CHONDROCYTE RESPONSE TO *IN VITRO* MECHANICAL INJURY AND CO-CULTURE WITH JOINT CAPSULE TISSUE

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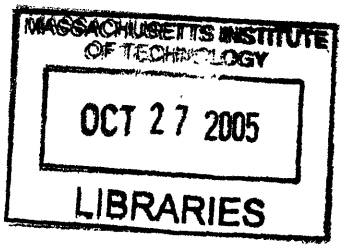

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ABSTRACT

Acute traumatic joint injury in young adults leads to an increased risk for the development of osteoarthritis (OA) later in life irrespective of surgical intervention to stabilize the injured joint. Although the mechanism by which injury leads to joint degeneration remains to be elucidated, several injury-related factors may contribute to the development of OA. These factors include but are not limited to altered mechanical loading and initiation of a cellular response in cartilage or other joint tissues at the time of the injury. Three *in vitro* models of joint injury were investigated to separately evaluate the effects on cartilage of mechanical overloading and damage to joint capsule and synovial lining. Models of injury included (1) mechanical injury to cartilage explants alone, (2) co-culture of normal cartilage explants with an excised specimen of joint capsule tissue, and (3) co-culture of mechanically injured cartilage explants with excised joint capsule tissue. These models have been shown previously to result in matrix damage and decreased biosynthesis by the chondrocytes.

We measured gene expression levels of matrix molecules and matrix proteases and found them to be expressed in control cartilage at levels ranging over five orders of magnitude, and to be differentially regulated in these three models of joint injury. Expression of matrix molecules including collagen II and aggrecan were unaffected by injurious compression or co-culture with joint capsule tissue during the first 24 hours; however, the combination of injurious compression followed by co-culture resulted in a ~50% decrease in expression by 24 hours. Matrix proteases aggrecanase-2 (ADAMTS-5) and stromelysin (MMP-3) showed increased expression of 40-250-fold by 12 hours following injurious compression and 6-12-fold during 24 hours of co-culture with joint capsule tissue. Aggrecanase-1 (ADAMTS-4) and collagenase-3 (MMP-13) showed larger magnitude increases in expression during co-culture (6-8-fold; 6-24 hours) compared to injurious compression (2-4-fold; 6-24 hours). Expression of transcription factors, c-fos and c-jun, was rapidly increased by injurious compression (40-100-fold within one hour) but was less affected by co-culture with joint capsule tissue (increased 3-5-fold; 1-24 hours). Expression level results displayed a general trend toward matrix degradation in the models of joint injury with specific differences apparent between the models. Analysis of matrix protein fragments in the same injury models showed cleavage of aggrecan at the aggrecanase site in the interglobular domain by 16 days following injurious compression and during co-culture with joint capsule tissue. Equilibrium and dynamic stiffness of cartilage explants were decreased by 30-35% immediately after injurious compression but were unaffected through 16 days of co-culture with joint capsule tissue. Specific changes in gene expression and activity of matrix proteases observed in these injury models may be indicative of some of the molecules responsible in the initial phase of cartilage degradation observed clinically following joint injury.

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CHAPTER 1: INTRODUCTION

1.1. THE KNEE

The knee occurs at the junction of three bones: the femur, tibia, and patella. At the ends of the bones is a layer of articular cartilage, which cushions the joint, distributes load to the underlying bone, provides a low friction surface for movement, and contributes to lubrication (Ross, 2003). Additional cushioning is provided by the menisci, two C-shaped pieces of cartilage located between the femur and the tibia. There are four main ligaments in the knee serving to stabilize the joint. These are the anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), medial collateral ligament (MCL), and lateral collateral ligament (LCL). There are two main muscle groups responsible for extension, quadriceps, and flexion, hamstring. The knee is the largest synovial joint in the body. During level walking, the force across the tibiofemoral joint ranges between two and four times body weight while the force across the patellofemoral joint does not exceed 50% of body weight. Peak force transmission across the joint increases as damage occurs to the menisci, articular cartilage, and subchondral bone (Standring, 2005).

1.2. CARTILAGE STRUCTURE

Cartilage is an avascular tissue composed of chondrocytes in an extensive extracellular matrix, which is produced and maintained by the cells. The cartilage matrix is well adapted for weight bearing and is 60-80% water. Additionally, the matrix includes proteoglycans, type II collagen, and hyaluronic acid. Cartilage extracellular matrix permits diffusion of substances between blood vessels in the surrounding connective tissue and the chondrocytes, maintaining the viability of the tissue. The matrix of hyaline cartilage appears glassy in the living state explaining its name derived from the Greek *hyalos* meaning glass. Chondrocytes are located throughout the cartilage matrix in spaces called lacunae. When damaged, chondrocytes have a limited capacity to repair the matrix (Ross, 2003).

Many different types of collagen are expressed in articular cartilage to form the complex fibrillar structure of the collagen network. This network varies in structure from the articular surface to the deep zones of cartilage and from the pericellular space surrounding chondrocytes to the interterritorial matrix. In articular cartilage, the collagen network is composed predominantly of Type II collagen fibrils present as heteropolymers with collagen IX molecules covalently linked to the surface of the fibrils and collagen XI forming a filamentous template at the fibril core. Collagen XI serves to regulate fibril diameter through retained N-propeptide domains. In addition to the abundant collagen fibrils, Type III collagen is found in normal and osteoarthritic human articular cartilage, colocalized with collagen II. Type VI collagen comprises <1% of the collagen in the matrix and is concentrated around chondrocytes. Types XII and XIV collagens are also present in the matrix of cartilage but are not covalently bound to collagen fibrils (Eyre, 2004).

In addition to collagen, the extracellular matrix of articular cartilage is composed of a wide variety of proteoglycans. Aggrecan is the most abundant proteoglycan in cartilage and is largely responsible for the ability of the tissue to resist compression. Aggrecan has an amino-terminal globular domain that interacts with hyaluronan and link protein to form large, multimolecular complexes. Chondroitin sulfate and keratin sulfate chains are covalently attached along the aggrecan core protein in three glycosaminoglycan attachment domains (Roughley, 2001).

The cartilage matrix also contains other proteoglycans that do not form aggregates, as well as noncollagenous and nonproteoglycan-linked glycoproteins. These small regulatory and structural proteins influence interactions between the chondrocytes and the matrix and can be valuable clinically as markers of cartilage turnover and degradation. Examples of such proteins are anchorin CII, tenascin, and fibronectin, which help anchor chondrocytes to the matrix (Ross, 2003).

Because chondrocytes are dispersed, the matrix acts as a signal transducer for the embedded cells; loads applied to cartilage create mechanical, electrical, and chemical signals that direct cellular synthetic activity. Normal matrix turnover relies on the ability of chondrocytes to detect changes in matrix composition and respond to these changes by synthesizing appropriate new molecules. As the body ages, matrix composition changes and chondrocytes loose their ability to respond to their environment (Ross, 2003).

1.3. JOINT CAPSULE, SYNOVIUM, LIGAMENTS

The joint capsule is a fibrous membrane of variable thickness extending from the patellar tendon to the collateral ligament on both sides of the knee (Standring, 2005). The loose connective tissue of the capsule is composed of sparse collagen fibers, proteoglycans, and hyaluronic acid. The matrix allows diffusion of oxygen and nutrients from the small vessels present throughout tissue and diffusion of carbon dioxide and metabolic waste back to vessels. Many of the cells occurring in loose connective tissue are transient wandering cells that migrate from local blood vessels in response to stimuli. This tissues serves as a site of inflammatory and immune reactions and can undergo considerable swelling (Ross, 2003).

The internal surface of the capsule is lined by a thin tissue known as the synovium. The synovial membrane of the knee is the most extensive and complex synovium in the body. It extends several centimeters above the patella and is separated from the patellar tendon by an infrapatellar fat pad. The synovium covers the fat pad and projects into the joint. At the sides of the joint, the synovial membrane descends from the femur and lines the capsule as far as the menisci, whose surfaces have no synovial lining. The cruciate ligaments are partially surrounded by synovial membrane. Following acute or chronic trauma, the synovial membrane can become thickened and inflamed (Standring, 2005).

Cruciate ligaments, termed cruciate because they cross, are located in the center of the knee joint. The anterior cruciate ligament (ACL) is attached to the anterior intercondylar area of the tibia and ascends, twisting on itself, to attach high of the lateral femoral condyle. The posterior cruciate ligament (PCL) is attached to the posterior tibia extending to the medial femoral condyle. The PCL is thicker and stronger than the ACL and its rupture is less common and usually better tolerated (Standring, 2005). Ligaments are dense connective tissue and consist mainly of densely packed collagen fibers arranged in parallel. Fibroblasts that produce and maintain the fibers are packed and aligned between fiber bundles (Ross, 2003).

1.4. OSTEOARTHRITIS

Arthritis and chronic joint symptoms occur in more than 70 million people in the United States or one out of every three adults. Arthritis is the leading cause of disability in the United States with costs for care reaching nearly $22 billion in 1995 with additional costs of $60 billion attributed to loss of productivity (Praemer, 1999). The prevalence of arthritis and chronic joint symptoms increases with age and in 2001 occurred in 19% of people age 19-44, 42% of people age 44-64, and 59% of those over 65 years old. Prevalence of arthritis also increases with increasing body weight occurring in 27% of underweight and normal weight individuals, 34% in those who are overweight, and 45% of obese people (Bolen, 2002).

Osteoarthritis (OA), a degenerative disease in which the cartilage in the joint is degraded and joint movement becomes painful, is the most common form of arthritis and occurs in a variety of joints including the knee, hip, hand/fingers, spine, and foot. Symptomatic OA occurs in the knee in 6% of adults in the hip in 3% of adults (Felson, 2000). It remains unclear whether OA is one disease or many disorders with a similar final pathway (Felson, 2000). Clinically, the most compelling definition of the disease is one that combines the pathology of the disease with pain during joint use (many people with radiographic and pathologic changes of OA do not

experience symptoms). The cause of pain in OA is unknown. Pathology of osteoarthritis involves the whole joint and includes focal and progressive hyaline cartilage loss with concomitant changes in bone underneath the cartilage including development of marginal outgrowths, osteophytes, and increased thickness of the bony envelope (bony sclerosis). Soft tissue structures in and around the joint are also affected including modest inflammation of the synovium, weakening of the bridging muscle in the joint, and loosening of the ligaments. Clinically, symptoms in a joint affected by OA may include pain, inflammation, swelling, stiffness, loss of range of motion, and weakness.

Treatment of OA remains challenging with no cure currently available for the disease. Physical activity can decrease pain, improve function, and delay disability caused by OA. In overweight patients, disease progression can be slowed by weight loss. NSAIDS (non-steroidal anti-inflammatory drugs) are used to reduce pain and inflammation in the joint. Dietary supplements including glucosamine and chondroitin sulfate are used with reported success. Physical therapy to improve flexibility and strengthen muscle is also used as a treatment for OA. In the end stage of disease, surgery is often the only option. Arthroscopy is performed to remove bone spurs, cysts, damaged lining, or loose fragments from the joint. Other surgical options include joint fusion, which eliminates joint flexibility and joint replacement, which maintains joint flexibility but can be problematic over time.

1.5. CLINICAL JOINT INJURY

In addition to age and body weight, joint injury is a well established risk factor for the development of OA particularly in the knee and hip (Davis, 1989; Roos, 1995; Gelber, 2000). Risk for OA is not affected by surgical intervention to stabilize the joint following ACL rupture (Lohmander, 2004; von Porat, 2004). A prospective study performed at John's Hopkins Medical School showed by 65 years of age the cumulative incidence of knee OA was 14% in participants

who had a knee injury during adolescence and young adulthood compared to 6% in those who did not (Gelber, 2000). Joint injuries typically include anterior cruciate ligament (ACL) or meniscus tears with ACL tears most common in patients under 30 and meniscus tears most common over the age of 30 (Roos, 1995). A study of a group of female soccer players who sustained an ACL injury showed by 12 years after injury (mean age 31 years at assessment), radiographic changes in knee index occurred in 82% with 51% fulfilling criteria for radiographic knee OA. Of these, 75% had symptoms affecting their knee-related quality of life and 42% had symptomatic, radiographic knee OA (Lohmander, 2004).

Clinical studies of knee injury have shown marked changes in the joint environment. Analysis of synovial fluid shows increased levels of matrix degrading enzymes (Lohmander, 1993a), increased markers of matrix turnover (Lohmander, 1993a; Lohmander, 1993b; Lohmander, 1999; Lohmander, 2003), and elevated levels of inflammatory cytokines (Irie, 2003) in injured joints compared to synovial fluid from uninjured controls. The origin of these factors in the synovial fluid is unknown and may include one or more of cartilage, synovium, ligament, tendon, and bone. It is likely that this altered joint environment is involved as either a cause or an effect in the pathology of OA development.

1.6. JOINT INJURY MODEL SYSTEMS

To study joint injury in more detail, *in vivo* and *in vitro* model systems have been developed. *In vivo* animal studies of joint injury have shown changes in both the cartilage and synovium after transection of the anterior cruciate ligament (ACLT). The ACLT model of joint injury results in changes analogous to those seen during OA progression including full-thickness loss of articular cartilage, osteophyte formation, cartilage surface fibrillation, chondrocyte cloning, hyperplasia of synovial lining, mononuclear cell infiltration, and joint capsule fibrosis (Brandt, 1991). In addition to gross morphological changes in the joint, ACLT leads to changes

in expression of matrix molecules and enzymes in cartilage and synovium (Takahashi, 1999; Le Graverand, 2002). Recent studies have specifically shown aggrecanase-2 to be responsible for cartilage degradation in mouse models of OA (Glasson, 2005; Stanton, 2005).

In vitro injury models include impact loading of articular cartilage and co-culture of cartilage with damaged synovium or joint capsule tissue to model soft tissue tears. Models in which cartilage tissue is overloaded result in cell and tissue damage including cell death (Quinn, 1998; Chen, 1999; Torzilli, 1999; Loening, 2000; Chen, 2001; D'Lima, 2001; Kurz, 2001; Thibault, 2002; Patwari, 2003; DiMicco, 2004). Co-culture of cartilage with synovial or joint capsule tissue also results in cell and tissue damage in the cartilage (Fell and Jubb, 1977; Jubb and Fell, 1980; Vankemmelbeke, 1999; Patwari, 2001). The combination of mechanical injury of cartilage followed by co-culture with joint capsule results in further reduction in biosynthesis than that measured after injury or co-culture alone (Patwari, 2001).

1.7. THESIS OBJECTIVES

We sought to gain further understanding of the mechanisms involved in joint injury by studying the effects on chondrocytes of established *in vitro* models of injury to cartilage. We hypothesized that specific changes would occur at the level of gene expression in response to injurious stimuli. Real-time PCR was used to quantitatively measure gene expression levels of 24 genes central to cartilage matrix maintenance in free swelling control cartilage explants. We then measured expression levels of these genes at six time points within the first 24 hours in multiple models of joint injury. Data from the mechanical injurious compression model of cartilage injury was compared to data from the injury model involving co-culture of cartilage with joint capsule tissue. Additionally, effects of these two stimuli applied individually were compared to measured gene expression changes in response to the combination of injurious compression followed by co-culture with joint capsule tissue. This experimental design made possible determination of

specific molecules that are affected by injurious compression, co-culture, or the combination of these stimuli. We next sought to measure protein levels and activity of catabolic enzymes in the joint injury models. We hypothesized that matrix damage observed in these injury models was the result of activity of specific catabolic enzymes able to cleave cartilage extracellular matrix molecules. As well, we sought to determine the effect on equilibrium and dynamic stiffness of cartilage tissue explants in each of the injury models. Overall, the goal of this project was to determine if there is a general shift in cellular activity toward matrix repair or degradation following events of joint injury and to identify specific enzymes that may be involved in these processes.

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CHAPTER 2: MECHANICAL INJURY OF CARTILAGE EXPLANTS CAUSES SPECIFIC TIME DEPENDENT CHANGES IN CHONDROCYTE GENE EXPRESSION

2.1. INTRODUCTION

Acute traumatic joint injury in young adults leads to an increased risk for the development of osteoarthritis (OA) later in life (Davis, 1989; Gelber, 2000; von Porat, 2004) despite efforts to intervene in this process by surgically stabilizing injured joints (Roos, 1995). Although the mechanism by which injury leads to tissue degeneration remains to be elucidated, several injury-related factors may contribute to the development of OA. These factors include but are not limited to instability in the joint due to ligament, tendon, or meniscus tear, and/or initiation of a cellular response in cartilage or other joint tissues at the time of the injury.

Previous clinical studies have shown an increase in MMP-3 and TIMP-1 protein levels as well as an increase in proteoglycan and type II collagen fragments in the synovial fluid of patients following a tear in the anterior cruciate ligament (ACL) or meniscus from 1 day to 20 years after the injury (Lohmander, 1994; Lohmander, 2003). During the first week after ACL injury, there was also a significant increase in synovial fluid levels of TNF-α and IL-1β. By three weeks after injury, the levels of these cytokines decreased to those seen in samples from patients with chronic arthritis (Irie, 2003). Gene expression studies comparing normal and OA cartilage have shown upregulation of MMP-13 in late-stage OA, while MMP-3 is downregulated (Bau, 2002); as well, BMP-2 is increased in OA cartilage and co-localizes with newly synthesized procollagen II suggesting anabolic remodeling of the tissue (Fukui, 2003).

Using the lapine ACL transection model to study the pathogenesis of OA *in vivo*, investigators have found an increase in MMP-3 expression level after 9 weeks (Takahashi, 1999), and location specific changes in mRNA levels of several genes after 3 and 8 weeks (Le Graverand, 2002). Expression of collagen II, aggrecan, biglycan, MMP-1, MMP-3, MMP-13,

and TIMP-1 all increased during the development of OA in this animal model, while decorin and fibromodulin showed decreased expression (Le Graverand, 2002).

Because loading variables are difficult to control *in vivo*, a number of investigators have developed *in vitro* models to isolate cartilage and study tissue and cellular level effects of mechanical injury. Mechanical loads applied *in vitro* range from single compressions of up to 50% strain (Torzilli, 1999; D'Lima, 2001; Ewers, 2001; Kurz, 2001; Patwari, 2003; DiMicco, 2004) to large amplitude cyclic compression at varying frequencies (~0.05 Hz to 0.3 Hz) for up to 2 hours (Chen, 1999; Chen, 2001; Thibault, 2002; Chen, 2003). Injurious mechanical compression of cartilage *in vitro* can damage the extracellular matrix, leading to increased water content (Chen, 1999; Torzilli, 1999; Loening, 2000; Kurz, 2001), decreased stiffness (Loening, 2000; Kurz, 2001), increased hydraulic permeability (Thibault, 2002), GAG loss to the culture medium (Quinn, 1998; Loening, 2000; D'Lima, 2001; Ewers, 2001; Kurz, 2001; Thibault, 2002; Patwari, 2003; DiMicco, 2004), collagen loss to the medium (Thibault, 2002), and temporary denaturation of collagen in the tissue (Chen, 1999; Torzilli, 1999; Thibault, 2002; Chen, 2003). In addition, injurious mechanical compression can lead to cell death by both apoptosis and necrosis (Quinn, 1998; Torzilli, 1999; Loening, 2000; Chen, 2001; D'Lima, 2001; Chen, 2003), as well as decreased matrix biosynthesis rates in the remaining viable cells after injury (Kurz, 2001).

Though many studies have focused on the effects of *in vitro* injurious compression on cartilage tissue, the resulting modulation of chondrocyte gene transcription has not been fully elucidated. The objective of this study was to quantify the effects of cartilage injury *in vitro* on 24 genes central to cartilage maintenance, including genes encoding macromolecules of the extracellular matrix (ECM), proteases that can cleave ECM proteins and their natural inhibitors, transcription factors, and cytokines previously shown to affect cartilage metabolism. Using real-time PCR, we measured levels of mRNA of these molecules at six time points after acute

mechanical injury. We observed distinct changes in the pattern and kinetics of expression that may suggest a role for certain catabolic processes associated with eventual cartilage degradation.

2.2. MATERIALS AND METHODS

Tissue Harvest. Articular cartilage explant disks were harvested from the femoropatellar grooves of 1-2 week old calves using previously developed methods (Sah, 1989). In brief, 9 mm diameter cartilage-bone cylinders were drilled perpendicular to the cartilage surface. These cylinders were then placed in a microtome holder and the most superficial ~200 μm layer was removed to obtain a level surface. Up to three sequential 1 mm slices were cut from each cylinder, and 4 disks (1 mm thick, 3 mm diameter) were cored from each slice using a dermal punch, giving 48 disks in total from each joint. These disks were then equilibrated in culture medium for 2 days (low glucose DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a 37°C, 5% CO_2 environment.

Injurious Compression. After equilibration of the explants, a custom-designed incubator-housed loading apparatus (Figure 2.1A) (Frank, 2000) was used to injuriously compress 36 cartilage disks from each joint while the remaining 12 served as free swelling controls. Cartilage samples to be injured were placed individually into a polysulfone chamber which allows radially-unconfined compression of the disk by impermeable platens (Figure 2.1B) (Kurz, 2001; Patwari, 2003; DiMicco, 2004). The measured thickness of the cartilage disk just prior to loading was recorded, and the zero-strain position was identified by the point of first contact between the loading platen and the cartilage surface. The injury protocol consisted of a single displacement ramp to a final strain of 50% at a velocity of 1 mm/s (strain rate 1.0/s in displacement control), followed by immediate removal of the displacement at the same rate (Figure 2.1C). Application of these strain and strain rate parameters resulted in an average peak stress of ~20 MPa; this loading protocol has been shown previously to produce damage to the ECM, a significant decrease in cell viability, a decrease in cell biosynthesis by remaining viable

cells, and an increase in GAG loss to the medium in similar bovine cartilage explants (Quinn, 1998; Loening, 2000; Kurz, 2001; Patwari, 2003; DiMicco, 2004). After injury, the disks were placed into fresh culture medium (described above). Groups of 6 cartilage disks were removed from culture at 1, 2, 4, 6, 12, and 24 hours, flash frozen in liquid nitrogen, and stored at -80°C. Two groups of 6 free swelling disks were frozen at 4 and 24 hours to serve as controls. Explant disks in each group of 6 specimens were purposely matched across depth and location along the joint surface to prevent bias based on location; as a result, each experimental condition represents an average of specimens within the joint surface.

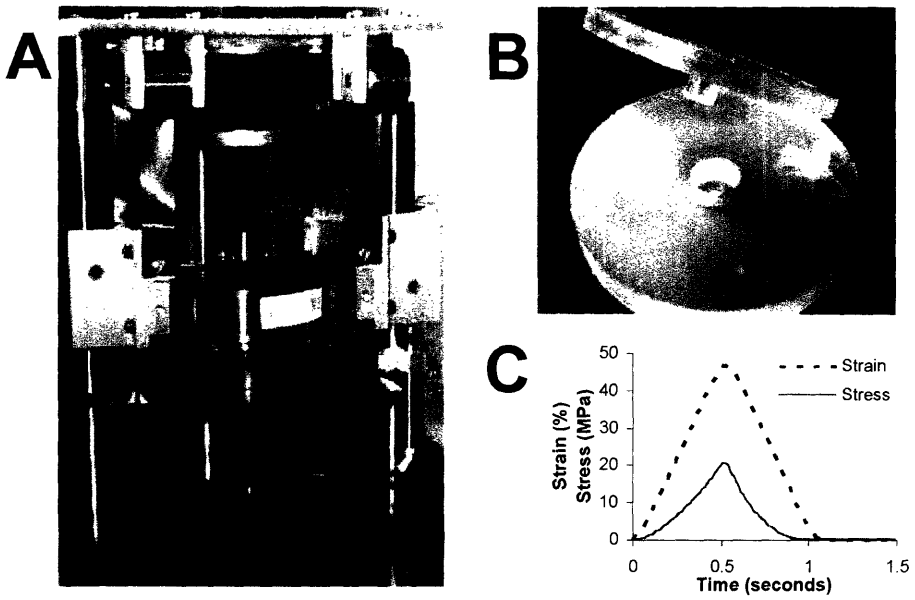

Figure 2.1: Loading device and example of compression waveforms. A) An incubator housed loading apparatus was used to apply injurious compression in displacement control to individual cartilage disks. The load and displacement were recorded by transducers during loading. B) Polysulfone chamber used to hold cartilage disks during loading in unconfined compression. C) Representative data acquired during compression to 50% strain at a strain rate of 1.0/s. Peak stress reached a maximum value of 20.7 MPa.

RNA Extraction. RNA was extracted from the 6 pooled cartilage disks by first pulverizing the tissue and then homogenizing in Trizol reagent (Invitrogen, CA) to lyse the cells. Extracts were then transferred to Phase Gel Tubes (Eppendorf AG, Germany) with 10% v/v chloroform and spun at 13,000g for 10 min. The clear liquid was removed from above the phase

gel and RNA was isolated from the sample using the RNeasy Mini Kit (Qiagen, CA). Genomic DNA was removed by a DNase digestion step (Qiagen, CA) during purification. Absorbance measurements were read at 260 nm and 280 nm to determine the concentration of RNA extracted from the tissue and the purity of the extract. The average 260/280 ratio of absorbencies was 1.86±0.12. Reverse transcription of equal quantities of RNA (2.5 μg) from each sample was performed using the Amplitaq-Gold RT kit (Applied Biosystems, CA).

Real-time PCR. Real-time PCR was performed using the Applied Biosystems 7700 instrument and SYBR Green Master Mix (Applied Biosystems, CA). Primers were designed to bovine sequences for matrix molecules (collagen II, aggrecan, link protein, fibronectin, fibromodulin, and collagen I), proteases (MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, ADAMTS-5), protease inhibitors (TIMP-1, TIMP-2), cytokines (TNF-α, IL-1β), housekeeping (β-actin, GAPDH), transcription factors (c-fos, c-jun, sox 9), and growth factors (IGF-1, IGF-2, TGF-β) using Primer Express software (Applied Biosystems, CA). Standard curves for amplification using these primers were generated; all primers demonstrated approximately equal efficiency, with standard curve slopes ~1 indicating a doubling in cDNA quantity each cycle. Expression levels in injured samples were normalized to those of free swelling control samples for each gene.

Statistical Analyses. In each experiment, expression levels measured in injured sample groups were normalized to those of free swelling control groups for each gene; expression data are presented as the average of three replicate experiments (± SE). Changes in gene expression levels in the injured samples with respect to free swelling controls at the 4 hour and 24 hour time points were examined using a non-parametric t-test (Troyanskaya, 2002). The t-test was made non-parametric by estimating the p-values from permuted data sets (Good, 2000); the t-statistic was calculated from each of the permuted data sets to create a distribution of possible values. Using this method, all changes in expression that were 5-fold or greater were found to be statistically significant. Changes between 2 and 5-fold were also found to be significant with

three exceptions (c-fos at 4 hours and 24 hours, and c-jun at 24 hours); in certain instances, lower fold changes were also found to be significant.

Gene Clustering. To distinguish the main expression trends, a k-means clustering algorithm was applied to the injury time course data (Eisen, 1998; Jain, 1999; Dougherty, 2002; Fitzgerald, 2004). Each gene was grouped based on the correlation of the time course expression profile to a set of randomly chosen starting genes. Group profiles were then calculated as the average of the expression profiles of the genes in each group. The correlation between each gene and group profile was calculated and the genes regrouped in an iterative fashion until the groupings settled. To ensure that an optimal clustering solution for the twenty-four genes was found, the algorithm was run sufficient times to cover every possible selection of starting genes. Each set of randomly chosen starting genes produced a deterministic grouping of the genes, with each gene paired with the highest correlating group profile. The optimal solution was chosen as the grouping that had the highest overall correlation of genes to group profiles, by averaging over all the genes (see (Fitzgerald, 2004) for details). The number of groups was varied from three to six and five groups were chosen to best represent the trends. To determine the distinctiveness of the main expression trends, the final group profiles were compared using a comparison means Student's t-test. The Euclidean distance between two group profiles represented the difference of means, and the average squared distance of the genes within a group to the group profile represented the variance. The number of genes in each group corresponded to the degrees of freedom for that group.

2.3. RESULTS

Gene expression levels in uninjured control cartilage disks. Real-time PCR was used to determine the gene expression levels of 24 genes of interest in uninjured control cartilage disks for comparison to mechanically injured disks. Levels of expression of the tested genes varied over five orders of magnitude, as seen in Figure 2.2, with data normalized to the level of the lowest expressed gene, ADAMTS-4 (aggrecanase-1). Extracellular matrix molecules as well as sox 9, a transcription factor promoting expression of matrix molecules in cartilage, showed the highest levels of expression. Genes typically used as internal controls (e.g., GAPDH, β-actin) showed intermediate levels of expression, while certain cytokines, matrix metalloproteinases, and transcription factors displayed relatively lower levels of expression. ADAMTS-4 and ADAMTS-5 (aggrecanase-2) showed the lowest levels of expression of the genes tested.

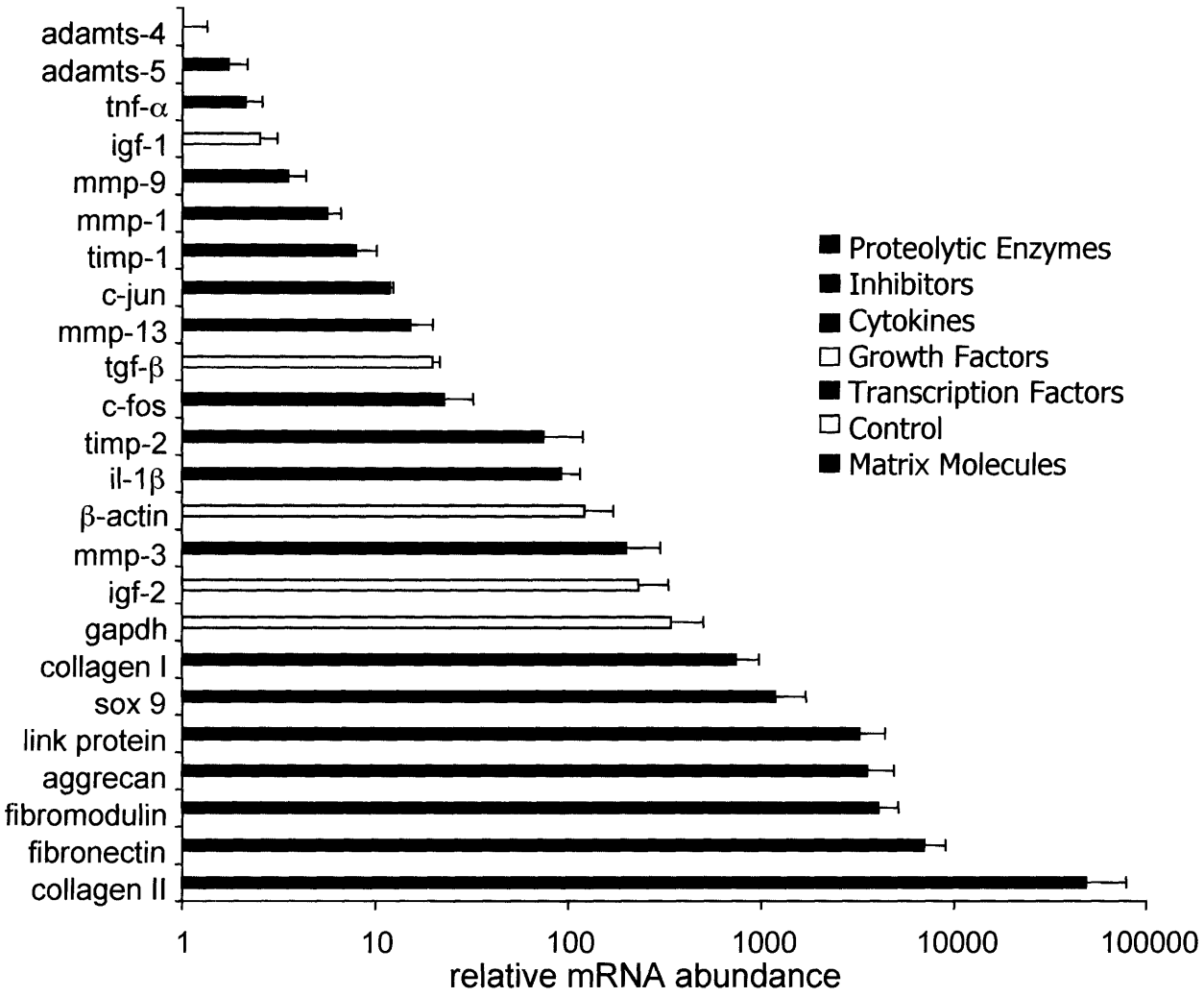

Figure 2.2: Free swelling expression levels of 24 genes ranked by relative abundance. Medium was changed 2 days after harvest and samples were taken 4 and 24 hours after medium change for gene expression quantification. Levels at the two time points were averaged to give a single value for each tissue sample. Expression levels were normalized to expression of ADAMTS-4, the least abundant gene measured. Data are reported as Mean ± SE for n = 3 replicate experiments using tissue from 3 different joints.

Effects of injurious compression on gene expression. Levels of expression in non-injured free swelling controls (Figure 2.2) changed selectively in response to injurious compression. P-values comparing expression levels after injury to control free swelling expression levels were calculated at the 4 and 24 hour time points and are shown in Table 2.1. While expression levels of some genes remained unchanged in response to injury, others exhibited dramatic differences compared with their free-swelling controls. GAPDH and β-actin

increased in expression ~4-fold over free swelling levels after compression (Figure 2.3). Because these genes showed changing expression levels within the 24 hours after loading, they were not used as internal controls to normalize the data acquired for the other genes. Rather, all expression levels were instead normalized by using a fixed quantity of extracted RNA for reverse transcription as described above in 2.2. MATERIALS AND METHODS. By using a fixed quantity of RNA from each sample, decreased cell viability in injuriously compressed cartilage should not affect the levels of expression reported; rather changes in expression should represent changes within the viable cells remaining in the tissue.

Table 2.1: List of group members generated by k-means clustering with p-values comparing expression in injured and control cartilage. Gene expression profiles were iteratively clustered into five groups using k-means clustering. Groups were formed based on gene to group profile correlation. To compare control and injured cartilage, p-values were calculated from t-tests performed at the time points 4 hours and 24 hours after injury; bold represents p-value <0.05, * represents p-value <0.01.

