CARTILAGE MECHANOBIOLOGY AND TRANSCRIPTIONAL EFFECTS OF COMBINED MECHANICAL COMPRESSION AND IGF-1 STIMULATION ON BOVINE CARTILAGE EXPLANTS

Cameron A Wheeler
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May 2006

Signature of Author_____
Cameron A Wheeler
Department of Biological Engineering

Certified by_____
Alan J. Grodzinsky, Thesis Advisor
Professor of Mechanical, Electrical, and Biological Engineering

Accepted by_____
Ram Sasisekharan, Graduate Program Co-Chairman
Professor of Biological Engineering

Abstract

Background: Investigators have focused on mechano-regulation of upstream signaling and responses at the level of gene transcription, protein translation and post-translational modifications. Intracellular pathways including those involving integrin signaling, mitogen activated protein kinases (MAPKs), and release of intracellular calcium have been confirmed in several laboratories.

Studies with IGF-1: Insulin-like growth factor-1 (IGF-1) is a potent anabolic factor capable of endocrine and paracrine/autocrine signaling. Previous studies have demonstrated that mechanical compression can regulate the action of IGF-1 on chondrocyte biosynthesis in intact tissue; when applied simultaneously, these stimuli act by distinct cell activation pathways. Our objectives were to elucidate the extent and kinetics of the chondrocyte transcriptional response to combined IGF-1 and static compression in cartilage explants. ***Discussion:*** Clustering analysis revealed five distinct groups. TIMP-3 and ADAMTS-5, MMP-1 and IGF-2, and IGF-1 and Collagen II, were all robustly co-expressed under all conditions tested. In comparing gene expression levels to previously measured aggrecan biosynthesis levels, aggrecan synthesis is shown to be transcriptionally regulated by IGF-1, whereas inhibition of aggrecan synthesis by compression is not transcriptionally regulated. ***Conclusion:*** Many genes measured are responsive the effects of IGF-1 under 0% compression and 50% compression. Clustering analysis revealed strong co-expressed gene pairings. IGF-1 stimulates aggrecan biosynthesis in a transcriptionally regulated manner, whereas compression inhibits aggrecan synthesis in a manner not regulated by transcriptional activity.

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Chapter 1

Mechanobiology: Introduction, Background, and Significance*

* This chapter has appeared as a review paper in Current Opinions in Orthopaedics (*Wheeler, Cameron A; Fitzgerald, Jonathan B; Grodzinsky, Alan* **Cartilage mechanobiology: the response of chondrocytes to mechanical force.** Current Opinion in Orthopedics. 16(5):346-353, October 2005.)

1.1 Purpose of the Review

A comprehensive understanding of chondrocyte mechanobiology is critically important for a clear understanding of the etiopathology and treatment of osteoarthritis (OA) as well as for the long-term survival of tissue engineered implants for cartilage repair.

Recent Findings

A large body of evidence has emerged documenting the effects of various mechanical loading modalities on chondrocyte biosynthesis and gene expression. Many physical forces and flows occur in cartilage during loading *in vivo*. For example, dynamic compression of cartilage results in deformation of cells and the extracellular matrix, hydrostatic pressurization of the tissue fluid, pressure gradients and the accompanying flow of fluid within the tissue, and streaming potentials and currents induced by fluid convection of counter-ions through the negatively charged extracellular matrix (ECM). In addition, local changes in tissue volume caused by compression also lead to alterations in matrix water content, ECM fixed charge density, mobile ion concentrations, and osmotic pressure. Any of these mechanical and physicochemical phenomena in the micro-environment of chondrocytes may affect cellular metabolism. While specific components of certain mechanotransduction pathways have been identified, the exact mechanisms by which mechanical forces influence the biological activity of chondrocytes are not yet fully understood. New genomic and proteomic technologies and methodologies including systems biological analyses are being applied to better understand cellular mechanotransduction.

Summary

Investigators have focused on mechano-regulation of upstream signaling and responses at the level of gene transcription, protein translation and post-translational

modifications. Intracellular pathways including those involving integrin signaling, mitogen activated protein kinases (MAPKs), and release of intracellular calcium have been confirmed in several laboratories.

1.2 Introduction

Articular cartilage is an avascular, aneural, alymphatic tissue that provides a low friction weight bearing surface for joint locomotion. During joint loading *in vivo*, cartilage is subjected to mechanical stresses and strains that span a wide range of amplitudes and frequencies [1, 2]. Peak stresses can reach 10–20 MPa (100–200 atm) during activities such as stair climbing [3]. While compressive strains of 15% – 40% may occur in response to long-term or "static" loads within the physiological range [1], compressions of only a few percent occur during normal ambulation (e.g., the "dynamic" strains that occur at walking frequencies of ~1 Hz). Chondrocytes occupy 3% to 5% of tissue volume in adult human cartilage [1]. These cells maintain a mechanically functional extra-cellular matrix (ECM) by mediating the synthesis, assembly, and degradation of proteoglycans (PGs), collagens, glycoproteins, and other matrix molecules. It is well known that chondrocytes can sense and respond to their mechanical environment; however, the mechanotransduction pathways by which mechanical forces influence the biological activity of chondrocytes are not fully understood.

1.3 Systems for Studying Chondrocyte Mechanotransduction

Since mechanotransduction mechanisms are difficult to quantify *in vivo*, model systems such as cartilage explant organ culture and three dimensional chondrocyte/gel culture have been used. Cartilage explants preserve native tissue structure and cell-matrix interactions and thereby enable quantitative correlations between mechanical loading parameters and biological responses such as gene expression and biosynthesis. Muir [4]

emphasized the important but complex role of the native ECM and chondrocyte-ECM interactions in chondrocyte response to load; thus, investigators [4] have cautioned that the use of isolated, plated chondrocytes that are depleted of ECM must be approached with care regarding the potential for chondrocyte dedifferentiation and the interpretation of the results in relation to the behavior of cartilage. In native tissue, however, the coupling between mechanical, chemical, and electrical forces and flows within the ECM can complicate the identification of specific physical stimuli, necessitating specialized experimental and theoretical modeling approaches. Therefore, three-dimensional agarose [5], alginate [6], and other scaffold culture systems have also been used to study chondrocyte response to mechanical compression[7-9], hydrostatic pressure [10], stretch [11], physicochemical stimuli (pH and osmolarity [12], and electrical currents [13]). Finally, a variety of specialized, incubator-housed instruments have been developed to mimic mechanical stimuli found *in vivo* and apply components of compression, shear, stretching, hydrostatic or osmotic pressure to explants, isolated cells, or cell-encapsulated gel constructs *in vitro* [14-17], shown schematically in Fig. 1.1.

