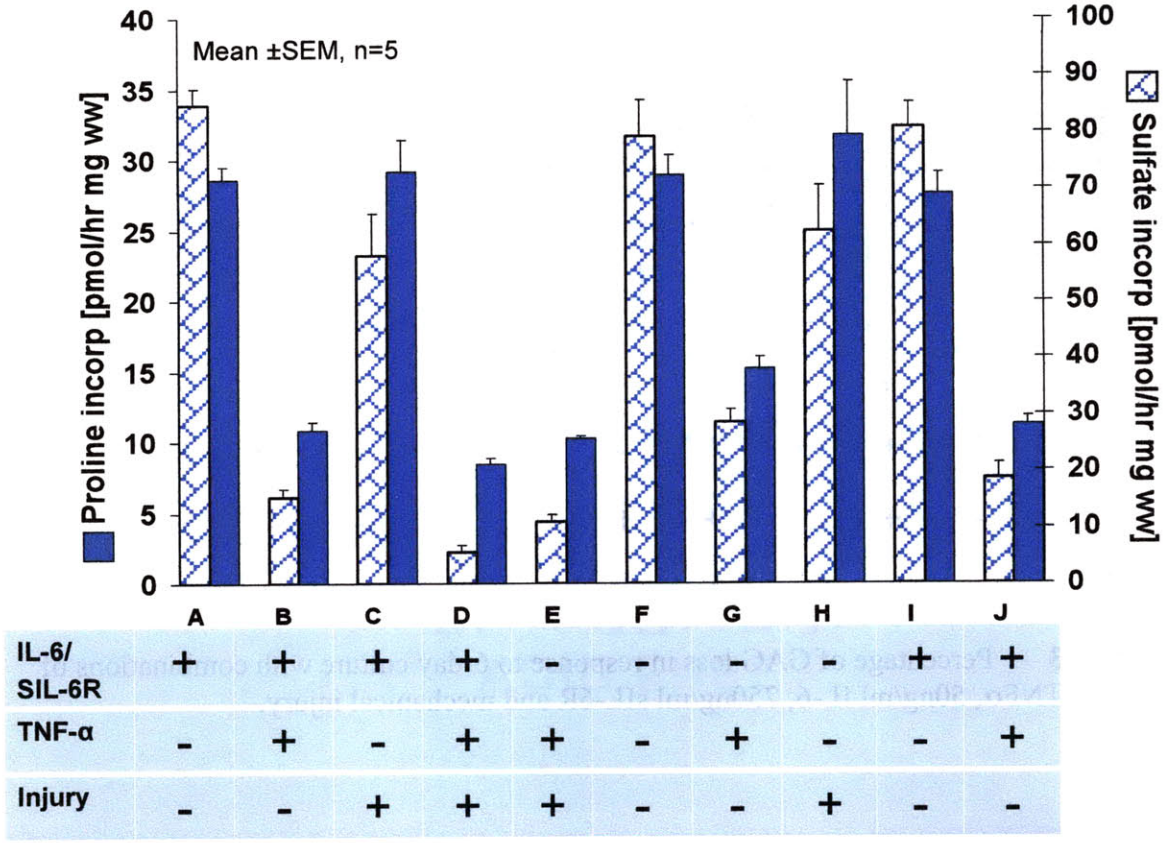


Figure A.2 Chondrocyte biosynthesis during days 4-6 in response to treatments with combinations of IL-6/sIL-6R (50/250 ng/mL), TNFα (100 ng/mL), and mechanical injury.

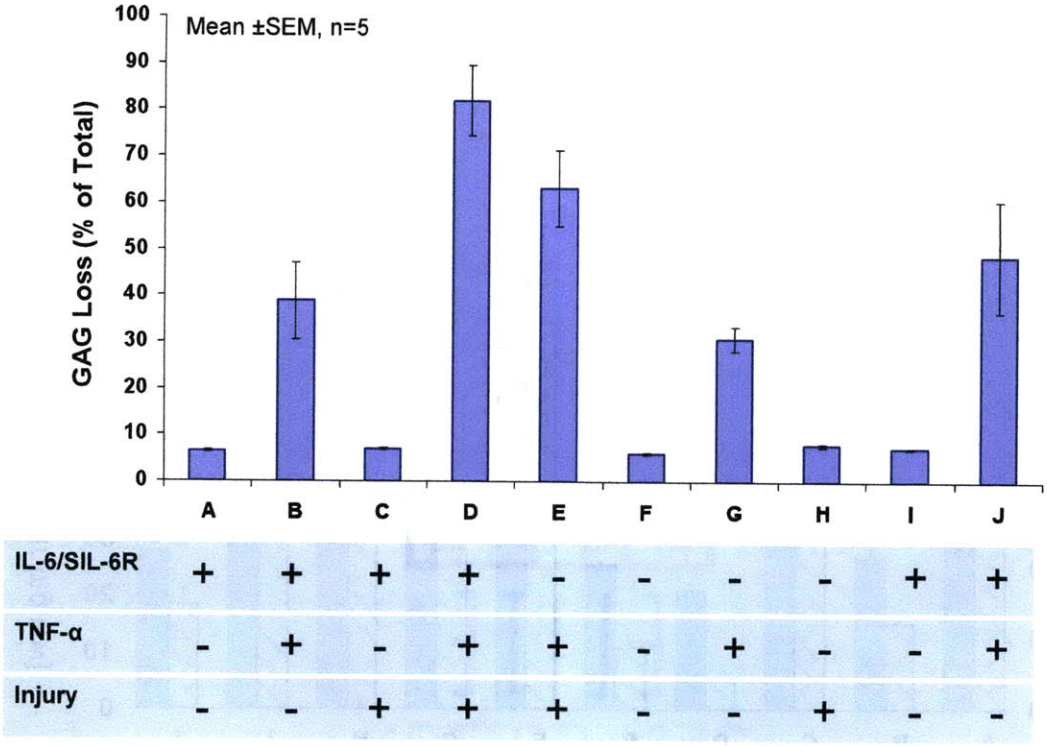


Figure A.3 Percentage of GAG loss in response to 6-day culture with combinations of 100ng/ml TNFα, 50ng/ml IL-6, 250ng/ml sIL-6R and mechanical injury.

A.1.4 Discussion

This is a preliminary study investigating the combined effect of injury with cytokine treatments. We found that injury formed interactions with both IL-6 and with TNFα by significantly increasing GAG release.

Conditions involving TNFα, either alone or in combination with other factors, showed very large increases in GAG release. The amount of total cumulative GAG loss observed in Group G (TNFα alone) and Group J (IL-6/sIL-6R + TNFα) were very close to the GAG release reported in the Flannery paper [2]. However, we did not find the significant effect of

the combination of IL-6/sIL-6R with TNFα over TNFα alone, as shown in the Flannery paper. This is possibly due to the high concentration of TNFα used in this study.

We further tested whether IL-6 would interfere with the TNFα/injury interaction. Here we report that IL-6 did not have any significant effects on TNFα/injury interaction in terms of the GAG release ($p=0.32$), but it was able to reduce biosynthesis. The lack of difference in GAG loss could be due to the fact that by 6 days, the combination resulted in almost 100% GAG loss, thereby minimizing the difference between conditions D and E. The combination of TNFα, IL-6/sIL-6R and mechanical injury caused the most severe GAG loss among all the treatment conditions. TNFα concentration used in this experiment was 100ng/mL, which contributed to the large degradation of cartilage matrix and possibly masked the potentiating effects of IL-6 and mechanical injury.

The observations from this experiment led to the use of lower TNFα concentration (25ng/ml) in the later experiments.

A.2. Effect of blocking endogenous IL-6 in the in vitro models of cartilage degradation

The goal of this study was to test the hypothesis that endogenous IL-6 was involved in the process of aggrecan degradation induced by other pro-inflammatory cytokines and injury. We used an anti-IL-6 Fab fragment (provided by Centocor) to block endogenous IL-6 in cartilage treated with TNFα, IL-1 and mechanical injury. As the following preliminary studies suggested, pre-incubation of cartilage with the anti-IL-6 Fab was necessary for the inhibition of GAG loss in treatment conditions.