Group 1			Group 2			Group 3			Group 4			Group 5		
	4hr	**24hr**		**4hr**	**24hr**		**4hr**	**24hr**		**4hr**	**24hr**		**4hr**	**24hr**
MMP-3	**0.01**	**0.05**	c-fos	0.53	0.30	MMP-1	0.07	**0.01**	IGF-1	0.16	0.08	Collagen II	0.46	0.09
ADAMTS-5	*	*	c-jun	**0.03**	0.26	MMP-9	0.83	**0.04**	IGF-2	0.15	0.49	Aggrecan	**0.05**	**0.02**
TGF-β	*	**0.03**				MMP-13	**0.03**	*	ADAMTS-4	**0.03**	**0.02**	Fibromodulin	0.09	0.72
						Collagen I	**0.02**	**0.05**				Link Protein	0.12	0.18
						TIMP-1	**0.02**	**0.03**				IL-1β	0.09	0.25
						TIMP-2	0.69	*						
						Fibronectin	0.49	**0.04**						
						sox 9	*	0.92						
						GAPDH	0.14	*						
						β-actin	0.08	**0.03**						
						TNF-α	0.91	0.33						

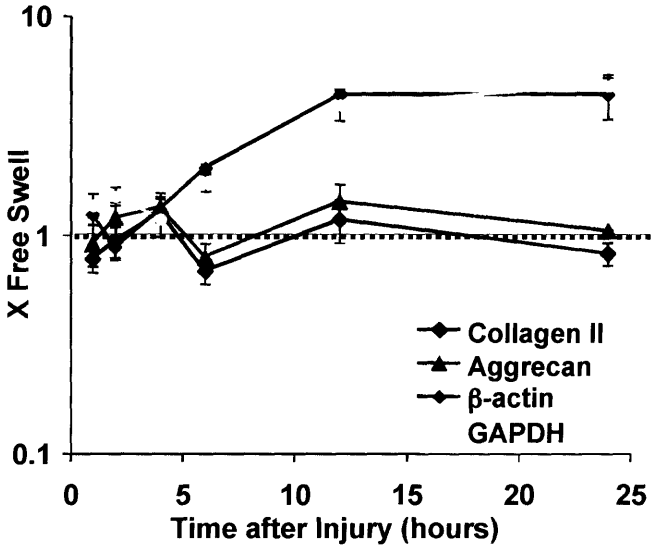

Figure 2.3: Changes in expression level of matrix molecules, β-actin, and GAPDH genes after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

Extracellular matrix molecules showed no change in expression levels greater than 2-fold during the 24 hours immediately following compression. Collagen II and aggrecan (Figure 2.3) as well as fibromodulin and link protein (data not shown) did not change their expression during the 24 hours immediately following injury. Fibronectin increased ~2-fold at the 12 and 24 hour time points (data not shown).

The most dramatically changing gene in this study was MMP-3, which increased in expression ~250-fold over the free swelling control level following injurious compression (Figure 2.4). MMP-3 expression began to increase within 2 hours after injury, peaked by 12 hours, and decreased to a ~50-fold increase over free swelling level by 24 hours. MMP-13, however, showed only a ~2-fold increase over free swelling level during the 24 hours after injury (Figure 2.4), and MMP-1 and MMP-9 increased by ~6-fold and ~4-fold, respectively, over their free swelling levels (data not shown). Like MMP-3, ADAMTS-5 also showed a dramatic increase in expression to ~40-fold over the free swelling level by 12 hours after injury, and

remained elevated at ~10-fold over free swelling by 24 hours (Figure 2.4). In contrast, ADAMTS-4, increased only ~2-3-fold over free swelling levels and showed little variation with time in the 24 hours after injury (data not shown). TIMPs, the endogenous tissue inhibitors of metalloproteinases, were also affected by injurious compression. TIMP-1 increased to ~12-fold over free swelling levels by 12 hours and remained elevated by 24 hours after injury (Figure 2.4). TIMP-2, which is expressed at an overall higher level than TIMP-1 in free swelling cartilage (Figure 2.2), was increased by only ~2-fold at 12 and 24 hours after injury (data not shown).

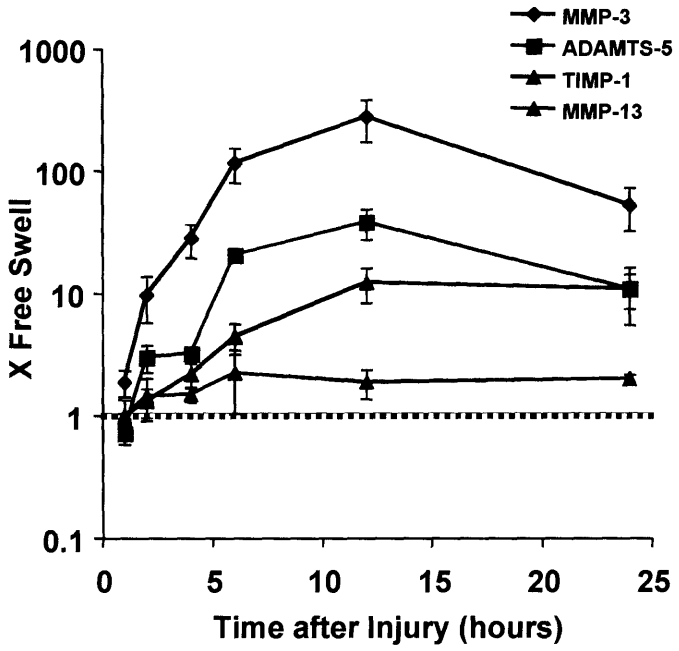

Figure 2.4: Changes in expression level of matrix proteases and tissue inhibitor of matrix metalloprotease-1 after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

The immediate response transcription factors, c-fos and c-jun, responded to injury with a rapid increase in expression (~120-fold for c-fos and ~40-fold for c-jun) within the first hour after injury (Figure 2.5). By 4 hours, both genes returned to ~3-fold over free swelling levels and

remained moderately elevated for 24 hours. Another transcription factor, sox 9, which promotes transcription of matrix molecules, did not change expression level significantly during the 24 hours following injurious compression (Figure 2.5). This is consistent with the observed lack of change in expression levels for the matrix molecules shown in Figure 2.3.

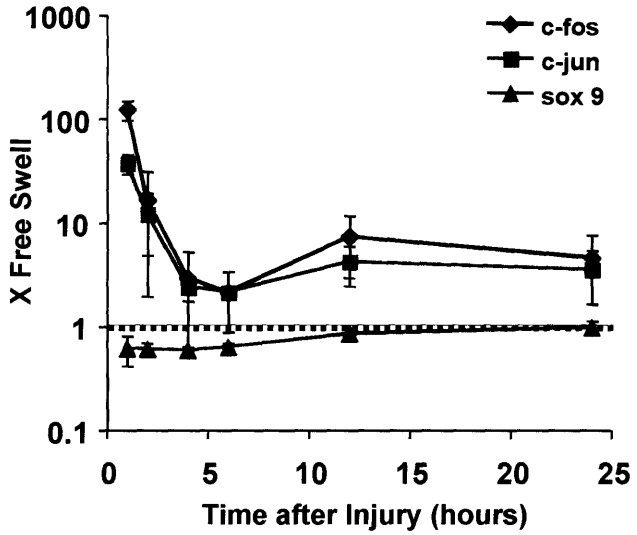

Figure 2.5: Changes in expression level of transcription factors after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at reverse transcription step. Mean ± SE (n=3).

Selected growth factors also showed specific changes in expression level in response to injurious compression. TGF-β increased expression in the first 4 hours after injury to a peak value ~7-fold over free swelling levels, remained elevated through 12 hours, and then decreased to ~4-fold over the free swelling value by 24 hours (Figure 2.6). Insulin-like growth factors IGF-1 and IGF-2 (Figure 2.6) and, similarly, the cytokines IL-1β and TNF-α (Figure 2.7) showed little variation with time (not exceeding 2-fold changes compared to non-injured controls) in the 24 hours immediately following injurious compression.

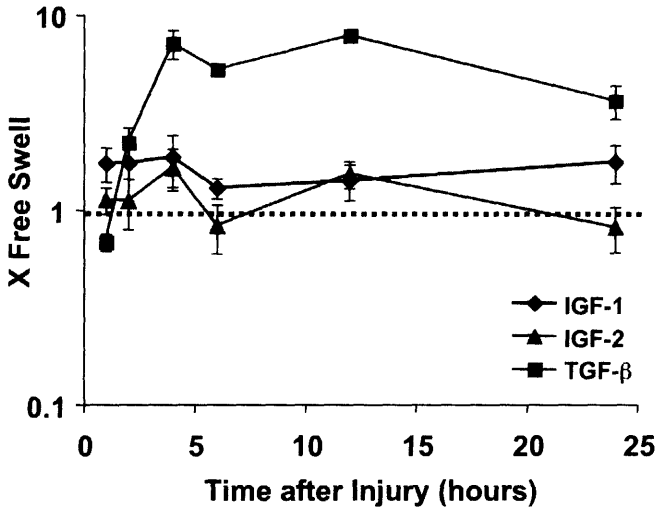

Figure 2.6: Changes in expression level of growth factors after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at reverse transcription step. Mean ± SE (n=3).

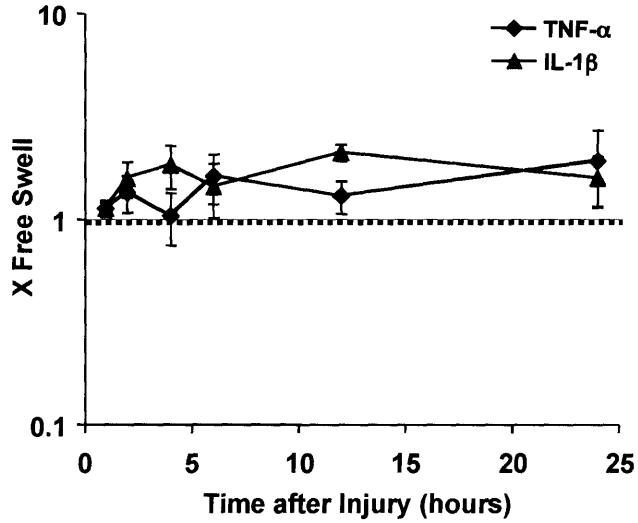

Figure 2.7: Changes in expression level of cytokines after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at reverse transcription step. Mean ± SE (n=3).

Clustering and statistical analyses of gene expression profiles. Clustering analysis revealed five groups with distinct temporal expression profiles induced by injury. The group expression profiles are shown in Figure 2.8, and the corresponding group members are listed in Table 2.1 along with the associated p-values that were calculated from t-tests performed at the time points 4 hours and 24 hours after injury to compare control and injured cartilage. In general, the group expression profiles reflect the main traits of the individual genes within each group with average correlation coefficients of 0.90, 1.00, 0.89, 0.77 and 0.88 for groups 1-5 respectively. Comparison of means Student's t-tests revealed that the group expression profiles of Figure 2.8 were distinct. The unique profile of Group 2 was significantly different from expression profiles of Groups 3, 4, and 5 ($p<0.05$) and Groups 1 and 3 were also significantly different ($p=0.006$). Groups 1 and 2 expression profiles were found to be not significantly different from each other primarily due to the low number of genes within each of these groups.

2.4. DISCUSSION

A single injurious compression of cartilage has been shown previously to decrease extracellular matrix biosynthesis rates, compromise mechanical properties, and reduce chondrocyte viability (Torzilli, 1999; Loening, 2000; D'Lima, 2001; Kurz, 2001; Patwari, 2003; DiMicco, 2004). We undertook this study to determine if changes also occur at the level of gene expression and to determine if the changes are general or specific to certain genes. Analysis of samples was performed using real-time PCR, which allows the measurement of many genes to be achieved in a high-throughput manner using a relatively small sample volume. We observed significant changes in the expression of several catabolic and anabolic genes in response to mechanical injury, and used k-means clustering (Fitzgerald, 2004) to further analyze gene expression patterns and co-regulation of specific genes that may result from injury.

Gene group behavior. Clustering resulted in separation of the genes into five groups that displayed distinct behavior after injury (Figure 2.8, Table 2.1). Group 1 contained MMP-3, ADAMTS-5, and TGF-β for which large increases in expression level occurred at early times (within 4 hours) following injury. In addition to directly cleaving matrix molecules, MMP-3 has been implicated as a member of activation cascades of matrix-degrading enzymes, including other MMPs. Stimulation of these three genes immediately after injury may represent an attempt to remodel the damaged matrix by removing some of the matrix molecules or by activating latent molecules in the matrix. The transcription factors c-fos and c-jun (Group 2) showed an immediate transient upregulation followed by a rapid decline within 4 hours. c-fos and c-jun are members of the activating protein-1 (AP-1) family of genes that were shown previously to activate MMPs in a chondrocyte cell line after IL-1β treatment (Vincenti and Brinckerhoff, 2002). This is consistent with the activation of several MMPs in Groups 1 and 3 of this study, observed at time points following the increased expression of c-fos and c-jun immediately after injury.

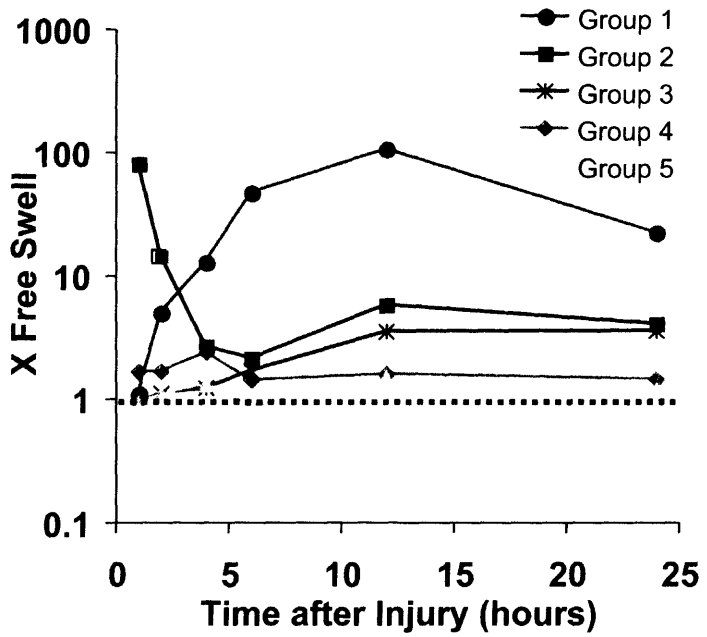

Figure 2.8: Group expression profiles generated by k-means clustering. The main temporal gene expression patterns induced by injury of cartilage explants. Group profiles were calculated by averaging the expression profiles of genes within each group.

Group 3 represents the slowly increasing expression pattern seen for MMPs (other than MMP-3) and their inhibitors, as well as TNF-α, fibronectin, collagen I, GAPDH, and β-actin. Although expression of sox-9 remained below free swelling levels for all time points tested (Figure 2.5), this gene clustered into Group 3 because its expression increased from 0.6 to 1.0-fold of free swelling controls during the 24 hours after injury, as was the case for Group 3 overall. Further investigation is required to determine the extent to which these molecules may affect cartilage behavior after injury in this system, since the changes in expression were relatively low.

Group 4 (IGF-1, IGF-2, and ADAMTS-4) and Group 5 (collagen II, aggrecan, fibromodulin, link protein, and IL-1β) showed expression patterns that did not vary significantly with time after injury. Thus, any immediate alterations in ECM biosynthesis that may result from mechanical injury are unlikely to be related to events at the level of matrix gene transcription

(Group 5). Any rapid initial repair of matrix immediately after acute mechanical injury is also not likely to be associated with changes in expression of Group 4 genes.

Comparison to previous studies *in vitro* and *in vivo*. The effects of mechanical injury on the expression of MMP-3 and MMP-13 (Figure 2.4) are similar to trends reported previously (Patwari, 2003) using Northern analysis of similar cartilage explants subjected to the same injury protocol of Figure 2.1C. In that study, injury caused a significant increase in MMP-3 expression over controls (10-fold) but no change in MMP-13 in the first 24 hours. By comparison, when the data of Figure 2.4 are averaged over the full 24 hour period, MMP-3 appears upregulated 80-fold while MMP-13 expression changed less than 2-fold. Taken together, these two studies show similar differential changes in the expression of MMP-3 and MMP-13, obtained using both Northern and real-time PCR techniques. *In vivo* studies of OA progression following joint injury have also reported changes in MMP gene expression levels. Le Graverand et al., using a lapine ACL transection model of OA, found 2-3 fold increased chondrocyte expression of MMP-3 and 10-30 fold increased expression of MMP-13 (Le Graverand, 2002), which differs in the relative increases between MMP-3 and MMP-13 seen here and in previous *in vitro* studies (Patwari, 2003). This difference may be related, in part, to the presence of other tissues in the *in vivo* model, such as ligaments, tendons, and synovium, which are not included in the present *in vitro* mechanical compression model of injury to cartilage alone.

It is also informative to compare the observed changes in gene expression reported here to changes in protein levels reported previously in response to mechanical injury of cartilage *in vitro*. Immunohistochemical analyses of adult (23 month old) bovine articular cartilage disks subjected to a rapid ramp displacement to 50% strain and held for 5 minutes revealed an increase in MMP-1, MMP-3, and MMP-13 as well as a decrease in TIMP-1 and TIMP-2 (Pufe, 2004). A study applying compressive loading to immature bovine tissue measured increased synthesis and activity of MMP-2 and MMP-9 after 1-16 hours of loading while no change was measured in TIMP-1 or TIMP-2 synthesis (Blain, 2001). Porcine cartilage disks from 3-6 month old animals

subjected to a cutting injury showed an increase in synthesis of MMP-1, MMP-3, and TIMP-1, while collagen synthesis remained unchanged (Vincent, 2002). Interestingly, our study also revealed changes in the expression of MMPs and TIMPs, but no change in collagen II expression. In addition, compression injury was found previously to increase fibronectin protein synthesis (Chen, 1999), and the compression injury used here caused an increase in fibronectin gene expression (Table 2.1). Specific differences found in these studies may be due to regulation at the level of translation as well as to the different injury models used (e.g., cutting vs. compression).

In vivo studies at the protein level also show certain parallels to the results reported here. Lohmander et al. analyzed human synovial fluid after ACL or meniscus injury and found increases in MMP-3 and TIMP-1 protein levels within 1 day after injury that persisted in the injured joint for 20 years (Lohmander, 1994). Similarly, increased chondrocyte mRNA levels of MMP-3 and TIMP-1 were found here after cartilage injury and in the lapine ACL transection model (Le Graverand, 2002). Irie et al. recently found elevated levels of the inflammatory cytokines IL-1β and TNF-α in human joints within 24 hours after ACL injury (Irie, 2003). In the current study, cartilage injury did not cause an increase in chondrocyte expression of IL-1β or TNF-α. While the major source of the increased cytokine, MMP, and TIMP levels seen in the synovial fluid of injured human joints could be from the synovium or tissues other than cartilage, it is informative to be able to identify specific changes in chondrocyte gene and protein expression for comparison.

Comparison to studies of OA tissue. Changes in expression of proteases and cytokines have been found during the progression of OA. Bau et al. (Bau, 2002) compared chondrocytes isolated from patients with normal articular cartilage to chondrocytes from patients with early and late stage OA. MMP-13 and ADAMTS-4 expression increased in late stage OA, while MMP-3 expression was the highest of the genes tested and was downregulated in OA (Bau, 2002). Murata et al. measured IL-1α and IL-1β gene expression (RT-PCR) and protein levels

(ELISA) in OA chondrocytes isolated from cartilage obtained during joint arthroplasty (Murata, 2003). They reported a decrease in IL-1α and IL-1β transcript in cells from advanced OA tissue compared to cells from tissue displaying only moderate degeneration. This decrease in expression was accompanied by a decrease in protein level in advanced OA. In contrast, we found that the expression of IL-1β was not significantly altered by acute compression injury *in vitro* (Figure 2.7). Other distinct and important differences were observed in gene expression exhibited by OA tissues versus that in explants subjected to acute mechanical injury. For example, collagen I and collagen II exhibit increased expression levels with the progression of OA (Gebhard, 2003), while no significant change in the expression of collagen II was observed; here, following compression injury, collagen I increased expression 2.5-fold by 24 hours and was found to be statistically significant. It should be emphasized that the focus of the present study is on immediate changes after injury (within the first 24 hours), while OA develops over a time span of many years and involves the pathology of the whole joint (Buckwalter and Mankin, 1998). It will be important to expand such *in vitro* studies to include longer culture periods after injury. In addition, *in vitro* models of whole joint injury that involve injured cartilage in the presence of exogenous cytokines or injured cartilage co-cultured with other injured joint tissues (Patwari, 2001; Patwari, 2003) may give additional insight into cellular pathways underlying chondrocyte response to injury.

Comparison to non-injurious loading. Investigators have studied the effects of static compression on cartilage explants as well as chondrocyte-seeded gels to determine if changes occur at the levels of gene expression and protein synthesis in these model systems. Cartilage explants were compressed very slowly to 25% and 50% strain and maintained in compression for up to 24 hours. This low strain-rate protocol does not alter cell viability (Kim, 1996), in contrast to the marked increase in cell death and matrix damage typically observed after injurious loading. These samples were compared to unloaded controls. Results by real-time PCR showed a transient increase in mRNA levels for aggrecan and collagen II, as well as other matrix proteins,

followed by a down regulation below control levels by 24 hours (Fitzgerald, 2004). The down regulation of aggrecan and collagen II expression was shown to be dose dependent by Northern analysis with 50% compression causing a greater decrease than 25% (Ragan, 1999). Radiolabel incorporation into proteoglycans and collagen also decreased with increasing static compression (Ragan, 1999). Transcription of many matrix proteases, including MMP-3, increased with loading duration 3- to 16-fold by 24 hours of loading (Fitzgerald, 2004). Transcription factors c-fos and c-jun were transiently upregulated by 6- to 35-fold after 1 hour of loading (Fitzgerald, 2004). Results similar to those seen for matrix molecule gene expression in cartilage explants were obtained in a cell-seeded construct. Primary chondrocytes were seeded in collagen I gels and subjected to static compression of 0%, 25%, or 50% for up to 24 hours (Hunter, 2002). Results using competitive and real-time PCR showed inhibition of collagen I, collagen II, and aggrecan mRNA expression. Radiolabel incorporation of proline and sulfate were also inhibited by the application of static compression (Hunter, 2002). In both of these experimental systems, changes in mRNA expression level correlated with changes that occurred at the protein level. Notably, changes seen in response to static compression were markedly different from those observed in injurious loading scenarios. In the current study, matrix molecules did not change in expression level as was seen in static compression; also, the magnitude of increased expression for degradative enzymes and transcription factors were higher in response to injurious compression compared to non-injurious static compression. Taken together, the response of chondrocytes to mechanical compression appears to depend on the specific parameters (rate, amplitude, and duration) of the applied compression.

An unexpected result in the current study was the relatively high level of collagen I expression in free swelling control tissue (Figure 2.2). Type I collagen molecules can be found in diseased or damaged articular cartilage; however, it is not an abundant protein in healthy cartilage. While collagen I expression was, indeed, ~60-fold lower than that of collagen II, mRNA levels were higher than most of the other non-ECM genes studied here. Relatively high

levels of collagen I expression were previously reported for 6 month old porcine cartilage, and found to be ~3-fold lower than collagen II expression (Fehrenbacher, 2003). The tissue used in the current study was obtained from newborn bovines, and collagen I expression may vary widely with age and species. Additionally, cells from the sparse blood vessels present in newborn cartilage tissue could contribute to the expression of collagen I, as seen in the control data of Figure 2.2.

One limitation of our study is that samples from different locations within the femoropatellar groove were pooled; thus, it was not possible to determine if tissue from different depths and locations along the groove would react differently to injurious compression. Explant disks in each group of 6 specimens were purposely matched across depth and location along the joint surface to prevent bias based on location; as a result, gene expression data represent an average of specimens within the joint surface. Another limitation is the use of newborn tissue. We and others have previously studied the effects of injury on cell viability and ECM degradation in the presence and absence of exogenous cytokines using both immature and adult cartilage from bovine and human joint surfaces and found that certain responses vary with age (Kurz, 2001; Patwari, 2003; Kurz, 2004). It will be important to extend the present study to identify any age- or disease-dependence of changes in gene expression caused by mechanical injury.

In summary, injurious compression caused time dependent changes within 4 to 24 hours in the expression of specific catabolic and anabolic genes that can regulate matrix remodeling and turnover, while many extracellular matrix molecules were unaffected. Ongoing studies are focused on determining whether these changes at the level of gene expression result in changes in protein levels in the cartilage, and whether the high upregulation of ADAMTS-5 and MMP-3 in response to injurious mechanical compression may be associated with cell-mediated changes in the proteolytic cleavage of ECM molecules over extended times after injury.

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CHAPTER 3: CO-CULTURE OF INJURED OR UNINJURED CARTILAGE WITH JOINT CAPSULE TISSUE LEADS TO SPECIFIC CHANGES IN GENE EXPRESSION LEVELS IN CHONDROCYTES

3.1. INTRODUCTION

Analysis of synovial fluid after joint injury shows increased levels of matrix degrading enzymes (Lohmander, 1993a), increased markers of matrix turnover (Lohmander, 1993a; Lohmander, 1993b; Lohmander, 1999; Lohmander, 2003), and elevated levels of inflammatory cytokines (Irie, 2003) compared to synovial fluid from uninjured controls. The origin of these factors in the synovial fluid is unknown and could include one or more of cartilage, synovium, ligament, tendon, and bone. Following joint injury, young adults are at an increased risk for the development of osteoarthritis (OA) (Davis, 1989; Roos, 1995; Gelber, 2000) irrespective of surgical intervention to stabilize the joint (Lohmander, 2004; von Porat, 2004). Taken together, clinical data suggests that the joint environment is altered after injury leading to an increased likelihood of developing OA.

In vivo animal studies of joint injury have shown changes in both the cartilage and synovium after transection of the anterior cruciate ligament (ACLT). The ACLT model of joint injury results in changes analogous to those seen during OA progression including full-thickness loss of articular cartilage, osteophyte formation, cartilage surface fibrillation, chondrocyte cloning, hyperplasia of synovial lining, mononuclear cell infiltration, and joint capsule fibrosis (Brandt, 1991). In addition to gross morphological changes in the joint, ACLT leads to increased expression of collagen II, aggrecan, MMP-1, MMP-3, MMP-13 and decreased expression of decorin and fibromodulin in cartilage (Takahashi, 1999; Le Graverand, 2002); synovial cells in the ACLT joint increase expression of MMP-3 and IL-1β (Takahashi, 1999).

In vitro injury models in which cartilage tissue is overloaded result in damage to the extracellular matrix evidenced by increased water content (Chen, 1999; Torzilli, 1999; Kurz,

2001), decreased stiffness (Loening, 2000; Kurz, 2001), increased hydraulic permeability (Thibault, 2002), GAG lost to the culture medium (D'Lima, 2001; Kurz, 2001; Patwari, 2003; DiMicco, 2004), and temporary denaturation of collagen II (Chen, 1999; Torzilli, 1999; Thibault, 2002). Additionally, cells within the matrix have been shown to decrease biosynthesis rates (Kurz, 2001) and undergo cell death by necrosis and apoptosis (Torzilli, 1999; Loening, 2000; Chen, 2001; D'Lima, 2001). In the 24 hours following injurious compression, degradative enzymes able to cleave extracellular matrix molecules increase gene expression from 5-250-fold above control expression levels. During the same period after loading, expression of matrix molecules remains constant at a level equal to that in control uninjured cartilage (described in detail in CHAPTER 2).

Culturing cartilage in the presence of damaged joint tissues has also been used as an *in vitro* model of joint injury. This type of model focuses on the potential effect on cartilage of factors released from other tissues present in the joint. Co-culture of cartilage with synovial or joint capsule tissue results in a decrease in biosynthesis rate (Jubb and Fell, 1980; Patwari, 2001) and loss of staining for proteoglycan and collagen in the cartilage (Fell and Jubb, 1977). Mechanical injury of cartilage followed by co-culture with joint capsule results in further reduction in biosynthesis than that measured after injury or co-culture alone (Patwari, 2001). Synovial or capsular tissue generates soluble proteoglycan degrading activity resulting in cleavage of aggrecan at the aggrecanase site in the interglobular domain. Treatment with an inhibitor of IL-1β and TNF-α, ACITIC, did not inhibit GAG release caused by co-culture; however, release was blocked by treatment with EDTA (Vankemmelbeke, 1999). Conditioned medium from either capsule tissue culture or fibroblast cultures established from explants of joint capsule caused GAG release from cartilage (Ilic, 2000). Aggrecanase-2 (ADAMTS-5) is expressed and is active in normal bovine and osteoarthritic human synovium (Vankemmelbeke, 2001). Neither aggrecanase activity nor ADAMTS-5 production by synovial cells was induced

by IL-1α or retinoic acid (previously shown to upregulate aggrecanase activity in cartilage (Flannery, 1999)) (Vankemmelbeke, 1999; Vankemmelbeke, 2001).

Previous studies have shown synovium and capsular tissue release cartilage matrix degrading enzymes as well as cause a general decrease in biosynthesis rates in the chondrocytes. The effects on chondrocyte gene expression were studied using *in vitro* models of joint injury. Models included co-culture of cartilage with joint capsule tissue (including synovium) either with or without additional mechanical injury of the cartilage. The objectives of this study were to (1) determine if co-culture of cartilage with joint capsule tissue including synovium can selectively affect gene expression of components of the extracellular matrix, matrix degrading enzymes, inhibitors, growth factors, and inflammatory cytokines; (2) determine the effect of injurious compression on gene expression in co-cultured cartilage; (3) measure specific cleavage of aggrecan in each of the injury models and compare this to treatment with exogenous IL-1.

3.2. MATERIALS AND METHODS

Tissue Harvest and Culture. Articular cartilage explant disks were harvested from the femoropatellar grooves of 1-2 week old calves using previously developed methods (Sah, 1989). In brief, 9 mm diameter cartilage-bone cylinders were drilled perpendicular to the cartilage surface. These cylinders were then placed in a microtome holder and the most superficial ~200 μm layer was removed to obtain a level surface. Up to three sequential 1 mm slices were cut from each cylinder, and 4 disks (1 mm thick, 3 mm diameter) were cored from each slice using a dermal punch, giving 48 disks in total from each joint. Joint capsule tissue was cut from the medial side in the joint immediately proximal to the articular cartilage. Full thickness samples consist of fibrous tissue with a single layer of synovial tissue on the cartilage facing side. Joint capsule tissue was cut into 36 pieces ~5 mm square using a razor. Cartilage disks and joint capsule samples were then equilibrated separately in culture medium for 2 days (low glucose DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a 37°C, 5% CO_2 environment. Serum was replaced with 1% ITS for samples used in Western Analysis.

Injurious Compression, Co-culture, and Exogenous IL-1α Treatment. Injurious compression was performed using a custom-designed incubator-housed loading apparatus (Frank, 2000) to injuriously compress cartilage disks as described previously (Kurz, 2001; Patwari, 2003; DiMicco, 2004). The injury protocol consisted of a single unconfined compression displacement ramp to a final strain of 50% at a velocity of 1 mm/s (strain rate 1.0/s in displacement control), followed by immediate removal of the displacement at the same rate. Application of this strain resulted in an average peak stress of ~20 MPa and has been shown previously to result in cell death and tissue matrix damage (Quinn, 1998; Loening, 2000; Kurz, 2001; Patwari, 2003; DiMicco, 2004) when applied to similar bovine cartilage explants. For co-

culture samples, cartilage tissue was cultured in the same well of a tissue culture plate with a piece of joint capsule tissue for the duration of the experiment. The two pieces of tissue were not in contact during culture. Where indicated, cartilage was treated with recombinant human IL-1α (R&D Systems, MN) at 10 ng/ml. IL-1α was added at the time of medium change every two days throughout the duration of the culture. Cartilage maintained in free swelling culture without joint capsule tissue or exogenous cytokine served as controls for each experiment.

For gene expression analysis, groups of 6 cartilage disks from the experimental treatment group were removed from culture at 1, 2, 4, 6, 12, and 24 hours, flash frozen in liquid nitrogen, and stored at -80°C. Groups of six free swelling uninjured disks were frozen at 4 and 24 hours to serve as controls.

For Western analysis, conditioned medium was collected 2, 4, 6, and 8 days after initiation of treatment (either injurious compression, co-culture, both compression and co-culture, or IL-1α treatment) at the time of medium change and stored at -20°C. Medium was collected from free swelling cartilage concurrently to serve as control.

For each experiment, explant disks in each treatment or control group were purposely matched across depth and location along the joint surface to prevent bias based on location; as a result, each experimental condition represents an average of specimens within the femoropatellar groove.

RNA Extraction. RNA was extracted from the 6 pooled cartilage disks by first pulverizing the tissue and then homogenizing in Trizol reagent (Invitrogen, CA) to lyse the cells. Extracts were then transferred to Phase Gel Tubes (Eppendorf AG, Germany) with 10% v/v chloroform and spun at 13,000g for 10 min. The clear liquid was removed from above the phase gel and RNA was isolated from the sample using the RNeasy Mini Kit (Qiagen, CA). Genomic DNA was removed by a DNase digestion step (Qiagen, CA) during purification. Absorbance measurements were read at 260 nm and 280 nm to determine the concentration of RNA extracted from the tissue and the purity of the extract. The average 260/280 ratio of absorbencies was

1.91±0.10. Reverse transcription (RT) of equal quantities of RNA (25 ng per μl RT volume) from each sample was performed using the Amplitaq-Gold RT kit (Applied Biosystems, CA).

Real-time PCR. Real-time PCR was performed using the MJ Research Opticon 2 instrument and SYBR Green Master Mix (Applied Biosystems, CA). Primers were designed for matrix molecules (collagen II, aggrecan, link protein, fibronectin, fibromodulin, and collagen I), proteases (MMP-1, MMP-3, MMP-9, MMP-13, ADAMTS-4, ADAMTS-5), protease inhibitors (TIMP-1, TIMP-2), cytokines (TNF-α, IL-1β), housekeeping (β-actin, GAPDH), transcription factors (c-fos, c-jun), and growth factor (TGF-β) using Primer Express software (Applied Biosystems, CA). Standard curves for amplification using these primers were generated; all primers demonstrated approximately equal efficiency, with standard curve slopes ~1 indicating a doubling in cDNA quantity each cycle.