1.4 Chondrocyte biosynthesis and gene expression

Static compression (Fig. 1.1a) of animal and human cartilage explants [18, 19] as well as high *hydrostatic pressure* applied to chondrocyte monolayers [20] can cause a dose-dependent decrease in the biosynthesis of proteoglycans, collagens, and other ECM proteins as quickly as one hour after application of compression. Complete recovery of biosynthesis can occur after release of compression, but at different rates for different ECM macromolecules [21], strongly suggesting that specific transduction pathways are involved. In contrast, *dynamic compression and shear* (Fig 1.1b,c) [18, 22, 23] and cyclic hydrostatic compression [20, 24] can markedly upregulate ECM biosynthesis in a manner

dependent on compression amplitude and frequency [20, 22], as well as the developmental stage and the depth from the articular surface of the cartilage sample [14, 25, 26]. Tissue-level and cell-level quantitative autoradiography have been used to visualize the spatial distribution of newly-synthesized ECM molecules in response to compression and shear [23, 27, 28], and to compare with the theoretically predicted profiles of physical stimuli, highlighting the roles of ECM and cell deformation as well as intratissue fluid flow (shown schematically in Fig. 1.1).

Mechanical forces can also influence aggrecan gene expression [29-33] and the transcription of many matrix proteins and proteases in chondrocytes and other connective tissue cells [33-36]. Investigators have also found that *fluid shear flow* [37-40] can alter aggrecan synthesis and the expression of aggrecan, TIMP-1, IL-6 and MMP-9. The induction of MMP-9 gene expression appeared to be mediated via the JNK signaling pathway [38], and the aggrecan promoter via the ERK pathway [39]. While the fluid velocities in these experiments were much higher than physiological for cartilage, the resulting shear stresses may be relevant. When isolated bovine and human chondrocytes were *cyclically stretched on flexible membranes*, aggrecan and type II collagen mRNA expression were increased [40], consistent with a role for cell deformation and membrane perturbation. Cyclic (1 Hz square wave) uniaxial stretch (5% elongation) of embryonic chick sternal chondrocytes seeded into a 3D collagen sponge induced expression of Indian hedgehog (Ihh) and also upregulated bone morphogenic proteins 2 and 4 downstream of Ihh which, in combination, stimulated cell proliferation [11]. Interestingly, mechanical induction of Ihh mRNA was abolished by blocking stretch activated channels [11].

1.5 Upstream Signaling

Investigators have been trying to map the sequential intracellular signaling pathways through which mechanical forces can modify the gene expression of specific molecules. Major roles have been identified for certain classical signaling pathways including those involving integrins, mitogen activated protein kinases (MAPKs), and release of intracellular calcium.

Integrin signaling pathways

Evidence suggests that integrins can convert extracellular mechanical stimuli into intracellular signals in a variety of cell types [41]. In chondrocytes, the alpha $\alpha5\beta1$ fibronectin-binding integrins have been implicated as part of a mechanotransduction complex that involves tyrosine protein kinases, cytoskeletal proteins, ion channels, and second-messenger signaling cascades [42, 43]. Researchers have also shown that the $\alpha5\beta1$ integrin complex is present in OA chondrocytes, but results in different downstream effects when activated or blocked compared to normal chondrocytes [44]. Application of hydrostatic pressure to chondrocyte monolayers in a manner that induced strain on the culture dish and plated cells caused interleukin-4 (IL-4) secretion via $\alpha5\beta1$ integrin and subsequent intracellular calcium release followed by cell hyperpolarization [42, 45]. One possible connecting link is the N-methyl-D-aspartate (NMDA) receptor, since integrin signaling has been shown to influence the activity of this receptor in other cells [46]. NMDA is phosphorylated by protein kinases including protein kinase C (PKC) and phosphotidylinositol 3-kinase (PI3K) [47]. Salter el al. observed that the NMDA receptor induced depolarization in OA chondrocytes and hyperpolarization in normal chondrocytes, suggesting a possible alteration in chondrocytic mechanotransduction as a consequence of the function of the NMDA receptor during OA [48].

Mitogen activated protein kinase pathways

Investigators have been trying to map the sequential intracellular signaling pathways through which mechanical forces may modify chondrocyte gene expression of specific molecules. Several recent studies have demonstrated a role for mitogen activated protein kinases (MAPKs) [49, 50] which can alter matrix gene expression and changes in matrix production by chondrocytes within compressed cartilage and in chondrocyte monolayers [51]. This family of ubiquitous signaling molecules includes extracellular-signal regulated protein kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and p38. Activated MAP kinases are thought to translocate to the nucleus, where they may induce phosphorylation of transcriptional factors and eventual upregulation of various genes.

Fanning et al. [52] examined the effects of slow ramp-and-hold compression of cartilage explants to final static strains up to 50% that were held for a range of compression durations; these compression conditions were found previously to inhibit chondrocyte biosynthesis but not to affect cell viability. Mechanical compression caused (1) a rapid induction of ERK1/2 phosphorylation at 10 min followed by a rapid decay, as well as a sustained level of ERK2 phosphorylation that persisted for at least 24 hrs; (2) phosphorylation of p38 in strictly a transient fashion, with maximal phosphorylation occurring at 10 min; and (3) stimulation of SEK1 phosphorylation with a maximum at the relatively delayed time point of 1hr and with a higher amplitude than ERK1/2 and p38 phosphorylation. (SEK1is an immediate upstream specific activator of JNKs 1,2 and 3 [53], and the JNK and p38 kinases together constitute the SAPK sub-family of MAPKs [54]). Fanning et al. [50] proposed that the rapid activation of ERK1/2 and p38 may be due to the cell deformation, fluid flow and pressurization, while the SEK1 pathway was activated only under static compression without fluid flow or pressurization [50]. Thus, it was suggested that the initial transient ERK1/2 response was due to the dynamic components of static compression, consistent with the results of Li et al. [49], who found a significant upregulation of ERK1/2 activation in response to dynamic compression.