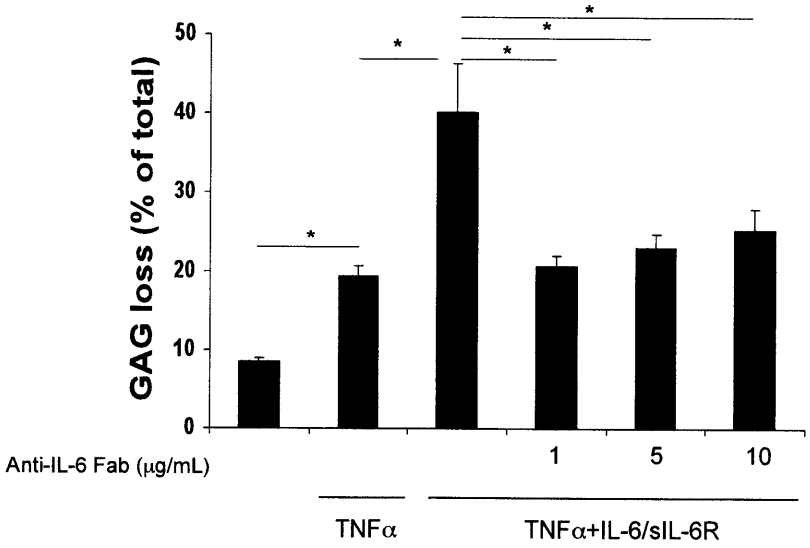


Figure A.4 Percentage of GAG loss from cartilage in response to 6-day culture with TNFα (25 ng/ml) and IL-6/sIL-6R (50/250 ng/ml), in the presence of anti-IL-6 Fab (1, 5, 10 μg/ml). IL-6/sIL-6R treatment significantly potentiated the GAG loss induced by TNFα. The anti-IL-6 Fab fragment at 1, 5 and 10 μg/mL effectively blocked the effect of exogenous IL-6. Mean±SEM (n=5 cartilage disks). *=$p<0.05$.

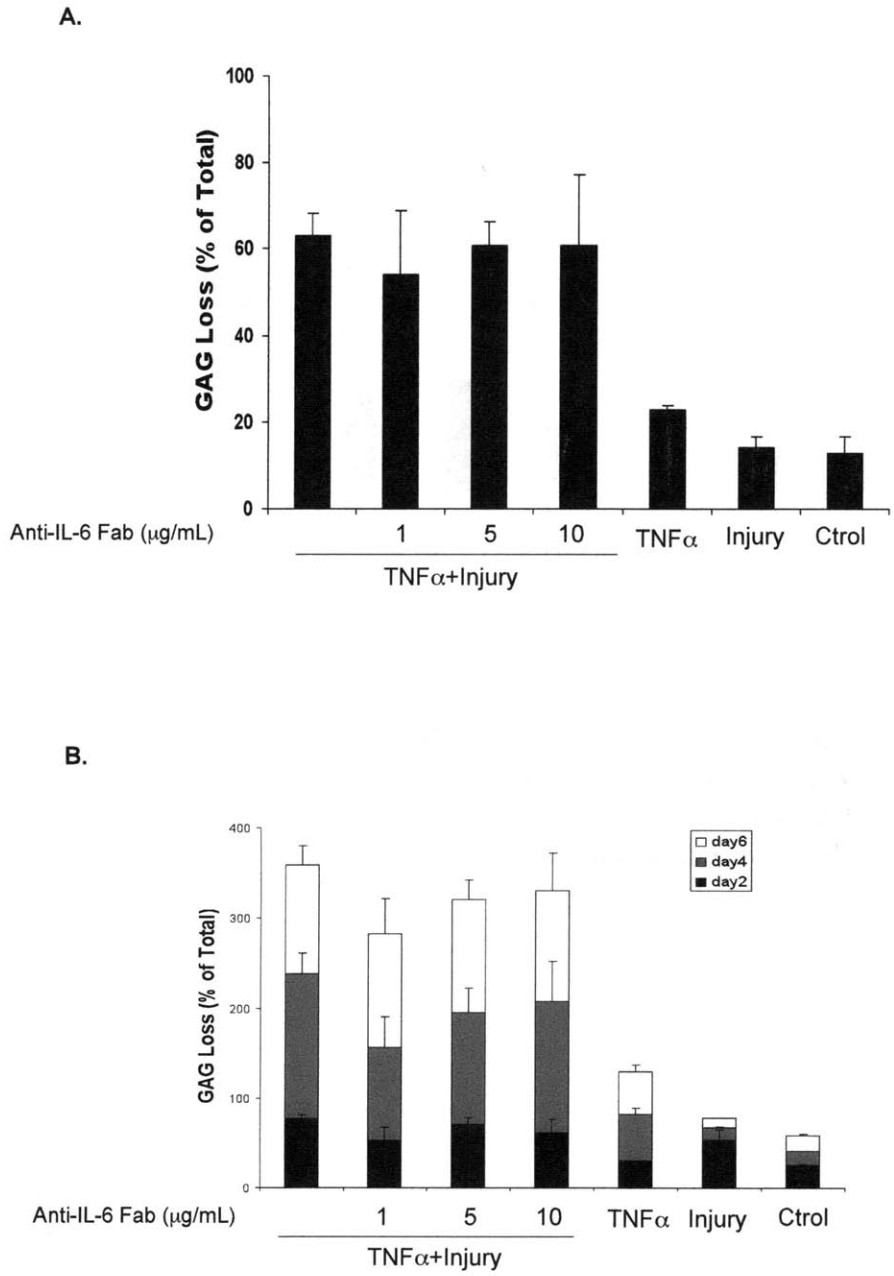


Figure A.5 **A,** Percentage and **B,** cumulative GAG loss in response to 6-day culture with TNFα (25 ng/ml) following mechanical injury, in the presence of anti-IL-6 Fab (1, 5, 10 μg/ml). Cartilage samples were mechanically injured on day 0, then immediately placed in culture medium containing TNFα (25 ng/ml), and anti-IL-6 Fab (1, 5, 10 μg/ml). GAG loss from cartilage treated with TNFα and mechanical injury were not affected by the Fab fragment. Mean±SEM (n=4 cartilage disks).

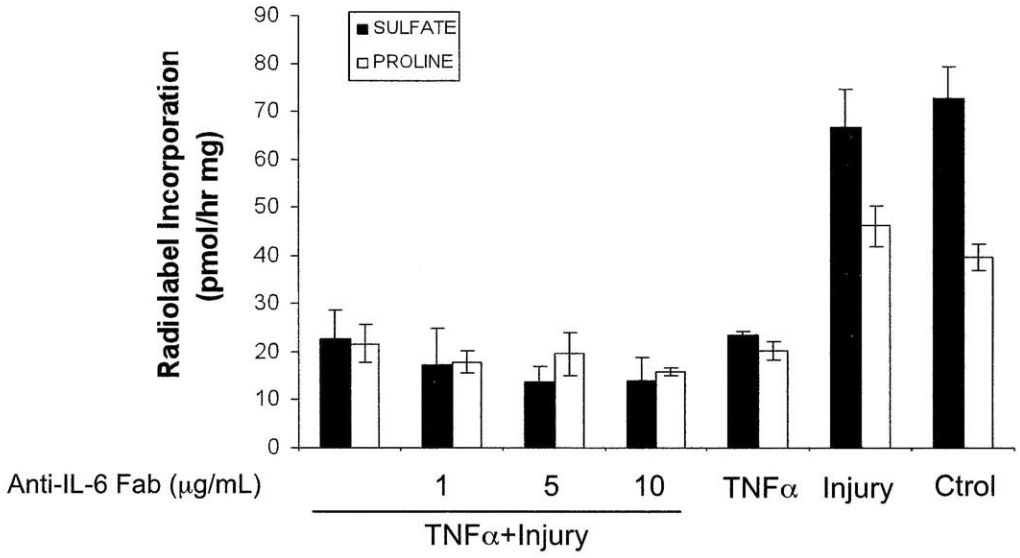


Figure A.6 Chondrocyte biosynthesis in response to 6-day culture with TNFα (25 ng/ml) following mechanical injury, in the presence of anti-IL-6 Fab (1, 5, 10 μg/ml). Cartilage samples were mechanically injured on day 0, and were immediately placed in culture medium containing TNFα and anti-IL-6 Fab. Anti-IL-6 Fab fragment did not inhibit the biosynthesis reduction caused by TNFα plus mechanical injury treatment. Mean±SEM (n=4 cartilage disks).

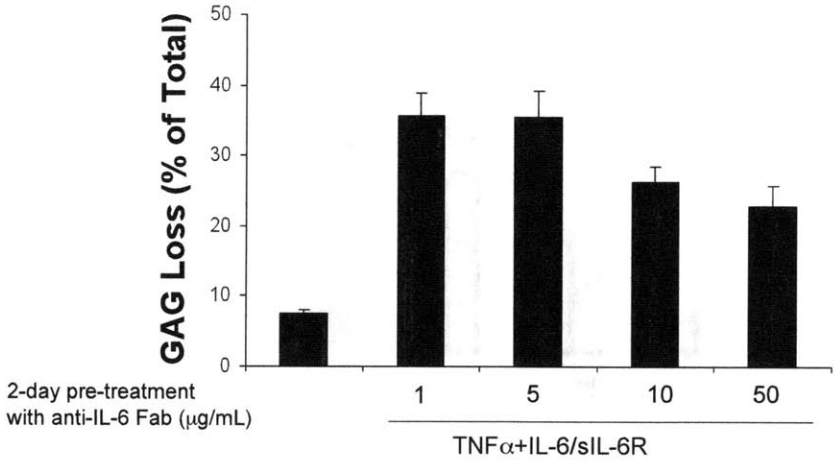


Figure A.7 Percentage of GAG loss from cartilage pre-treated for 2 days with anti-IL-6 Fab then stimulated with the combination of TNFα (25 ng/ml) and IL-6/sIL-6R (50/250 ng/ml). Mean±SEM (n=4 cartilage disks).