Western Analysis for Aggrecan. After addition of protease inhibitors (5mM EDTA, 0.1mM PMSF, and 5mM NEM), culture medium was ethanol precipitated (3 volumes ethanol with 5mM sodium acetate) overnight at -20°C. Samples were then spun at 13000g for 30min at 4°C. Supernatant was removed and pellet was dried in a Speedvac. The pellet was then dissolved in digestion buffer (50mM sodium acetate, 50mM Tris, 10mM EDTA, pH 7.6) and treated with protease-free chondroitinase-ABC, keratanase II, and endo-β-galactosidase to remove GAG chains from the core protein (Seikagaku, Japan). After digestion, samples were dried in Speedvac and resuspended in Tris-Gly SDS 2x sample buffer (BioRad, CA) with reducing agent (DTT). Samples were boiled and then run on 4-15% gradient Tris-HCl gels (BioRad, CA) and transferred to nitrocellulose membrane at 4°C. Membranes were then blocked and incubated with primary antibody (one of anti-G1, anti-G3, or anti-NITEGE diluted 1:2000 in 5% non-fat milk) overnight. Anti-mouse or anti-goat (depending on primary used) secondary antibody incubation for 1 hour was followed by development using Western Lightning kit (PerkinElmer, MA). Membranes were imaged for chemiluminescence with Fluorochem (Alpha Innotech, CA).

Statistical Analyses. In each gene expression experiment, expression levels measured in treated sample groups (co-culture with joint capsule or injurious compression followed by co-culture) were normalized to those of free swelling control groups for each gene; expression data are presented as the average of three replicate experiments (± SE). Changes in gene expression levels in the experimental treatment samples with respect to free swelling controls at the 4 hour and 24 hour time points were examined using a non-parametric t-test (Troyanskaya, 2002). The t-test was made non-parametric by estimating the p-values from permuted data sets (Good, 2000); the t-statistic was calculated from each of the permuted data sets to create a distribution of possible values. Using this method, all changes in expression that were 4-fold or greater were found to be statistically significant with one exception (ADAMTS-5 at 24 hours in injurious compression followed by co-culture model) with several lower magnitude changes also found to be significant.

Gene Clustering. To distinguish the main expression trends, a k-means clustering algorithm (Eisen, 1998; Jain, 1999; Dougherty, 2002; Fitzgerald, 2004) was applied to the combined data set of time course data for expression following injurious compression, co-culture with joint capsule, and injurious compression followed by co-culture. Each gene was grouped based on the correlation of the time course expression profile in the three injury models to a set of randomly chosen starting genes. Group profiles were then calculated as the average of the expression profiles of the genes in each group. The correlation between each gene and group profile was calculated and the genes regrouped in an iterative fashion until the groupings settled. To ensure that an optimal clustering solution for the twenty-one genes was found, the algorithm was run sufficient times to cover every possible selection of starting genes. Each set of randomly chosen starting genes produced a deterministic grouping of the genes, with each gene paired with the highest correlating group profile. The optimal solution was chosen as the grouping that had the highest overall correlation of genes to group profiles, by averaging over all the genes (see

(Fitzgerald, 2004) for details). The number of groups was varied from three to six and five groups were chosen to best represent the trends.

3.3. RESULTS

Co-culture of cartilage with full thickness joint capsule tissue results in specific changes in gene expression levels of matrix molecules, catabolic enzymes, and cytokines in the chondrocytes. Changes in expression during co-culture differ in uninjured cartilage tissue and mechanically injured cartilage.

Typical normalization genes are affected by injury models. Expression of GAPDH and β-actin responded to co-culture with joint capsule tissue with increases in expression of 12-fold and 2-fold respectively (Figure 3.1A). With additional mechanical injury to the cartilage, increases in expression during co-culture with joint capsule tissue were 7-fold and 4-fold respectively (Figure 3.1B). Because these molecules responded to co-culture with or without injurious compression, we did not normalize the expression data of other genes tested to the expression of either GAPDH or β-actin. Instead, all data was normalized only to the total quantity of RNA in each sample as described in 3.2. MATERIALS AND METHODS.

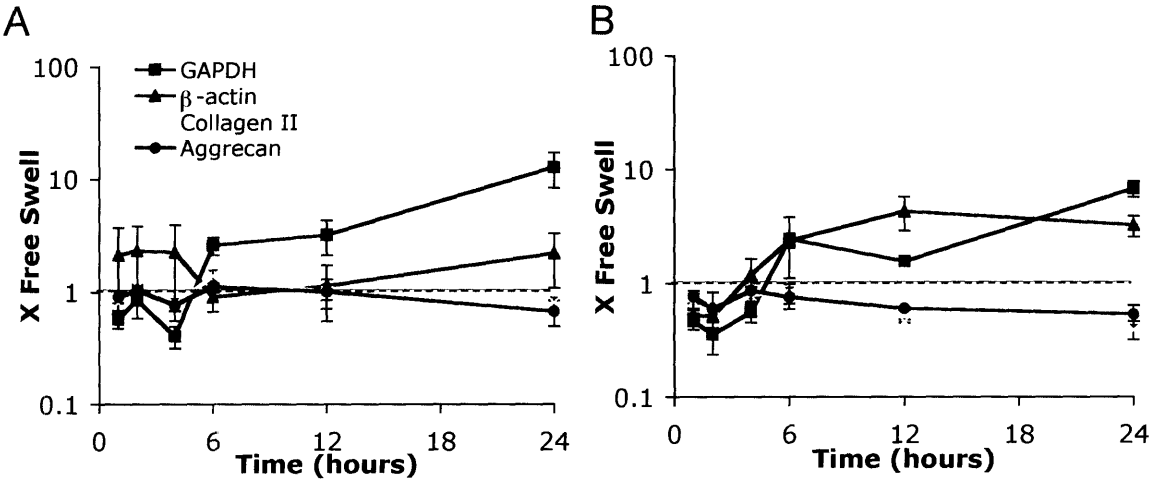

Figure 3.1: Changes in expression level of matrix molecules, GAPDH, and β-actin during co-culture of uninjured and injured cartilage with joint capsule tissue. Values on the y-axis represent fold change from free swelling control levels with a value of 1 indicating similar expression during co-culture of (A) uninjured cartilage or (B) mechanically injured cartilage to the level measured in free swelling, non-co-cultured conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

Gene expression of extracellular matrix molecules. Matrix molecules, collagen II and aggrecan, remained between 65-140% control levels during 24 hours of co-culture with joint capsule tissue (Figure 3.1A). When cartilage was first subjected to mechanical injury and then co-cultured with joint capsule, collagen II and aggrecan decreased expression to 40% and 53% of control levels respectively by 24 hours (Figure 3.1B). Additionally, fibromodulin responded to co-culture and mechanical injury with a decrease in expression at all time points to 30-60% control levels while co-culture alone did not significantly affect expression (data not shown). Link protein increased expression to 130% during the first 4 hours of co-culture, followed by decrease to 50% by 24 hours; injury followed by co-culture resulted in a non-significant decreased expression to 70% controls at all time points (data not shown). At all time points during co-culture of injured or uninjured cartilage, fibronectin was expressed between 60% and 115% of expression levels in free-swelling controls (data not shown).

Gene expression of catabolic enzymes and inhibitors. In contrast to the either stable or decreased expression seen for components of the extracellular matrix, enzymes able to cleave either collagen II or aggrecan showed selective increases in expression during co-culture with joint capsule tissue. Stromelysin (MMP-3) showed the largest increase in expression of 16-fold during 24 hours of co-culture (Figure 3.2A) and a peak 140-fold increase 12 hours after mechanical injury followed by co-culture with joint capsule (Figure 3.2B). Aggrecanase-2 (ADAMTS-5) increased expression 6-fold over control during co-culture with joint capsule tissue (Figure 3.2A); mechanical injury of the cartilage followed by co-culture resulted in a peak in expression 85-fold over control at 12 hours (Figure 3.2B). Aggrecanse-1 (ADAMTS-4) increased to a peak of 6-fold and 10-fold by 6 hours of co-culture in uninjured and injured cartilage respectively, returning to baseline by 24 hours in both cases (Figure 3.2). Collagenase-3 (MMP-13) increased to 7-fold above control levels by 6 hours of co-culture, decreasing to 4-fold over control by 24 hours of co-culture with joint capsule tissue (Figure 3.2A); no response was seen when cartilage was first mechanically injured (Figure 3.2B). Collagenase (MMP-1) did not

change expression level during co-culture with joint capsule in either injured or uninjured cartilage (Figure 3.2). Gelatinase B (MMP-9) decreased to 30% control level in injured cartilage co-cultured with joint capsule tissue but showed no response to co-culture alone (data not shown). Tissue inhibitors of metalloproteinase (TIMP) are able to bind to and inactivate MMPs. TIMP-1 did not respond to co-culture with joint capsule tissue with a change of expression in either uninjured or injured cartilage (data not shown). TIMP-2 increased expression ~2-fold from 4 hours to 24 hours after co-culture in both uninjured or injured cartilage (data not shown).

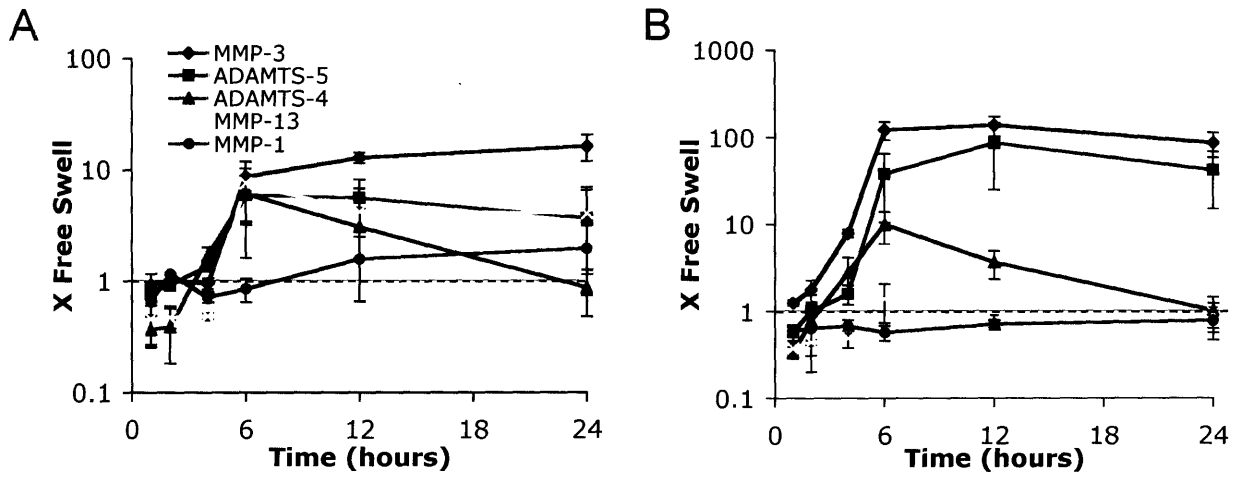

Figure 3.2: Changes in expression level of matrix proteases during co-culture of uninjured and injured cartilage with joint capsule tissue. Values on the y-axis represent fold change from free swelling control levels with a value of 1 indicating similar expression during co-culture of (A) uninjured or (B) injured cartilage to the level measured in free swelling, non-co-cultured conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

Gene expression of cytokines, growth factors, and transcription factors. Inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), were differentially regulated compared to transforming growth factor β (TGF-β), during co-culture of uninjured or mechanically injured cartilage with joint capsule tissue. IL-1β increased gradually during co-culture with joint capsule tissue from 50% to 2-fold above the control level between 1 hour and 24 hours (Figure 3.3A); mechanical injury followed by co-culture with joint capsule

caused an immediate decrease in expression to 15% control expression which returned to baseline by 6 hours (Figure 3.3B). TNF-α did not change expression level significantly in either model at any of the time points measured (Figure 3.3). TGF-β did not respond to co-culture alone (expressed between 60-150% control at all time points) but showed an increase in expression of 5-fold between 4 hours and 12 hours after mechanical injury followed by co-culture with joint capsule tissue, decreasing to 2-fold above controls by 24 hours (Figure 3.3B). Expression levels of transcription factors, c-fos and c-jun, were increased 2-5-fold during 24 hours of co-culture with joint capsule tissue of uninjured cartilage (Figure 3.4A). One hour after mechanical injury and co-culture with joint capsule, expression levels of c-fos and c-jun were increased to 110-fold and 40-fold of controls respectively decreasing to 1.5-5.5-fold over controls by 12 and 24 hours of culture (Figure 3.4B).

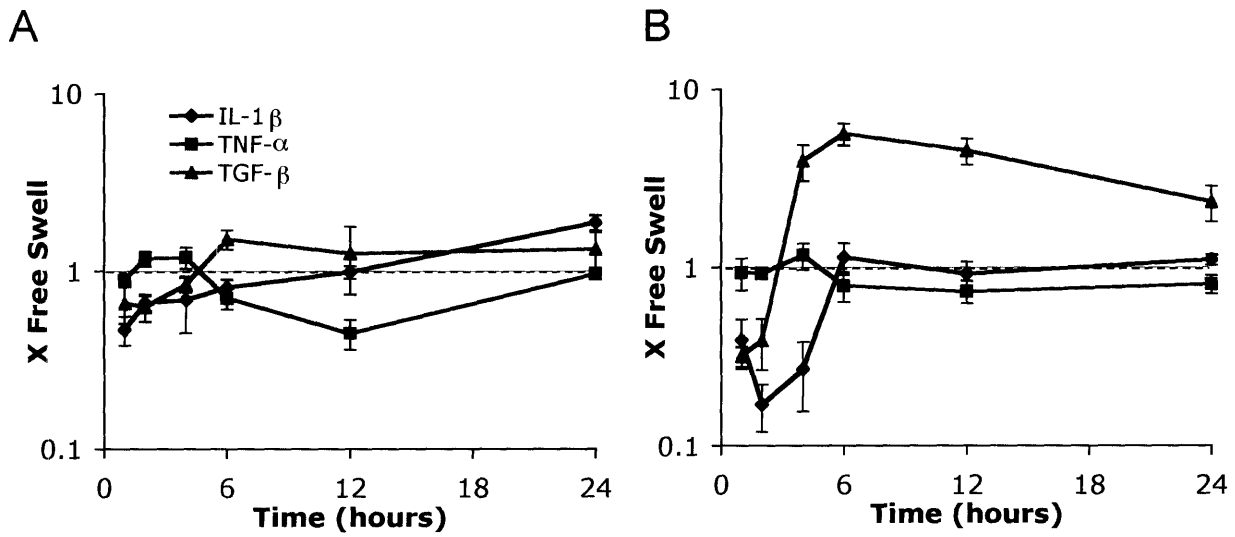

Figure 3.3: Changes in expression level of cytokines and TGF-β during co-culture of uninjured and injured cartilage with joint capsule tissue. Values on the y-axis represent fold change from free swelling control levels with a value of 1 indicating similar expression during co-culture of (A) uninjured or (B) injured cartilage to the level measured in free swelling, non-co-cultured conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

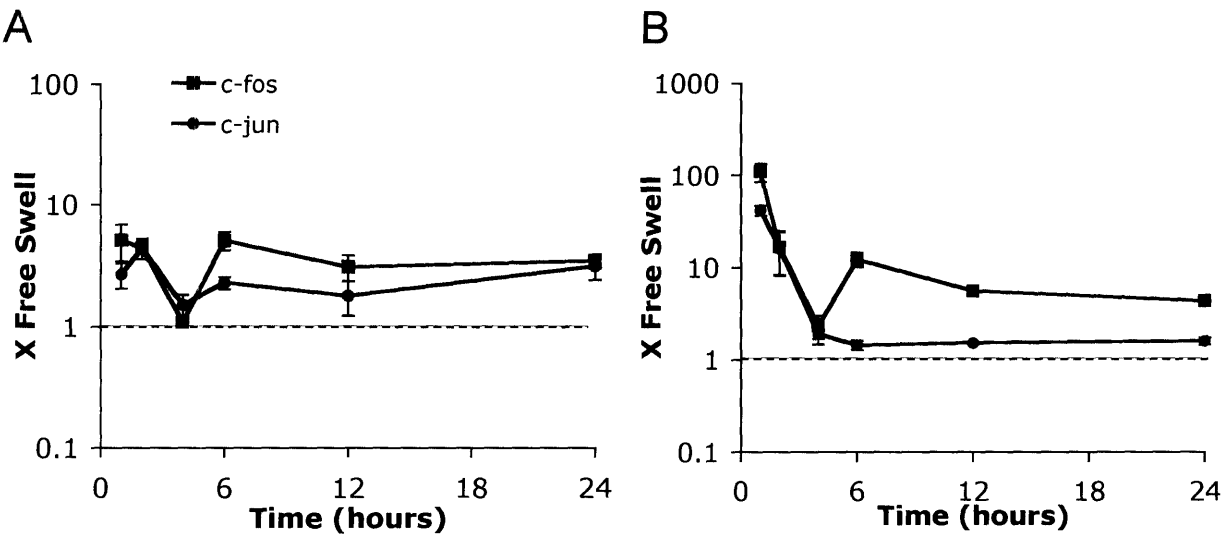

Figure 3.4: Changes in expression level of transcription factors during co-culture of uninjured and injured cartilage with joint capsule tissue. Values on the y-axis represent fold change from free swelling control levels with a value of 1 indicating similar expression during co-culture of (A) uninjured or (B) injured cartilage to the level measured in free swelling, non-co-cultured conditions. Six cartilage disks were pooled for each time point for each experiment. All samples were normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

Aggrecan fragment analysis. To determine if the increase in expression level measured by real-time PCR of enzymes able to cleave cartilage matrix molecule aggrecan resulted in cleavage and release of this matrix component to the culture medium, conditioned medium from cultures was analyzed by Western blotting. Conditioned medium from either uninjured or injured cartilage cultures in the presence or absence of joint capsule tissue as well as samples containing joint capsule tissue alone (no cartilage) were immunoblotted with antibodies to globular domains of aggrecan (anti-G1 and anti-G3) and the N-terminal neoepitope resulting from cleavage by aggrecanase at amino acid 373 (anti-NITEGE). Medium samples collected on day 4 stained positively for anti-NITEGE in all conditions that included joint capsule tissue in the culture (Figure 3.5, lanes 4-8) as well as for cartilage treated with exogenous IL-1α. Similar results were seen with anti-G1 and anti-G3 antibodies (data not shown). Medium collected on days 2, 6, and 8 showed similar results to that seen on day 4 for all anti-bodies (data not shown). Importantly, because culture medium from joint capsule tissue alone stained positively for aggrecan cleavage

products, it is not possible from this analysis to determine if co-culture resulted in increased damage to the cartilage extracellular matrix through cleavage at this aggrecanase site.

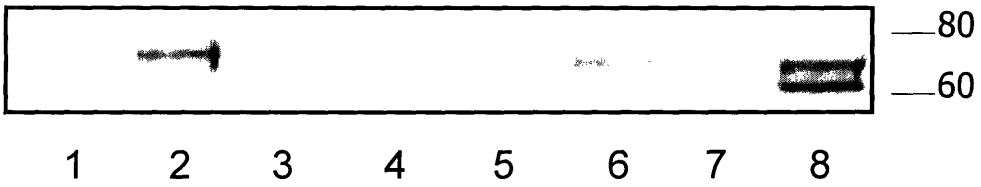

Figure 3.5: Western blot for aggrecanase fragments in *in vitro* models of joint injury. Anti-NITEGE antibody blot of conditioned medium from free-swell control (lane 1), IL-1α treated cartilage (lane 2), mechanically injured cartilage (lane 3), mechanically injured cartilage co-cultured with joint capsule tissue (lane 4), uninjured cartilage co-cultured with live (lane 5) or dead (lane 6) joint capsule tissue, live (lane 7) or dead (lane 8) joint capsule tissue cultured alone. Dead joint capsule tissue was subjected to three cycles of freeze-thaw.

3.4. DISCUSSION

Co-culture of cartilage tissue explants with joint capsule or synovial tissue has been shown previously to result in decreased chondrocyte biosynthesis rates and loss of collagen II and aggrecan from the tissue extracellular matrix (Fell and Jubb, 1977; Jubb and Fell, 1980; Patwari, 2001). We undertook this study to determine if co-culture with joint capsule tissue results in changes in gene expression levels in chondrocytes. Real-time PCR analysis was used to measure expression of 21 molecules involved in both cartilage matrix production and degradation at six time points from one hour to 24 hours during co-culture with joint capsule tissue. Gene expression levels were also measured in cartilage first subjected to injurious mechanical compression and then co-cultured with joint capsule tissue to determine if gene expression changes in this combinatorial injury model differ from those measured following injurious compression of cartilage or during co-culture of uncompressed cartilage with joint capsule tissue. We observed significant changes in expression of several molecules in response to each of the different injury models of several molecules. Clustering analysis was performed to summarize the expression levels of genes in the three models of cartilage and joint injury.

Gene expression group behavior. Gene expression profiles of expression levels in the 24 hours following mechanical injurious compression, co-culture with joint capsule tissue, or the combination of injurious compression followed by co-culture with joint capsule tissue were clustered to group genes based on their response to all three of these injury models (Figure 3.6, Table 3.1). Clustering the three data sets together allowed groups to be formed which show similar expression level responses to the different *in vitro* models of injury. The behavior of the individual groups showed levels of response with varied kinetics and magnitudes to each of the three joint injury models.

Group 1 (Figure 3.6A, Table 3.1) contained matrix molecules aggrecan, collagen II, and fibromodulin and showed expression levels within 70-120% of control following either injurious

compression or co-culture with joint capsule tissue. In contrast to what was seen with either stimulus alone, genes in Group 1 decreased within 1 hour to 70% control and further decreased to 50% control at 12 and 24 hours following the combination of injurious compression and co-culture with joint capsule tissue. In contrast, Group 2 (Figure 3.6B, Table 3.1) contained genes with a variety of functions in chondrocytes (collagen I, link protein, fibronectin, MMP-1, MMP-9, TIMP-1, TIMP-2. IL-1β, TNF-α). On average, the expression levels of the genes in Group 2 increased 3.5-fold in response to injurious compression but remained between 70-120% of control levels during co-culture with joint capsule in either uninjured or mechanically injured cartilage.

Group 3 (Figure 3.6C, Table 3.1) which contained matrix proteases (MMP-13 and ADAMTS-4) and GAPDH (a molecule typically used as a control in gene expression studies) showed a general increase in expression in response to injurious compression between 1 hour and 24 hours after loading from 1.5-fold to 3-fold over free swell control. Co-culture of either uninjured or mechanically injured cartilage resulted in an immediate decrease in expression of the genes in Group 3 at 1 hour and 2 hours to 40-50% control levels followed by increased expression of 6-fold and 5-fold above controls of uninjured and injured cartilage respectively. Gene members of Group 3 showed a larger magnitude response to co-culture with joint capsule compared to the response seen for injurious compression without co-culture. This may suggest higher sensitivity of these molecules to factors released from joint capsule tissue compared to the response to mechanical injurious compression.

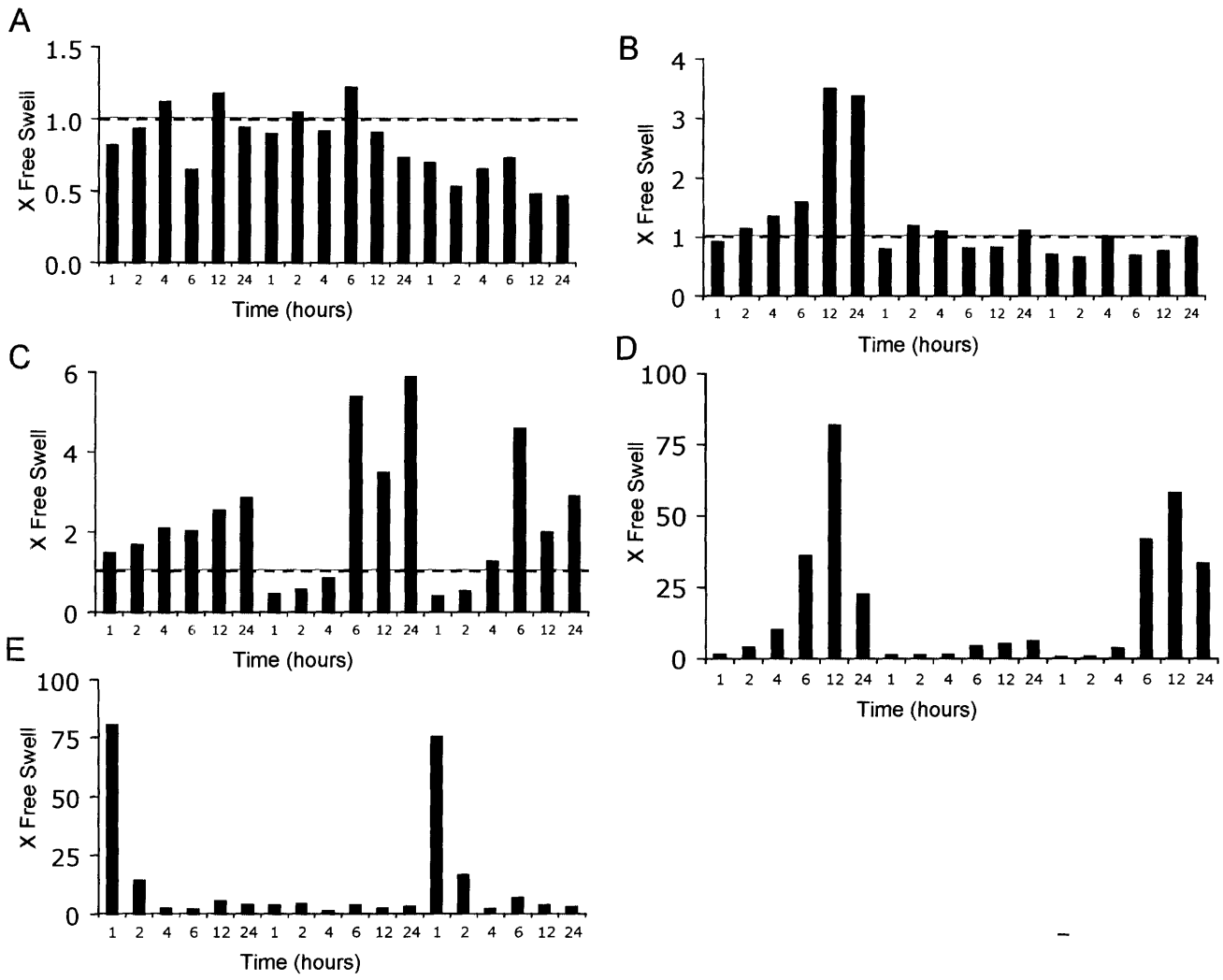

Figure 3.6: Group expression profiles generated by k-means clustering. The main temporal gene expression patterns in cartilage explants induced by injurious compression, co-culture with joint capsule tissue, and injurious compression followed by co-culture with joint capsule tissue. The gene expression data set for clustering consisted of expression levels at six time points each after mechanical injury (dark blue), during co-culture with joint capsule (light blue) and after mechanical injury followed by co-culture with joint capsule tissue (red). Expression data was clustered into five groups. A) Group 1, B) Group 2, C) Group 3, D) Group 4, and E) Group 5 with members listed in Table 3.1. Group profiles were calculated by averaging the expression profiles of genes within each group.

Table 3.1: List of group members generated by k-means clustering with p-values comparing expression in co-cultured and control cartilage. Gene expression profiles in cartilage following injurious compression and co-culture with joint capsule tissue of uninjured and injured cartilage were iteratively clustered into five groups using k-means clustering. Groups were formed based on gene to group profile correlation. To compare control and co-cultured cartilage, p-values were calculated from non-parametric t-tests performed after 4 hours and 24 hours of co-culture of uninjured or injured cartilage with joint capsule tissue compared to control expression levels; bold represents p-value <0.05, * represents p-value <0.01.

	p-values				
	Co-culture		Co-culture + Injury		
Gene	4 hour	24 hour	4 hour	24 hour	Group
Aggrecan	**0.03**	0.06	0.56	**0.02**	1
Collagen II	**0.03**	**0.03**	0.16	**0.01**	1
Fibromodulin	0.90	0.15	*	*	1
Collagen I	**0.04**	*	0.50	**0.04**	2
Fibronectin	0.32	0.95	0.30	0.74	2
IL-1β	0.28	**0.01**	**0.01**	0.20	2
Link Protein	**0.01**	**0.01**	0.75	0.36	2
MMP-1	**0.02**	0.58	**0.03**	0.52	2
MMP-9	**0.02**	0.65	0.43	*	2
TIMP-1	0.99	0.68	0.23	0.12	2
TIMP-2	**0.03**	**0.04**	**0.03**	**0.03**	2
TNF-α	0.33	0.70	0.45	0.09	2
ADAMTS-4	0.06	0.87	0.28	0.99	3
GAPDH	**0.01**	**0.02**	**0.02**	**0.02**	3
MMP-13	*	0.39	**0.01**	0.89	3
ADAMTS-5	0.10	0.06	0.18	0.15	4
β-actin	0.50	0.38	0.75	**0.04**	4
MMP-3	0.77	**0.01**	*	**0.05**	4
TGF-β	**0.04**	0.39	**0.02**	**0.05**	4
c-fos	0.29	*	0.08	**0.01**	5
c-jun	0.17	**0.02**	0.09	**0.03**	5

Molecules in Group 4 showed increases in expression in response to all three of the injury models (Figure 3.6D, Table 3.1). The four genes in Group 4 varied from lower magnitude responses (β-actin and TGF-β: 4-8-fold increase following injurious compression with or without co-culture, ~2-fold increase during co-culture with joint capsule tissue) to larger magnitude responses (ADAMTS-5 and MMP-3: 40-250-fold increase following injurious compression with or without co-culture, 6-16-fold during co-culture with joint capsule tissue). In contrast to Group 3, which showed larger increases in the two models including co-culture with

joint capsule, Group 4 showed increases with larger magnitude in the two models including mechanical injurious compression with all members showing a peak in expression 12 hours after injurious compression. As a group, these molecules were more sensitive to injurious compression than co-culture with joint capsule tissue. The matrix proteases, ADAMTS-5 and MMP-3, showed large increases in expression and may result in degradation of the tissue matrix in these *in vitro* injury models. Increased expression of the growth factor TGF-β may be indicative of an effort to repair tissue damage in response to mechanical injury by increasing biosynthesis of matrix molecules. Alternatively, increased expression of TGF-β may represent an additional activator of degradative processes as this molecule has been seen previously to activate aggrecanases (Moulharat, 2004) and collagenase-3 (Moldovan, 1997) in human chondrocytes and cartilage explants and lead to synovial hyperplasia and proteoglycan loss from cartilage following intra-articular injection (Elford, 1992).

Transcription factors c-fos and c-jun (Group 5; Figure 3.6E, Table 3.1) showed an immediate transient upregulation followed by rapid decrease within 4 hours in response to injurious compression both alone and followed by co-culture with joint capsule tissue. In uninjured cartilage co-cultured with joint capsule tissue, c-fos and c-jun showed a moderate (2-4-fold) upregulation at all time points. These transcription factors are members of the activating protein-1 (AP-1) family of genes and have been shown previously to activate MMPs in a chondrocyte cell line following IL-1β treatment (Vincenti and Brinckerhoff, 2002).

Medium analysis in co-culture systems. Aggrecan protein fragments were released from full-thickness joint capsule tissue cultured in the absence of cartilage. Aggrecan production by joint capsule tissue has been previously reported as a result of cartilaginous differentiation of the joint capsule which occurs when the hip is subjected to increased mechanical loading during dislocated hip arthropathy (Yutani, 1999). To determine if cartilage matrix is degraded in an *in vitro* co-culture model system, it is therefore necessary to look more specifically at the protein content of the cartilage tissue itself rather than more generally in the tissue culture medium

where proteins will be present from all tissues included in the co-culture system. Future studies will address this issue in the mechanical injurious compression and co-culture models of injury used here. This finding additionally highlights the need to carefully consider possible sources of proteins measured in synovial fluid samples obtained from injured or arthritic joints. It must be considered that the proteins present in the synovial fluid may have been released from a large number of tissue sources particularly following a joint injury which disrupts the normal integrity of one or more of the following: ligaments, tendons, meniscus, articular cartilage surface, or synovial lining.

Comparison to *in vitro* co-culture studies. Early studies showed breakdown of the extracellular matrix of cartilage when co-cultured with minced synovial tissue. Co-culture resulted in near complete depletion of collagen II and proteoglycan from the cartilage by 14 days (Fell and Jubb, 1977). Matrix damage was most severe for live cartilage in contact with synovium with no effect seen of dead synovium. Damage was less severe, though above that seen in isolated cartilage, when cartilage was at a distance from living synovium. These results suggest an active role of the cartilage in the co-culture model (larger effect seen for live cartilage compared to dead cartilage) but do not identify specific catabolic factors that may be responsible for the observed matrix degradation. Here, we measured increased expression of specific matrix proteases in the chondrocytes activated by co-culture with joint capsule tissue (MMP-3, MMP-13, ADAMTS-4, ADAMTS-5) as well as an additional factor activated by co-culture following mechanical injury (TGF-β). These factors may play a role in degrading both collagen and proteoglycan components of the extracellular matrix in these models.

In addition to matrix degradation, co-culture with synovium also results in decreased biosynthesis rate, measured by $^{35}SO_4$ incorporation, in cartilage when cultured in contact or at a distance from synovium for 8 days. Tissue was shown to recover biosynthesis rate over 4 days cultured in isolation following 8 days of co-culture (Jubb and Fell, 1980). Mechanical injurious compression followed by 6 days of co-culture caused further decrease in incorporation rates of

$^{35}SO_4$ and ^{3}H-proline compared to 6 days of co-culture without initial compression (Patwari, 2001). Within the first 24 hours of co-culture with joint capsule tissue, we did not measure a significant decrease in expression levels of collagen II or aggrecan. It remains possible that expression of these molecules is reduced during longer incubation periods. Consistent with a more substantial reduction in biosynthesis rates during co-culture with joint capsule that followed injurious compression (Patwari, 2001), expression of collagen II and aggrecan were reduced to ~50% control levels within 24 hours in this joint injury model. Injurious compression of cartilage alone also did not decrease expression of matrix molecules within 24 hours (described in detail in CHAPTER 2).

Degradative capacity of synovium. Previous studies report the presence of matrix proteases able to cleave components of cartilage matrix in synovium tissue as well as by cells derived from synovium of normal and arthritic joints. Factors that regulate cartilage maintenance including IL-1, TNF-α, and TGF-β additionally regulate expression of proteases in synovium though differences are seen when compared to the effects on cartilage and chondrocytes.

Both synovium and joint capsule tissue generate a soluble aggrecan degrading activity when cultured *in vitro* (Vankemmelbeke, 1999; Ilic, 2000). This activity causes cleavage at the aggrecanase site in the interglobular domain of aggrecan. Aggrecanase activity is blocked by EDTA but not by inhibitors of IL-1β, TNF-α, or by ACITIC (inhibits trypsin-like serine proteinases and is able to block cytokine-mediated cartilage breakdown (Bryson, 1998)) (Vankemmelbeke, 1999). Higher aggrecanase cleavage potency was measured in medium from co-culture of synovium with either live or dead cartilage than that seen from synovium alone (Vankemmelbeke, 1999). This suggests that in addition to aggrecanase activity released directly from synovium, there is additional release of a factor(s) retained in the cartilage matrix that causes increased aggrecan cleavage.