ATP and Ca^{2+}

Ion channels have been identified as another important factor in mechanotransduction, including effects of cell stretching on chondrocyte hyperpolarization and depolarization [55]. ATP has been shown to be involved in signaling in many cell types. Under compressive conditions, bovine chondrocytes can release ATP [56, 57] which, in the extracellular space, can then bind to membrane receptors and initiate a signaling cascade including stimulation of an increase in proteoglycan synthesis [58]. While ATP can induce anabolic signaling in normal chondrocytes, OA chondrocytes do not show upregulation of matrix production. Mechanical stimulation can also increase the concentration of intracellular calcium ions, derived either from intracellular stores or from the extracellular space and transported into the cell via stretch activated ion channels. While hyperosmotic stress can initiate intracellular Ca^{2+} signaling in chondrocytes [59], Erickson et al. demonstrated that the stretch activated ion channels were not necessarily responsible for Ca^{2+} transients under these conditions. Cell volume was also shown to decrease under hyperosmotic stress and, hence, the stretch effect was explained by an inhomogeneity in the cell surface [59]. The role of intracellular calcium in native cartilage explants was studied by Vahlmu and Raia [60]; using blockers of intracellular Ca^{2+} and protein kinase C, they demonstrated that regulation of aggrecan mRNA levels under creep compression involved Ca^{2+}/calmodulin and myo-inositol 1,4,5-triphosphate signaling processes. Fitzgerald et al. also found that compression of cartilage explants induces multiple time-dependent gene expression patterns that involve intracellular calcium and cyclic AMP [61].

1.6 Pro-inflammatory pathways in normal and injurious compression

Acute traumatic joint injury increases the risk for subsequent development of OA [62]. In order to quantify the events following cartilage and joint injury, investigators have turned to a variety of *in vitro* and animal models. Studies have shown that threshold levels of compressive strain, strain rate, and peak stress can cause cartilage matrix disruption, tissue swelling, cell necrosis and apoptosis, and increased loss of matrix macromolecules [63-70]. As a baseline control for changes in gene expression in bovine calf cartilage explants, mRNA levels measured in non-injured free swelling tissue was found to vary over five orders of magnitude, with matrix molecules being the most highly expressed of the genes tested and cytokines, matrix metalloproteinases (MMPs), aggrecanases (ADAMTSs), and transcription factors showing lower levels of expression [71]. While the matrix molecules showed little change in expression after injurious compression, MMP-3 increased ~250-fold, ADAMTS-5 increased ~40-fold, and TIMP-1 increased ~12-fold over free swelling levels [66]. In addition, injurious compression results in a decrease in biosynthetic rates in the remaining viable cells, and these viable cells no longer respond to the stimulatory effects of moderate dynamic compression seen in normal cartilage [70]. Taken together, these studies suggest that mechanical overload can cause long-term cell mediated changes in matrix quality and turnover.

Deschner et al. recently summarized the interaction between loading and inflammatory pathways [72], which may be activated by excessive loads and inhibited by moderate cyclical loading [42, 45, 73]. Thus, these interactions appear to depend on the magnitude and loading rate (frequency). Mechanical forces can influence production of NO [16, 74, 75], PGE2 [37], and IL-6 [76]. Interestingly, cross-talk between NO and PGE2 pro-inflammatory pathways, and between NOS2 and COX2 (upstream of NO and PGE2), can be regulated by mechanical stimuli [77]. These pathways have been traditionally associated with inflammatory cytokines such as IL-1, an initiator of cartilage degradation [42, 45, 73, 78-80]. Dynamic compression (15% strain amplitude, 1Hz, 48 hr) could

inhibit NO synthesis by equine chondrocytes in agarose gel constructs [81] ,and could inhibit NO and PGE2 release by superficial zone equine chondrocytes stimulated by IL-1β [82].

Cell microenvironment and organelle morphology

Loading of cartilage (Fig. 1.2a) produces cellular deformation [83, 84] in proportion to the local deformation of the ECM, and in a manner consistent with the depth-dependent compressive properties of the bulk tissue [85]. Deformations within the pericellular matrix (Fig. 1.2b) also affect the physicochemical microenvironment of the chondrocyte [28, 86] and may, in turn, signal the cell to modulate its biosynthetic response. Deformation-induced fluid flow in the pericellular region enhances transport of soluble factors to cell receptors, and alters the local concentration of mobile ions leading to electrochemical changes such as shifts in pH[87]. Cell-surface connections to the ECM enable pericellular deformations to be transmitted through the cell membrane to intracellular organelles via cytoskeletal elements such as actin microfilaments, microtubules, and intermediate filaments[20, 83, 88]. Compression can also dramatically affect the morphology of intracellular organelles that regulate cell biosynthesis and metabolism by altering gene transcription, intracellular transport and trafficking, and protein translation and post-translational processing. Using chemical fixation, high-pressure freezing, and electron microscopy, Szafranski et al. [89] observed that compression of bovine cartilage explants caused a concomitant reduction in the volume of the extracellular matrix, chondrocyte, nucleus, rough endoplasmic reticulum, and mitochondria. Interestingly, however, the Golgi apparatus was able to resist loss of intraorganelle water and retain a portion of its volume relative to the remainder of the cell. These combined results suggested the hypothesis that organelle volume changes were driven mainly by osmotic interactions while shape changes were mediated by

structural factors, such as cytoskeletal interactions that may be linked to extracellular matrix deformations. The observed volume and shape changes of the chondrocyte organelles and the differential behavior between organelles during tissue compression provides evidence for an important mechanotransduction pathway linking translational and post-translational events. For example, since the Golgi is the site of post-translational modifications of aggrecan (e.g., glycosylation and sulfation) [89, 90], changes in Golgi morphology and function with compression may play a critical role in the known changes in GAG chain length and sulfation caused by compression [21]. Such changes in GAG and aggrecan structure, which also occur naturally with age (Fig. 1.2c) may profoundly influence aggrecan function. Such functional mechanical changes can now be measured directly using atomic force microscopy methodologies [91, 92] (Fig. 1.2d).