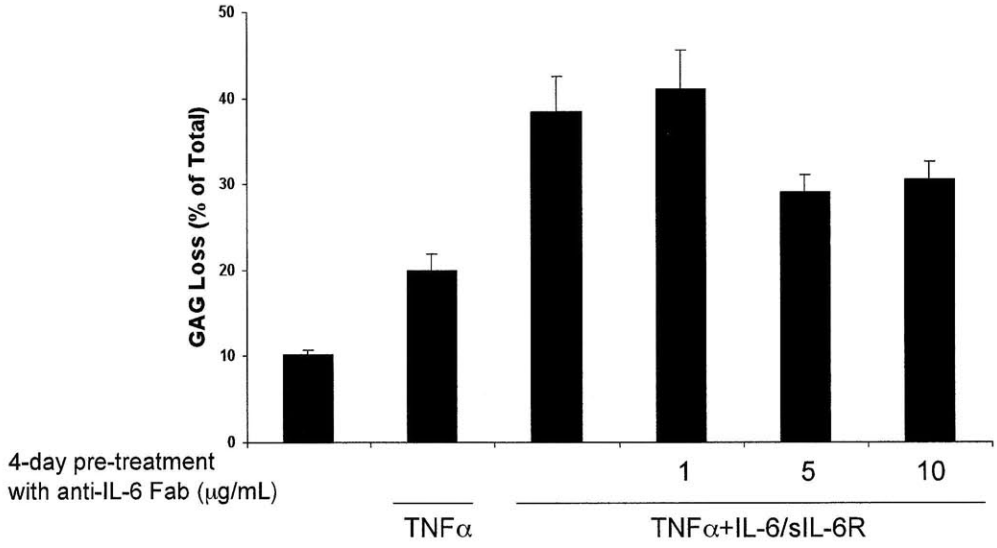


Figure A.8 Percentage of GAG loss from cartilage pre-treated for 4 days with anti-IL-6 Fab then stimulated with the combination of TNFα (25 ng/ml) and IL-6/sIL-6R (50/250 ng/ml). Mean±SEM (n=4 cartilage disks).

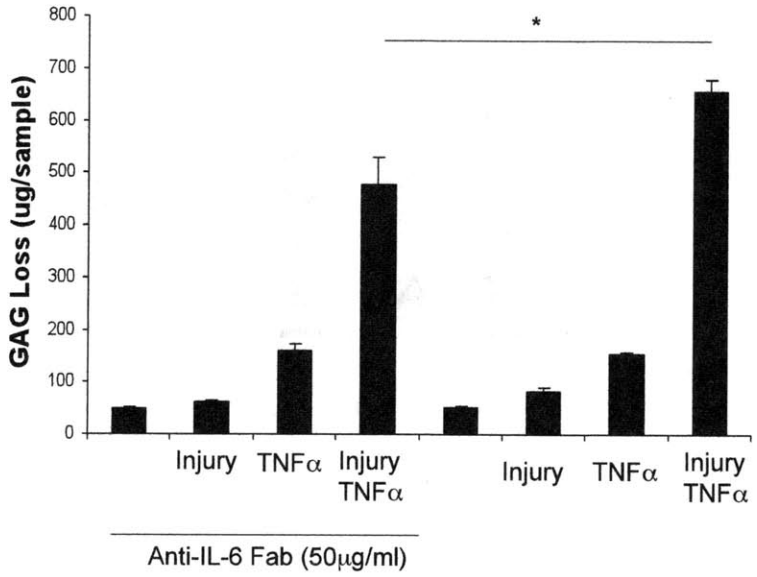


Figure A.9 Effect of blocking endogenous IL-6 in cartilage treated with TNFα and mechanical injury. Half of the cartilage samples were pre-incubated with 50μg/ml anti-IL-6 Fab for 6 days prior to receiving treatments with mechanical injury, TNFα, or both. In these conditions, the anti-IL-6 Fab was supplied in the medium throughout the experiment. Mean±SEM (n=5 cartilage disks). The cumulative GAG loss (6 days) in the condition of TNFα+injury+anti-IL-6 Fab accounted for 84.2% of total GAG content, whereas the GAG loss in the condition of TNFα+injury represented 90.7% of total GAG content.

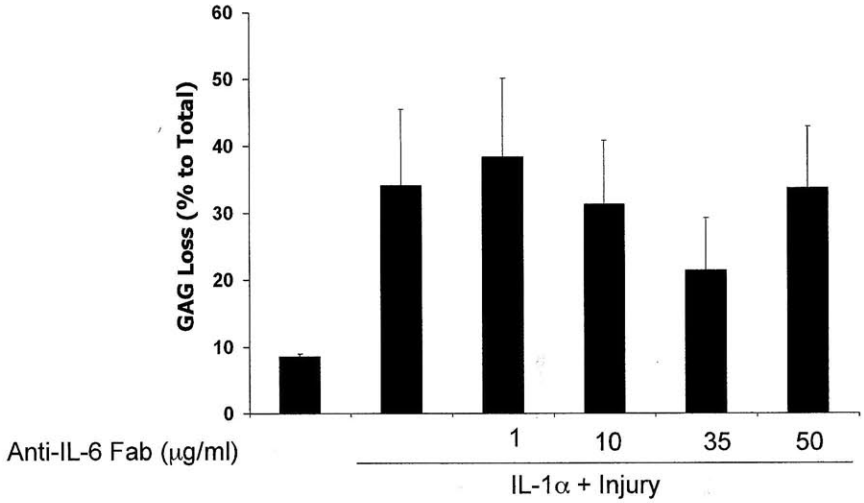


Figure A.10 Effect of anti-IL-6 Fab on GAG loss induced by the combined treatments with IL-1α (10ng/ml) and mechanical injury. Cartilage subjected to the anti-IL-6 Fab treatments were pre-incubated with the Fab for 2 days and they continued to receive the same concentration of Fab until the end of the experiment. Mean±SEM (n=5 cartilage disks).

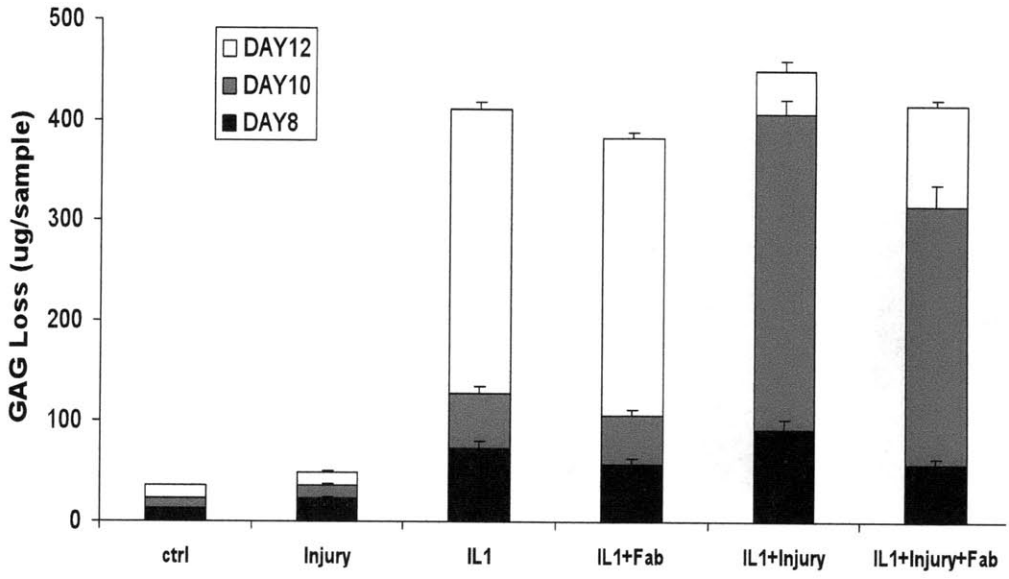


Figure A.11 Effect of anti-IL-6 Fab in cartilage treated with IL-1α and mechanical injury. Cartilage subjected to the anti-IL-6 Fab (35μg/ml) treatments were pre-incubated with the Fab for 6 days prior to the treatments with IL-1α (10μg/ml), mechanical injury and Fab. Mean±SEM (n=5 cartilage disks).