Joint capsule fibroblasts express aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) at the mRNA level (Ilic, 2000). Aggrecanase-2 is present at the protein level in

human arthritic and bovine normal synovium (Vankemmelbeke, 2001; Yamanishi, 2002). Within the synovium, aggrecanase-2 is concentrated in the synovial lining and around blood vessels (mostly pericellular, some matrix staining observed) (Vankemmelbeke, 2001). In synovium from arthritic joints, aggrecanase-2 is predominantly present in a 53 kDa form compared to normal synovium in which it is found predominantly in a 70 kDa form (Yamanishi, 2002). Aggrecanase-2 gene expression and activity are not regulated by IL-1α, all-*trans*-retinoate, TNF-α, or TGF-β (Vankemmelbeke, 2001; Yamanishi, 2002). Two of the same factors, IL-1α and retinoic acid, increase activity of aggrecanase-2 in cartilage without a concomitant change at the gene expression level (Flannery, 1999). In contrast to aggrecanase-2, TGF-β (but not IL-1 or TNF-α) increases mRNA and protein expression of aggrecanase-1 in fibroblast-like synoviocytes (FLS) (Yamanishi, 2002). Similarly, in cartilage from normal and OA joints, TGF-β also causes an increase in expression of aggrecanase-1, but not aggrecanase-2 (Moulharat, 2004).

A recent study showed the ability of immune cell microparticles to regulate gene expression in synovial fibroblasts. Microparticles produced by either activated or apoptotic T cells and monocytes were shown to increase mRNA expression of MMP-1 (10-70-fold), MMP-3 (8-80-fold), MMP-9 (4-25-fold), MMP-13 (3-30-fold) but not MMP-2, MMP-14, TIMP-1, TIMP-2, or TIMP-3 in human synovial fibroblasts from normal (obtained at trauma), osteoarthritic, or rheumatoid arthritic joints (Distler, 2005). MMP activity as well as inflammatory mediators IL-6 (4-25-fold), IL-8 (8-35-fold), and monocyte chemoattractant protein (MCP)-1 (2-6-fold) and MCP-2 (3-25-fold) were also increased after treatment of synovial fibroblasts with immune cell microparticles (Distler, 2005). Treatment with a TNF-α antibody did not reduce MMP induction in synovial fibroblasts caused by microparticles. IL-1 receptor antagonist (IL-1Ra) reduced MMP and inflammatory cytokine production by ~30%; similar reduction in MMP and cytokines was seen in IL-1Ra treated fibroblasts not exposed to immune cell microparticles suggesting this decrease is due to blockade of endogenous IL-1

produced by the synovial fibroblasts rather than a blockade of the effect of the microparticles (Distler, 2005).

Taken together, these studies outline the ability of a number of factors produced by synovial cells to act as degradative factors in the joint environment. Further study is required to determine mechanisms involved in regulation and downstream effects of these factors following joint injury that may lead to the progression of OA.

Comparison to *in vivo* injury models. Anterior cruciate ligament transection (ACLT) results in joint degeneration similar to that seen during the development of osteoarthritis including full-thickness loss of articular cartilage, osteophyte formation, cartilage surface fibrillation, chondrocyte cloning, hyperplasia of synovial lining, mononuclear cell infiltration, joint capsule fibrosis, duplication of the tide mark, and capillary invasion of the calcification front (Brandt, 1991). In addition to macroscopic changes in the joint indicative of OA, ACLT results in specific changes in gene expression levels of several molecules in both cartilage and synovium. Collagenase-3 (MMP-13) is increased ~15-30-fold above control 3 weeks after ACLT decreasing to ~7-16-fold above controls by 8 weeks post-ACLT. Other matrix proteases, collagenase (MMP-1) and stromelysin (MMP-3), were either not changed or increased ~2-3-fold in cartilage from different sites within the joint following ACLT (Le Graverand, 2002). In another study, MMP-3 expression was increased 9 weeks after ACLT in both the cartilage and synovium; IL-1β was increased in synovium after ACLT and not detected in cartilage (Takahashi, 1999). Though the experimental models are quite different, it is interesting to note that we also measured increased expression in chondrocytes of both MMP-3 and MMP-13 but not IL-1β during co-culture of cartilage with joint capsule tissue. Together, these studies suggest that IL-1 present in joint fluid following injury and during OA progression is not likely produced by the cartilage but is rather the product of a different tissue in the joint, possibly synovium. MMP expression by chondrocytes is increased in both *in vivo* and *in vitro* models of injury.

Three to eight weeks following ACLT, matrix molecules collagen II and aggrecan increase expression ~2-4-fold in cartilage while decorin and fibromodulin expression are decreased (Le Graverand, 2002). We measured no change or decreased expression of these same matrix molecules within 24 hours of co-culture of either uninjured or mechanically injured cartilage with joint capsule tissue.

In our *in vitro* models of joint injury, the magnitude of the change in expression measured for aggrecanases, ADAMTS-4 and ADAMTS-5, were quite different with large increases measured for ADAMTS-5 expression compared to that measured for ADAMTS-4. Interestingly, ADAMTS-5 was recently identified as the primary aggrecanase responsible for cartilage degradation in murine models of OA. A joint instability model showed reduction in pathology, proteoglycan release, and cleavage at the aggrecanase site in the interglobular domain (IGD) in ADAMTS-5 deficient mice with no protection seen for ADAMTS-4 deficiency (Glasson, 2005). Similarly, an inflammatory model of OA showed decreased proteoglycan loss and increased matrix staining for proteoglycan in ADAMTS-5 deficient mice (Stanton, 2005). These studies suggest that ADAMTS-5 is primarily responsible for proteoglycan breakdown seen during OA progression with ADAMTS-4 playing at most a minor role.

Comparison to clinical injury. Long term clinical studies have noted an increased risk for the development of osteoarthritis following ACL tear (Davis, 1989; Roos, 1995; Gelber, 2000; Lohmander, 2004; von Porat, 2004). The risk for disease is not affected by surgical repair of the ligament (Lohmander, 2004; von Porat, 2004). In addition, following ACL tear, collagen II denaturation/degradation occurs in the low weight bearing intercondylar notch similar to the level of degradation seen in late stage OA (Price, 1999). This suggests collagen II degradation is an early event following ACL injury and is unlikely to be a direct result of altered mechanical loading. Taken together, these findings provide evidence that restoration of normal joint loading is alone insufficient to prevent progressive degradation of cartilage following joint injury. Motivated by these and similar findings, studies have been undertaken to identify changes that

occur in the joint environment following injury that initiate or accelerate matrix degradation and lead to the development of OA. Analysis of synovial fluid after ACL or meniscus tear shows elevated levels of a variety of factors indicative of both breakdown: including degraded aggrecan and collagen II, MMP-3, and collagenase, as well as evidence of repair: TIMP-1 and aggrecan synthesis (Lohmander, 1993a; Lohmander, 1993b; Lohmander, 1994; Lohmander, 1999; Lohmander, 2003). Additionally, both inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-8) and suppressors of inflammation (IL-1Ra and IL-10) are present at increased levels in the first week after joint injury (Irie, 2003). These studies highlight the complexity of events following joint injury and motivate controlled *in vitro* studies to isolate particular factors and determine the effect of each in the degradation of cartilage tissue.

We analyzed gene expression response to two *in vitro* models of joint injury. Our goal was to isolate the effects on cartilage of two factors: damaged joint capsule / synovial lining tissue and mechanical overloading. We also sought to understand the interaction between these two factors by studying the combination model of mechanical injurious compression followed by co-culture with joint capsule tissue. Using real-time PCR, we measured changes in expression of extracellular matrix components and matrix proteases and observed a general trend towards activation of tissue degradation processes in our models. This approach makes possible the observation of differential effects on chondrocytes caused by mechanical loading and joint capsule tissue damage.

Our study is limited by inclusion of only two tissue types (joint capsule and cartilage). It is likely that other tissues and fluids present in the joint have a significant effect on disease progression following joint injury. Another limitation is the short duration of the study with measurements made only in the first 24 hours after initiation of each injury model. We sought to focus first on early time points to measure immediate responses, however, it will be important in future studies to determine the long term trends in each of these models. A third limitation is the young age of the tissue used in this study. It is possible that tissue from mature animals will

behave differently to the injury models and this should be considered when making comparisons to other studies.

One caveat stems from our identification of aggrecan fragments released from joint capsule tissue. It is vital that appropriate outcome measurements be used which correctly attribute proteins to their source in analyses studying multiple tissues. In addition, aggrecan measured in synovial fluid, though certainly indicative of general joint damage, may not be a specific measure of cartilage matrix degradation. Again, the tissue used for this study was from newborn calves and this finding may depend on tissue age and species.

In summary, our study demonstrates specific changes that occur within the chondrocytes during co-culture with joint capsule tissue. These changes are affected by inclusion of mechanical injurious compression of the cartilage before co-culture. It is possible to separate the effects of soluble factors released from joint capsule tissue from effects of mechanical loading using these *in vitro* model systems. Future studies are planned to measure cartilage degradation and protein levels of specific matrix proteases that respond to injury models at the expression level.

3.5. REFERENCES

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CHAPTER 4: CHANGES IN CARTILAGE TISSUE COMPOSITION AND STIFFNESS FOLLOWING *IN VITRO* MODELS OF JOINT INJURY

4.1. INTRODUCTION

Cartilage tissue provides resistance to tension and compression in weight bearing joints through its dense extracellular matrix composed predominantly of type II collagen fibrils and highly negatively charged proteoglycans. The major proteoglycan present in cartilage extracellular matrix is composed of an aggrecan core protein with 80-100 covalently bound glycosaminoglycan (GAG) chains. Cartilage matrix is maintained by the chondrocytes present in the matrix. Chondrocytes respond to mechanical load with changes in the biosynthetic rates of matrix molecules. Low amplitude dynamic compression (Sah, 1989) and simple shear (Jin, 2001) increase biosynthetic rates in cartilage explants while injurious compression (Kurz, 2001) and static compression to 25-50% strain decreases biosynthesis (Ragan, 1999). During the development of osteoarthritis, cartilage matrix is degraded and is no longer functionally able to withstand normal levels of loading within the joint. Changes in the molecular structure of the cartilage matrix, resulting from specific cleavage by matrix degrading enzymes, are likely responsible for the degradation of the tissue and loss of ability to withstand normal mechanical loads.

Following clinical injury of the knee, joint synovial fluid contains markers of cartilage tissue degradation (Lohmander, 1993a; Lohmander, 2003) with specific cleavage of aggrecan occurring at the aggrecanase site in the interglobular domain (IGD) (Glu^{373}-Ala^{374}) (Lohmander, 1993b). Additionally, cleavage of aggrecan at the aggrecanase site in the IGD is the predominant catabolic event in cellular mediated aggrecan breakdown in cartilage explants (Sandy, 1991) and primary bovine chondrocytes (Lark, 1995) following IL-1 treatment. It has been shown that joint injury leads to increased risk for subsequent development of osteoarthritis (Davis, 1989; Roos, 1995; Gelber, 2000; Lohmander, 2004). *In vitro* models of joint injury have been developed to

study some aspects of injury, including mechanical overload and soft tissue tear, on cartilage. Mechanical overload results in matrix damage, decreased biosynthetic activity of the chondrocytes, cell death, and compromised mechanical properties (Quinn, 1998; Chen, 1999; Torzilli, 1999; Loening, 2000; Chen, 2001; D'Lima, 2001; Ewers, 2001; Kurz, 2001; Thibault, 2002; Chen, 2003; Patwari, 2003; DiMicco, 2004; Kurz, 2004). Co-culture of cartilage with synovium or joint capsule tissue cut from the joint also leads to matrix damage and decreased biosynthesis activity (Fell and Jubb, 1977; Jubb and Fell, 1980; Vankemmelbeke, 1999; Patwari, 2001).

Specific aggrecan cleavage products and the enzymes involved in catabolism in *in vitro* models of joint injury have not yet been fully elucidated. We undertook this study to determine if aggrecanase specific cleavage occurs in cartilage tissue explants following mechanical injurious compression and during co-culture with joint capsule tissue. Secondly, we sought to determine if matrix damage during co-culture with joint capsule tissue results in a measurable change in the mechanical properties of cartilage as has been previously measured following mechanical injurious compression (Kurz, 2001). We hypothesize that a change in mechanical properties at the tissue level will result in an altered loading environment for the chondrocytes within the matrix, which will in turn affect synthesis of both matrix molecules and matrix proteases.

4.2. MATERIALS AND METHODS

Tissue Harvest and Culture. Articular cartilage explant disks were harvested from the femoropatellar grooves of 1-2 week old calves using previously developed methods (Sah, 1989). In brief, 9 mm diameter cartilage-bone cylinders were drilled perpendicular to the cartilage surface. These cylinders were then placed in a microtome holder and the most superficial ~ 200 μm layer was removed to obtain a level surface. Up to three sequential 1 mm slices were cut from each cylinder, and 4 disks (1 mm thick, 3 mm diameter) were cored from each slice using a dermal punch, giving 48 disks in total from each joint. Joint capsule tissue was cut from the medial side proximal to the articular cartilage of the condyle. Full thickness samples consist of fibrous tissue with a single layer of synovial tissue on the cartilage facing side. Joint capsule tissue was cut into 36 pieces ~5mm square using a razor. Cartilage disks and joint capsule samples were then equilibrated separately in culture medium for 2 days (low glucose DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a 37°C, 5% CO_2 environment.

Injurious Compression and Co-culture with Joint Capsule Tissue. Injurious compression was performed using a custom-designed incubator-housed loading apparatus (Frank, 2000) to injuriously compress cartilage disks as described previously (Kurz, 2001; Patwari, 2003; DiMicco, 2004). The injury protocol consisted of a single unconfined compression displacement ramp to a final strain of 50% at a velocity of 1 mm/s (strain rate 1.0/s in displacement control), followed by immediate removal of the displacement at the same rate. Application of this strain resulted in an average peak stress of ~20 MPa and has been shown previously to result in cell death and tissue matrix damage (Quinn, 1998; Loening, 2000; Kurz, 2001; Patwari, 2003; DiMicco, 2004) when applied to similar bovine cartilage explants. For co-culture samples, cartilage tissue was cultured in the same well of a tissue culture plate with a

piece of joint capsule tissue for the duration of the experiment. The two pieces of tissue were not in direct contact during culture. Cartilage maintained in free swelling culture without joint capsule tissue served as controls for each experiment. For each experiment, explant disks in each treatment or control group were purposely matched across depth and location along the joint surface to prevent bias based on location; as a result, each experimental condition represents an average of specimens within the femoropatellar groove.

Mechanical Property Measurement. Compressive mechanical properties of the cartilage were measured 2, 4, 8, and 16 days after mechanical injurious compression, co-culture with joint capsule tissue, or injurious compression followed by co-culture. Uninjured free swelling disks cultured in the absence of joint capsule tissue served as experimental controls. Individual disks were subjected to unconfined uniaxial compression in a polysulfone chamber. Three sequential compression ramps to 10%, 12.5%, and 15% strain were applied; equilibrium stress was attained at each step. The equilibrium compressive stiffness was calculated using linear regression of the equilibrium stress v. stain data at these three points. Dynamic compressive stiffness was measured during application of sinusoidal compression with amplitude 0.5% strain at five frequencies ranging from 0.01 Hz to 1.0 Hz superimposed on 15% static compressive offset strain.

Tissue Digestion and GAG Measurement. Following measurement of cartilage tissue mechanical properties, cartilage disks were digested in 1 ml protease K solution (100 μg/ml in 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 8) at 60°C for 18 hours. Glycosaminoglycan (GAG) content of tissue digests was measured using the dimethylmethylene blue (DMMB) dye-binding assay.

Western Analysis. Aggrecan was extracted from cartilage in 4 M guanidine hydrochloride/50 mM sodium acetate/5 mM EDTA/15 mM benzamidine/1 μg/ml pepstatin A/0.3 M aminohexanoic acid/0.1 mM PMSF by rocking for 48 hours at 4°C. Tissue extract was then ethanol precipitated (3 volumes ethanol with 5 mM sodium acetate) overnight at -20°C. Following precipitation, samples were spun at 13000 g for 30min at 4°C. Supernatant was

removed and pellet dried in a Speedvac. The pellet was then dissolved in digestion buffer (50 mM sodium acetate, 50 mM Tris, 10 mM EDTA, pH 7.6) and treated with protease-free chondroitinase-ABC, keratanase II, and endo-β-galactosidase to remove GAG chains (Seikagaku, Japan). After digestion, samples were dried and resuspended in Tris-Gly SDS 2x sample buffer (BioRad, CA) with reducing agent (30 μg/ml DTT). Samples were denatured and run on 4-15% gradient Tris-HCl gels (BioRad, CA) and then transferred to a nitrocellulose membrane at 4°C. Membranes were blocked and incubated with anti-G1, anti-G3, or anti-NITEGE primary antibody (1:2000) overnight. Anti-mouse or anti-goat secondary antibody incubation for 1 hour was followed by development using Western Lightning kit (PerkinElmer, MA); membranes were imaged for chemiluminescence with Fluorochem (Alpha Innotech, CA).

Statistical Methods. A paired Student's t-test assuming unequal variance was used to calculate p-values.

4.3. RESULTS

Models of joint injury result in GAG loss from cartilage tissue. Biochemical measurement of the glycosaminoglycan (GAG) content of cartilage following mechanical injury, co-culture with joint capsule tissue, and the combination of mechanical injury followed by co-culture with joint capsule showed depletion of GAG from the matrix in each of the injury models by 16 days in culture (Figure 4.1). The GAG content of control free swelling cartilage did not change significantly over 16 days in culture. Following mechanical injury, a non-significant 5% decrease in GAG was seen within the first two days after injurious compression. GAG was significantly decreased after mechanical injury to 90% of control levels by 4 days in culture and continued to decrease reaching 76% of control by 16 days. Co-culture with joint capsule tissue also resulted in loss of GAG from cartilage tissue. In contrast to mechanical injury, which caused an immediate decrease in GAG, during the first eight days of co-culture with joint capsule tissue, GAG in the cartilage remained within 4% of control levels. By eight days of co-culture, GAG decreased to 85% of control and further decreased to 76% by 16 days. The combination of mechanical injury followed by co-culture resulted in the largest magnitude decease in GAG by 16 days in culture to 62% of control levels with little change seen in the first 4 days of culture.

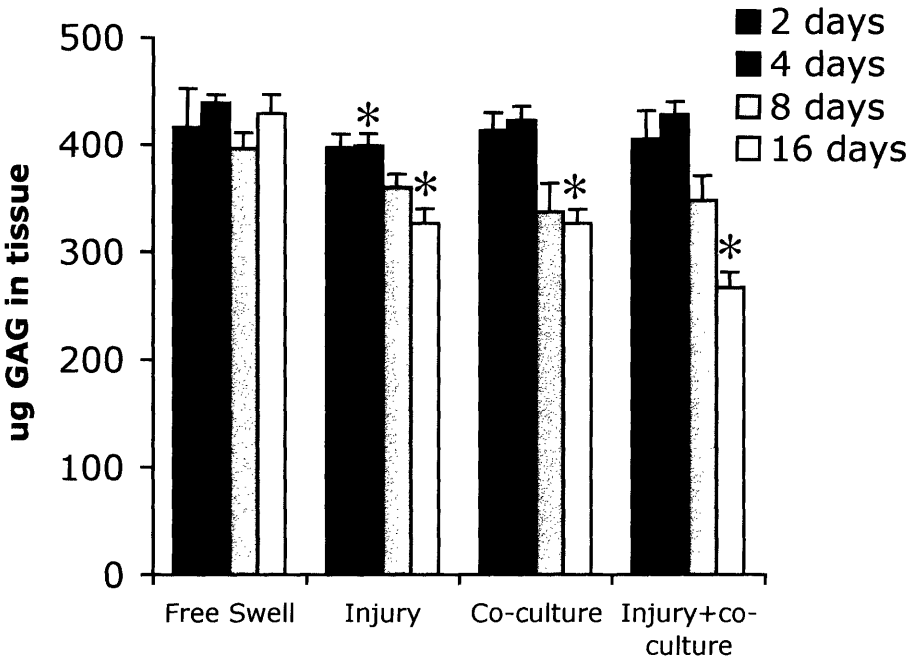

Figure 4.1: Glycosaminoglycan (GAG) content of cartilage subjected to mechanical injurious compression and co-culture with joint capsule tissue. Cartilage tissue explants were subjected to mechanical injurious compression, co-culture with joint capsule tissue, or the combination of mechanical injurious compression followed by co-culture with joint capsule tissue. Tissue was digested and GAG content of the digest was measured by DMMB dye binding assay. * indicates p-value ≤ 0.05 compared to free swell control. Mean ± SE (n=4).

Injury models result in specific cleavage of aggrecan at the aggrecanase site in the IGD. Tissue extracts were analyzed by Western blotting to detect aggrecan protein content of cartilage explants following mechanical injurious compression, co-culture with joint capsule tissue, and the combination of injurious compression followed by co-culture with joint capsule tissue. Antibodies that bind to the globular domains at the N-terminus (anti-G1) and the C-terminus (anti-G3) showed cleavage of the aggrecan core protein in response to the different models of joint injury (Figure 4.2). Each of the tissue samples contained predominantly high molecular weight aggrecan that migrated above the 200 kDa molecular weight standard and had an intact G1 domain (Figure 4.2A). Aggrecanase cleavage in the interglobular domain (IGD) results in an N-terminal fragment that runs as a doublet with molecular weight between 60 and

80 kDa. Injurious compression resulted in accumulation of G1 fragments of aggrecan in the tissue that are the product of specific cleavage by aggrecanase at this site (Figure 4.2B, lane 2). Compared to free swell control, injurious compression did not cause additional loss of the G3 domain from the tissue (Figure 4.2C, lane 2), indicative of full-length aggrecan with intact G1 and G3 domains being present following mechanical injury. Co-culture of cartilage with joint capsule tissue resulted in aggrecanase cleavage products in the tissue (Figure 4.2B, lane 3), similar to that seen for injurious compression. In contrast to mechanical compression, however, co-culture with joint capsule tissue resulted in loss of the G3 domain from the tissue (Figure 4.2C, lane 3). The combination of mechanical injurious compression followed by co-incubation with joint capsule tissue also resulted in aggrecanase cleavage as was seen for both compression and co-culture alone (Figure 4.2B, lane 4). The combination of compression followed by co-incubation led to substantial loss of G3 domain from the tissue compared to either compression or co-culture alone (Figure 4.2C, lane 4).

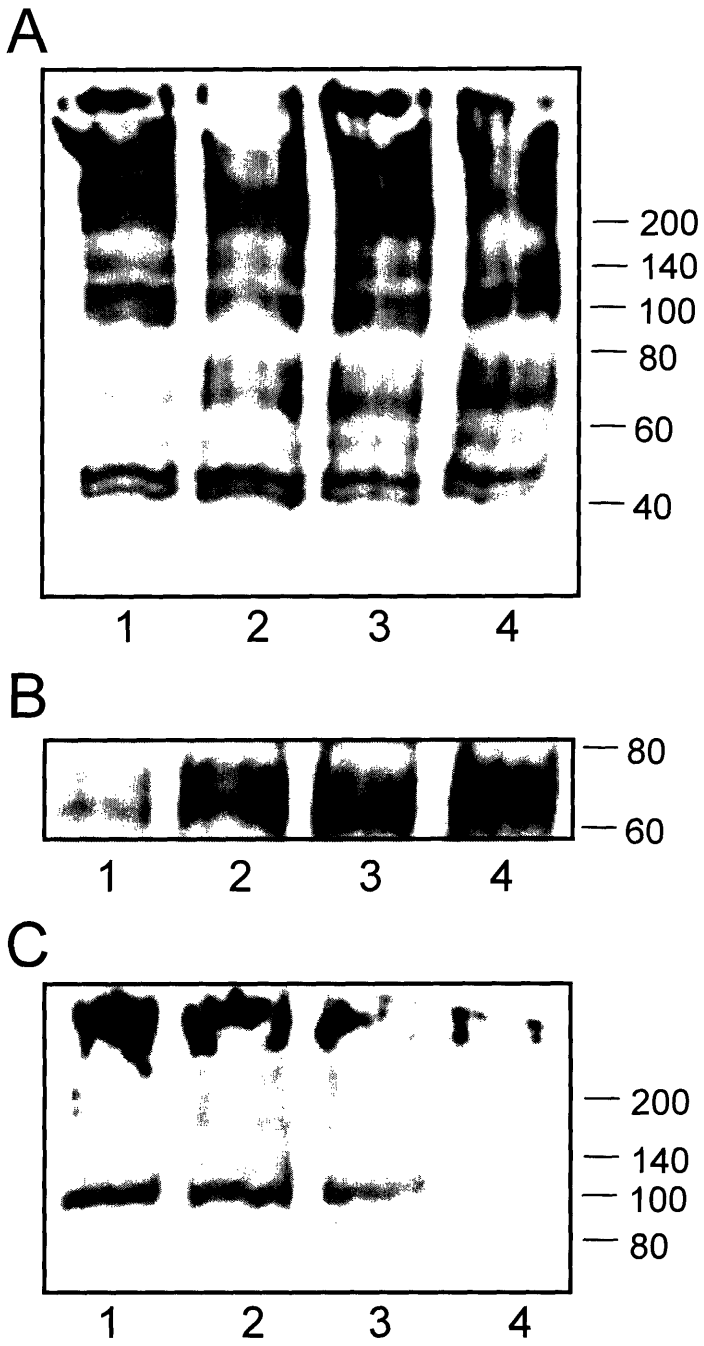

Figure 4.2: Western blot for aggrecan core protein in cartilage tissue extract following mechanical injurious compression and co-culture with joint capsule tissue. Cartilage tissue extract samples were probed with anti-G1 primary antibody (A and B) or anti-G3 antibody (C). Sample extracts are from free swell control cartilage (lane 1), cartilage following injurious mechanical compression (lane 2), cartilage co-cultured with joint capsule tissue (lane 3), and cartilage subjected to injurious compression followed by co-culture with joint capsule (lane 4). Molecular weigh standards shown to the right in units of kDa.

Mechanical properties of cartilage are affected by time in culture and injurious compression. Measurements of equilibrium and dynamic stiffness of cartilage explants were measured in unconfined compression using a custom designed incubator housed loading apparatus (Frank, 2000). Equilibrium stiffness decreased by 49% in control samples by sixteen days in free swelling culture (Figure 4.3A). Injurious compression resulted in a further significant decrease in cartilage equilibrium stiffness of 32-35% measured two and four days after injury (Figure 4.3A). Co-culture of cartilage with joint capsule tissue did not affect the equilibrium stiffness of the cartilage during 16 days of co-culture. Surprisingly, the combination of injurious compression followed by co-culture with joint capsule tissue also did not decrease equilibrium stiffness as was observed for injurious compression alone (decreased 18-20%, not significant). Dynamic stiffness measured at 0.3 Hz showed a continuous decrease with time in culture in control samples of 36% from day 2 to day 16 (Figure 4.3B). Similar to that seen for equilibrium stiffness, dynamic stiffness was decreased following injurious compression (13-38%). This effect continued through 16 days in culture. Co-culture with joint capsule tissue did not result in a decrease in cartilage tissue dynamic stiffness. In contrast to equilibrium stiffness, cartilage subjected to injurious compression followed by co-culture with joint capsule tissue showed a decrease of 22-30% in dynamic stiffness from two to sixteen days of culture (Figure 4.3B).

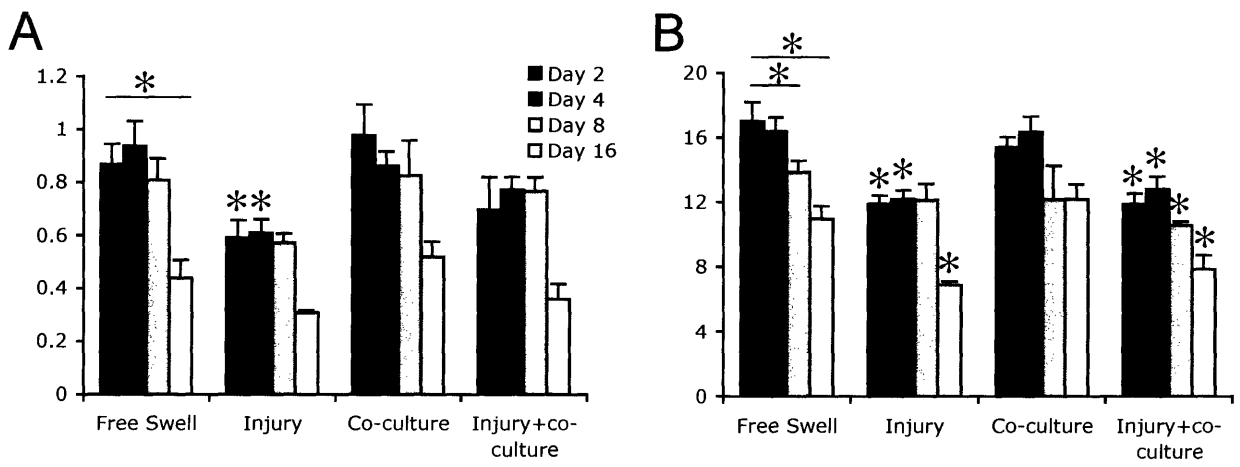

Figure 4.3: Equilibrium and dynamic stiffness of cartilage following injurious compression and co-culture with joint capsule tissue. Mechanical properties of cartilage explant disks were measured in unconfined compression. A) Equilibrium stiffness was measured by application of successive ramp and hold steps in displacement at 10%, 12.5%, and 15% strain. Equilibrium stiffness reported in MPa. B) Dynamic stiffness was measured by application of 0.5% strain amplitude sinusoidal compression at 0.3 Hz. Dynamic stiffness reported in MPa. * indicates p-value ≤ 0.05 compared to free swell control. Mean ± SE (n=4).

4.4. DISCUSSION

Cartilage matrix is composed largely of water, proteoglycan and type II collagen molecules. The primary proteoglycan present in cartilage consists of an aggrecan core protein with 80-100 GAG chains attached along the protein backbone. These GAG chains are highly negatively charged and provide cartilage with the ability to resist compression within the joint. Through the progression of joint diseases (e.g. osteoarthritis), matrix is broken down and cartilage looses the ability to respond to loads within the joint. Using *in vitro* models of joint injury, we show loss of GAG from cartilage tissue explants following either mechanical injurious compression or co-culture of cartilage with joint capsule tissue. Additionally, Western blotting of tissue extracts show cleavage of the aggrecan core protein at the aggrecanase specific site in the IGD leading to accumulation of aggrecan fragments with the NITEGE amino acid sequence at the C-terminus resulting from cleavage at the Glu^{373}-Ala^{374} bond. Finally, the equilibrium and dynamic stiffness of the tissue was found to decrease with time in culture and following injurious compression but was not affected by co-culture with joint capsule tissue.

Matrix degradation in *in vitro* models of joint injury. A number of studies have previously reported GAG release to the culture medium (Quinn, 1998; Loening, 2000; D'Lima, 2001; Ewers, 2001; Thibault, 2002; Patwari, 2003; DiMicco, 2004) and decreased sulfate incorporation rate (Quinn, 1998; Chen, 1999; Torzilli, 1999; Kurz, 2001) following injurious compression. In agreement with previous studies, we measured a decrease in GAG content of cartilage tissue explants following injurious compression. Previous studies have also reported GAG loss from the matrix (Fell and Jubb, 1977) and decreased biosynthesis (Jubb and Fell, 1980; Patwari, 2001) during co-culture of cartilage with synovial or joint capsule tissue. By eight days in co-culture with joint capsule tissue, no significant decrease in the GAG content of the cartilage was measured. However, by sixteen days in co-culture, there was a significant decrease in GAG content of the cartilage.

Aggrecanase activity in models of joint injury. Both injurious compression and co-culture with joint capsule tissue caused significant increases in gene expression of aggrecanase-1 and aggrecanase-2 within 24 hours (see CHAPTER 2 and CHAPTER 3 for details). Aggrecanse-1 (ADAMTS-4) increased 2-fold following injurious compression, 6-fold during co-culture, and 10-fold following injurious compression and co-culture. Aggrecnase-2 (ADAMTS-5) showed larger magnitude increases of 40-fold following injurious compression, 6-fold during co-culture, and 85-fold following injurious compression and co-culture. By sixteen days in culture, aggrecanase cleavage fragments are present in cartilage tissue explants subjected to all three of these models of joint injury above the level seen in control free swell cartilage. We hypothesize that this accumulation of aggrecanase specific cleavage fragments results from increased production of one or both of the aggrecanases by the chondrocytes in response to injurious compression and co-culture with joint capsule tissue. Additionally, synovial tissue is a source of aggrecanase-2 protein (Vankemmelbeke, 2001). In the two models that include co-culture with joint capsule tissue (includes synovial lining), aggrecanase cleavage in the cartilage may also result from aggrecanase present in the synovium that is released to the medium and able to diffuse into the cartilage explant.

Comparison to previous measurements of mechanical properties. Previous measurement of cartilage tissue stiffness in unconfined compression of similar cartilage tissue explants reported a dynamic stiffness of 10-15 MPa (Buschmann, 1999). This is in agreement with the stiffness measured in control samples through sixteen days in culture. Korhonen and co-workers report a Young's Modulus on the order of 1 MPa for adult bovine articular (Korhonen, 2002); this is in agreement with the equilibrium stiffness measured here in unconfined compression. Injurious compression decreased cartilage stiffness measured immediately (Loening, 2000) and six hours after loading (Kurz, 2001). We show that this decrease in stiffness following mechanical injury persists for four days in culture. By eight and sixteen days after

injury, the stiffness of injured tissue continued to be lower than control tissue samples however this difference was not statistically significant.