1.7 Systems biology approaches

Real time PCR and gene clustering analyses have been used to study intermediate-size gene sets thought to be involved with cartilage mechanotransduction. Fitzgerald et al. [33] examined the kinetics of mechano-regulation of gene transcription in response to static compression of bovine calf cartilage explants for periods between 1-24 hours in the presence or absence of an intracellular calcium chelator or an inhibitor of cyclic AMP activated protein kinase A. Cluster analysis of the data revealed four main expression patterns: two groups that contained either transiently upregulated or duration-enhanced expression profiles could each be subdivided into genes that did or did not require intracellular calcium release and cyclic AMP activated protein kinase A for their mechano-regulation. Transcription levels for aggrecan, type II collagen, and link protein were upregulated approximately 2 to 3-fold during the first 8 hrs of 50% compression and subsequently down-regulated to levels below that of free-swelling controls by 24hrs.

Transcription levels of matrix metalloproteinases-3,9,13, aggrecanase-1 and the matrix protease regulator cyclooxygenase-2 increased with the duration of 50% compression 2 to 16-fold up to by 24 hrs. Thus, transcription of proteins involved in matrix remodeling and catabolism dominated over anabolic matrix proteins as the duration of static compression increased. These approaches are also being used to study responses to dynamic compression and tissue shear of cartilage explants.

Researchers have begun to integrate genomic and proteomic approaches with the computational tools of systems biology for applications in musculoskeletal research, including medical diagnostics, and drug discovery [93]. DNA microarray technology is being used to explore the complex feedback loops in transcription factors and layered signaling pathways underlying the mechanotransduction as well as the pathobiology of osteoarthritis. Aigner et al. [94-96] examined transcript levels of matrix components and matrix degrading proteinases using DNA arrays. By comparing normal chondrocytes with early and late stage OA chondrocytes, they examined expression trends involving up and down regulation of MMPs, TIMPS, proteoglycans, and collagens [96]. Such approaches can be directly applied to the study of mechanotransduction. While DNA arrays can sample large numbers of genes, they are limited in their sensitivity and they do not measure posttranscriptional regulation or modifications [94]. While recognizing these limitations, the potential of such profiling approaches is clear [95, 97], since the results can be used to formulate hypotheses about specific molecules and mechanisms in ways that are complementary to the traditional one-gene or one-protein hypothesis-testing approach.

1.8 Conclusion

Chondrocytes can sense and respond to mechanical forces in an extraordinarily sensitive and robust manner. These cells can distinguish between compression, tension

and shear deformation of the surrounding ECM, and respond in a manner that varies with the rate (frequency) of loading. Recent studies have identified several intracellular signaling pathways that are involved in chondrocyte mechanotransduction and the regulation of cartilage and exhibit levels of overlap or crosstalk in their signaling. These complex signals are responsible for activation of ECM molecules proteinases, inflammatory factors, and regulatory proteins which govern tissue homeostasis. Significant technical advances have enabled the study of transduction mechanisms by chondrocytes within their native, dense ECM. Advanced genomic and proteomic technologies should lead to a further rapid increase understanding the fundamental link between chondrocyte mechanobiology, physiology, and tissue homeostatis in health and disease, with direct application to cartilage repair and tissue engineering.

Acknowledgements

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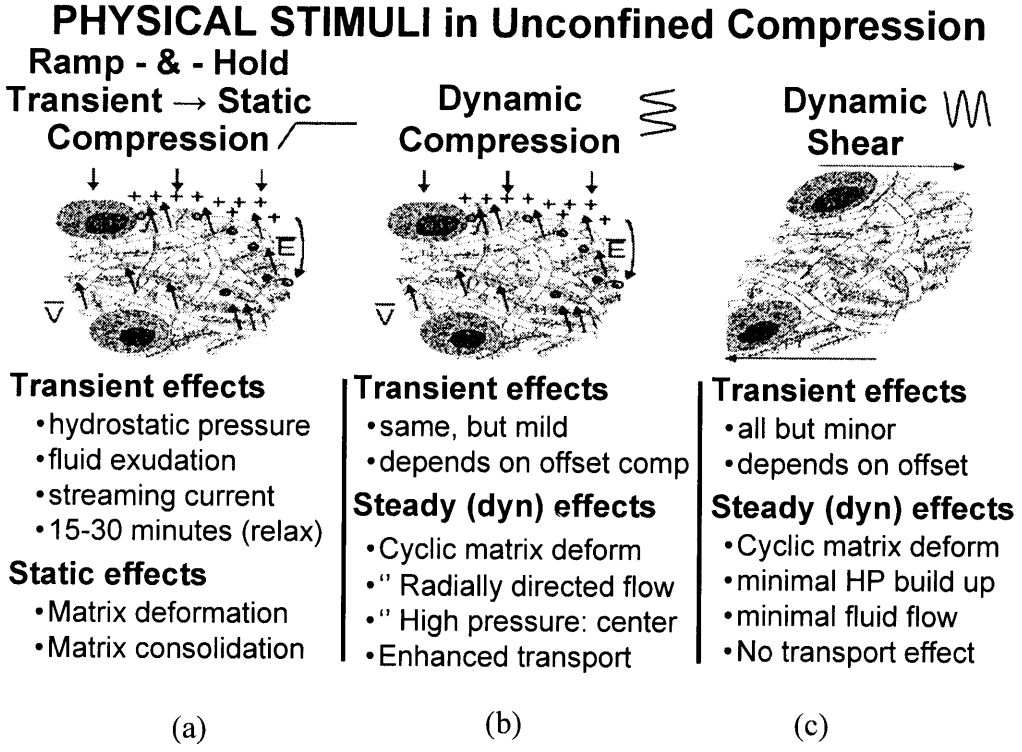

Figure 1.1: Schematic of physical forces and flows occurring during mechanical loading of cartilage in vivo, that can be stimulated in vitro by means of (a) static compression, (b) dynamic compression, and (c) dynamic tissue shear.