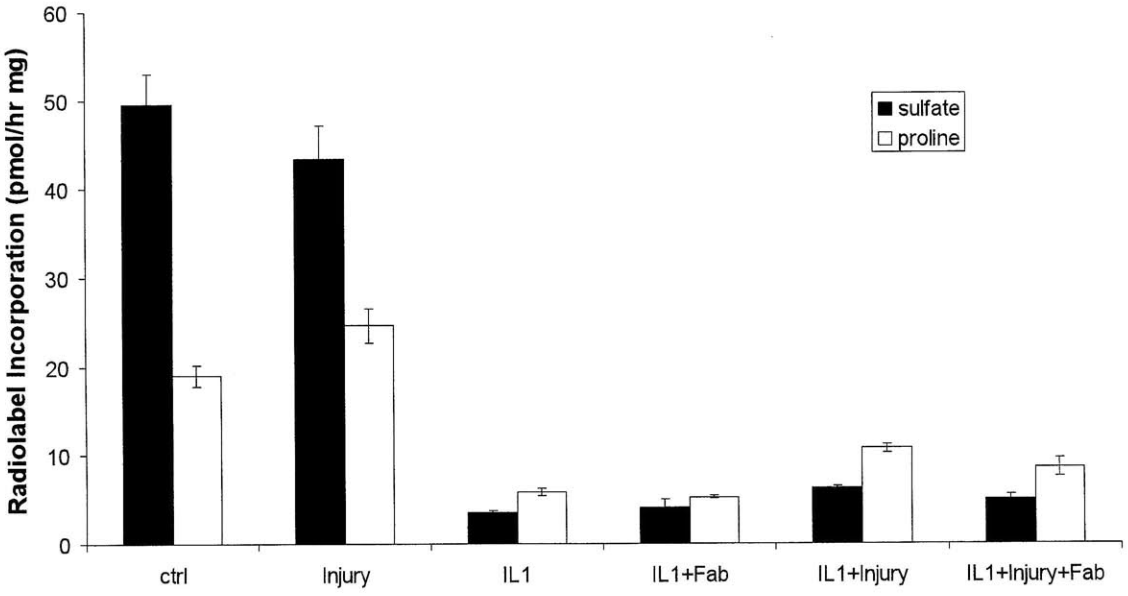


Figure A.12 Chondrocyte biosynthetic response to IL-1α, mechanical injury and anti-IL-6 Fab treatment during days 4-6. Cartilage subjected to the anti-IL-6 Fab (35μg/ml) treatments were pre-incubated with Fab for 6 days prior to another 6-day treatments with IL-1α (10μg/ml), mechanical injury and Fab. Mean±SEM (n=5 cartilage disks).

APPENDIX B

Preliminary Studies

B.1 Co-culture of injured cartilage with synovial tissue

Synovial tissues can release cartilage matrix degrading enzymes and pro-inflammatory cytokines. Previous studies have shown mechanical injury of cartilage followed by co-culture with joint capsule resulted in biosynthesis inhibition(1). The goal of this study was to compare the difference between cartilage co-cultured with joint capsule (full thickness capsular tissue) and those with synovial membrane.

Cartilage subjected to mechanical injury was compressed with 50% final strain with 1/second strain rate. Both joint capsules and synovial membranes were excised using 5mm dermal punch. In the co-culture condition, each cartilage was incubated with 1 piece of joint capsule or synovial membrane for 6 days.

In general, joint capsule and synovial membrane did not cause significant GAG loss from cartilage, irrespective of injury. Both were capable of suppressing chondrocyte biosynthesis. Since there was no significant difference between the two types of tissues in proteoglycan degradation and synthesis, and it is easier to harvest and culture synovial membranes than joint capsule, we suggest using synovial membrane in the co-culture model.

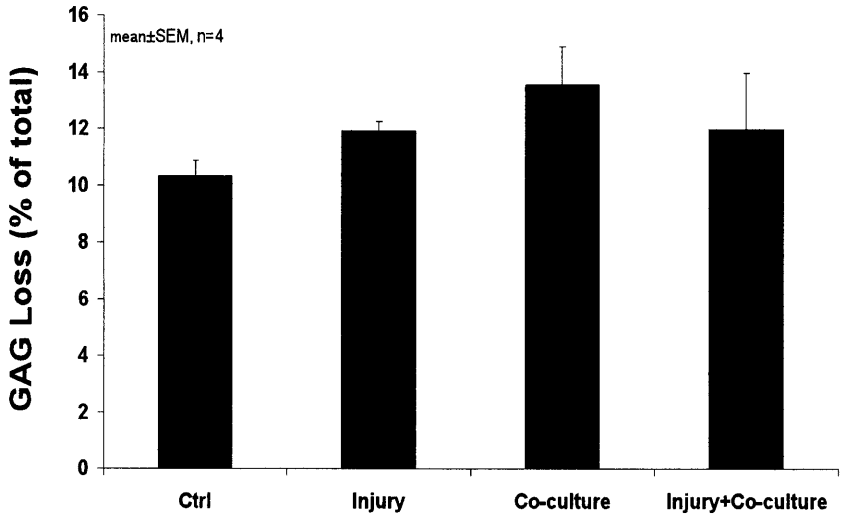


Figure B.1 Percentage of GAG loss from cartilage samples subjected to mechanical injury, co-culture with joint capsule, and both treatments. Cartilage was in culture for a total of 6 days.

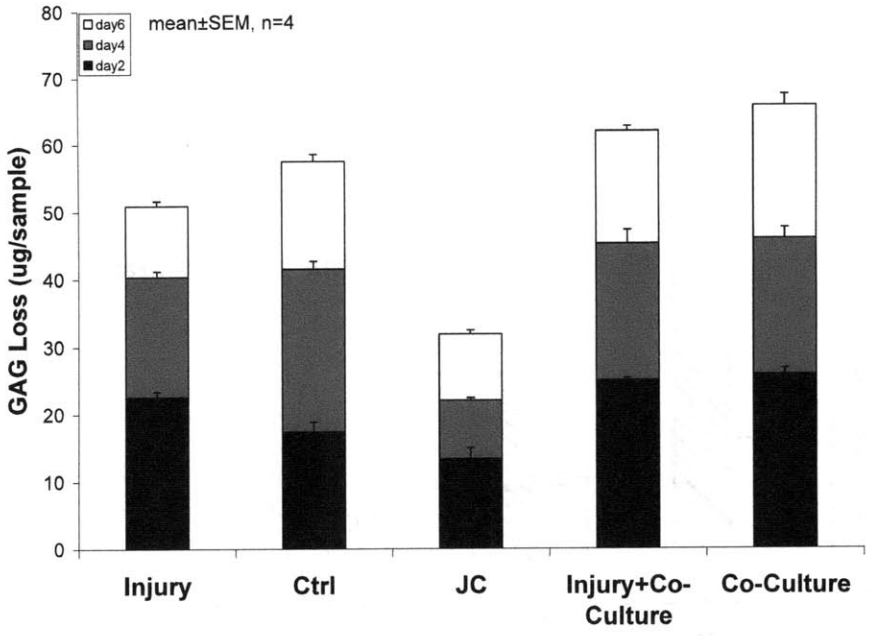


Figure B.2 Cumulative GAG loss from cartilage samples subjected to mechanical injury, co-culture with joint capsule (JC), and both treatments. Joint capsule tissue alone released approximately 35μg of sGAG during the 6-day culture.

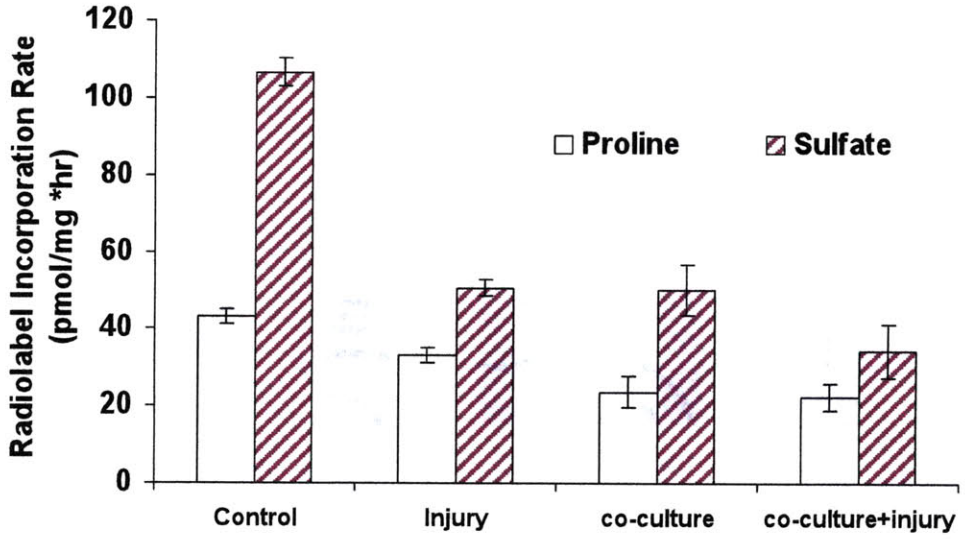


Figure B.3 Sulfate and proline incorporation rates measured during days 4-6 from cartilage tissues subjected to mechanical injury, co-culture with joint capsule and both treatments. Both mechanical injury and co-culture with joint capsule suppressed chondrocyte biosynthesis. In each condition, n=4 cartilage explants.