Relationship between GAG content and cartilage stiffness. Glycosaminoglycan (GAG) molecules in cartilage are highly negatively charged and account for 50% of the tissue stiffness (Buschmann and Grodzinsky, 1995). In addition to charge repulsion of GAGs during compression, tissue stiffness depends on steric interactions of proteins in the matrix as well as higher order organization of molecules in the tissue. Stiffness of cartilage is likely to decrease immediately after mechanical injurious compression before significant GAG is lost from the tissue due to large-scale structural damage of the explant, which can be observed macroscopically. Subsequent GAG loss would lead to further reduction in tissue stiffness in this model. It is interesting to note that although co-culture of cartilage with joint capsule tissue results in decreased GAG in the cartilage explant and specific cleavage of aggrecan at the aggrecanase site, no change in the stiffness of the tissue was measured. Also noteworthy, GAG content of free swell control tissue did not change with time in culture while stiffness decreased. It is possible the structure of proteoglycans and association of matrix components within the tissue are altered during culture both alone and with joint capsule tissue resulting in a lack of correlation between tissue stiffness and GAG content. Further study will be necessary to fully determine how a change in molecular content affects tissue level properties. Future studies are planned to measure the collagen content of the tissue explants and further characterize aggrecan and collagen fragments. These studies may lead to candidate matrix proteases responsible for damage seen in each model of joint injury.

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CHAPTER 5: SUMMARY AND CONCLUSIONS

Cartilage degeneration that occurs clinically following joint injury presents an interesting area of research with a multitude of factors that potentially contribute to the observed pathology. We utilized *in vitro* models of joint injury to separately study the effects on cartilage of mechanical overloading and of soluble factors released from damaged joint capsule and synovium. We also combined these two models by first injuriously compressing the cartilage and then co-culturing it with joint capsule tissue to elucidate interactions between these two aspects of joint injury. Analysis was performed by measuring changes in gene expression, protein abundance, and activity of enzymatic cleavage of matrix molecules. Additionally, we measured the mechanical properties of the cartilage in each of the models to determine the overall effect on tissue structure and function.

Chapter 2 describes the effects of a single injurious compression (50% strain, 100%/s strain rate) on gene expression of matrix molecules, matrix proteases, protease inhibitors, transcription factors, growth factors, and cytokines. While expression of matrix molecules was not effected by injurious compression, matrix proteases, ADAMTS-5 and MMP-3, increase to a peak of 40-fold and 250-fold respectively above control expression levels 12 hours after injury. Transcription factors, c-fos and c-jun, were also highly regulated by injurious compression increasing by 120-fold and 40-fold respectively within 1 hour after injury. TIMP-1, an inhibitor of matrix proteases increased 12-fold over control. Surprisingly, expression of β-actin and GAPDH were affected by injury making them unsuitable for use as normalization genes in this study as is often done in gene expression studies. A k-means clustering algorithm was used to group the gene expression profiles into five groups that showed similar changes in expression following injury. The relative level of expression in free swelling control samples of each of the genes is also reported. Expression in control samples varied over five orders of magnitude with matrix molecules most highly expressed and the aggrecanase enzymes having the lowest expression levels of the genes tested.

Chapter 3 extends the analysis of gene expression in cartilage injury models by reporting changes in expression levels during co-culture of cartilage with joint capsule tissue. This model aims to investigate the effect on cartilage of damage to other joint tissues including joint capsule, synovial lining, ligaments, or tendons. The joint capsule tissue used in our studies includes the synovial lining from the joint as well as the underlying fibrous tissue. Because the capsule tissue is cut from the joint, it is understood to be damaged with the potential to release soluble factors that may have an affect on the cartilage when co-cultured. We compared co-culture of normal, uncompressed cartilage to cartilage that was first injuriously compressed and then co-cultured with joint capsule tissue to determine if these two treatments interact. Co-culture with joint capsule tissue did not affect expression of matrix molecules, similar to what was seen in the injurious compression model. Interestingly, injurious compression followed by co-culture led to a 50% decrease in expression of collagen II and aggrecan within 24 hours. Matrix proteases, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5, all increased 6-9-fold by six hours of co-culture with some molecules continuing to increase expression through 24 hours of co-culture and others returning to baseline. Addition of injurious compression before co-culture with joint capsule resulted in varying levels of responses by these proteases; MMP-3 and ADAMTS-5 were highly upregulated to 85-130-fold control, ADAMTS-4 increased 10-fold, and no change was measured in MMP-13 expression. Again, expression of β-actin and GAPDH was affected by the injury models precluding them from use for normalization. Study of the effects on cartilage of mechanical injurious compression and co-culture with joint capsule separately and then together allows the response of cartilage to these two different factors to be isolated. As well, the interaction of mechanical loading and soluble factors present in the joint can be determined. Finally, analysis of culture medium conditioned with joint capsule tissue contained aggrecan protein fragments. This finding highlights the need to fully characterize the experimental system under study in order to correctly identify the origin of any molecules present in the culture medium containing multiple tissues.

Chapter 4 describes degradation of the cartilage tissue matrix in each of the injury models discussed in Chapters 2 and 3. Both injurious compression and co-culture of cartilage with joint capsule tissue cause a decrease in the GAG content of the cartilage. The combination of injurious compression followed by co-culture results in a larger magnitude decrease in GAG content when compared to either treatment alone. Western analysis showed accumulation of aggrecanase specific cleavage products in cartilage explants following injurious compression, co-culture with joint capsule, or the combination. Interestingly, while injurious compression either alone or in combination with co-culture resulted in a decrease in the equilibrium and dynamic stiffness of the cartilage, co-culture with joint capsule tissue in the absence of mechanical injury did not affect cartilage stiffness. These results motivate further investigation of the tissue structure of cartilage over time in culture and in response to these injury models.

In conclusion, we measured specific cellular responses to each of the joint injury models. Responses varied in magnitude and were time dependent. These studies have identified a subset of molecules that appear to be involved in the eventual loss of cartilage from the joint surface following injury. Additionally, we have been able to highlight differences in the effects caused by mechanical overload of cartilage tissue, damage to other joint tissues, and the combination of these factors. We found that an altered mechanical or chemical environment results in changes in gene expression and specific matrix damage with the combination of these events causing more substantial damage to the tissue than each factor alone.

APPENDIX A: EXPERIMENTAL PROTOCOLS

A.1. INJURIOUS COMPRESSION USING THE INCUDYN

For experiments involving mechanical injury to cartilage explants, a custom built incubator housed loading apparatus (IncuDyn; Frank, 2000) was used. This machine allows application of distance controlled or load controlled waveforms in real time as programmed by the user. For the experiments described in this Thesis, the IncuDyn was used in displacement control to apply 50% strain at a strain rate of 100%/s to cartilage explants cut to 3 mm diameter, 1 mm thick disks.

For loading, cartilage explants were placed in a single-well polysulfone chamber (designed and machined at MIT) and mechanically compressed individually as described in Table A.1. This loading protocol includes one step of machine set-up (Find bottom of chamber) that calibrates the machine to set the zero displacement location to be equal to the bottom of the well in the loading chamber. Because the chamber has an indentation of 130 μm below the surface reached by the platen when lowered, an offset of this magnitude is included when setting the zero displacement location. After setting the zero displacement, the platen is raised to 1.35 mm to allow space for the cartilage explant to be inserted into the machine without initial compression. After calibration, a single cartilage explant (3 mm diameter, 1 mm thick) is placed in the polysulfone loading chamber and the chamber placed in the IncuDyn. A compression ramp is then applied, lowering the platen until the force exceeds 50 g (indicating the platen is now in contact with the explant). The displacement of the platen is then stopped and the location is recorded as the sample thickness. The parameter DispRelative is then set to zero and serves as an input to the compression signal applied during injurious compression. After measuring and recording the sample thickness, a ramp to 50% compression is applied, mechanically injuring the explant. After loading, the platen is raised to 1.35mm to allow the loading chamber to be removed from the IncuDyn and the next sample to be inserted.

Table A.1: Injurious compression protocol (File: C:\Users\Jenny\Injury.pro). The IncuDyn machine in the Grodzinsky Lab at MIT was used to mechanically injure cartilage explants for gene expression and matrix degradation analyses.

Message	Description
Find bottom of chamber	Compression ramp: -2 mm in 60 sec, 10 samples/sec
	Terminate when force reaches –500 g
	Set signal: displacement = offset 130 μm
Find top of plug	Offset load = 0 g
	Compression ramp: -2 mm in 60 sec, 10 samples/sec
	Terminate when force reaches –50 g
	Set parameter: thickness = displacement
	Offset DispRelative = 0 m
Injure	Compression arbitrary (C:\Users\spike.arb): amplitude = -55%, in 1 sec, end 1.5 sec, 100 samples/sec
	Goto 1.35 mm, velocity 500 μm/s

One important consideration when choosing parameters for a loading protocol is an inconsistency between the programmed strain amplitude and the strain amplitude recorded during loading of the explant. When programmed to apply 50% strain, the IncuDyn consistently records a strain of 45%; when programmed to apply 55% strain, the recorded strain is 50%. Previous studies performed in the lab using mechanical injury of cartilage report peak stress ~20-22 MPa with gross damage to the tissue in the form of an elliptical sample shape or cracks visible immediately after loading. With the strain set to 50%, gross damage does not occur as often and peak stress is typically ~14-18 MPa. To more closely match previous experiments, the strain setting was increased to 55% for studies reported in CHAPTER 4. This level of strain more often resulted in visible tissue damage and a strain ~20 MPa. For experiments reported in CHAPTER 2 and CHAPTER 3, strain was set to 50%.

A.2. JOINT CAPSULE TISSUE HARVEST AND CULTURE

For experiments involving co-culture of cartilage with joint capsule tissue, capsular tissue was harvested at the time of cartilage explant harvest. Co-cultured cartilage and joint capsule tissues were always harvested from the same joint. Full thickness capsular tissue was first cut from the joint before drilling cartilage-bone cylinders and was placed in a petri dish in medium with the synovial lining facing down. This tissue was then cut into samples for use in co-culture experiments inside the tissue culture hood. Procedures are described below in more detail.

After securing the joint in the vice grip (following removal of femoral head), the outermost layer of tissue (~100 μm) was removed in multiple stages with blade changes with the goal of uncovering a sterile layer of tissue. The joint surface was then washed with sterile PBS. With a new blade, the patellar tendon was cut ~5 cm below the patella. This cut was then extended laterally to produce an opening across the entire front of the joint. The cut was full thickness and extended into the joint space at approximately the position of the meniscus. Using a scalpel blade, a cut was then made full thickness through the capsule tissue medial to the patellar tendon extending upward to the transition between capsular tissue and muscle. A cut was then made medial and then downward along the transition between capsule (white) and muscle (pink) to result in a piece of capsular tissue removed from the joint. Cutting this piece of tissue should expose the medial condyle and include the layer of synovial tissue in contact with the cartilage in the native joint. This piece of tissue was placed synovium side down in a petri dish containing medium. This was then repeated on the lateral side. Care was taken to include the synovial tissue on samples from both the medial and lateral side.

In the culture hood, tissue was cut with a scalpel into pieces ~4-5 mm across. For experiments described in CHAPTER 3, full thickness joint capsule tissue including the fibrous, collagen rich tissue as well as the loose connective tissue and synovium was used for co-culture experiments (~5 mm thick). This tissue quickly dulls razor blades when cutting and requires

~1.5-2 ml of medium, changed every two days, for culture. Due to conversations with Ernst Hunziker (University of Bern, Switzerland) discussing samples fixed and sent to his laboratory for analysis by histology (Figure A.1), it was decided to use only the loose connective tissue separated from the fibrous tissue. It is thought that this tissue included the majority of reactive cells/factors seen to have an effect in co-culture experiments. Tissue pieces still included the synovial layer. In order to remain as consistent as possible with previous experiments, this tissue was cut into pieces ~4-5 mm across (~0.5 mm thick) with the thinking that this would include the same volume of synovial cells. These samples required less culture medium; 0.75 ml for two days was sufficient.

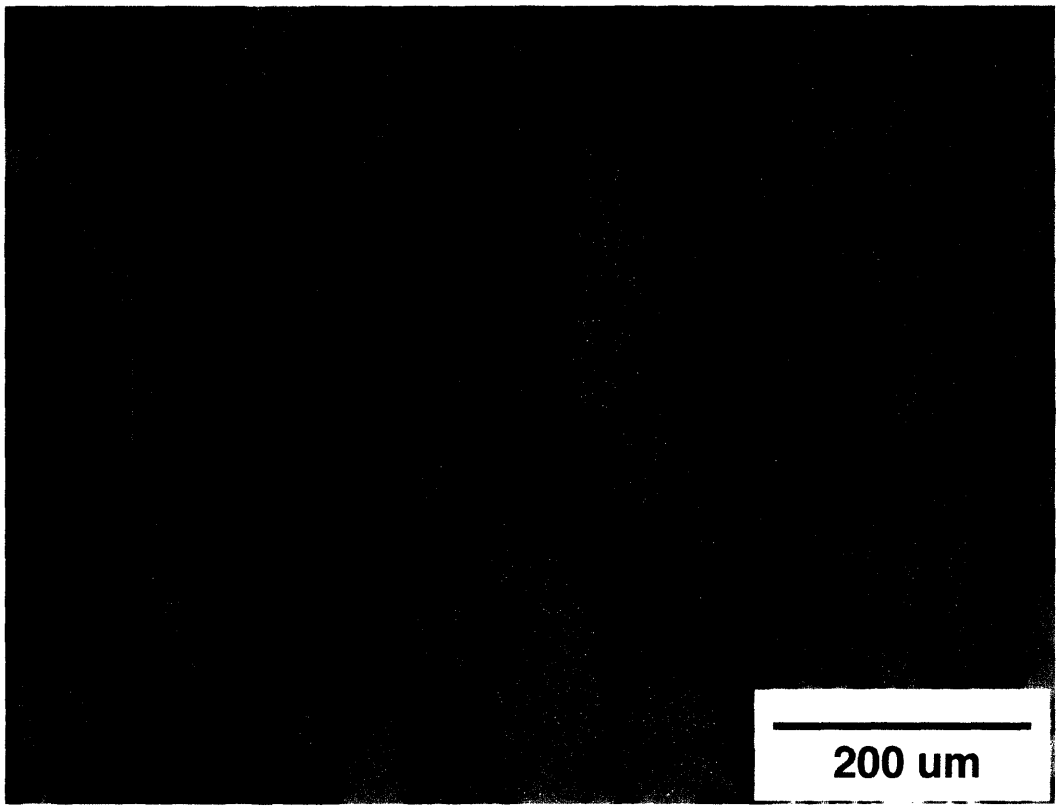


Figure A.1: Histology of joint capsule tissue used in co-culture experiments. Full thickness joint capsule tissue was harvested from the medial side of the patellar tendon. Tissue was fixed in 10% formalin and prepared for histology in the Hunziker Lab in Bern, Switzerland. For experiments in CHAPTER 3, full thickness tissue was used in co-culture experiments; for experiments in CHAPTER 4, only the synovial lining and loose connective tissue (appearing on the right side of the above image) was used for co-culture.

A.3. REAL-TIME PCR

A.3.1. SUPPLIES

Low profile multiplate 96, white	MLL-9651	MJ Research
8-strip ultra clear caps	TCS-0803	MJ Research
12-strip 0.2ml thin-wall tubes	TBS-1221	MJ Research
2-20µl LTS multipipette, 12 channels		Rainin
Precision pipette tips, sterilized	RT-L10F	Rainin
SYBR Green PCR master mix	4309155	Applied Biosystems
Primers	supply sequence	MWG Biotech
RNase, DNase free water	10977-015	Gibco

A.3.2. SUPPLIERS

Gibco
Grand Island, NY
(716) 774-6700
www.invitrogen.com

MJ Research Inc
Watertown, MA
(888) 735-8437
sales@mjr.com

Applied Biosystems
Foster City, CA
(800) 638-5800
www.appliedbiosystems.com

Rainin
Woburn, MA
(800) 472-4646
pipets@rainin.com

MWG Biotech
High Point, NC 27265
(877) 694-2832
info@mwgbiotech.com

A.3.3. PRIMERS

- Designed using Applied Biosystems Primer Express software available on the Applied Biosystems real-time PCR machine
- Ordered from MWG Biotech: scale 0.01, lyophilized
- Water added (DNase, RNase free) to concentration of 100 pmol/μl as indicated in the package insert.
- Stock diluted 10x for use in real-time PCR reactions:
  20 μl forward primer
  20 μl reverse primer
  160 μl water

A.3.4. PROCEDURE

1. Wipe lab bench, pipettes, pipette tip boxes with 70% Ethanol to clean.
2. Fill an ice bucket to top with ice.
3. Place two black plate holder trays in the ice (if you can find them). One will be used to hold the plate, the other will hold the strips of tubes for mixing reagents.
4. Place one strip of 8 tubes vertically in the plate holder, one strip of 11 horizontally.
5. Pipette 90 μl master mix into each of the 11 horizontal tubes.
6. Add 13.5 μl of one of the 11 primers to be tested into each tube across the row mixing with the pipette.
7. Pipette 84 μl water into each of the 8 vertical tubes.
8. Add 18 μl of one of the 8 samples (cDNA) to be tested into each tube down the column mixing with the pipette.
9. Open a new box of Rainin multipet pipette tips.
10. Place a new 96 well multiplate into the second plate holder.
11. Set the multipet to 8.5 μl.
12. Get one full column of tips (8) from the box (will be left with 8 rows of 11 tips).
13. Mix the column of 8 samples with the pipette and pipette 8.5 μl of solution into each column in the plate.
14. Remove tips from pipette.
15. Set the multipet to 11.5 μl.
16. Get row of tips (11) from the box.
17. Mix row of 11 master mix/primer with the pipette and pipette 11.5 μl of solution into each row in the plate, mix when adding to the plate with the cDNA and water, do not fully plunge pipette – will lead to bubbles in samples, change tips between each row.
18. Collect 20 μl total volume from leftover master mix/primer solutions and pipette into the 'no template control' well.
19. Use black cap tool to cover each well with a clear cap (even empty wells must be covered). Do not touch tops of caps as optical reading is taken through the cap and fingerprints may interfere.
20. Spin plate in plate spinner in the BioMicro Center for ~1 min.
21. Use real-time PCR machine to analyze samples.

A.3.5. ANALYZING THE DATA

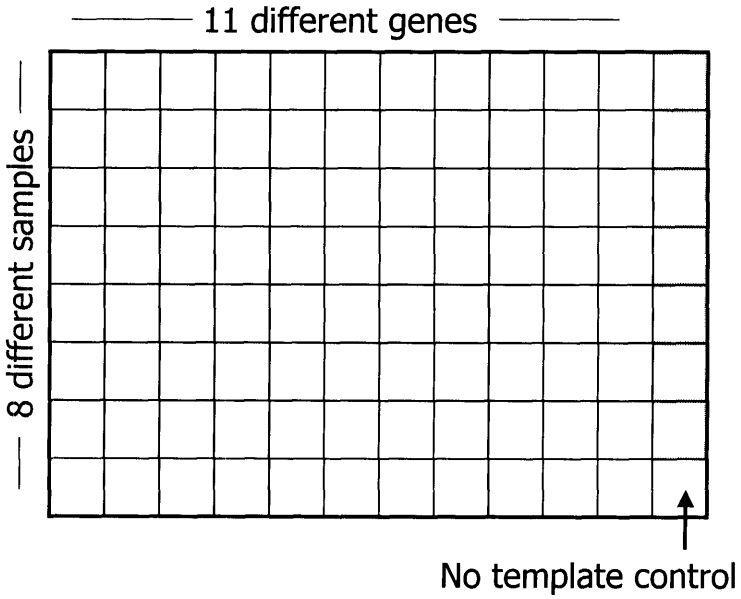
1. Subtract baseline (average florescence in cycles 1-10) from all samples.
2. Choose threshold: should be in the linear range of increasing signal, if possible, use the same threshold for all genes (MUST use the same threshold within one gene for all samples).
3. Export C_T for each well into an Excel spreadsheet.
4. Calculate relative quantity of cDNA in each sample as $Quantity=2^{(40-CT)}$.
5. To compare relative expression levels in two samples, use the ratio of $Quantity_1$ to $Quantity_2$. This gives the fold change in expression between the two samples

A.3.6. VOLUME CALCULATIONS

Per well:		Total needed:
10μl master mix	x 9 →	90μl master mix
1.5μl mixed forward/reverse primer		13.5μl mixed primer
7μl RNase, DNase free water	x 12 →	84μl water
1.5μl cDNA		18μl cDNA

A.3.7. PLATE SET-UP

This set-up allows testing of 8 experimental conditions for 11 different genes with room for a no template control (NTC)



A.3.8. THERMOCYCLE PROTOCOL

Temp (°C)	Time (min)	
50	2	
95	10	
94	1/4	 Repeat 40 cycles
60	1	

Reading is taken at the end of the minute at 60°C.

PCR is followed by a melting curve: measure florescence at each degree between 60°C and 90°C. This allows verification that primers are producing a single product with the expected melting temperature.

A.4. AGGRECAN EXTRACTION, MEDIUM PREPARATION, AND WESTERN BLOTTING

A.4.1. GUANIDINE HCL EXTRACTION

4 M guanidine
0.3 M amino-caproic acid (Sigma A2504 "AHA")
50 mM Na acetate
15 mM benzamidine
5 mM EDTA
5 mM iodoacetic acid (Sigma I2512 "IAA") can substitute NEM
0.1 mM AEBSF (Sigma A9456) can substitute PMSF
1 μg/ml pepstatin A, pH 6.5 (store as 100x in methanol) (Sigma P4265)

- Chop cartilage disks (1-2) at room temp or pulverize cartilage disks (>3) in liquid N_2, add 500-1000 μl GuHCl solution with protease inhibitors. Incubate 48 hours, 4°C, with agitation.

A.4.2. GAG MEASUREMENT

- Use DMMB assay; need to get 100 μg GAG per sample

A.4.3. ETHANOL PRECIPITATION (ETHANOL PRECIPITATE EITHER CULTURE MEDIUM OR TISSUE EXTRACT FROM ABOVE)

5 mM Na acetate in absolute ethanol

If ethanol precipitating medium, add 5 mM EDTA, 0.1 mM PMSF, and 5 mM NEM from concentrated stock solutions to medium before EthOH precipitating.

- Add 3 volume of ethanol (with Na acetate) to medium or tissue extract and store at -20°C overnight
- Pellet the precipitate:
    - Centrifuge at 13,000 rpm for 30 min.
    - Discard supernatant. Cover tubes with parafilm, poke hole in top so gas can escape, freeze in liquid N_2 or –80C freezer, dry in Speedvac 30 min or lyophilize.

A.4.4. GAG DIGESTION

- Resuspend 100 µg GAG lyophilized sample in 100 µl Chase Buffer:
  0.05 M Tris HCl (or Tris Base - must adjust pH)
  0.05 M Na acetate
  0.01 M EDTA
  pH 7.6
  [note: for a 20 ml solution: 60 mg Tris base (MW 121.1), 41 mg Na acetate (MW 82.03), 37.2 mg EDTA (MW 372.2)]
- Assay GAG content again after ethanol precipitate is resuspended in buffer.
- Digest with protease-free chondroitinase ABC (25 mU/100 µg GAG) (Warner) for 3 hours in a water bath of 37°C.
- Add keratanase II (0.5 mU/100 µg GAG) and endobetagalactosidase (0.5 mU/100 µg GAG) (Seikagaku #100455, #100812, respectively… 1 mU/µl when rehydrated)
  [note: Dilute 2.5 µl into 20 µl water → 0.5 mU/5µl] and digest at 37°C for an additional 2 hours.

A.4.5. RUN THE WESTERN

- Take 10-20% of each sample and lyophilize or dry in Speedvac.
- Make 400 µl sample buffer
  0.012 g dithiothreitol (DTT, Cleland's reagent, Sigma D0632) [DTT stock: 154.5 mg in 1 ml 10 mM sodium acetate + 250 µl DI water for final concentration: 30 mg/ml] (Note: this is for a reducing gel, for non-reducing, leave out DTT, replace with water)
  100 µl 12 M urea [7.2 g urea (MW 60.06) into 10 ml water]
  200 µl Tris-Gly SDS 2x sample buffer (BioRad Laemmli #161-0737)

- Resuspend each sample in 15 µl sample buffer
- Make standards; mix 4 µl + 11 µl sample buffer – can use either or both of the following
  Rainbow Visible Standard 220kDa to 30kDa; Amersham RPN 756
  Biotinylated protein ladder; Cell Signaling #7727
- Denature proteins (samples and standards): boil 5 minutes at 100°C
- Use 4-15% (gradient) Tris-HCl gel (BioRad Ready Gel, 10 wells, 30 µl, #161-1104)
- Assemble gel:
  - Cut off the bottom of the thin plastic cover on the gel cassette. Make sure the rubber gasket has notch facing out. Slide the cassette over the thick plastic plate (with the wells facing upward and away from the thick plastic). Tighten screws. Snap into main chamber with wells pointed inward.

- Load samples on gel:
    - Place the inner chamber inside the outer chamber (the gel should be run with the entire apparatus in an ice bath).
    - Fill the inner chamber with enough running buffer to cover the wells but not spill over, check to see if the inner chamber is leaking, tighten screws if necessary.
        - Running buffer:
          50 mM Tris base (6 g/L; MW121.1)
          384 mM glycine (28.8 g/L; MW75.02)
          2 g/L SDS
          *store in fridge
    - Carefully remove the comb.
    - Load samples. Don't introduce air bubbles.
    - Fill outer chamber with running buffer above the level of the wells (this will minimize leakage from the inner chamber)
    - Connect red-red, black-black.
    - Start at 100 V. Check to make sure the electrodes are connected properly (or the bands may migrate upward and out of the gel.). After a few minutes, the bands should be migrating down.
    - Run at 130-150V for 40-60 min in a cold room or an ice bath.
      (note: Higher voltage may lead to slanted bands)

A.4.6. TRANSFER

- Prepare transfer buffer
  25 mM Tris base (3.03 g/L; MW 121.1)
  192 mM glycine (14.4 g/L; MW 75.02)
  20% methanol
  *store at 4°C

- Remove gel:
    - Break cassette, cut off wells
- Create blotting sandwich:
    - Immerse all components in transfer buffer to **remove all air bubbles**. Leave in fluid for **5 minutes** or roll all air bubbles out by applying pressure to the paper and sponge.
    - Loading order:
        - Black side down
        - Sponge
        - Blotting Paper (Sigma P4431, 7x10cm)
        - Gel
        - Nitrocellulose Membrane (cut to size before this step)
        - Blotting Paper
        - Sponge
        - Close cassette

- Load Transfer Apparatus:
    - Place black side to the black side of the inner chamber. Place ice block in chamber. Fill chamber until the membrane and gel are covered.
    - Add magnetic stirrer to keep fluid moving and cooled.
- Transfer at 70V for 2 hr in cold room with ice block in buffer to keep solution cool.

A.4.7. BLOCKING

- Prepare Tris-buffered saline (TBS) and TBS with Tween 20 (TBS-T)
  20 mM Tris base (2.42 g/L; MW 121.1)
  0.137 M NaCl (8 g/L)
  pH 7.6 (will need to add lots of HCl)

  Add 0.1% Tween20 (1 mL/L) for TBS-T
  *store in fridge

- Blocking solution:
  TBS-T with 1% dry nonfat milk (0.2 g into 20 ml TBS-T)
- Block membrane in blocking solution for 5-10 min., room temperature on shaker.

A.4.8. PRIMARY ANTIBODY PROBE

- Prepare TBS-T with 5% dry nonfat milk (1.0 g in 20 ml TBS-T)
- Incubate overnight with primary antibody at 4°C on a shaker
  (note: exact dilution is dependent on each antibody, ~1:1000-3000)
  anti-G1 from J. Sandy: 1:2000
  anti-G3 from J. Sandy: 1:2000
  anti-NITEGE from Wyeth: 1:2000
  anti-ADAMTS-4 (6hAgg1P3/30) from Wyeth: 1:2000
  anti-ADAMTS-5 from Wyeth (2 different Ab - 1hAgg2P3/3 better for use in non-reducing conditions and 3hAgg2P3/34 which works equally well in reducing and non-reducing but not as good as 1h in non-red): 1:2000 for either

A.4.9. SECONDARY ANTIBODY PROBE

- Wash in 15-20 ml TBS-T for 5 min. (x2)
- Incubate in TBS-T with 5% milk plus secondary antibody for 1 hour at room temperature on shaker
  Dilution: 1:2000 (10µl:20ml) for HRP-linked anti-rabbit IgG (for J. Sandy primaries); Cell signaling #7074 or HRP-linked anti-mouse IgG (for Wyeth primaries); Cell Signaling #7076
  Include anti-biotin HRP-linked Ab (Cell Signaling #7075) if using biotinylated standards (10µl:20ml)

  Note: Longer incubations than 1 hour can lead to non-specific binding

A.4.10. WASH

- Wash in TBS-T for 10 min. (x3)
  Note 1: On the last wash, get ready to develop the membrane. If you wait too long, the antibodies will wash off.
  Note 2: If you need to keep the membrane in fluid, store in TBS (no Tween).

A.4.11. DEVELOP USING WESTERN LIGHTNING (PERKINELMER NEL104)

- Get the following equipment ready:
  Aluminum foil
  15 ml conical tube
  Small plastic box
  P1000 pipette and tips
  Forceps
  Western Lightning kit
  Gloves
  Nitrocellulose membrane

- Pipette 1 ml of each of the two Western Lightning solutions into 15 ml tube. Wear gloves, the chemicals are toxic. Vortex to mix.
- Pour solutions into small plastic box.
- Place membrane face down in box on top of solution.
- Close box and cover with foil.
- Incubate 1 min.
- Place the membrane face up in imager (Dedon lab – 2nd floor 500 Tech Square – Alpha Innotech Flourochem 8900)
- User name: JOHN K; password: BASS
- Click ACQUIRE
- With door open, arrange membrane in center of field, adjust camera so membrane fills window; close door.
- Filter in position 1 (for chemiluminescence)
- Click MOVIE SETUP; then LOAD SETUP.
- Load G1 movie, or 10min movie (for NITEGE or enzyme gels).
  G1: total frames=5, lengths=10s, 20s, 30s, 1m, 1m.
  10min: total frames=7; lengths=10s, 20s, 30s, 1m, 2m, 3m, 3m.
  sensitivity/resolution: medium/medium
  stack frames checked
  noise reduction checked
- Click GO to begin imaging
- SAVE movie stack (will save image as it appears after each additional stacked frame as a separate file).

A.5. MECHANICAL PROPERTY TESTING

Objective: Measure mechanical properties of cartilage tissue samples using the IncuDyn. Measurements made in compression and shear, static and dynamic. Initiated to determine if co-culture with joint capsule tissue results in a measurable change in the mechanical properties of either injured or uninjured cartilage. Injury has been shown to decrease the stiffness of cartilage (Kurz, 2001).

A.5.1. USING THE INCUDYN

SETUP THE LOADING MACHINE:

1. Attach lid of loading chamber to the rod on the top of the machine with three screws.
2. If loading in shear, attach bottom of chamber to the floor of the loading machine. The stage rotates during shear loading and so needs to be attached to the chamber to apply a load.
3. Move the lid down to be in contact with the bottom of the chamber (load to –100g to –2kg). This may require moving the load cell up or down to be in the dynamic range of the instrument.
4. Once the lid is in contact with the bottom chamber, move the load cell to be near the lower end of its dynamic range (since this is the lowest position the lid will reach during loading, you will maximize the measurable range).

COMPRESSION:
Test one plug at a time (3 mm diameter, 1 mm thick)
Use clear polycarbonate chamber.

1. Load protocol file (Jenny/mech prop.dyn).
2. Open output file.
3. Find bottom of chamber.
4. Raise lid.
5. Place plug in center of chamber, cover with 100 μl medium.
6. Lower lid to engage load cell but not load the plug.
7. Double click "Find top of plug" step in protocol. This lowers the lid until the cartilage is in contact with the lid and sets the sample thickness to this height.
8. Double click "Mechanical testing starts here" step. The program will run through the entire loading protocol to test static and dynamic mechanical properties in compression.

SHEAR:
Test four plugs at a time (each plug: 3 mm diameter, 1 mm thick)

1. Load protocol file (Jenny/shear mech prop.dyn).
2. Open output file.
3. Find bottom of chamber.
4. Raise lid.
5. Place each plug in center of chamber, cover with 100μl medium.
6. Lower lid to engage load cell but not load the plug.
7. Double click "Find top of plug" step in protocol. This lowers the lid until the cartilage is in contact with the lid and sets the sample thickness to this height.
8. Double click "Mechanical testing starts here" step. The program will run through the entire loading protocol to test static and dynamic mechanical properties in shear.

A.5.2. TESTING PROTOCOL

COMPRESSION:

1. –10% ramp compression in 60 seconds, hold for 240 seconds (300 sec total time).
2. –2.5% ramp compression to –12.5% in 20 seconds, hold 160 seconds.
3. –2.5% ramp compression to –15% in 20 seconds, hold for 160 seconds.
4. Sine wave amplitude 0.5% at frequencies: 0.01, 0.03, 0.1, 0.3, 1.0 Hz.

Total testing time: ~25 min to setup and run protocol.

SHEAR:

1. –15% ramp compression in 60 seconds, hold for 240 seconds.
2. 1% ramp shear in 60 seconds, hold for 240 seconds.
3. 1% ramp shear to 2% in 60 seconds, hold for 240 seconds.
4. 1% ramp shear to 3% in 60 seconds, hold for 240 seconds.
5. Shear sine wave amplitude 0.5% at frequencies: 0.01, 0.03, 0.1, 0.3, 1.0 Hz.
6. Shear goto Angle = 0. (note: this step is necessary to remain in the dynamic range of the shear load cell).

Total testing time: ~35 min to setup and run protocol.

A.5.3. ANALYZING THE DATA

To extract the information from the .dyn files for mechanical properties calculations, a macro was written in Visual Basic in Excel that copies the thickness, and "data" lines from the output files into a separate spreadsheet. The program also calculates stiffness at each static step and each frequency. It is then necessary to plot the static compression data as stress v. strain and

calculate the slope of the line, which represents the stiffness of the sample, using linear regression analysis.

Table A.2: Parameter order in "data" lines of IncuDyn output file. During application of displacement, the IncuDyn measures and records parameters in a specified output file. This file can be exported into Excel for data analysis.

Static Loading	Entry	Dynamic Loading
Date/Time	1	Date/Time
0	2	Frequency (Hz)
Time (seconds)	3	Static displacement (meters)
Displacement (meters)	4	Displacement amplitude (meters)
Load (grams)	5	Displacement phase angle
	6	Total harmonic distortion of displacement
	7	Static load (grams)
	8	Load amplitude (grams)
	9	Load phase angle
	10	Total harmonic distortion of load

MACRO CODE: Written in VISUAL BASIC; runs in Microsoft Excel.