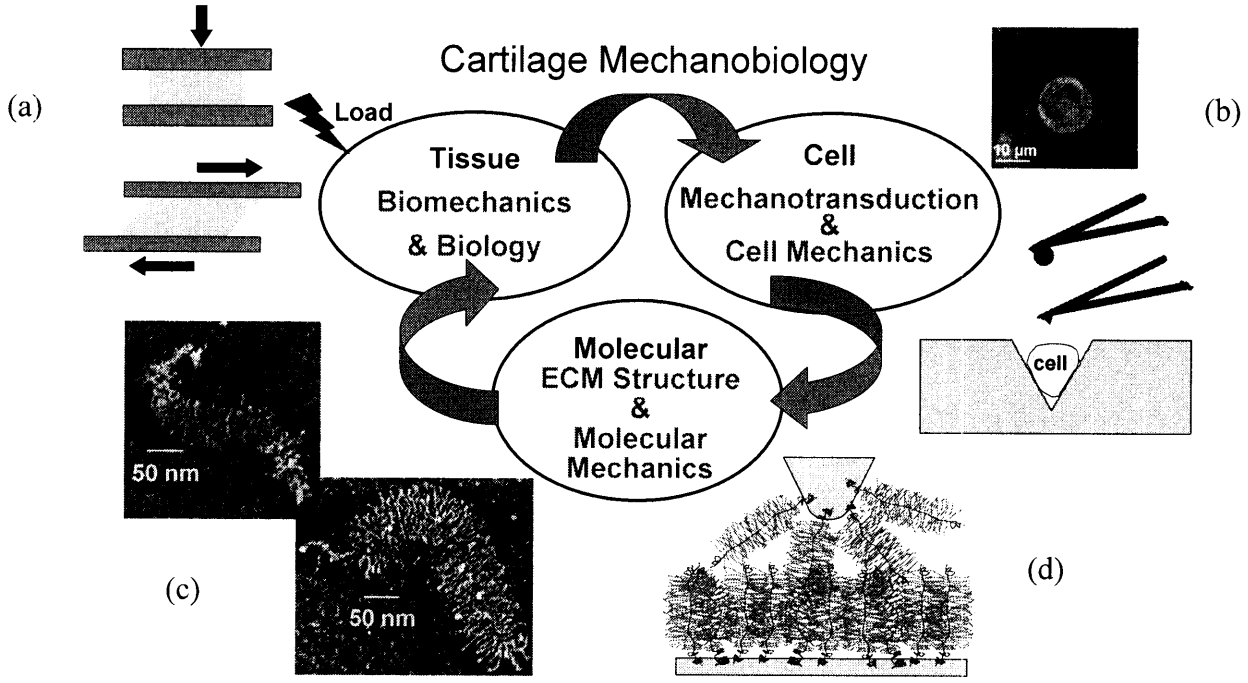

Figure 1.2 : Loading of cartilage explants (a), or direct mechanical stimulation of cells (b) can produce mechanical stimuli that may be sensed by the cell and its pericellular microenvironment (b). These mechanical stimuli may alter the rate of synthesis as well as the molecular structure of ECM molecules such as aggrecan (c) which, in turn, could ultimately affect tissue-level biomechanical properties in a feedback fashion (a). New cell-level and molecular-level measurement techniques, such as those based on atomic force microscopy, are being used to quantify the molecular mechanical properties of ECM macromolecules (d) as well as cellular mechanical properties (b).

Chapter 2

TRANSCRIPTIONAL EFFECTS OF COMBINED MECHANICAL COMPRESSION AND IGF-1 STIMULATION ON BOVINE CARTILAGE EXPLANTS*

* This chapter is in preparation for submission to the Journal of Orthopaedics Research (Wheeler, Cameron A and Grodzinsky, Alan J.)

2.1 Abstract

Introduction*:** Insulin-like growth factor-1 (IGF-1) is a potent anabolic factor capable of endocrine and paracrine/autocrine signaling. Numerous studies have shown that chondrocytes produce this important growth factor, and that IGF-1 can stimulate ECM biosynthesis by chondrocytes in native cartilage and tissue engineered constructs. Previous studies have demonstrated that mechanical compression can regulate the action of IGF-1 on chondrocyte biosynthesis in intact tissue; when applied simultaneously, these stimuli act by distinct cell activation pathways. Our objectives were to elucidate the extent and kinetics of the chondrocyte transcriptional response to combined IGF-1 and static compression in cartilage explants. ***Methods: Cartilage explants were harvested as performed previously in our lab. Cartilage plugs were placed in 50% compression and 0% compression with or without 300 ng/ml of IGF-1. RNA was obtained and measured by real-time PCR for 2, 8, 24, 32, 48 hours after treatment and compression.

Results and Discussion: Transcript levels in response to compression alone agreed with previously published data. Key matrix molecules, aggrecan and collagen II, responded positively to the addition of IGF-1 without compression, but this effect was abrogated when compression was applied in combination with IGF-1 treatment. Clustering analysis revealed five distinct groups. TIMP-3 and ADAMTS-5, MMP-1 and IGF-2, and IGF-1 and Collagen II, were all robustly co-expressed under all conditions tested. These co-expressed molecules suggest inherent regulation and positive feedback in chondrocyte gene expression. In comparing gene expression levels to previously measured aggrecan biosynthesis levels, aggrecan synthesis is shown to be transcriptionally regulated by IGF-

1, whereas inhibition of aggrecan synthesis by compression is not transcriptionally regulated.

Conclusion: Many genes measured are responsive the effects of IGF-1 under 0% compression and 50% compression. Clustering analysis revealed strong co-expressed gene pairings. IGF-1 stimulates aggrecan biosynthesis in a transcriptionally regulated manner, whereas compression inhibits aggrecan synthesis in a manner not regulated by transcriptional activity.