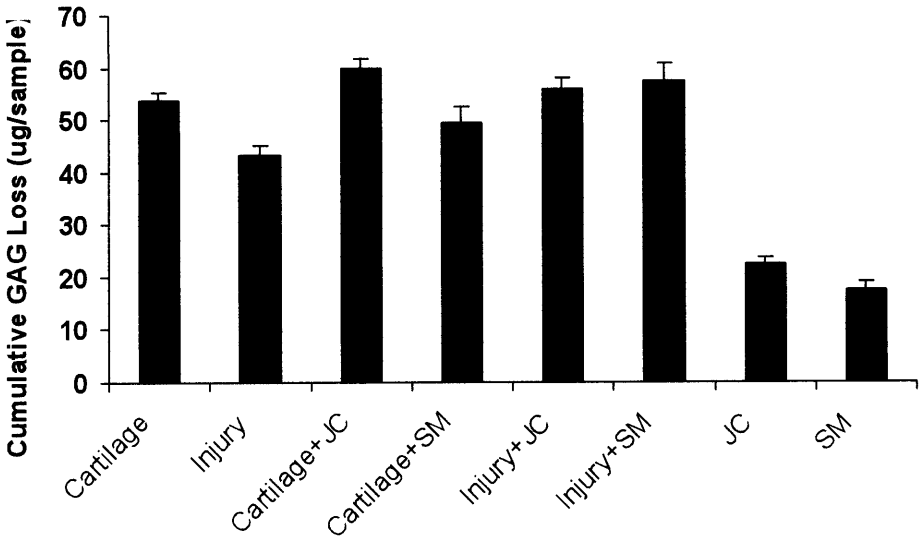


Figure B.4 Cumulative GAG loss from cartilage treated with mechanical injury, followed with co-culture with either joint capsule (JC) or synovial membrane (SM) for 6 days. Values are mean and SEM, n=6 samples.

Collaborator: Sangwon Byun.

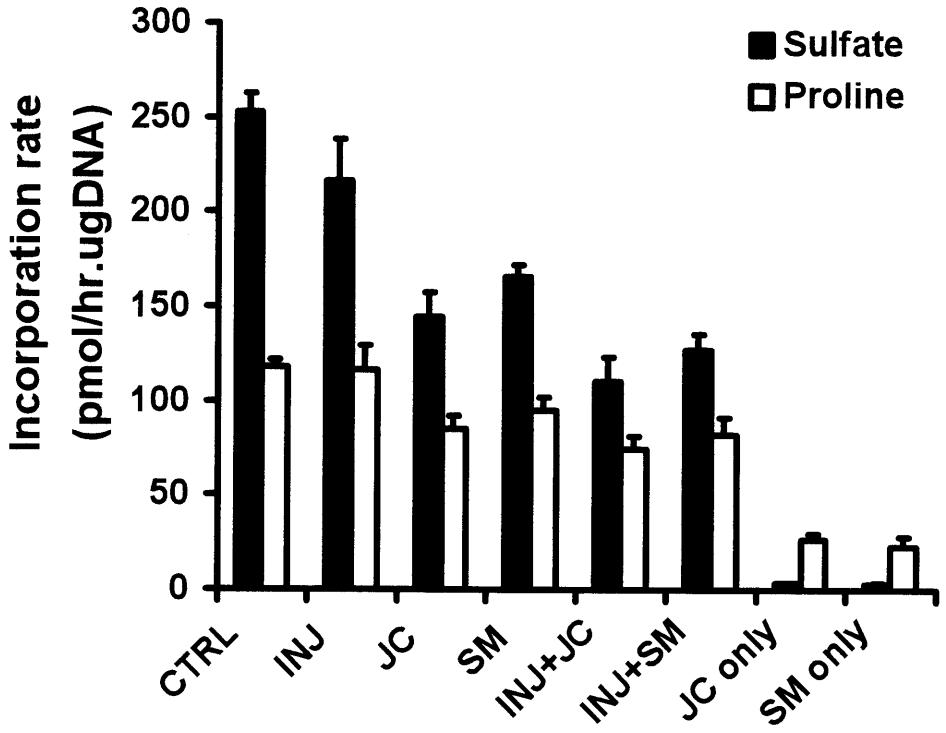


Figure B.5 Chondrocyte biosynthesis from cartilage treated with mechanical injury, followed with co-culture with either joint capsule (JC) or synovial membrane (SM). Both joint capsule and synovial membrane significantly reduced chondrocyte biosynthesis. Cells from joint capsule and synovial membranes also showed detectable sulfate and proline incorporation. Incorporation rates were measured from days 4-6. Values are mean and SEM, n=6 samples.

Collaborator: Sangwon Byun.

B.2 Effect of leptin in cartilage degradation

Obesity increases the susceptibility to the development of osteoarthritis (OA). The leptin levels in the synovial fluid of OA patients are similar or higher than those measured in serum(2). In vitro studies have demonstrated that leptin can decrease chondrocyte proliferation and increase the production of IL-1, MMP-9 and MMP13 in human chondrocytes(3).

The objectives of this study were to examine the effect of leptin in cartilage degradation induced by cytokines. We found higher concentration of leptin (200ng/ml) significantly increased the GAG loss induced by TNFα treatment, whereas lower concentrations of leptin suppressed the GAG loss caused by IL-1α treatment. Leptin produced no effect on nitrite release or chondrocyte biosynthesis. More studies are needed to confirm these findings and to further investigate the role of leptin in cartilage degeneration.

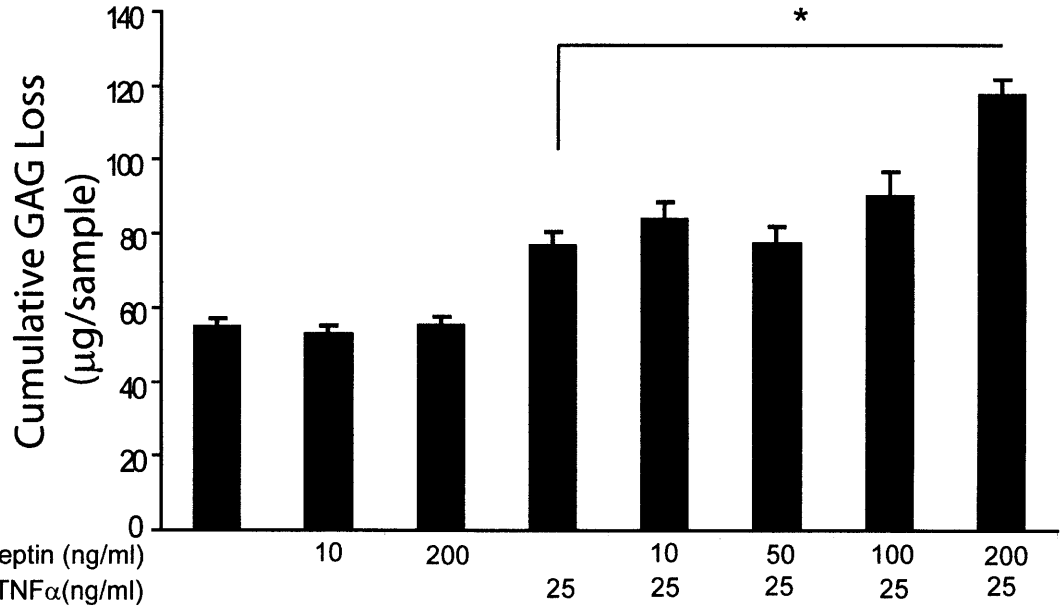


Figure B.6 Cumulative GAG loss in response to 6-day treatments with TNFα and leptin. Cartilage treated with 200ng/ml leptin significantly increased GAG loss comparing to samples with TNFα treatment only. Values are mean and SEM. In each condition, n=5 cartilage disks. *=$P<0.05$, only comparisons from selected hypothesis were shown.