COMPRESSION:

```
Sub getData()

' run this macro from the data file (output as .dyn from IncuDyn) after first Inserting a blank worksheet in
front of data worksheet
' sample area is 7.07mm2
' in addition to copying data from the .dyn data file, macro also calculates the stiffness at each dynamic
frequency
' for static stiffness, will need to plot stress v. strain (calculated by this macro) and calculate slope (by
fitting a line to the data points)

' declares variables to be used as counters (i,j,k) and the samples thickness (thick)
Dim i As Integer
Dim j As Integer
Dim k As Integer
Dim thick

i = 1
j = 1
k = 1

' loops through all lines of data in the output file - copies information of interest to worksheet 1
  For i = 1 To 10000
```

```
' copies thickness to worksheet 1 and sets variable "thick" equal to the sample thickness for later stiffness
calculations
    If Worksheets(2).Cells(i, 1).Value = "thickness" Then
       For k = 1 To 2
         Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
         thick = Worksheets(2).Cells(i, k).Value
       Next k
       j = j + 1

' copies data measured at the end of static compression steps to worksheet 1
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" And Worksheets(2).Cells(i, 3).Value = "0" Then

      For k = 1 To 5
        Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
      Next k
      Worksheets(1).Cells(j, 6) = -Worksheets(1).Cells(j, 4) / thick
      Worksheets(1).Cells(j, 7) = ((0.001 * 9.81) / (7.07 * 10 ^ -6)) * (Worksheets(1).Cells(j, 5).Value)
      j = j + 1

' copies dynamic data for lowest frequency to worksheet 1 - need starting position data as final step of
static compression as well as frequency data at 0.01Hz
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" And Worksheets(2).Cells(i, 3).Value = "0.01" Then

      Worksheets(1).Cells(j, 4) = Worksheets(2).Cells(i, 4).Value
      Worksheets(1).Cells(j, 5) = Worksheets(2).Cells(i, 8).Value
      Worksheets(1).Cells(j, 6) = -Worksheets(1).Cells(j, 4) / thick
      Worksheets(1).Cells(j, 7) = ((0.001 * 9.81) / (7.07 * 10 ^ -6)) * (Worksheets(1).Cells(j, 5).Value)
      j = j + 1
      For k = 1 To 11
        Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
      Next k
      Worksheets(1).Cells(j, 12) = Worksheets(1).Cells(j, 5) / (thick - Worksheets(1).Cells(j, 4))
      Worksheets(1).Cells(j, 13) = ((0.001 * 9.81) / (7.07 * 10 ^ -6)) * (Worksheets(1).Cells(j, 9).Value)
      Worksheets(1).Cells(j, 14) = Worksheets(1).Cells(j, 13) / Worksheets(1).Cells(j, 12)
      j = j + 1

' copies dynamic data for all higher frequencies
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" Then
      For k = 1 To 11
        Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
      Next k
      Worksheets(1).Cells(j, 12) = Worksheets(1).Cells(j, 5) / (thick - Worksheets(1).Cells(j, 4))
      Worksheets(1).Cells(j, 13) = ((0.001 * 9.81) / (7.07 * 10 ^ -6)) * (Worksheets(1).Cells(j, 9).Value)
      Worksheets(1).Cells(j, 14) = Worksheets(1).Cells(j, 13) / Worksheets(1).Cells(j, 12)
      j = j + 1

    End If

  Next i

End Sub
```

SHEAR:

```
Sub getShearData()

' run this macro from the data file (output as .dyn from IncuDyn) after first Inserting a blank worksheet in
front of data worksheet
' sample area is 4 times 7.07mm2 because four samples are tested at once in shear
' measurement assumes a 2.5in radius, because samples are at 1in from center, we divide by 2.5 to
account for this
' in addition to copying data from the .dyn data file, macro also calculates the stiffness at each dynamic
frequency
' for static stiffness, will need to plot stress v. strain (calculated by this macro) and calculate slope (by
fitting a line to the data points)

' declares variables to be used as counters (i,j,k) and the samples thickness (thick)
Dim i As Integer
Dim j As Integer
Dim k As Integer
Dim thick

i = 1
j = 1
k = 1

' loops through all lines of data in the output file - copies information of interest to worksheet 1
  For i = 1 To 16000

' copies thickness to worksheet 1 and sets variable "thick" equal to the sample thickness for later stiffness
calculations
    If Worksheets(2).Cells(i, 1).Value = "thickness" Then
       For k = 1 To 2
         Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
         thick = Worksheets(2).Cells(i, k).Value
       Next k
       j = j + 1

' copies channel to worksheet 1 - this allows you to know what the fields in the data line refer to
(measured by torque load cell or compression load cell)
    ElseIf Worksheets(2).Cells(i, 1).Value = "chan" Then
       For k = 1 To 6
         Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
       Next k
       j = j + 1

' copies data measured at the end of static compression steps to worksheet 1
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" And Worksheets(2).Cells(i, 3).Value = "0" Then

      For k = 1 To 5
        Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
      Next k
      Worksheets(1).Cells(j, 6) = Worksheets(1).Cells(j, 4) / (2.5 * thick)
```

```
        Worksheets(1).Cells(j, 7) = Worksheets(1).Cells(j, 5) / (0.0635 * 4 * 7.07 * 10 ^ -6)
        Worksheets(1).Cells(j, 8) = Worksheets(1).Cells(j, 7) / Worksheets(1).Cells(j, 6)
        j = j + 1

' copies dynamic data for lowest frequency to worksheet 1 - need starting position data as final step of
static compression as well as frequency data at 0.01Hz
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" And Worksheets(2).Cells(i, 3).Value = "0.01" Then

        Worksheets(1).Cells(j, 4) = Worksheets(2).Cells(i, 4).Value
        Worksheets(1).Cells(j, 5) = Worksheets(2).Cells(i, 8).Value
        Worksheets(1).Cells(j, 6) = Worksheets(1).Cells(j, 4) / (2.5 * thick)
        Worksheets(1).Cells(j, 7) = Worksheets(1).Cells(j, 5) / (0.0635 * 4 * 7.07 * 10 ^ -6)
        Worksheets(1).Cells(j, 8) = Worksheets(1).Cells(j, 7) / Worksheets(1).Cells(j, 6)
        j = j + 1
        For k = 1 To 11
          Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
        Next k
        Worksheets(1).Cells(j, 12) = Worksheets(1).Cells(j, 5) / (2.5 * thick)
        Worksheets(1).Cells(j, 13) = Worksheets(1).Cells(j, 9) / (0.0635 * 4 * 7.07 * 10 ^ -6)
        Worksheets(1).Cells(j, 14) = Worksheets(1).Cells(j, 13) / Worksheets(1).Cells(j, 12)
        j = j + 1

' copies dynamic data for all higher frequencies
    ElseIf Worksheets(2).Cells(i, 1).Value = "data" Then
      For k = 1 To 11
        Worksheets(1).Cells(j, k) = Worksheets(2).Cells(i, k).Value
      Next k
      Worksheets(1).Cells(j, 12) = Worksheets(1).Cells(j, 5) / (2.5 * thick)
      Worksheets(1).Cells(j, 13) = Worksheets(1).Cells(j, 9) / (0.0635 * 4 * 7.07 * 10 ^ -6)
      Worksheets(1).Cells(j, 14) = Worksheets(1).Cells(j, 13) / Worksheets(1).Cells(j, 12)
      j = j + 1

    End If

  Next i

End Sub
```

APPENDIX B: SUPPLEMENTARY DATA

B.1. REAL-TIME PCR GENE EXPRESSION DATA

B.1.1. GENE EXPRESSION IN CARTILAGE EXPLANTS FOLLOWING INJURIOUS MECHANICAL COMPRESSION

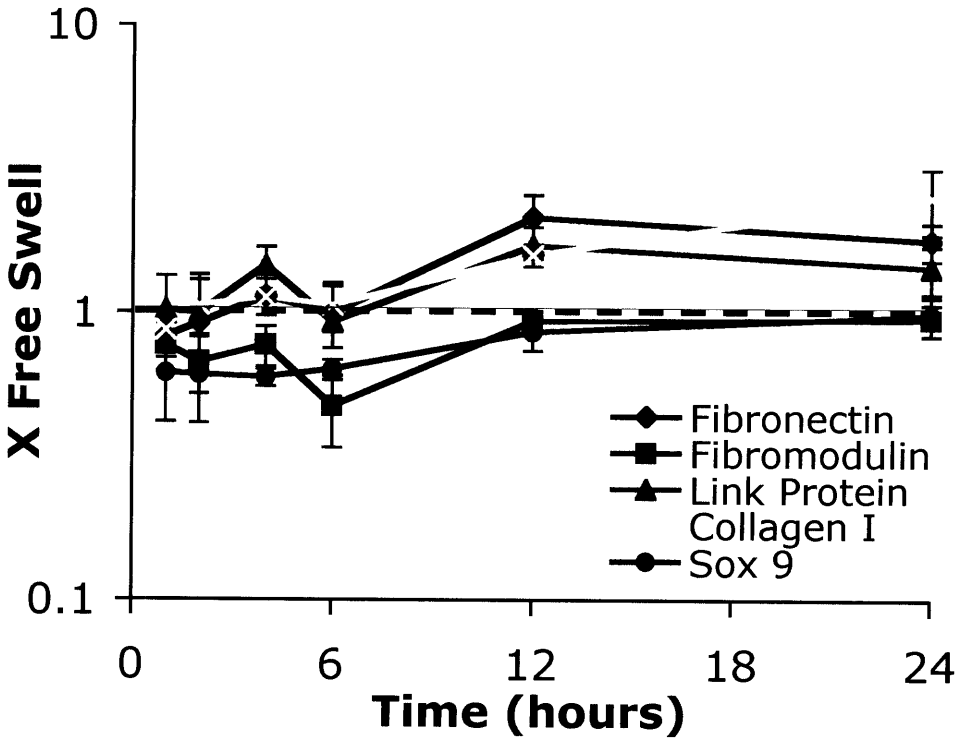


Figure B.1: Changes in expression level of matrix molecules (fibronectin, fibromodulin, link protein), collagen I, and transcription factor sox 9 genes after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

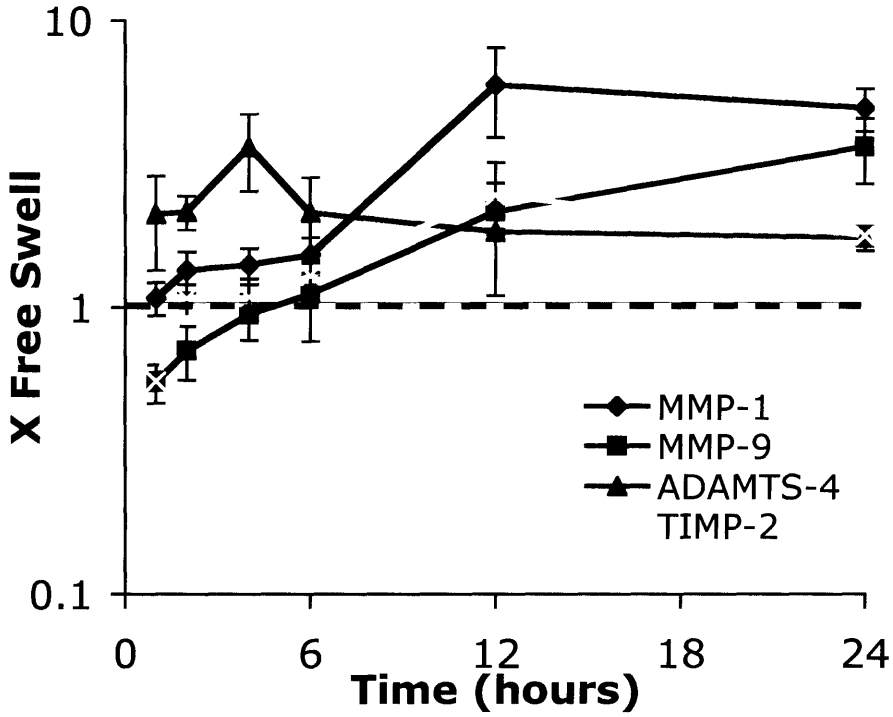


Figure B.2: Changes in expression level of matrix degrading molecules (MMP-1, MMP-9. ADAMTS-4) and inhibitor (TIMP-2) genes after injurious compression. Values on the y-axis represent fold change from free swelling levels with a value of 1 indicating similar expression after injury to the level measured in free swelling conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

B.1.2. GENE EXPRESSION IN CARTILAGE EXPLANTS DURING CO-CULTURE WITH JOINT CAPSULE TISSUE

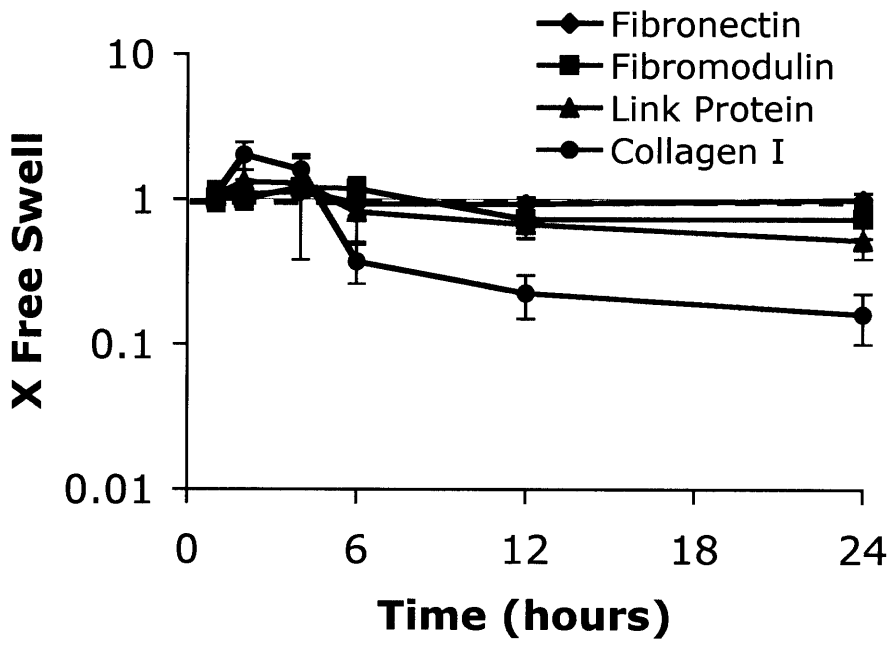


Figure B.3: Changes in expression level of matrix molecules (fibronectin, fibromodulin, link protein), and collagen I genes during co-culture with joint capsule tissue. Values on the y-axis represent fold change from non-co-cultured levels with a value of 1 indicating similar expression during co-culture to the level measured in control conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

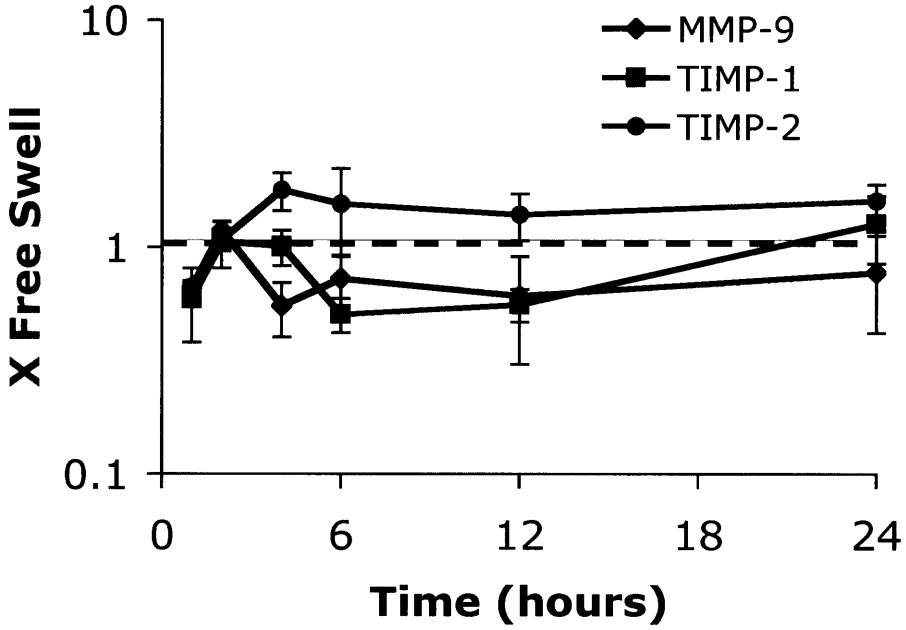


Figure B.4: Changes in expression level of matrix degrading molecule (MMP-9), and inhibitors (TIMP-1, TIMP-2) genes during co-culture with joint capsule tissue. Values on the y-axis represent fold change from non-co-cultured levels with a value of 1 indicating similar expression following injury and co-culture to the level measured in control conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

B.1.3. GENE EXPRESSION IN CARTILAGE EXPLANTS FOLLOWING INJURIOUS MECHANICAL COMPRESSION AND CO-CULTURE WITH JOINT CAPSULE TISSUE

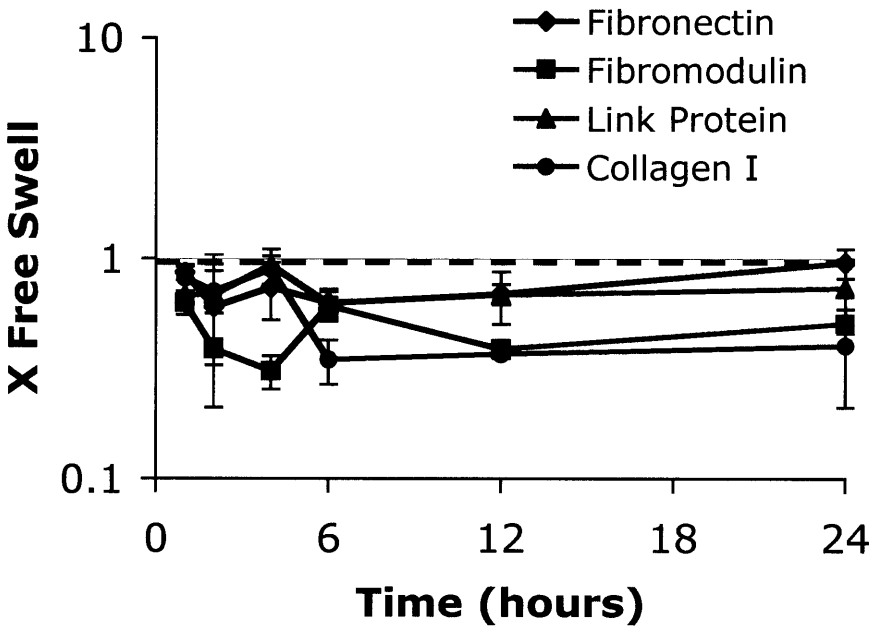


Figure B.5: Changes in expression level of matrix molecules (fibronectin, fibromodulin, link protein), and collagen I genes during co-culture with joint capsule tissue following injurious mechanical compression. Values on the y-axis represent fold change from free swelling, non-co-cultured levels with a value of 1 indicating similar expression following injury and co-culture to the level measured in control conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

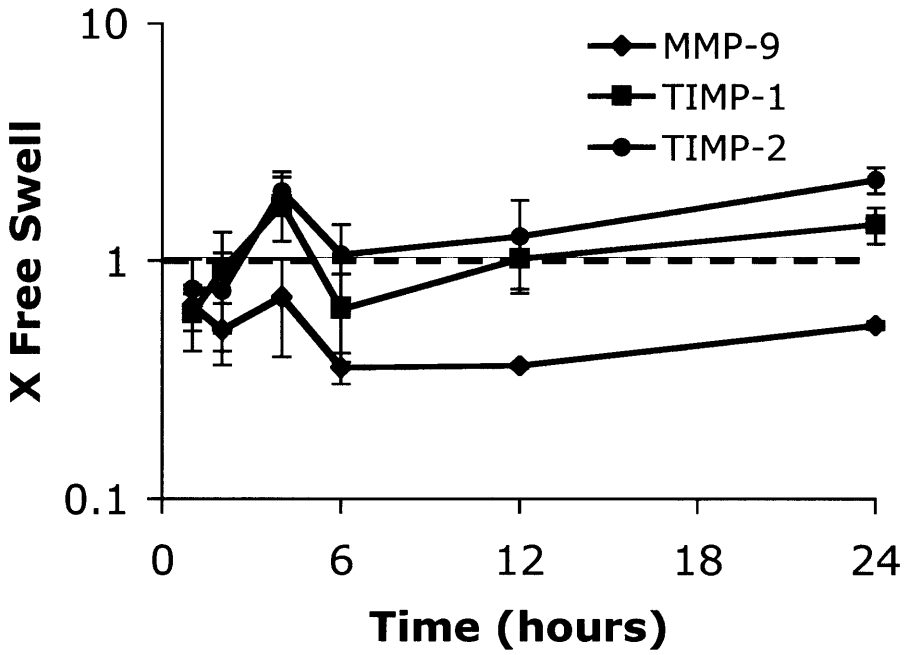


Figure B.6: Changes in expression level of matrix degrading molecule (MMP-9), and inhibitors (TIMP-1, TIMP-2) genes during co-culture with joint capsule tissue following injurious mechanical compression. Values on the y-axis represent fold change from free swelling, non-co-cultured levels with a value of 1 indicating similar expression following injury and co-culture to the level measured in control conditions. Six cartilage disks were pooled for each time point for each experiment. All samples normalized to total RNA at the reverse transcription step. Mean ± SE (n=3).

B.2. INTERPRETATION OF RELATIVE GENE EXPRESSION LEVELS

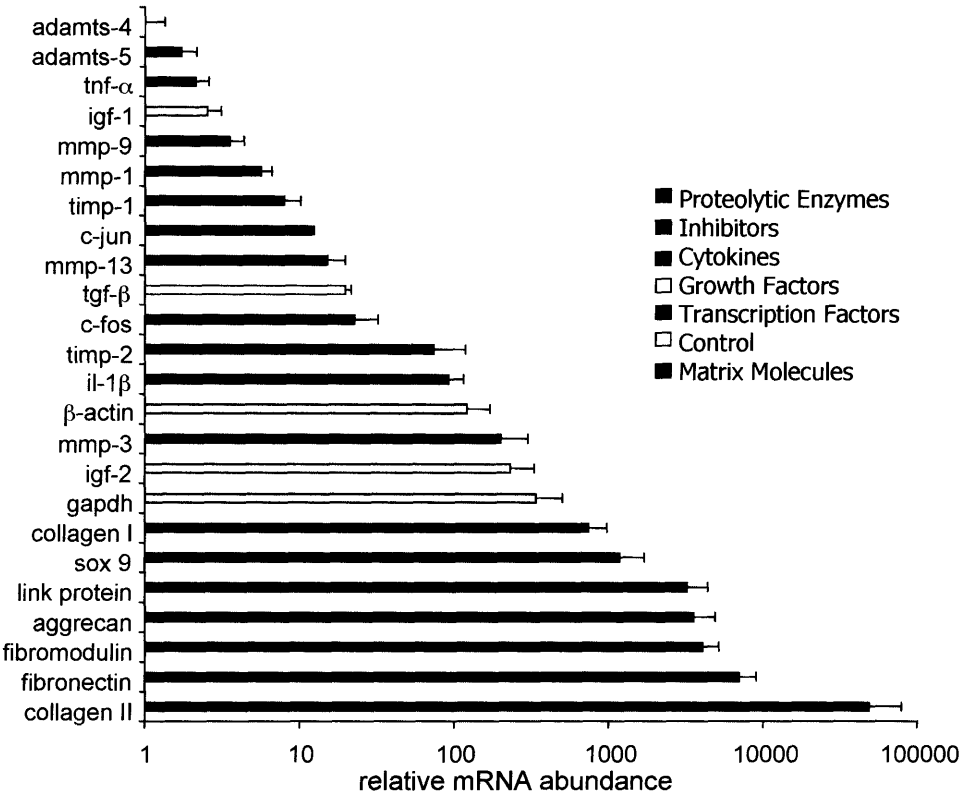


Figure B.7: Free swelling expression levels of 24 genes ranked by relative abundance. Expression levels were normalized to expression of ADAMTS-4, the least abundant gene measured. Data are reported as Mean ± SE (n = 3).

When comparing relative expression levels of different genes within a single cDNA sample as was done here, it was assumed that the efficiencies of the primers for the different genes had equal efficiencies. Standard curves were generated by Moonsoo Jin, Jon Fitzgerald, and myself to quantify the efficiency of each primer used in our real-time PCR studies. We determined that the efficiencies of each primer set were between 95% and 110%. When interpreting the data presented in Figure B.7, it must be considered that this variation in primer efficiencies will potentially result in low percentage inaccuracies in the reported level of expression for each gene and should serve as a caveat for quoting exactly the levels of expression reported.

B.3. WESTERN BLOTS

B.3.1. WESTERN BLOT FOR AGGRECAN G1 DOMAIN ON CONDITIONED MEDIUM IN INJURY MODELS

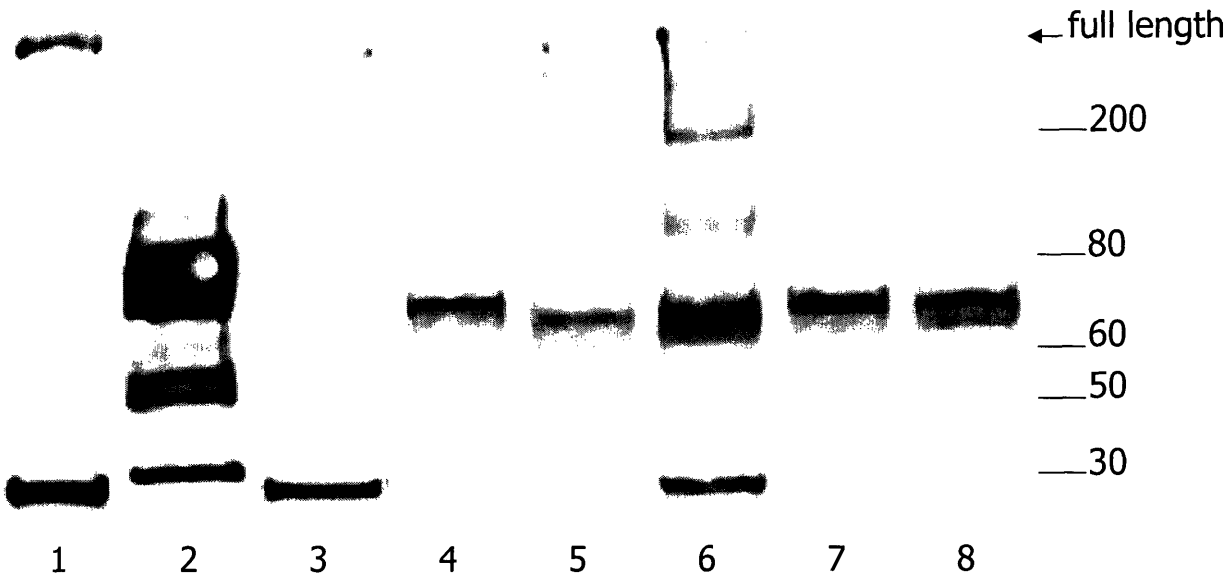


Figure B.8: Conditioned medium analysis for aggrecan fragments with intact G1 domain following treatment with IL-1α, mechanical injury, or co-culture with joint capsule tissue. Western blot for aggrecan using an anti-G1 antibody (J. Sandy) in conditioned medium samples from free-swell control (lane 1), IL-1α (10 ng/ml) treated cartilage (lane 2), mechanically injured cartilage (lane 3), mechanically injured cartilage co-cultured with joint capsule tissue (lane 4), uninjured cartilage co-cultured with live (lane 5) or dead (lane 6) joint capsule tissue, live (lane 7) or dead (lane 8) joint capsule tissue cultured alone. Treatment with exogenous IL-1α results in abundant release of matrix protein aggrecan fragments to the culture medium. Joint capsule tissue when cultured alone releases aggrecan protein fragments to the culture medium making interpretation of cartilage degradation by examining culture medium alone inconclusive.

B.3.2. WESTERN BLOT FOR AGGRECAN G3 DOMAIN ON CONDITIONED MEDIUM IN INJURY MODELS

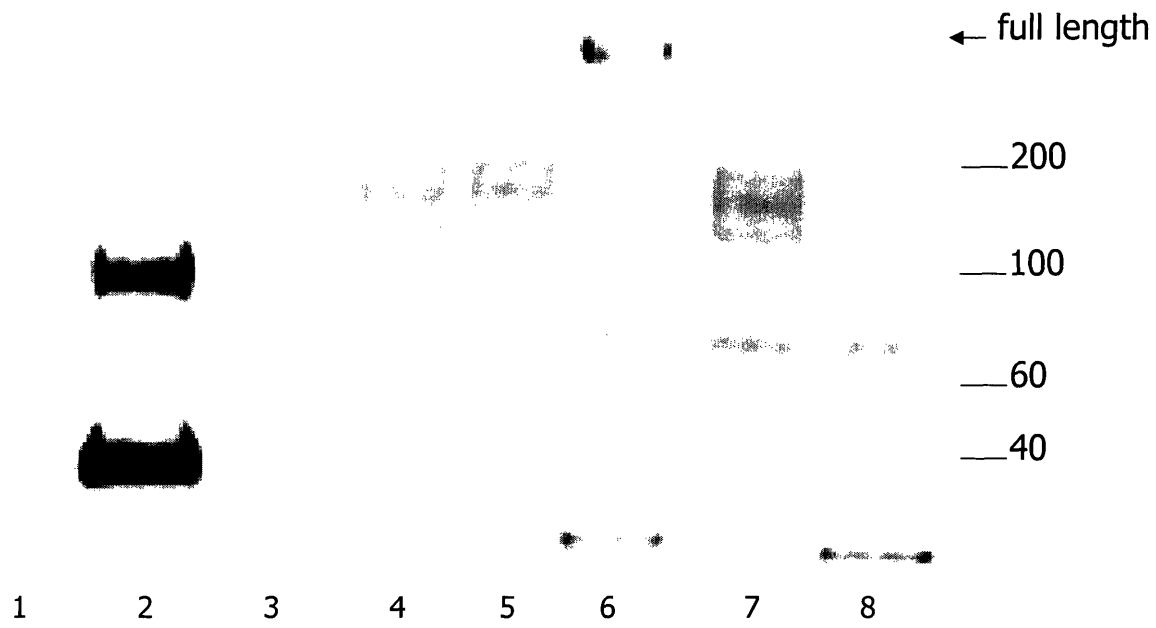


Figure B.9: Conditioned medium analysis for aggrecan fragments with intact G3 domain following treatment with IL-1α, mechanical injury, or co-culture with joint capsule tissue. Western blot for aggrecan using an anti-G3 antibody (J. Sandy) in medium from free-swell control (lane 1), IL-1α (10 ng/ml) treated cartilage (lane 2), mechanically injured cartilage (lane 3), mechanically injured cartilage co-cultured with joint capsule tissue (lane 4), uninjured cartilage co-cultured with live (lane 5) or dead (lane 6) joint capsule tissue, live (lane 7) or dead (lane 8) joint capsule tissue cultured alone. Treatment with exogenous IL-1α results in abundant release of matrix protein aggrecan fragments to the culture medium. Joint capsule tissue when cultured alone releases aggrecan protein fragments to the culture medium making interpretation of cartilage degradation by examining culture medium alone inconclusive.

B.3.3. WESTERN BLOT FOR ADAMTS-5 ON CONDITIONED MEDIUM FROM INJURY MODELS

Figure B.10: Conditioned medium analysis for aggrecanase-2 (ADAMTS-5) following treatment of cartilage with IL-1α, mechanical injury, or co-culture with joint capsule tissue. Western blot for aggrecanase enzyme, anti-ADAMTS-5 antibody (Wyeth Research, C. Flannery) in medium from free-swell control (lane 1), IL-1α (10 ng/ml) treated cartilage (days 1-2 of treatment lane 2; days 3-4 of treatment lane 3), mechanically injured cartilage (lane 4), mechanically injured cartilage co-cultured with joint capsule tissue (lane 5), uninjured cartilage co-cultured with live (lane 6) or dead (lane 7) joint capsule tissue, live (lane 8) or dead (lane 9) joint capsule tissue cultured alone, recombinant ADAMTS-5 (Wyeth Research; lane 10). Treatment with exogenous IL-1α results in release of ADAMTS-5 by days 3-4 to the culture medium. No enzyme was detected using Western blotting in any of the injury models tested due to either lack of production of this enzyme in these models or an enzyme protein level below the detection limit of the methods used.

B.3.4. WESTERN BLOT FOR ADAMTS-4 ON CONDITIONED MEDIUM FROM CO-CULTURE OF CARTILAGE WITH JOINT CAPSULE TISSUE

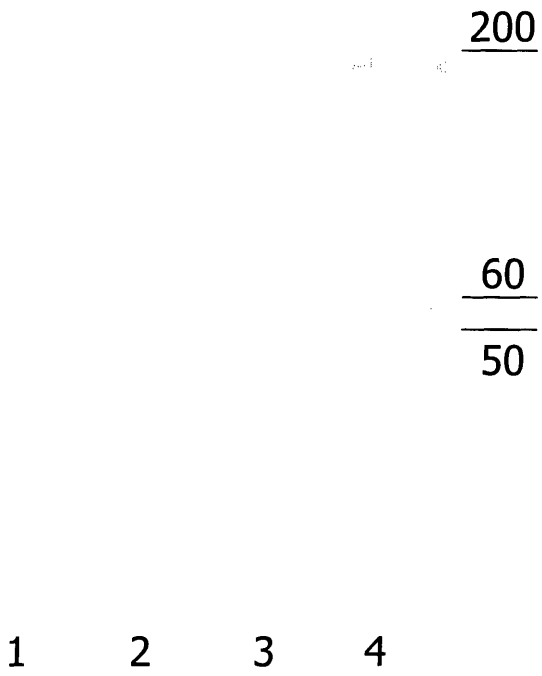


Figure B.11: Conditioned medium analysis for aggrecanase-1 (ADAMTS-4) following treatment of cartilage with IL-1α or co-culture with joint capsule tissue. Western blot (non-reducing conditions) for aggrecanase enzyme, anti-ADAMTS-4 antibody (C. Flannery) in medium from free-swell control (lane 1), IL-1α (10 ng/ml) treated cartilage (days 3-4 of treatment; lane 2), uninjured cartilage co-cultured with live joint capsule tissue (lane 3), live joint capsule tissue cultured alone (lane 4). Treatment with exogenous IL-1 results in release of ADAMTS-4 by days 3-4 to the culture medium. ADAMTS-4 was released to the medium from joint capsule tissue cultured alone; no additional release of enzyme was seen for cartilage co-cultured with joint capsule tissue. The high molecular weight band seen in the lanes containing medium from culture of joint capsule tissue may be due to non-specific binding of either the primary or secondary antibody to a protein present in the sample or may represent a high molecular weight form of ADAMTS-4 possibly bound to another protein.