2.2 Introduction:

Insulin-like growth factor-1 (IGF-1) is a 7.6 kDa, 8.5 PI, potent anabolic factor capable of endocrine and paracrine/autocrine signaling. While IGF-1 is primarily produced in the liver and transported throughout the body via the blood stream, numerous studies have shown that chondrocytes produce this important growth factor, and that IGF-1 can stimulate ECM biosynthesis by chondrocytes in native cartilage and tissue engineered constructs. The use of growth factors as therapeutics to reverse or inhibit cartilage degradation has been an underlying focus in cartilage research. The avascular, alymphatic, and aneural nature of cartilage suggests that the growth factor be administered though means of local delivery. Local delivery is complicated due to joint motion and cartilage structure which affects special and temporal diffusion rates.

To investigate the effects of exogenous IGF-1, many studies have examined how chondrocytes respond at the protein level as well as the gene transcript level. General protein levels have been measured using conventional radiolabel incorporation (35S, 3H), and show that chondrocytes respond in a dose dependent manner to IGF-1 [1-6]. The environment the chondrocytes are cultured in varies (explants, gels, monolayer), but generally chondrocyte biosynthesis levels are increased from 150% [4] to 2-6 fold control levels [3, 6-8]. Researchers have also examined particular proteins using western blot analysis. For example, MMP-13 was found to be suppressed in response to IGF-1 treatment [9]. When examining the transcript or gene levels in response to IGF-1, researchers have focused on specific molecules using real-time PCR and reverse transcription PCR. Type II collagen was shown in multiple studies to be significantly

upregulated by IGF-1 [3, 10-12]. Aggrecan transcripts have shown no significant increase [3, 4] or slight upregulation [12] with the addition of IGF-1 in the first 48 hours of IGF-1 treatment, yet were significant upregulation (130%) when treated for 1-3 weeks [10]. IGF-1 transcript levels were shown to peak at 24 hours after IGF-1 treatment, suggesting that IGF-1 has an autocrine response [1]. Sox-9, a transcription factor was also shown to have no significant response to IGF-1 [11].

Chondrocytes have been shown to be responsive to mechanical compression on both the protein and gene transcript levels. Static compression in vitro has been shown to decrease protein biosynthesis levels of type II collagen and proteoglycans in a dose dependent manner [5, 13]. In alginate and type I collagen gels seeded with chondrocytes, 50% static compression was shown to decrease radiolabel incorporation by nearly half in comparison to non-compressed controls [14, 15]. Similar findings have been shown in cartilage explants with proteoglycan and type II collagen synthesis decreasing within 1-2 hours of loading and remaining suppressed for the loading period (24 hrs) [16, 17]. When transcript levels were examined in response to static loading, type II collagen and aggrecan were shown to initially peak anywhere from 1 to 4 hours after compression, followed by a decrease to unaffected level of gene expression [13, 15, 18, 19]. Fitzgerald et al. have investigated 28 different ECM related molecules including matrix proteinases, tissue inhibitors of matrix metalloproteinases (TIMPs), growth factors, cytokines, and structural ECM molecules, under static compression for a 24 hour period and reported 4 distinct patterns associate with gene expression [19].

Bonassar et al. have examined the combination of IGF-1 treatment with static compression at a protein level and found when cartilage explants were treated with IGF-1 under 0% compression (cut thickness) a 2-3 fold increase was found over 48 hour [5]. Under static compression biosynthesis was decrease by 50% compared to non-compressed conditions. When compressed explants were treated with IGF-1, biosynthesis rates significantly increased, returning to levels comparable to non-compressed, non-treated explants. Thus compression diminished the effects of IGF-1, but did not altogether eliminate them. IGF-1 was still able to upregulate or rescues the synthesis rate of statically compressed cartilage explants.

Chondrocyte gene expression under a combination of IGF-1 treatment and static compression have not been examined thoroughly. Our objectives were to elucidate the extent and kinetics of the chondrocyte transcriptional response to combined IGF-1 and static compression in cartilage explants.

2.3 Methods:

Cartilage Harvest, Mechanical Loading, and Growth Factor Treatment: Cartilage-bone plugs were harvested from the patello-femoral groove of 1-2 week old calves. Cartilage disks (1mm thick X 3mm diameter) were cored and punch from the middle zone as described previously [20] and equilibrated for two days under free-swell conditions in the presence of serum-free feeding medium consisting of high glucose Dulbecco's modified essential medium supplemented with 10 nM Hepes Buffer, 0.1 mM nonessential amino acids, 20 μg/ml ascorbate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphoericin B. Five anatomically matched disks were separated for each time point (Figure 2.1), and placed in polysulfone loading chambers. Each time point consisted of four separate experiments. With cartilage disks matched for time, 8 disks were allocated to 0% compression (i.e., compressed to 1-mm cut thickness from free swelling), 50% compression, 0% compression + 300 ng/ml IGF-1, and 50% compression + 300 ng/ml IGF-1, the IGF-1 concentration found previously to maximally stimulate similar free-swelling calf cartilage explants [5]. At time zero, all chambers were slowly compressed to specified strains over a 3 minute period to avoid injurious effects of high strain rates. These strains were maintained for each of the four conditions for 2, 8, 24, 32, and 48 hours (Figure 2.1). Upon completion of loading time, disks were promptly removed, flash frozen in liquid nitrogen, and stored at -80° C.

RNA Extraction and Quantization, Primer Design, and Real-Time PCR: 8 disks for each time point and condition were taken from -80° C freezer and pulverized. In order to prevent RNA degradation, the pulverizing apparatus was constantly cooled using

liquid nitrogen. Once samples had been pulverized, Trizol (sigma, st. Louis) was added and homogenized to thoroughly break down the tissue. After chloroform was added, the mixture was transferred to pre-spun phase gel tubes, and spun at 13,000 rpm for 10 minutes at 4° C. Supernatant was removed, and RNA was extracted using Qiagen RNAeasy mini kit protocol with recommended DNase digest (Qiagen). RNA was stored in 50 µl of RNase free water under -80° C conditions. RNA quality and amount was quantified by using NanoDrop ND-1000 spectrophotometer. According to RNA measurements, 1µg of RNA was reverse transcribed using Applied Biosystems reagents as previously described [21]. Forward and reverse primers for 24 relent genes (Table 2.1) were designed based on bovine genomic sequences and standard curves were calculated as previously described [19]. Once cDNA was obtained, Real-Time PCR was performed using MJResearch Opticon2 instrument and SYBR Green Master Mix (SGMM, Applied Biosystems). SGMM was combined with RNase free water and cDNA and aliquated into MJResearch 96-well plate. Using a multi-pipette, a premixed solution of forward and reverse primer for 24 different genes was added to each well. Measured threshold values (Ct) were converted to RNA copy number according to previously calculated standard curves.