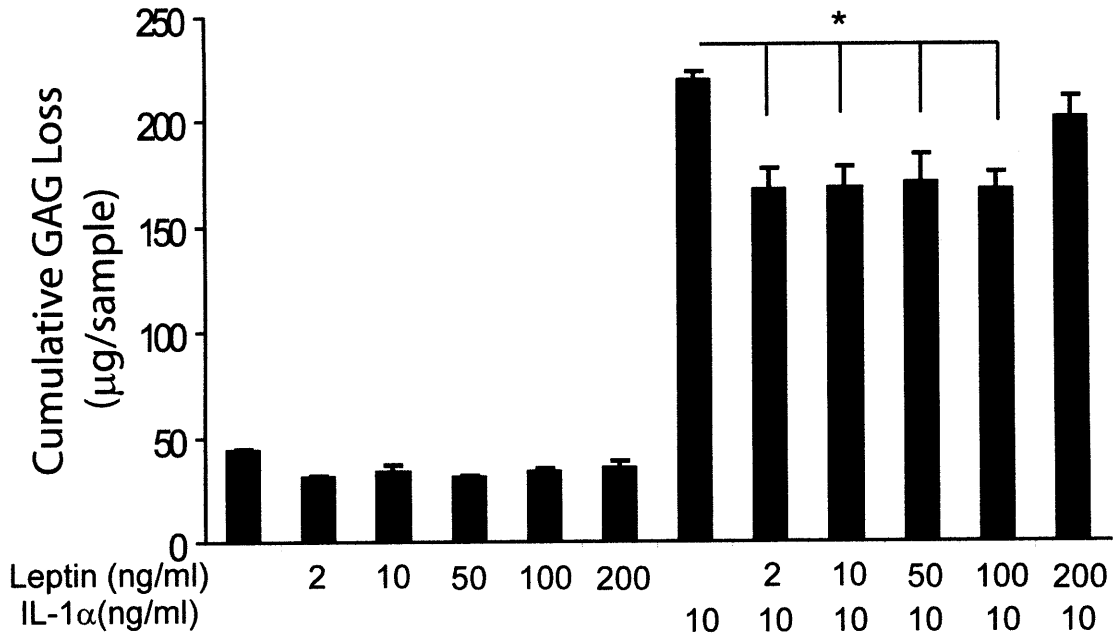


Figure B.7 Cumulative GAG loss in response to 6-day treatments with IL-1α and leptin. Cartilage treated with 2, 10, 50 and 100ng/ml leptin significantly decreased GAG loss induced by IL-1α treatment, whereas high concentration (200ng/ml) of leptin showed no effect on GAG loss induced by IL-1α. Values are mean and SEM (n=5 cartilage disks). *=P<0.05, only comparisons from selected hypothesis were shown.

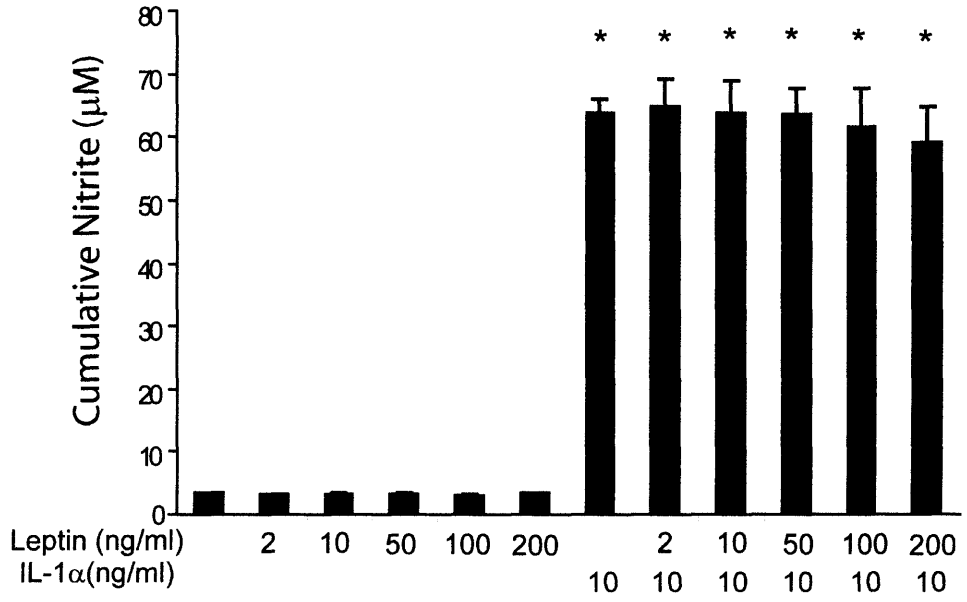


Figure B.8 Cumulative nitrite released to medium in response to 6-day treatments with IL-1α and leptin. IL-1α alone significantly increased nitrite formation, whereas leptin showed no effect on nitrite production. Values are mean and SEM (n=5 cartilage disks). *=$P<0.05$ vs. no-treatment control.

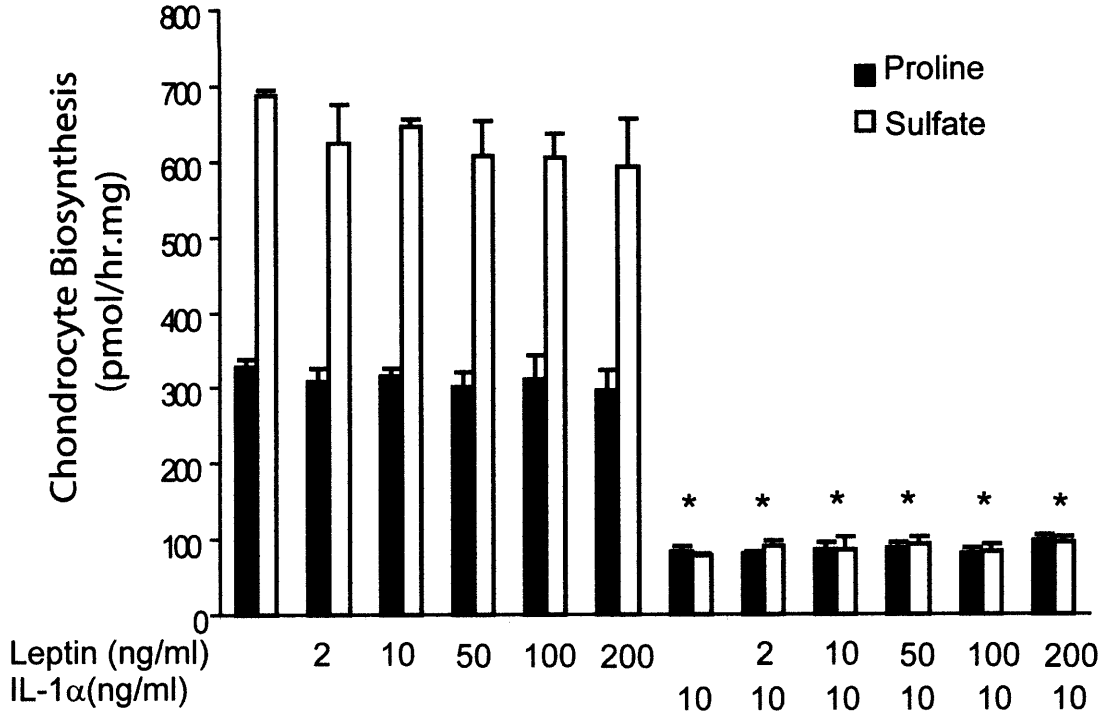


Figure B.9 Chondrocyte biosynthetic response to IL-1α and leptin (2, 10, 50, 100 and 200ng/ml). IL-1α significantly suppressed the biosynthesis of both proteoglycan and total protein. Leptin, at any concentration, produced no effect on chondrocyte biosynthesis, either in the presence or absence of IL-1α treatment. Mean±SEM (n=5 cartilage disks). *=$P<0.05$ vs. no-treatment control.

B.3 The role of P38 pathway in cartilage degradation

The P38 MAPK signaling pathways are known to be involved in the synthesis of inflammatory cytokines and matrix degrading enzymes(4). Blocking P38 pathways attenuated both cartilage degeneration and pain in animal model and suggested that p38 inhibitors may be beneficial for the treatment of osteoarthritis(5). The goal of this study was to investigate whether the P38 pathways signaling pathways were also involved in the cartilage degradation observed in the in vitro models of injury and whether the catabolic processes could be blocked using a P38 inhibitor.

We found P38 was phosphorylated in all of the treatment conditions, but more so in the conditions involving TNFα. The P38 inhibitor (SB 203580) dose-dependently reduced nitrite in cartilage treated with cytokines; however, contrary to what we expected, it increased both GAG loss and apoptosis. These studies suggested that P38 pathway was activated by the in vitro models of cartilage injury; however, blocking it may cause more deleterious response in cartilage. More studies are needed to understand the association between P38 pathway and matrix degradation.