B.4. ZYMOGRAMS TO MEASURE MMP-2, MMP-3, AND MMP-9 IN INJURY MODELS

METHODS: Zymography was used to analyze the presence of pro- and active MMP-2, MMP-9, and MMP-3 in culture medium following injurious mechanical compression and during co-culture with joint capsule tissue. Equal volumes of culture medium were mixed with loading buffer and electrophoresed under non-reducing conditions on a 10% SDS-polyacrylamide gel containing gelatin (Bio-Rad, #161-1113) for detection of MMP-2 and MMP-9, or a 12% gel containing casein (Bio-Rad, #161-1114) to identify MMP-3. Gels were washed 3 times for 20 minutes in 2.5% Triton X-100, rinsed in distilled water, then incubated in proteolysis buffer (50 mM Tris, 50 mM $CaCl_2$, 0.5 M NaCl, pH 7.8) for 16 hours on a shaker table at 37°C (Gilbert, 1997). Following proteolysis the gels were stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol for 1 hour and destained in 10% methanol, 7.5% acetic acid solution until bands were visible. Pro- and active forms of MMPs were identified by molecular weight. As a control experiment for activity on zymograms, 10 mM EDTA was added to the proteolysis buffer. Absence of proteolytic activity with the addition of EDTA supports the conclusion that MMPs are responsible for bands detected in the zymograms (Gilbert, 1997).

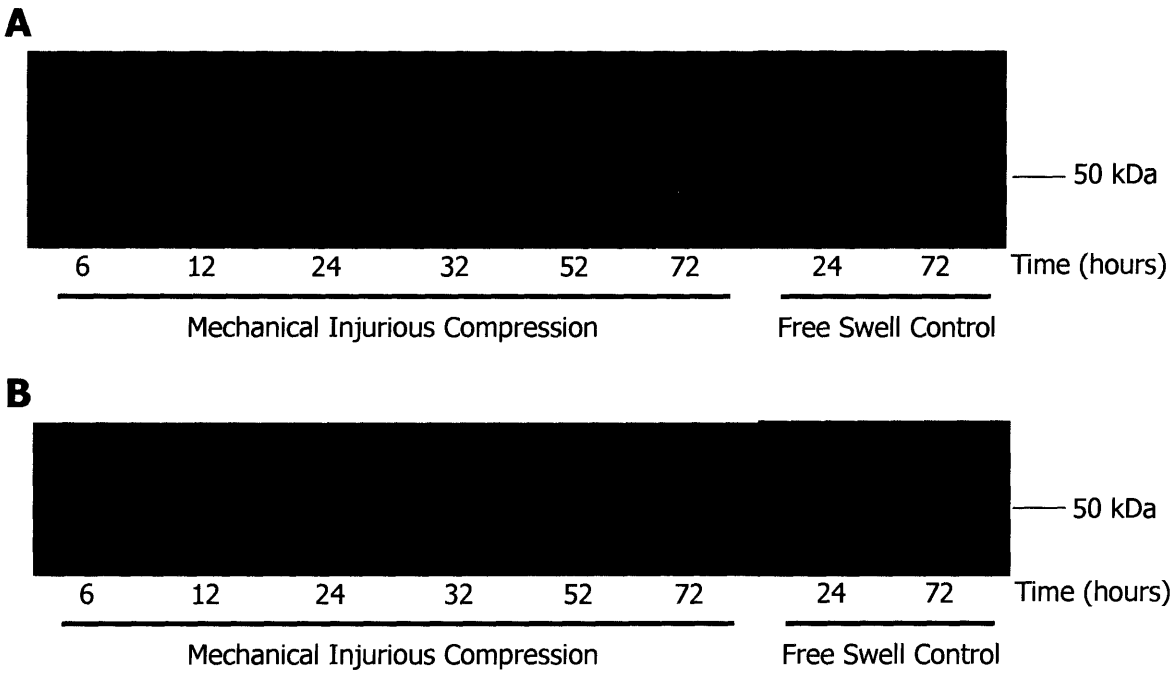


Figure B.12: Casein zymograms on conditioned medium from cartilage explants following mechanical injurious compression. Cartilage explants were either compressed to 50% strain at a strain rate of 100%/s or maintained in free swelling control culture. A) Following compression, explants were placed in fresh culture medium containing 10% fetal calf serum and cultured for the time indicated. B) Following compression, samples were placed in fresh culture medium containing 10% serum and 100 μg/ml cyclohexamide to block protein synthesis. The lack of an upregulation in MMP-3 protein in the medium (as was seen in A) indicates MMP-3 released to the medium following injurious compression was newly synthesized.

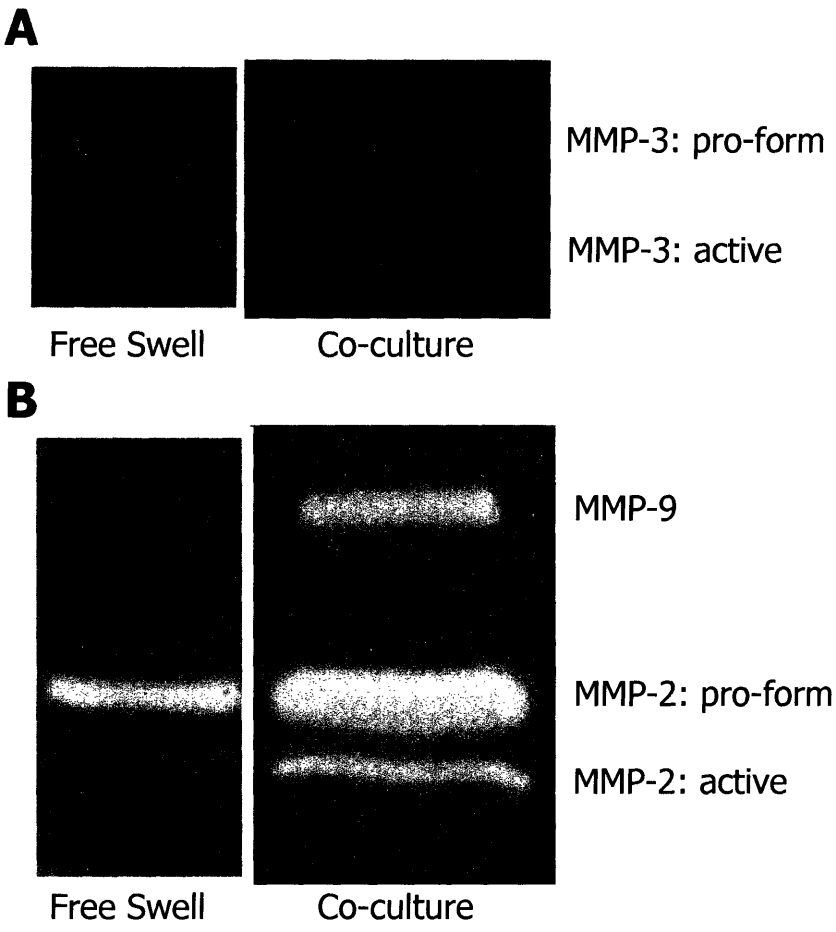


Figure B.13: Zymograms on conditioned medium from cartilage explants during co-culture with joint capsule tissue. Cartilage explants were either maintained in free swelling control culture or co-cultured with a piece of joint capsule tissue for 24 hours. A) Casein zymogram showing increased presence of MMP-3 in the culture medium during co-culture of cartilage with joint capsule tissue. B) Gelatin zymogram showing increased presence of MMP-2 and MMP-9 in the culture medium during co-culture of cartilage with joint capsule tissue.

B.5. HYDROXYPROLINE CONTENT OF CARTILAGE EXPLANTS FOLLOWING MECHANICAL INJURY AND CO-CULTURE WITH JOINT CAPSULE TISSUE

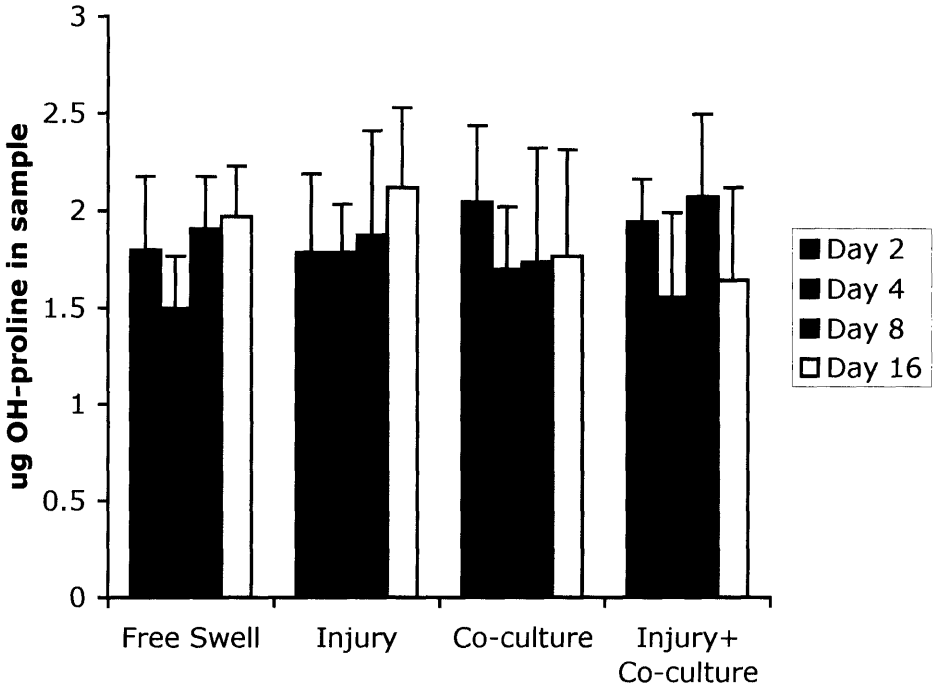


Figure B.14: Hydroxyproline content of cartilage tissue explants following mechanical injurious compression and co-culture with joint capsule tissue. Cartilage tissue explants were subjected to mechanical injurious compression, co-culture with joint capsule tissue, or the combination of mechanical injurious compression followed by co-culture with joint capsule tissue. Tissue was digested with Proteinase K and OH-proline content of the digest was measured by the hydroxyproline assay (Woessner, 1961; protocol from M. DiMicco). Mean ± SE (n=4).

B.6. GENE EXPRESSION DATA CLUSTERED USING K-MEANS CLUSTERING

Performed in collaboration with Jonathan Fitzgerald.

B.6.1. CLUSTERING ANALYSIS OF GENE EXPRESSION PROFILES FROM CARTILAGE EXPLANTS CO-CULTURED WITH JOINT CAPSULE TISSUE

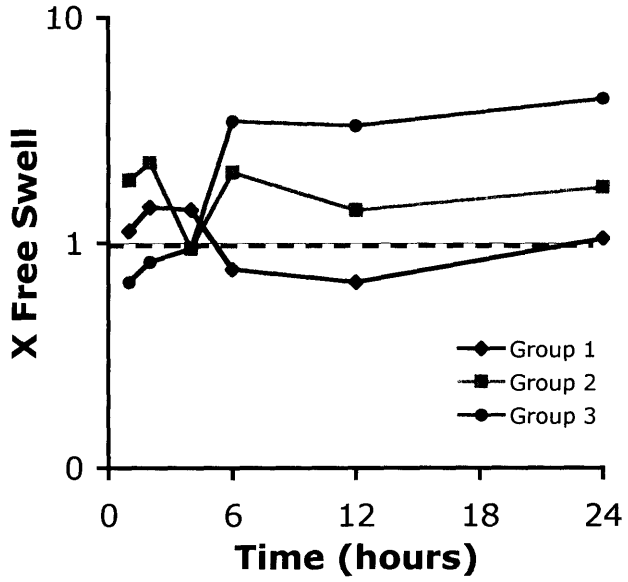


Figure B.15: Clustering analysis of gene expression in chondrocytes during co-culture of cartilage explants with joint capsule tissue. Group expression profiles generated by k-means clustering. The main temporal gene expression patterns induced by co-incubation of cartilage explants with joint capsule tissue. Group profiles were calculated by averaging the expression profiles of genes within each group.

Table B.1: Group members generated by k-means clustering. Temporal expression profiles for each group shown in Figure B.15.

GROUP 1	GROUP 2	GROUP 3
Fibronectin	Collagen II	MMP-1
Collagen I	Aggrecan	MMP-3
Fibromodulin	c-fos	MMP-13
β-actin	c-jun	ADAMTS-4
TIMP-1	MMP-9	ADAMTS-5
TNF-α	IGF-1	GAPDH
		TIMP-2
		IL-1β
		TGF-β
		IGF-2

B.6.2. CLUSTERING ANALYSIS OF GENE EXPRESSION PROFILES FROM MECHANICALLY INJURED CARTILAGE EXPLANTS CO-CULTURED WITH JOINT CAPSULE TISSUE

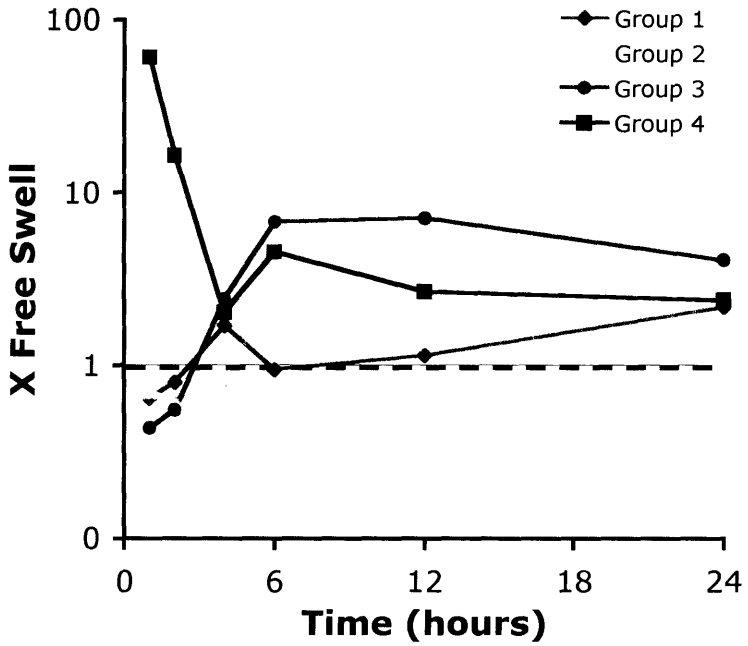


Figure B.16: Clustering analysis of gene expression in chondrocytes during co-culture of mechanically injured cartilage explants with joint capsule tissue. Group expression profiles generated by k-means clustering. The main temporal gene expression patterns induced in mechanically injured cartilage co-cultured with joint capsule tissue. Group profiles were calculated by averaging the expression profiles of genes within each group.

Table B.2: Group members generated by k-means clustering. Temporal expression profiles for each group shown in Figure B.16.

GROUP 1	GROUP 2	GROUP 3	GROUP 4
GAPDH	Collagen II	MMP-3	c-fos
TIMP-I	Aggrecan	ADAMTS-4	c-jun
TIMP-2	Fibromodulin	ADAMTS-5	
	Fibronectin	β-actin	
	Link Protein	TGF-β	
	Collagen I		
	MMP-1		
	MMP-9		
	MMP-13		
	TNF-α		
	IL-1β		

APPENDIX C: PRELIMINARY STUDIES

C.1. RNA ANALYSIS

Samples of RNA extracted from cartilage tissue were submitted for analysis on the Agilent 2100 Bioanalyzer in the BioMicro Center at MIT to evaluate the extraction protocol before beginning real-time PCR gene expression analysis in *in vitro* models of joint injury. The Agilent Bioanalyzer measures the integrity/purity and concentration of RNA. Samples were submitted twice with improvements made to the extraction protocol between submissions.

To improve the integrity of the RNA extracted from tissue samples, only two samples were taken from the –80°C freezer at a time for pulverizing. After these two samples were pulverized, they were returned to the freezer and the pulverizing equipment was re-frozen in liquid N_2 to ensure low temperature of the equipment while pulverizing all samples. Additionally, RNA extraction was only performed for a total of four samples at a time. This was done to reduce time for all steps throughout the extraction. Samples were spun on the benchtop, also to save time in sample transfer between the centrifuge, rather than at 4°C as had been done previously. The RNeasy kit was set-up before beginning extraction; β-mercaptoethanol was mixed with the RLT buffer. DNase was prepared for use before beginning extraction and kept on ice so that it was ready when needed. An overall effort was made to work quickly and keep samples at low temperature when possible.

Table C.1: Measurements of RNA concentration and purity. Samples were submitted to the BioMicro Center at MIT to assess purity and degradation in samples of RNA extracted from bovine articular cartilage. Improvements were made in the RNA extraction protocol to increase yield and maintain RNA integrity for use in real-time PCR.

Sample submission	Average concentration	rRNA Ratio [28S/18S]
Initial	74 ng/μl	0.86
After improvements	100 ng/μl	1.5

Improvements made to the extraction protocol resulted in an increase in the average concentration of RNA obtained from each sample (Table C.1). As well, the rRNA ratio increased to be closer to the ideal value of 2.0 that represents intact RNA with minimal degradation.

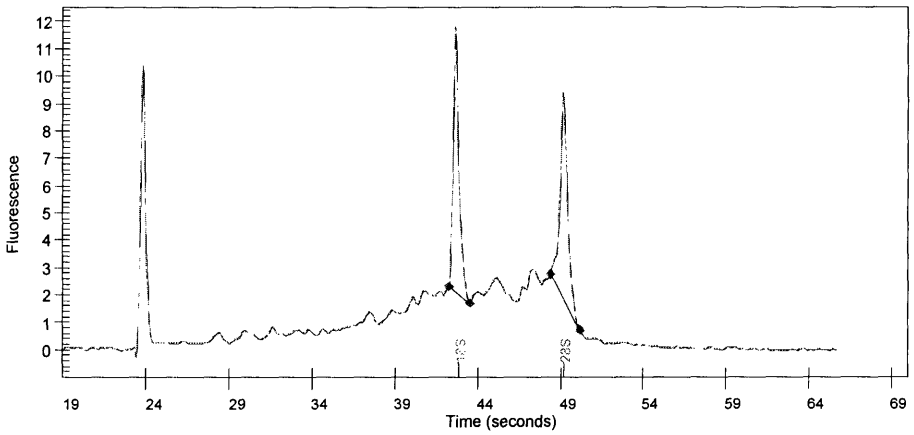


Figure C.1: Representative graph of Bioanalyzer data from initial submission of RNA to the BioMicro center at MIT. Shows degradation of RNA in sample which appears as intensity between the two peaks for 18s and 28s rRNA. Concentration: 52 ng/μl; rRNA ratio: 0.86.

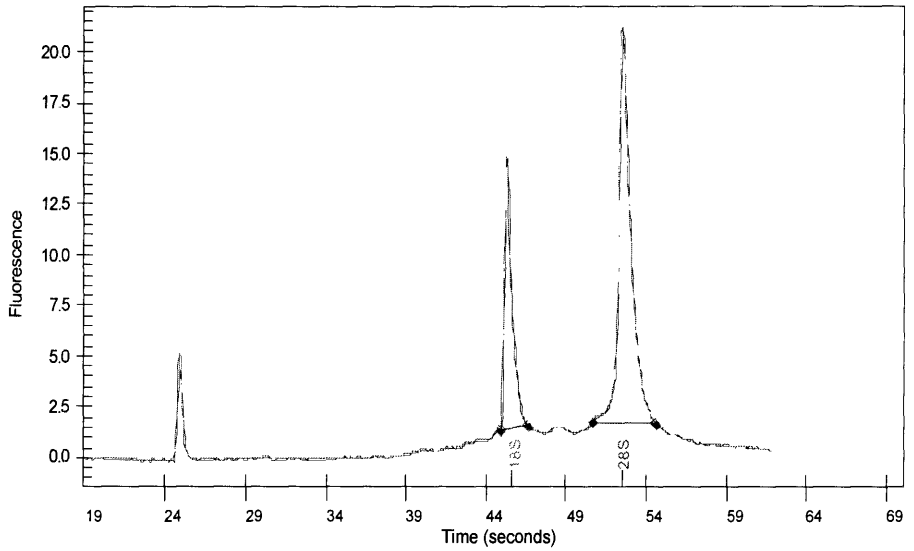


Figure C.2: Representative graph of Bioanalyzer data from second submission to the BioMicro center at MIT after making improvements to the RNA extraction protocol. The appearance of two peaks for rRNA with little intensity between the peaks signifies intact RNA. Concentration: 79 ng/μl; rRNA ratio: 1.99.

The concentration of RNA measured using the Agilent Bioanalyzer was in good agreement with the concentration measured using the UV Spectrophotometer in the Grodzinsky lab. After validating the quality of the RNA samples to be used for gene expression analysis using real-time PCR in the BioMicro center, the UV Spec in the Grodzinsky lab was used to measure RNA concentration and it was assumed that when the concentration of RNA was above 55 ng/μl and the ratio of absorbance at 260 nm and 280 nm was above 1.7 that the RNA was of suitable quality to use for gene quantification.

C.2. WESTERN BLOTS (APMA AND ADAMTS-4 TREATMENTS)

Before beginning experiments to measure aggrecan degradation, it was necessary to perform preliminary positive control studies to test the extraction protocol and antibodies being used. With this aim, cartilage tissue samples were treated with recombinant ADAMTS-4 and APMA. ADAMTS-4 was obtained from Wyeth research (C. Flannery) and cleaves aggrecan at the 'aggrecanase site' at amino acid 373. Cleavage at this site results in a neo-epitope C-terminus with sequence NITEGE (Caterson, 2000). The N-terminal fragment resulting from cleavage at this site (G1-NITEGE) runs on SDS-page gels as a doublet with molecular weight between 60 and 80 kDa. APMA (aminophenylmercuric acetate) was obtained from Sigma-Aldrich and is a known activator of matrix metalloproteinases (MMPs) which cleave aggrecan at the 'MMP site' at amino 341. Cleavage at this site results in a neo-epitope C-terminus with bovine sequence VDIPES (VDIPEN in human) (Caterson, 2000). The N-terminal fragment resulting from cleavage at this site (G1-VDIPES) runs between 50 and 60 kDa on SDS page.

Cartilage explants were treated with 10 μg/ml of ADAMTS-4 or 1 mM APMA for 16 hours. Aggrecan was extracted from the tissue and Western blotting performed as described in A.4. AGGRECAN EXTRACTION, MEDIUM PREPARATION, AND WESTERN BLOTTING.

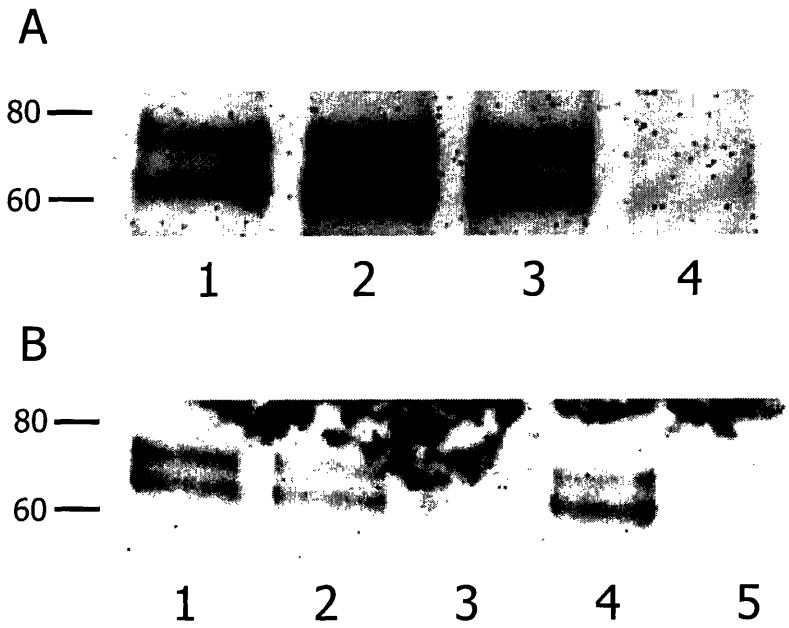


Figure C.3: Western blots for aggrecan fragments on tissue extracts from cartilage treated with 10 μg/ml recombinant ADAMTS-4 for 16 hours. Tissue extracts were run on 4-15% gradient SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with (A) anti-G1, or (B) anti-NITEGE primary antibodies. Lanes 1-3 in A and 1-4 in B show cartilage treated with ADAMTS-4; tissue extract from control (untreated) cartilage shown in lane 4 in A and lane 5 in B.

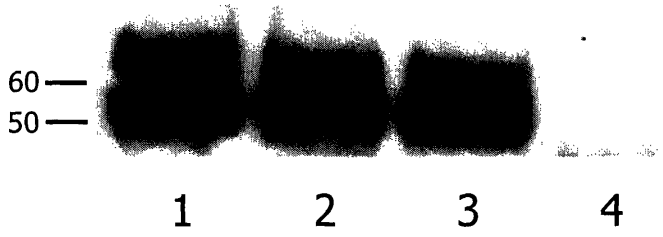


Figure C.4: Western blot for aggrecan fragments on tissue extracts from cartilage treated with 1 mM AMPA to activate MMPs. Tissue extracts were run on 4-15% gradient SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with anti-G1 primary antibody. Lanes 1-3 show cartilage treated with APMA; tissue extract from control (untreated) cartilage shown in lane 4.

APPENDIX D: GRODZINSKY LAB COLLABORATIVE PROJECTS

D.1. INVOLVEMENT OF ADAMTS-4 AND MT4-MMP IN AGGRECANOLYSIS OF IL-1 TREATED BOVINE CARTILAGE (P. Patwari)

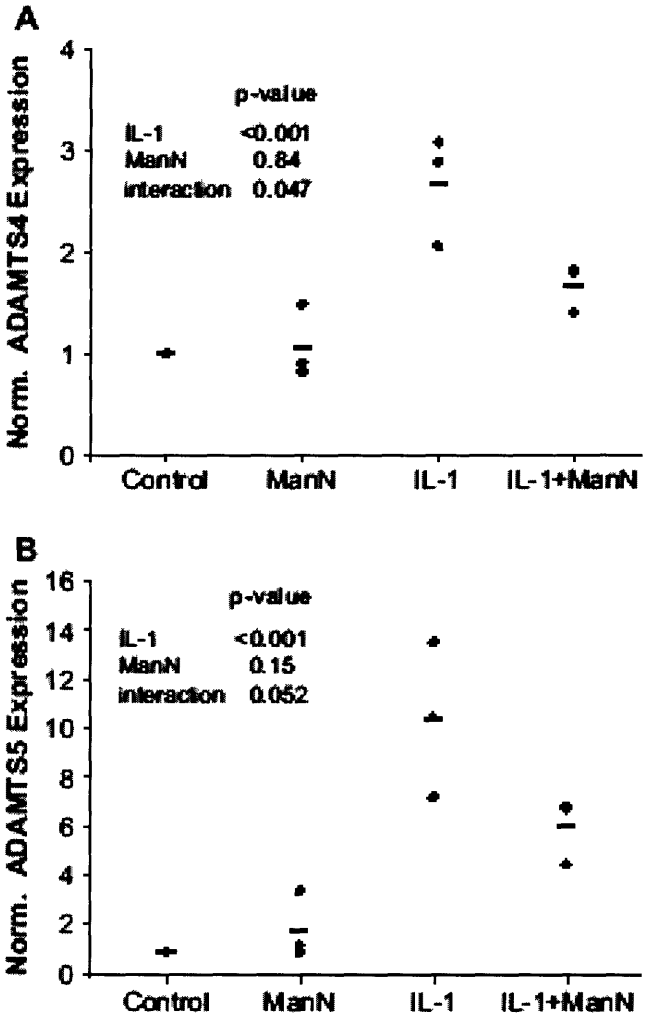


Figure D.1: Real-time PCR analysis of ADAMTS-4 and ADAMTS-5. Cartilage disks were incubated with no additives (control), 10 ng/ml IL-1α (IL-1), 1.35 mM mannosamine (ManN), or the combination (IL-1 + ManN). After 1 day of culture, mRNA was extracted and real-time PCR was performed. Expression levels for (A) ADAMTS-4 and (B) ADAMTS-5 are shown relative to untreated control disks for three replicate experiments as individual data points (circles) and mean (bars). The effect of ManN, IL-1, and the interaction between ManN and IL-1 was tested for significance by linear regression.

D.2. EXPRESSION OF DEGRADATIVE ENZYMES IN A DYNAMICALLY LOADED CHONDROCYTE-SEEDED PEPTIDE HYDROGEL (J. Kisiday)

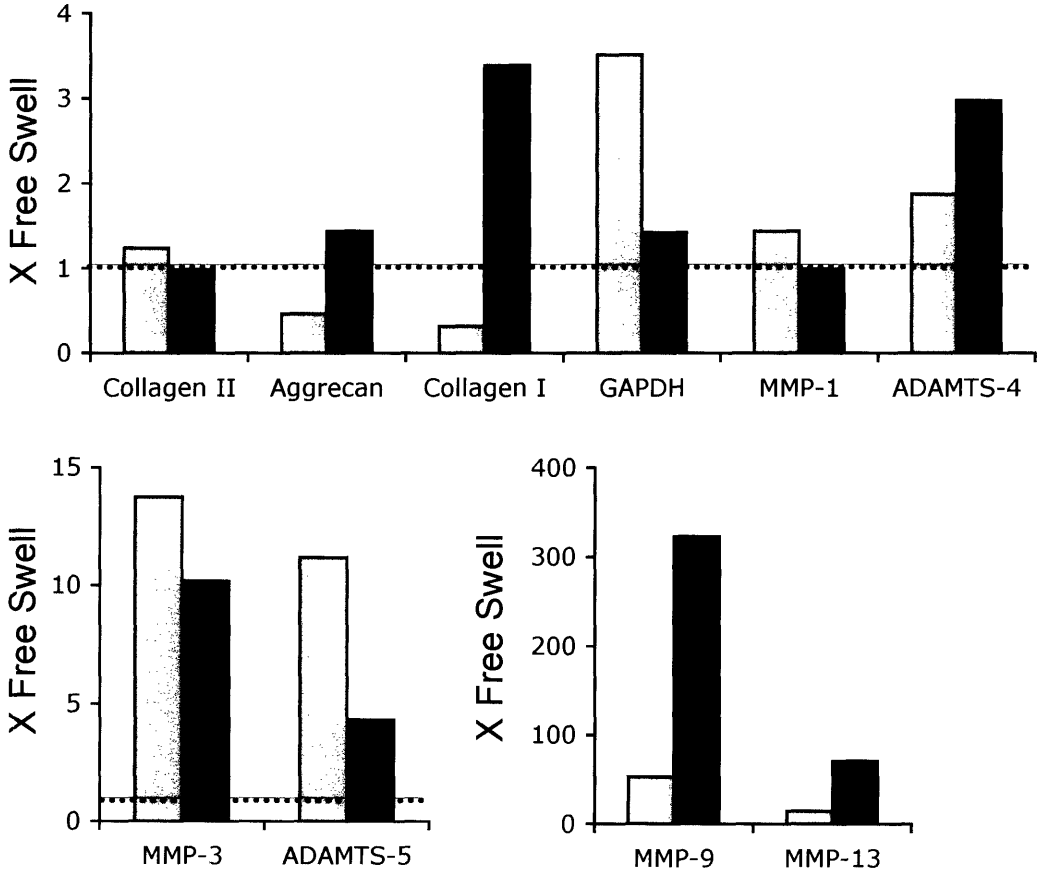


Figure D.2: Real-time PCR analysis of gene expression in dynamically loaded chondrocyte-seeded peptide hydrogel. 1-2 week old bovine chondrocytes were seeded at $30x10^6$ cells/ml into 0.36% self-assembling peptide scaffolds. Disks 1.6 mm thick, 12 mm diameter were punched after seeding and maintained in free swelling culture for 12-14 days before loading. Dynamic compression (2.5% strain amplitude, 0.3 Hz, 5% strain static offset) was applied in cycles of 4 repetitions of 45 min of loading/5 hr 15 min free swell followed by 24 hours without loading. Control gels were maintained as parallel free swell cultures. Samples were frozen for PCR on days 3 (light blue bars) and 13 (dark blue bars) within 30 min of the end of a loading cycle.

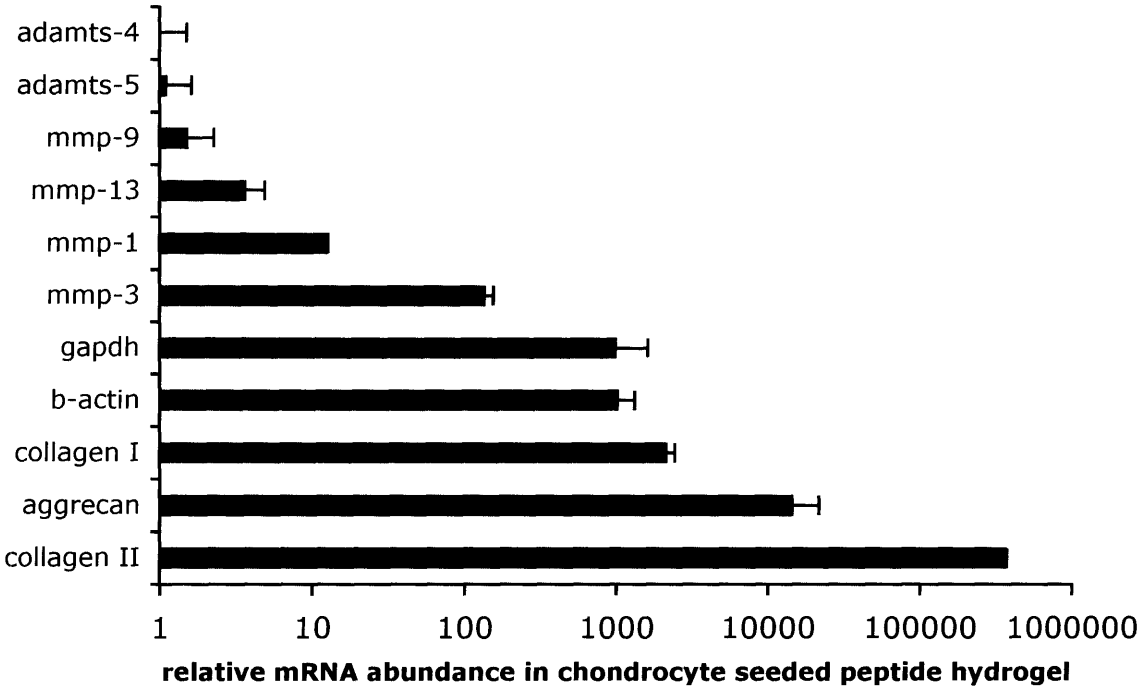


Figure D.3: Free swelling expression levels of 24 genes ranked by relative abundance in chondrocyte seeded peptide hydrogel. RNA was extracted from chondrocyte seeded self-assembling peptide hydrogel following 15-17 days in free swelling culture. Expression levels were normalized to expression of ADAMTS-4, the least abundant gene measured. Data are reported as Mean ± SE for n = 2 replicate experiments using tissue from 3 different joints.