Data Normalization and Statistical Analysis: Under each loading condition and time point, each gene RNA copy number was normalized to the 18s housekeeping gene from that same condition and time point [22]. To examine the time course of gene expression, 0% compression + IGF-1, 50% compression, and 50% compression + IGF-1 were normalized to 0% compression levels. Thus, if a gene expression value was below

or above 1, it represented a decrease or increase of gene expression respectively compared to 0% compression. Expression levels due to experimental error were removed. To assign statistical significance to expression levels, a non-parametric test was used to ensure unbiased results by avoiding the assumption of a parameter based distribution. The Wilcoxon sign ranked test was used to judge significance which incorporates the amount of data in the significance statistic. Significance was assigned if the Wilcoxon sign ranked test statistic produced a p-value less than 0.07. The minimum p-value using the Wilcoxon sign ranked test is 0.068 due to the fact that there are only four replicates. Thus, significance of 0.07 was chosen because with this non-parametric test and amount of data, 0.05 cannot be obtained.

Clustering Analysis: In order to understand general transcriptional patterns in the data, clustering analysis was performed on all normalized conditions (0% compression + IGF-1, 50% compression, and 50% compression + IGF-1) and time points (2, 8, 24, 32, 48 hours) over 23 genes. This resulted in a 15 x 23 matrix which was standardized by expression amplitude as described previously [19], in order to accentuate gene expression patterns as appose to expression magnitudes. The 15 gene expression array vectors were clustered using k-means clustering. Principle component analysis (PCA) was used to determine the components that contain the greatest variance in the expression data[23, 24]. Once the 15 principle components had been calculated, the k-means clustering algorithm was applied to the 15 principle components and clustered into k groups. The average and variance of each projected coordinate group was calculated to compose a group centroid. Centroid vectors were formed by combining the three main principal

components weighted by their projected centroid coordinate. The uniqueness of each Group's expression patterns were evaluated by the Wilcoxon sign ranked test.

2.4 Results:

Effects of static compression: Cartilage disks were subject to 2, 8, 24, 32, and 48 hours of compression applied in a ramp and hold fashion. The disks experience a peak stress when the compression is applied, followed by a slow stress relaxation due to the poroelastic properties of cartilage. The five time points were chosen to capture the kinetics of gene expression in repose to the changes in stress. Twenty-four different genes were measured at each one of the five time points and were normalized to 18s, a housekeeping gene, and 0% compression with no added IGF-1 was used as a control. 8 of the 23 genes measured were up-regulated for 3 or more of the time points examined. These included ADAM-TS5, MMP-13, MMP-3, TGF-β, c-Fos, c-Jun, and Sox-9 (Figure 2.2, Appendix A). Matrix metalloproteinase- 3 (MMP-3) was significantly up-regulated at 8, 24, 32, and 48 hours with a peak expression level of 30-fold compared to control at 32 hours (Figure 2.2A). ADAM-TS5 and MMP-13 displayed a transient increase in expression levels peaking at 48 hours with >6-fold and >17-fold up-regulation respectively (Figure 2.2B, Appendix A). TGF-β was consistently up-regulated 2.5-fold from 8 hours to 32 hours (Figure 2.2D). Supporting previously reported data[19], c-Fos and c-Jun were significantly up-regulated in response to 50% static compression at all time points measured (Appendix A). Interestingly, c-Fos, c-Jun and Sox-9 were all maximized at 8 hours (23-fold, 30-fold, and 6.8-fold respectively) (Appendix A). Displaying a transient decrease, IGF-1 was the only gene measured that showed a significant down-regulation in the presence of 50% strain for at least 3 of the measured time points. IGF-1 was significantly down-regulated by 50% at 24 hours and up to 70% at 48 hours (Appendix A).

Effects of IGF-1: To examine the effect of IGF-1 on cartilage explants, disks were incubated for 2, 8, 24, 32, and 48 hours with 300 ng/mL IGF-1 under 0% compression. 10 of the 23 genes were upregulated with the treatment of IGF-1. IGF-1 up-regulated for three or more time points, MMP-13, MMP-1, TNF-α, and IL-1β, which traditionally are thought to play catabolic roles in cartilage. MMP-13, TNF-α, and IL-1β were significantly up-regulated and peaked at 24 hours to the level of 8-fold, 2.5-fold, and 2.75-fold respectively (Figure 2.2B and Appendix A). MMP-1 and IGF2 were transiently up-regulated to a significant level until a 32 hour peak (4.75-fold and 3-fold respectively), after which expression levels returned to control (Figure 2.2I, 2.2E). Link and Aggrecan were also transiently up-regulated with a peak of 32 hours (4-fold and 2.5-fold respectively), but expression levels at 48 hours were still significantly above control (Figure 2.2F, 2.2G). As in 50% static compression, Sox-9 was significantly up-regulated at 8 hours to a comparable level of 6.3-fold (Appendix A). TIMP-3 and HSP90 were significantly upregulated with for all time points and both displayed an initial peak of expression (5-fold and 3-fold respectively), followed by a transient decrease of expression over time (Figure 2.2C, Appendix A). No genes measured were significantly down-regulated by the treatment of IGF-1.