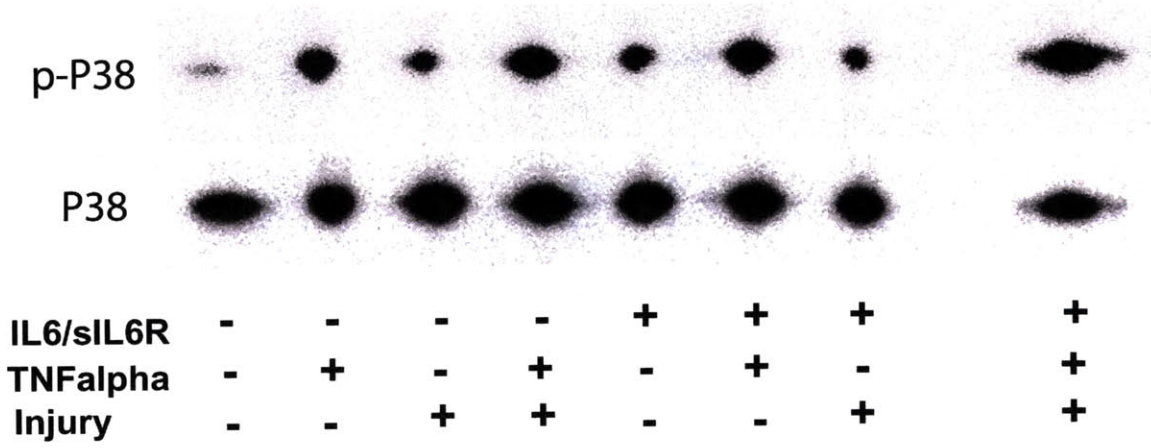


Figure B.10 Western analyses for phospho-P38 from both injured and uninjured cartilage treated with combinations of TNFα (25ng/ml), IL-6/sL-6R(50/250 ng/ml) for 4 days. Equal quantities of tissue extracts from each condition were run on a 4-15% gradient SDS-PAGE gel and immunoblotted with a primary antibody recognizing phospho-P38 (Cell Signaling).

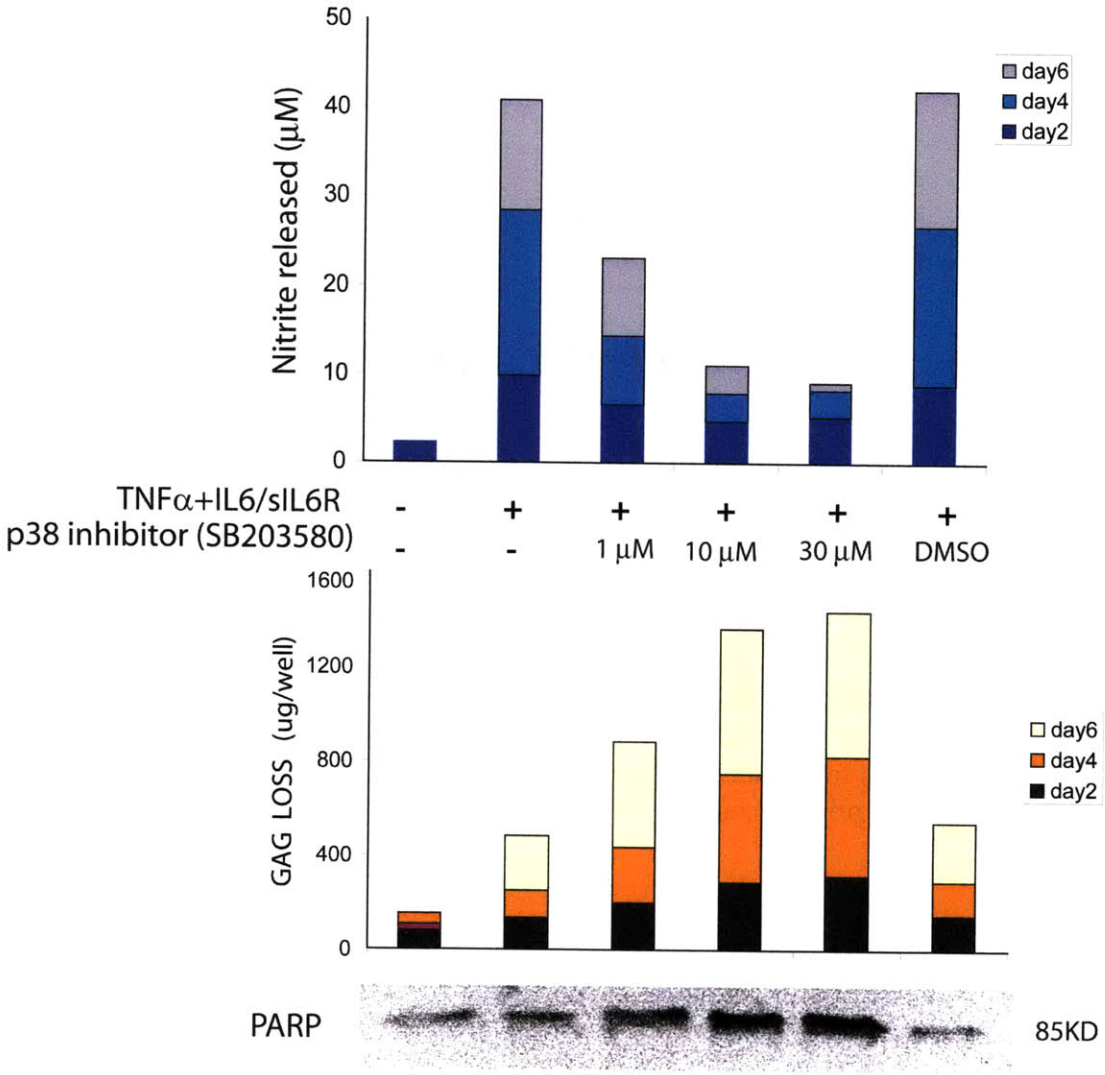


Figure B.11 Effect of blocking p38 pathway on nitrite release and GAG loss induced by TNFα and IL-6/sIL-6R. Cartilage disks were treated with both TNFα (25ng/ml) and IL-6/sIL-6R (50/250 ng/ml) for 6 days, in the presence of SD203580 (1, 10, 30 μM, Sigma). Blocking p38 pathway decreased nitrite (top), but increased GAG loss to medium (center) and level of PARP (bottom, Western blot) in cartilage. PARP (Poly ADP-ribose polymerase) is a protein involved in apoptosis.

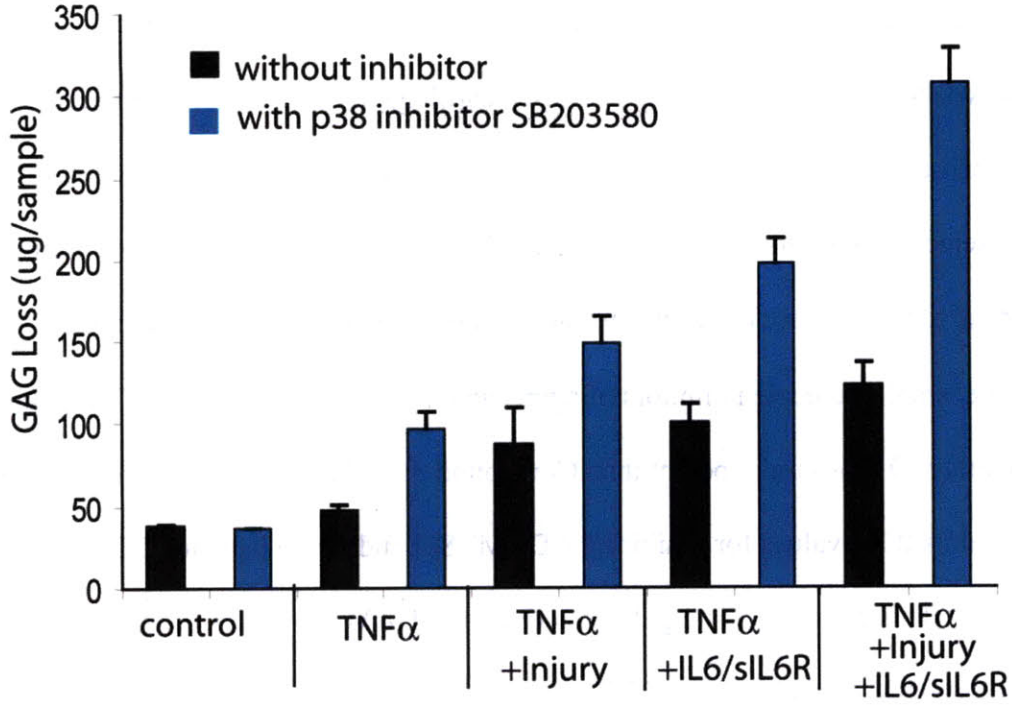


Figure B.12 Effect of SB203580 (10μM) on GAG loss from cartilage samples treated with combinations of TNFα (25ng/ml), IL-6/sIL-6R (50/250 ng/ml) and mechanical injury. Blocking P38 significantly increased GAG loss from all treatment conditions. Cartilage was in culture for 6 days. In each condition, n=6 cartilage samples.