D.3. ZYMOGRAPHY ON CONDITIONED MEDIUM SAMPLES IN COLLABORATION WITH THE KURZ LAB (J. Fay, Kiel, Germany)

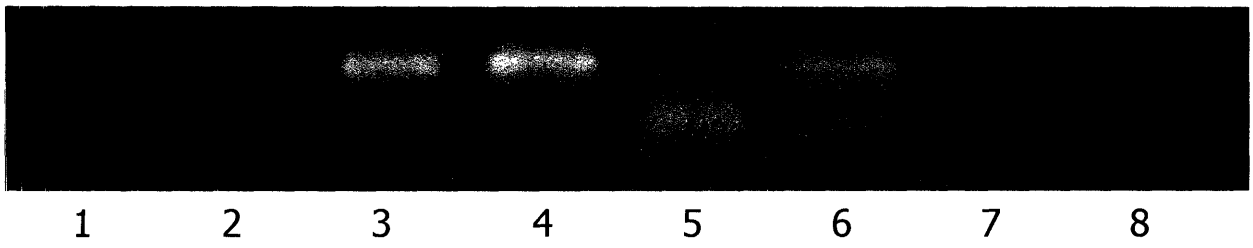


Figure D.4: Casein zymogram on conditioned medium from cartilage explants stimulated with bacterial supernatants. Medium was collected from cartilage explant cultures after 48 hours of stimulation with staph aureus or pseudomonas aeroginosa either in the presence or absence of superoxide-dismutase (SOD). Lanes: 1. Control; 2. Control + SOD; 3. Staph aureus stimulation;; 4. Staph aureus + SOD; 5. Pseudomonas aeroginosa stimulation; 6. Pseudomonas aeroginosa + SOD; 7. Pure (1:100) supernatant of Staph aureus; 8. Pure (1:100) supernatant of Pseudomonas. Bands were between 66 kDa and 45 kDa molecular weight markers.

Figure D.5: Gelatin zymogram on conditioned medium from cartilage explants stimulated with bacterial supernatants. Medium was collected from cartilage explant cultures after 48 hours of stimulation with staph aureus or pseudomonas aeroginosa either in the presence or absence of superoxide-dismutase (SOD). Lanes: 1. Control; 2. Control + SOD; 3. Staph aureus stimulation;; 4. Staph aureus + SOD; 5. Pseudomonas aeroginosa stimulation; 6. Pseudomonas aeroginosa + SOD; 7. Pure (1:100) supernatant of Staph aureus; 8. Pure (1:100) supernatant of Pseudomonas. Bands were between 66 kDa and 45 kDa molecular weight markers.

APPENDIX E: CENTOCOR COLLABORATIVE PROJECTS

All experiments were designed, performed, and analyzed with Mike DiMicco.

It has been shown previously that IL-1 and TNF-α interact with mechanical injury of bovine and human cartilage, inducing a synergistic release of GAG from the cartilage to the medium (Patwari, 2003). Likewise, it has been reported these cytokines act synergistically with IL-6/sIL-6R to induce greater GAG release from bovine cartilage than treatment with either IL-1α or TNF-α (Flannery, 2000; Rowan, 2001). Experiments described in this Appendix were designed to test if the combination of mechanical injury, IL-1/TNF, and IL-6 result in additional synergistic responses in release of GAG to the medium or altered biosynthesis rates and to test if co-culture with joint capsule tissue is affected by addition of exogenous IL-6 or IL-6 blockade.

E.1. INTERACTION BETWEEN IL-1, IL-6, AND MECHANICAL INJURY

Table E.1: Description of sample treatment groups to test for interactions between IL-6, IL-1α, and mechanical injurious compression.

Group	n	IL-6/sIL-6R, 50/250 ng/mL	IL-1α, 1 ng/mL	injury
A	4	yes	no	no
B	4	yes	yes	no
C	4	yes	no	yes
D	4	yes	yes	yes
E	4	no	no	no
F	4	no	yes	no
G	4	yes	no	no
H	4	yes	yes	no

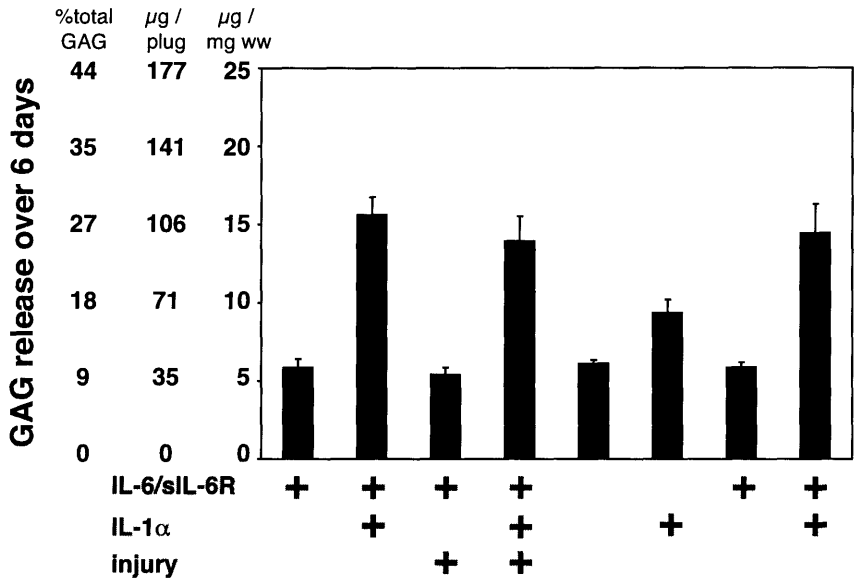


Figure E.1: Cumulative GAG released to the medium during six days in culture with combinations of exogenous IL-6, IL-1α, and mechanical injurious compression. Various units are plotted on the y-axis for comparison to previously published work. Mean±SE, n=3-4.

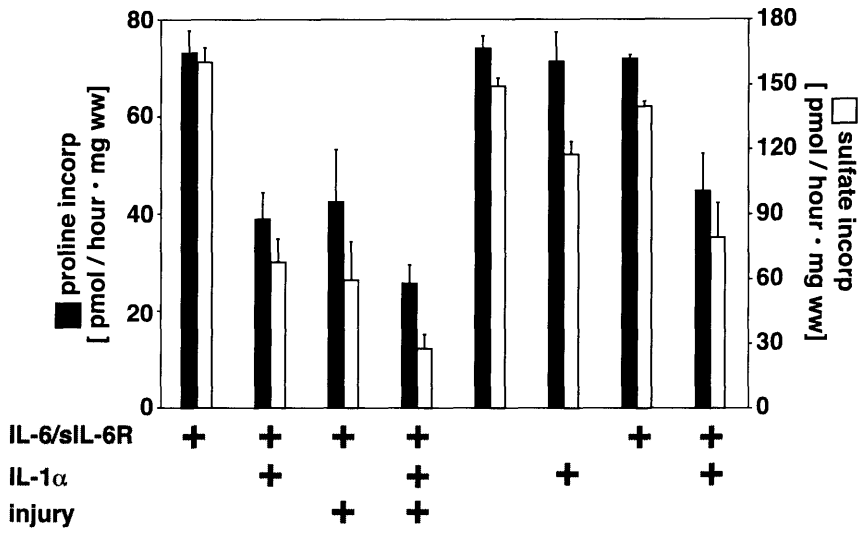


Figure E.2: Biosynthesis rates on days 5-6 in response to treatments with exogenous IL-6, IL-1α, and mechanical injurious compression. Mean±SE, n=3-4.

Treatment with IL-6/sIL-6R alone had no effect on GAG release, GAG biosynthesis, or protein biosynthesis, evaluated by comparing groups E and G. This lack of IL-6/sIL-6R effect on GAG release is in agreement with Flannery, 2000. Treatment with IL-1α resulted in 50% GAG release over the 6-day culture, and reduced sulfate (-21%) incorporation, with no effect on proline incorporation. Addition of injury did not affect the total GAG release from injured cartilage samples cultured in the presence of IL-6/sIL-6R for 6 days, but it did reduce both the proline and sulfate incorporations compared to treatment with IL-6/sIL-6R alone. The combination of IL-1α and IL-6/sIL-6R increased GAG release over either cytokine separately; this increase appeared to be more than additive in agreement with Flannery, 2000. There also appeared to be a synergistic reduction in sulfate and proline incorporations, which has not been previously reported. The addition of injury, however, did not appear to further increase the GAG release over treatment with the two cytokines, though injury further reduced both proline and sulfate incorporation.

There are two novel findings in this study. The first is that IL-1α and IL-6 act synergistically to reduce GAG and protein biosynthesis, in addition to the synergistic increase in GAG release previously observed, and seen again here. The second novel finding is that in contrast with previous studies where IL-1α and injury acted synergistically to increase GAG release, there was no such interaction in the presence of IL-6 and its soluble receptor (however, injury did further decrease both proline and sulfate incorporation compared with IL-1+IL-6-treated samples). Since this experiment did not compare samples injured with IL-1α and IL-6/sIL-6R to samples injured in medium with only IL-1α, it is not possible to conclusively determine that IL-6 interfered with the IL-1α/injury synergy, but it may be interesting to pursue this finding in future studies.

E.2. INTERACTION BETWEEN TNF-α, IL-6, AND MECHANICAL INJURY

Table E.2: Description of sample treatment groups to test for interactions between IL-6, TNF-α, and mechanical injurious compression.

Group	n	IL-6/sIL-6R, 50/250 ng/mL	TNF-α, 100 ng/mL	injury
A	4	yes	no	no
B	4	yes	yes	no
C	4	yes	no	yes
D	4	yes	yes	yes
E	4	no	no	no
F	4	no	yes	no
G	4	yes	no	no
H	4	yes	yes	no

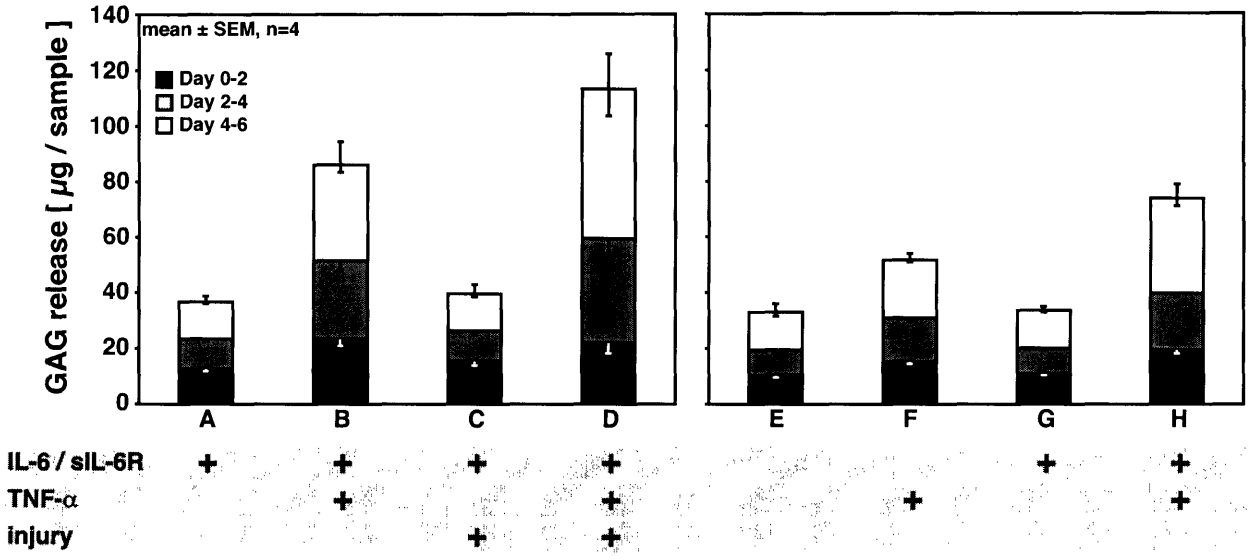


Figure E.3: Cumulative GAG released to the medium during six days in culture with combinations of exogenous IL-6, TNF-α, and mechanical injurious compression. Mean±SE, n=4.

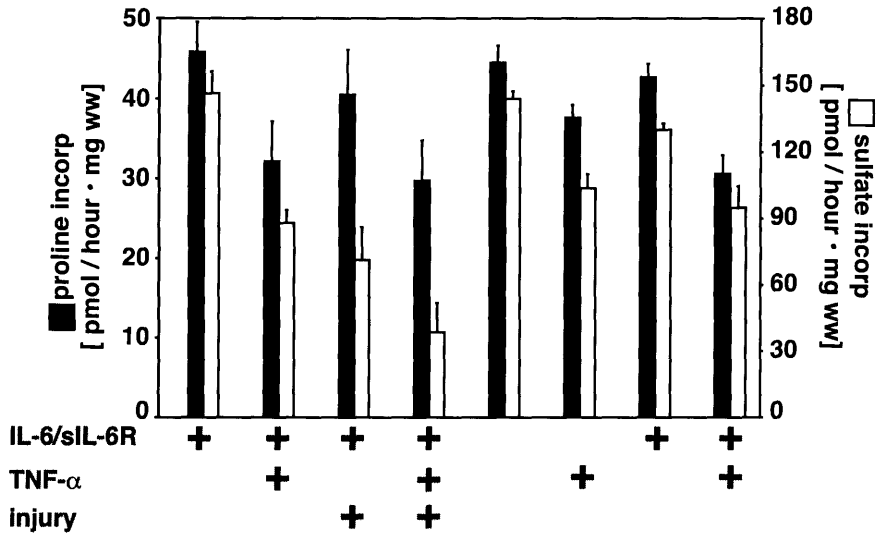


Figure E.4: Biosynthesis rates on days 5-6 in response to treatments with exogenous IL-6, TNF-α, and mechanical injurious compression. Mean±SE, n=4.

Treatment with IL-6/sIL-6R alone did not result in a significant release of GAG or change in proline incorporation but did reduce sulfate incorporation by 10% ($p<0.05$). Treatment with TNF-α significantly increased GAG release by 57% over controls ($p < 0.005$) and decreased proline and sulfate incorporation. Analysis by 2-way ANOVA (E, F, G, H) revealed significant effects of treatment with TNF-α ($p < 0.001$) and IL-6 ($p < 0.005$) on proline incorporation rate; there was also a significant interaction between the two cytokines ($p < 0.01$). Treatment with both cytokines resulted in GAG release that was higher than either IL-6 alone (by 120%, $p < 0.001$), or TNF-α alone (by 43%, $p < 0.01$). Addition of injury did not affect the total GAG release from injured cartilage samples cultured in the presence of IL-6 ($p = 0.5$). In the presence of IL-6, analysis by 2-way ANOVA indicated that there was a significant effect of treatment with TNF-α ($p < 0.001$), but no effect of injury ($p = 0.08$), and no interaction ($p = 0.15$). Treatment with TNF-α and injury was higher than injury alone (by 186%, $p < 0.001$), but was not higher than TNF-α alone ($p=0.12$).

The main objective of this experiment was to investigate the effect of including injury with cytokine treatments. In the presence of IL-6, the addition of injury did not affect GAG release. Furthermore, the increase in GAG release with IL-6, TNF-α, and injury can be attributed to the presence of TNF-α, since there was no main effect of injury ($p = 0.08$), nor an interaction between injury and TNF-α ($p=0.15$) in the presence of IL-6.

E.3. INTERACTION BETWEEN CO-CULTURE, IL-6, AND MECHANICAL INJURY

Table E.3: Description of sample treatment groups to test for interactions between IL-6, co-culture with joint capsule tissue, and mechanical injurious compression.

Group	n	IL-6/sIL-6R, 50/250 ng/mL	joint capsule	injury
A	4	yes	no	no
B	4	yes	yes	no
C	4	yes	no	yes
D	4	yes	yes	yes
E	4	no	no	no
F	4	no	yes	no
G	4	yes	no	no
H	4	yes	yes	no

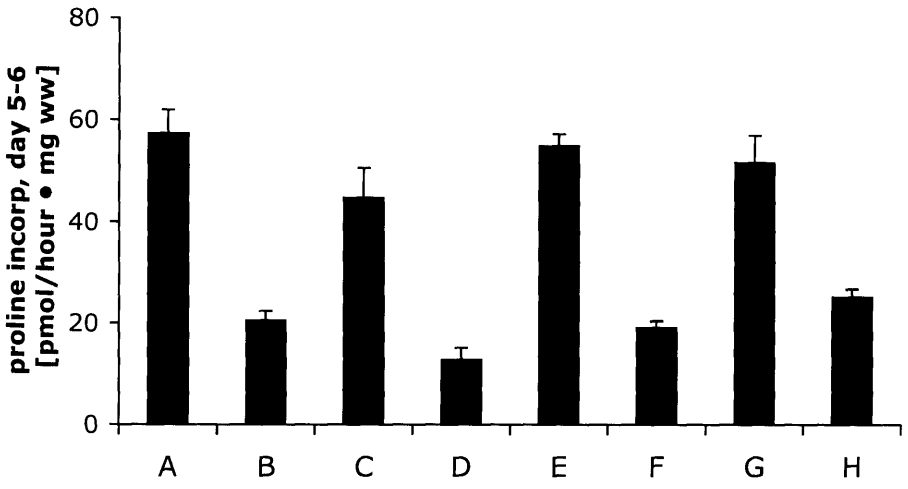


Figure E.5: Proline incorporation rate on days 5-6 in response to treatments with exogenous IL-6, co-culture with joint capsule tissue, and mechanical injurious compression. Mean±SE, n=4.

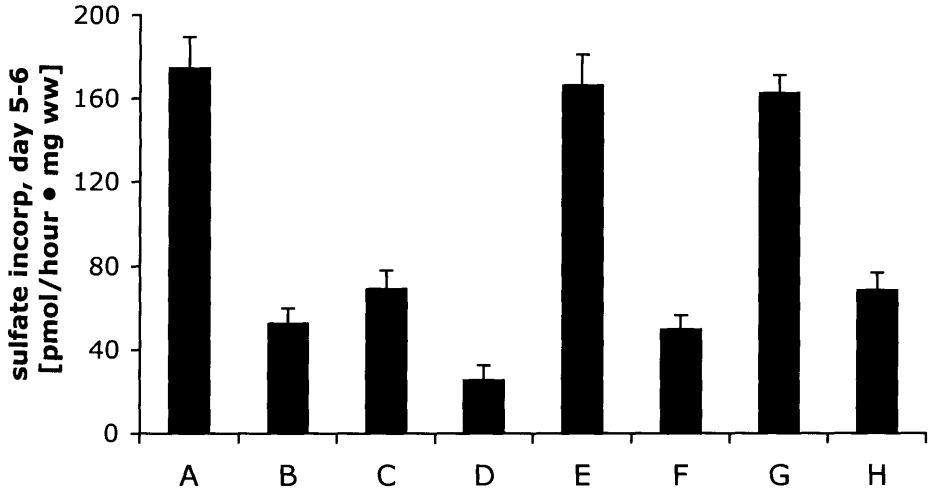


Figure E.6: Sulfate incorporation rate on days 5-6 in response to treatments with exogenous IL-6, co-culture with joint capsule tissue, and mechanical injurious compression. Mean±SE, n=4.

Treatment with IL-6/sIL-6R alone was assessed by comparing groups E and G, which were not significantly different in sulfate incorporation ($p = 0.84$) or proline incorporation ($p = 0.59$). Co-culture of cartilage with joint capsule tissue significantly decreased sulfate incorporation by 70% ($p < 0.005$), and proline incorporation by 65% ($p < 0.005$). Analysis by 2-way ANOVA (E, F, G, H) revealed no significant main effects of treatment with IL-6 on sulfate or proline incorporation (sulfate $p=0.95$, proline $p=0.66$). The effect of co-culture was significant ($p<0.001$ on both proline and sulfate incorporation). There was no significant interaction between IL-6 and co-culture on biosynthesis rates (proline $p=0.15$, sulfate $p=0.28$). Addition of injury did not affect the rate of proline incorporation in injured cartilage samples cultured in the presence of IL-6 ($p = 0.78$). However, there was a significant reduction in sulfate incorporation (-60%; $p < 0.02$). In the presence of IL-6, analysis by 2-way ANOVA indicated that there was a significant effect of co-culture on proline incorporation ($p < 0.001$), but no effect of injury ($p =$

0.36), and no interaction ($p = 0.71$). Both injury and co-culture affected sulfate incorporation, with no interaction ($p=0.06$).

The main objective of this experiment was to investigate the effect of including injury with co-culture and IL-6/sIL-6R treatments. In the presence of IL-6, the addition of injury significantly reduced sulfate incorporation as did co-culture with joint capsule. However there was no significant interaction between injury and co-culture in the presence of IL-6 on sulfate incorporation ($p=0.06$). Furthermore, the decreased proline incorporation with IL-6, co-culture, and injury can be attributed to the presence of joint capsule tissue, since there was no main effect of injury ($p = 0.36$), nor an interaction between injury and co-culture ($p>0.71$) in the presence of IL-6.

E.4. INTERACTION BETWEEN IL-6 AND MECHANICAL INJURY

Table E.4: Description of sample treatment groups to test for interactions between IL-6 and mechanical injurious compression.

Group	n	IL-6/sIL-6R, 50/250 ng/mL	injury
A	4	no	no
B	4	yes	no
C	4	no	yes
D	4	yes	yes

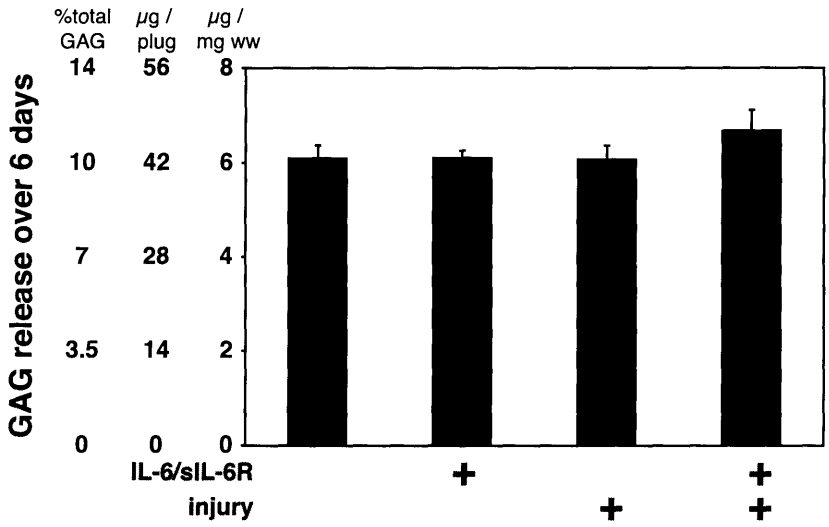


Figure E.7: Cumulative GAG released to the medium during six days in culture with combinations of exogenous IL-6 and mechanical injurious compression. Mean±SE, n=4.

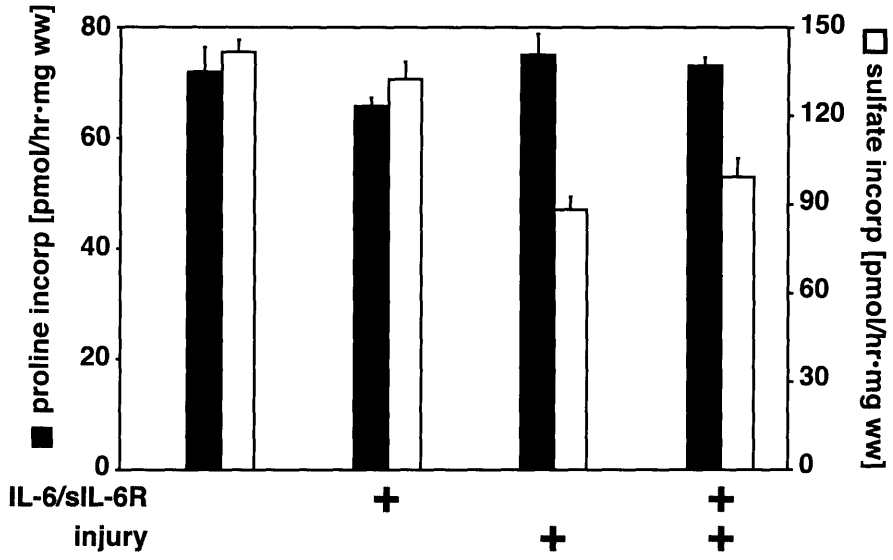


Figure E.8: Biosynthesis rates on days 5-6 in response to treatments with exogenous IL-6 and mechanical injurious compression. Mean±SE, n=4.

GAG release to the culture medium was measured over the 6 day culture period. As observed previously, the cumulative GAG release from injured samples was nearly identical to that from uninjured samples. Addition of IL-6/sIL-6R at 50/250 ng/ml had no effect on GAG release from free swelling controls and did not increase GAG release from injured samples.

Mechanical injury resulted in an approximately 40% decrease in sulfate incorporation; injury had no effect on proline incorporation in this study. There was no apparent effect of IL-6/sIL-6R treatment on biosynthesis rates.

E.5. EFFECT OF IL-6 BLOCKADE ON CO-CULTURE OF CARTILAGE WITH JOINT CAPSULE TISSUE

Table E.5: Description of sample treatment groups to test for an effect of IL-6 blockade on the reduction in biosynthesis observed during co-culture with joint capsule tissue.

Group	n	joint capsule	IL-6 mAb
A	4	no	no
B	4	yes	no
C	4	no	yes
D	4	yes	yes

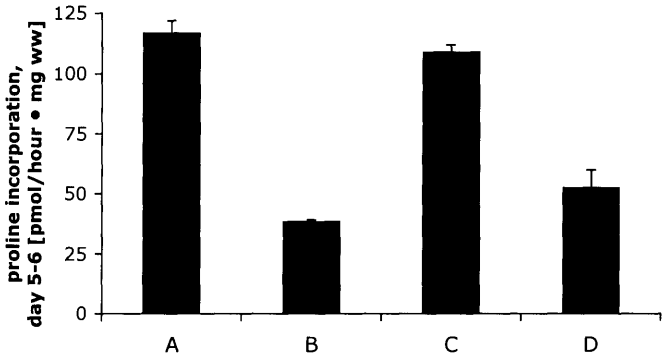


Figure E.9: Proline incorporation rate on days 5-6 during co-culture with joint capsule tissue and treatment with a monoclonal antibody to IL-6. Mean±SE, n=4.

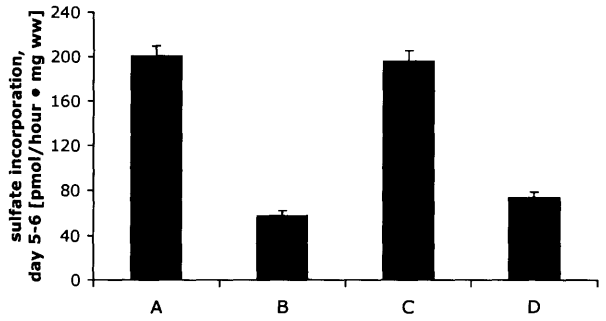


Figure E.10: Sulfate incorporation rate on days 5-6 during co-culture with joint capsule tissue and treatment with a monoclonal antibody to IL-6. Mean±SE, n=4.

Treatment with IL-6 mAb alone did not affect sulfate incorporation or proline incorporation. Co-culture of cartilage with joint capsule tissue significantly decreased sulfate incorporation by 71%, and proline incorporation by 67%. Addition of the IL-6 mAb to cartilage co-cultured with joint capsule resulted in a slight block of the biosynthesis reduction seen for co-culture alone (sulfate to 63% reduced compared to 71% for co-culture alone; proline to 55% reduction compared to 67% for co-culture alone).

The IL-6 mAb showed a partial restoration of biosynthesis levels of both proline and sulfate toward levels seen in control cartilage. A second set of experiments was carried out to increase the concentration of IL-6 mAb and test for more complete restoration of biosynthesis. Experiments were also performed in which the IL-6 Fab fragment to block IL-6 was used in place of the full-length mAb.

Table E.6: Description of sample treatment groups to test for an effect of IL-6 blockade with full length antibody or Fab fragment on reduction in biosynthesis observed during co-culture with joint capsule tissue.

Group	n	joint capsule	anti-IL-6 mAb [μg/mL]	anti-IL-6 Fab [μg/mL]
A	4	—	—	—
B	4	yes	—	—
C	4	yes	10	—
D	4	yes	100	—
E	4	yes	500	—
F	4	yes	—	4
G	4	yes	—	40
H	4	yes	—	200

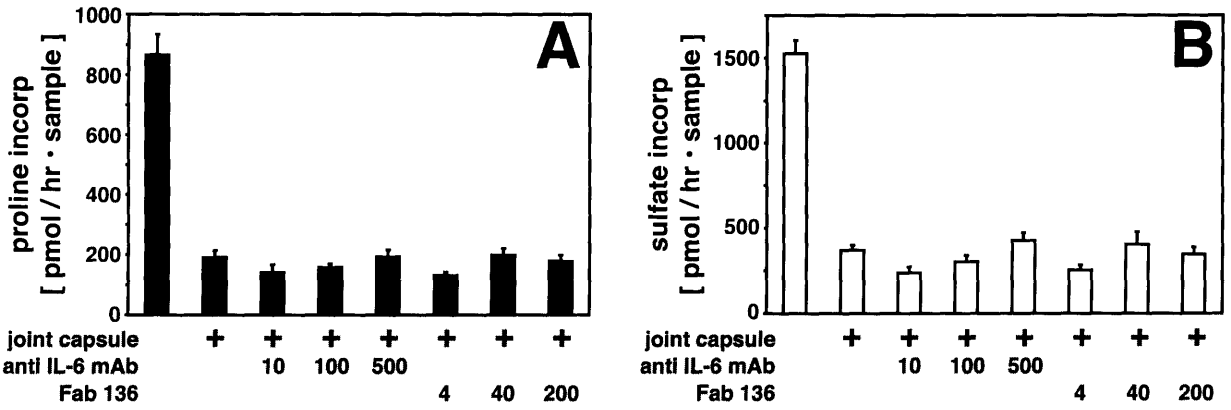


Figure E.11: Biosynthesis rates on days 5-6 during co-culture with joint capsule tissue and treatment with either a full length monoclonal antibody to IL-6 or the Fab fragment of the IL-6 antibody. Mean±SE, n=4.

Table E.7: Properties of IL-6 blocking antibodies provided by Centocor.

Parameter	anti IL-6 mAb	Fab 136
molecular mass (by SDS-PAGE)	148 kDa	48 kDa
EC_{50} vs 500 pg/mL rhIL-6	3.4 pM (50 ng/mL)	8.5 pM (41 ng/mL)

The presence of joint capsule in the same culture well with cartilage explants caused a ~80% reduction in both proline and sulfate biosynthesis, compared with cartilage cultured without joint capsule tissue present in the well. It had been previously determined that there was no effect of IL-6 mAb treatment alone on either proline or sulfate biosynthesis at 10 μg/mL. In the present experiment, the concentration of mAb was increased 10-fold and 50-fold, and in neither case was there an effect on the joint capsule-induced reduction of biosynthesis. To test whether the efficacy of IL-6 blockade in this system was related to the penetration of the large mAb molecules into cartilage during culture, we also tested solutions containing the smaller Fab 136 fragment of the anti-IL-6 mAb. At concentrations of 5, 40, or 200 μg/mL, there was no effect on the capsule-induced reduction in biosynthesis.

As has been seen previously, the co-culture of joint capsule tissue with cartilage is a potent inhibitor of chondrocyte biosynthesis. This experiment verified that using relatively high concentrations of anti IL-6 monoclonal antibodies, or their Fab fragments, had no effect on the reduction of biosynthesis in this model system.

Although it was not known whether IL-6 was involved in the biosynthesis effects observed with joint capsule co-culture, it is apparent that a soluble factor is involved that either directly or indirectly results in this reduction:

- A *direct mechanism* implies that a soluble factor acts directly on the cartilage, inducing a reduction in biosynthesis. Based on our previous experiments, in which IL-6 and its soluble receptor were added directly to the culture medium, this cytokine alone does not cause a change in biosynthesis, and therefore is not a direct mediator of this biosynthesis reduction (however, the fact that it interacted with exogenous recombinant IL-1α and TNF-α to induce a synergistic GAG release implies that the recombinant human proteins are active in a bovine tissue system). Moreover, the concentrations of antibodies used here were up to 10,000 times higher than the EC_{50} values quoted from Centocor for cell-based assays. At these high concentrations, the antibodies should be more than sufficient to bind any IL-6 in solution released from the joint capsule before it reaches the cartilage.
- An *indirect mechanism* of action would involve a soluble factor from the capsule inducing the production of one or more other molecules by chondrocytes, and it is these induced molecules that exert the inhibitory effect. If IL-6 were among these induced molecules, the neutralizing antibodies would need to be present within the tissue, at the cells, so that they could intercept the IL-6 before it could exert an effect. It is possible that at 48 kDa, even the Fab fragment is not sufficiently small to penetrate the cartilage matrix to a meaningful extent.

Finally, a recurring problem in the analysis of these experiments is the issue of cross-species reactivity between the tissue and any induced molecules (bovine), antibodies and their fragments (anti-human), and in previous studies, exogenous cytokines (human). It is possible that the antibodies used here do not recognize the bovine protein, and therefore, are not able neutralize it. Indeed, the sequence homology between bovine and human IL-6 is approximately 49%, and there are few long homologous stretches within the molecule. If the antibody and protein do not cross-react, bovine IL-6 in the culture medium (from the capsule) could not be bound by the antibody, and even if the Fab fragment could penetrate the cartilage, it would be unable to neutralize any processes dependent on induced IL-6.

In summary, the antibodies used here should be present at high enough concentrations to neutralize IL-6 dependent processes. That no effect was observed means that at least one of the following is true:

- IL-6 is not involved in the co-culture induced biosynthesis reduction
- The antibodies do not penetrate the cartilage, and therefore are ineffective against induced IL-6 activity
- The antibodies do not recognize bovine IL-6, and therefore have no activity in this system