B.4 Effect of blocking aggrecanases in cartilage degradation

From Chapter 2 of this thesis, we have seen that TNFα treatment and its combination with IL-6/sIL-6R and mechanical injury caused cartilage to release aggrecan fragments bearing NITEGE neo-epitope, suggesting GAG loss was due to aggrecanase activity. In this study, we used two selective inhibitors, Compound A and D (kindly provided by Micky Tortorella and John Sandy) to block aggrcanases in cartilage treated with TNFα. Both compounds are acyclic carboxylate, small molecule inhibitors of aggrecanases developed at Pfizer. Compound D is approximately 10 times more potent than Compound A. It has a molecular weight of 502 Daltons and its IC50 values for inhibiting ADAMTS-4 and -5 are 0.39 and 2.2 nM, respectively. This compound is highly selective for ADAMTS-4 and -5 and is inactive against MMPs and ADAMs (ADAM-8 and -17).

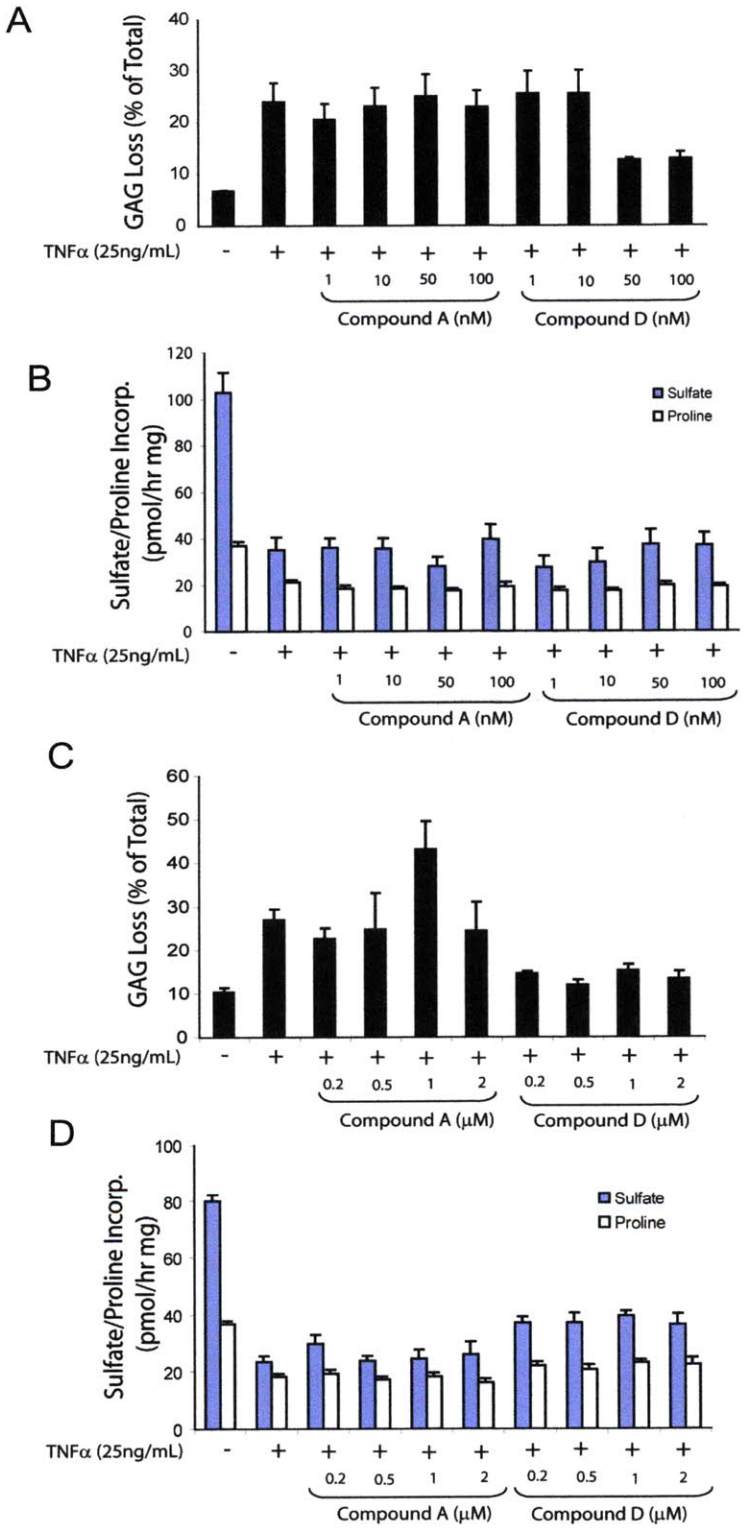


Figure B.13 GAG loss (A and C) and chondrocyte biosynthesis (C and D) in response to 6-day treatments with TNFα and different concentrations (1nM-2μM) of aggrecanase inhibitors (Compound A and Compound D). Biosynthesis was measured from days 4-6.

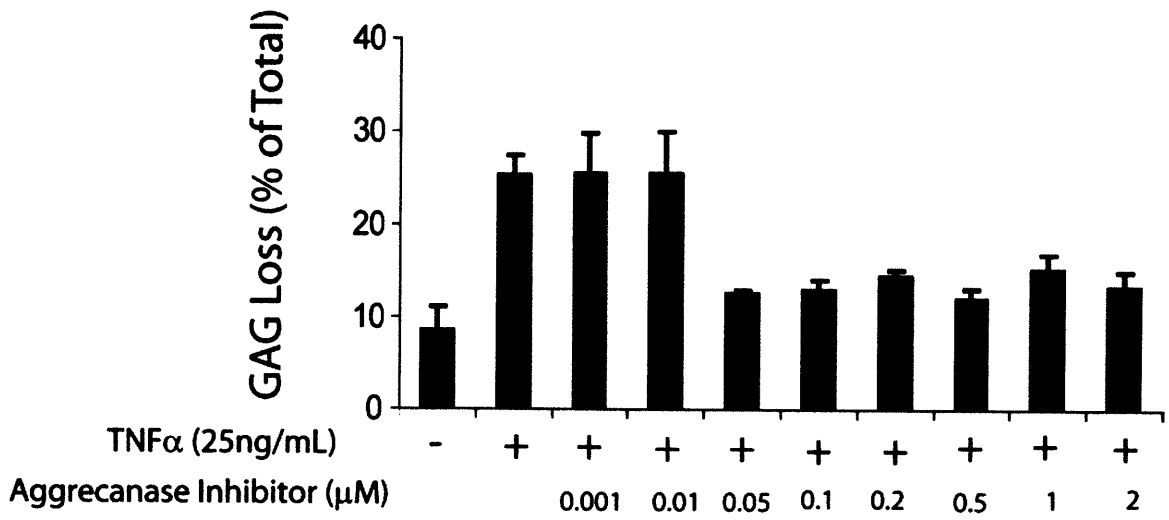


Figure B.14 Percentage of GAG loss in response to 6-day treatments with TNFα and an aggrecanase inhibitor (Compound D). Aggrecanase inhibitor at 50nM reduced the GAG loss caused by TNFα treatment by 76%.

B.5 References

1. Patwari P, Fay J, Cook MN, Badger AM, Kerin AJ, Lark MW, et al. In vitro models for investigation of the effects of acute mechanical injury on cartilage. Clin Orthop Relat Res 2001(391 Suppl):S61-71.
2. Presle N, Pottie P, Dumond H, Guillaume C, Lapicque F, Pallu S, et al. Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production. Osteoarthritis Cartilage 2006;14(7):690-5.
3. Simopoulou T, Malizos KN, Iliopoulos D, Stefanou N, Papatheodorou L, Ioannou M, et al. Differential expression of leptin and leptin's receptor isoform (Ob-Rb) mRNA between advanced and minimally affected osteoarthritic cartilage; effect on cartilage metabolism. Osteoarthritis Cartilage 2007;15(8):872-83.
4. Saklatvala J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol 2004;4(4):372-7.
5. Brown KK, Heitmeyer SA, Hookfin EB, Hsieh L, Buchalova M, Taiwo YO, et al. P38 MAP kinase inhibitors as potential therapeutics for the treatment of joint degeneration and pain associated with osteoarthritis. J Inflamm (Lond) 2008;5:22.