Effects of IGF-1 and static compression: Combining the treatment of 300 ng/mL of IGF-1 and static compression to a level of 50% strain, 11 of the 23 genes were significantly different than the 0% control for 3 or more time points. MMP-13, MMP-3, TGF-β, c-Fos, c-Jun, TIMP-3, and HSP90 were significantly up-regulated in at least 3 of

the measured time points. MMP-13 and MMP-3 were significantly upregulated 4 out of the 5 time points and had peak values of 28-fold and 33-fold respectively at 24 hours (Figure 2.2B, 2.2A). TGF-β and HSP90 were transiently upregulated to a peak level of 3.8-fold and 2.25-fold respectively, at 32 hours (Figure 2.2D, Appendix A). c-Fos and c-Jun were again significantly up-regulated for all time points with expression peaks at 24 and 8 hours (Appendix A). The peak value of c-Fos, 22-fold, was similar to the expression levels found in 50% static compression alone. Alternatively, the peak value of c-Jun had decreased from 30-fold under 50% static compression to 16-fold under 50% static compression with IGF-1. TIMP-3 showed a slow increase of expression which peaked at 24 hours to a level of 19-fold, followed by an up-regulated level of 10-fold (Figure 2.2C). Col2, IGF-2, TIMP-2, and Txnip were significantly down-regulated for 3 or more time points measured. IGF-2 and Txnip showed a transient decrease of expression over time and bottomed out at 48 hours decreasing 45% and 52% expression below control (Figure 2.2E, Appendix A). Col2 and TIMP-2 were drastically decreased under 50% static compression with IGF-1. Col2 was transiently down-regulated, decreasing expression levels by 43% at 24 hours, to 62% at 48 hours (Figure 2.2H). TIMP-2 was also transiently down-regulated, decreasing expression levels by 50% at 24 hours and 92% at 48 hours (Appendix A).

Comparing effects of IGF-1 under 0% and 50% compression: To elucidate the different effects of IGF-1, the effects of IGF-1 were isolated by normalized gene expression to like loading conditions. 50% static compression with IGF-1 was normalized by 50% static compression, and 0% static compression with IGF-1 was

normalized to 0% static compression. When examining the effects of IGF-1 under non-loaded conditions (0% static compression), 9 of the 23 genes were significantly altered for 3 or more time points by the addition of IGF-1. Under loading conditions (50% static compression), 4 of the 23 genes were significantly altered for 3 or more time points by the addition of IGF-1 (Appendix B). Of the significantly affected genes, aggrecan, IGF-2, and TIMP-3 were significantly affected by IGF-1 in both loaded and un-loaded conditions (Figure 2.3). Aggrecan, IGF-2, and Link were all up-regulated when treated with IGF-1 under un-loaded conditions, and down-regulated when treated with IGF-1 under loaded conditions (Figure 2.3A, 2.3B, 2.3C). Aggrecan in particular was significantly up-regulated for all time points by IGF-1 under un-loaded conditions, and significantly down-regulated for all time points by IGF-1 under loaded conditions (Figure 2.3A). In contrast, TIMP-3 was up-regulated significantly under both un-loaded and loaded conditions (Figure 2.3D). MMP-1 was up-regulated significantly with treatment of IGF-1 under un-loaded conditions, but was unaffected by the treatment of IGF-1 when loading conditions were present (Figure 2.3E).

Expression Trends and Groupings: After normalization of the data, using principle component analysis (PCA), the 15 dimensional space was reduced to a three dimensional space, by calculating three eigenvectors or principle components that represent 80% of the variance in the data. With three principle components, each standardized gene was projected in to the principle component space and can be visualized as shown in figure 2.4. All 15 dimensions of each gene were used in the k-means clustering technique to ensure that smaller gene variations were represented in the

grouping. After dividing the genes into 2 to 8 cluster groups and visually comparing the distinctness of the groups, 5 groups appeared to be an adequate number of groupings (Figure 2.4). These 5 groups contained 4 to 7 genes as shown in Table 2.2A. The mean expression level is represented by a centroid (Figure 2.4) and the mean expression profile of the 5 groups is shown in Figure 2.5.

Group 1 was significantly upregulated for all conditions and all time points (Figure 2.5A). Under treatment of IGF-1, a 2-fold increase was observed at 24 hours and under loading conditions a > 6-fold increase was observed at 24 hours as well. When combining the treatment of IGF-1 and compression, it appears the expression levels of IGF-1 treatment dominate initially (2 hours). At 24 hours there appears to be an additive effect of compression and IGF-1 treatment as the combined compression and IGF-1 levels are about 9-fold. (>6 + 2). At 32 and 48 hours, the effects of compression appear to dominate the effects of IGF-1 treatment. Group 2 under treatment, compression, and compression with treatment, are initially significantly upregulated, but return to control expression levels at 48 hours (Figure 2.5B). In both compression and treatment conditions, group 2 responds strongly by reaching > 3-fold and 2.5-fold respectively. When IGF-1 treatment and compression are combined, all significantly upregulated time points (2-32 hours) in treatment and compression are reduced with a peak value of 2-fold. Under treatment conditions, with a strong significant up-regulated for the first three time points, (peaked at > 3.5-fold) group 3 appears to be strongly upregulated by IGF-1 (Figure 2.5C). Under compression, group 3 has no significant initial changes, but at 48 hours in upregulated > 2-fold. When combining compression and treatment all time points are reduced to control levels, apart from 24 hours, which is still significantly

upregulated, but reduced to 2-fold from > 3.5-fold in treatment alone. Group 4 under IGF-1 treatment conditions is increasing significantly up-regulated until 32 hours (2.5-fold) before it returns to near control levels at 48 hours (Figure 2.5D). Under compression conditions, group 4 exhibits a near opposite effect as in the treatment conditions, by initially being up-regulated (3-fold), and decreasing over time to return to control levels. Previous data has shown that Aggrecan, a member of this group, acts in a similar expression profile with a initial upregulation with compression followed by a return to control levels [19]. Combining compression and treatment, no time points are significantly different than control, but artifacts of the initial peak at 2 hours from compression alone and peak at 32 hours from IGF-1 treatment alone appear. Group 5 showed very little change under IGF-1 treatment with a significant upregulation at 32 hours of 1.5-fold (Figure 2.5E). Under compression conditions, group 5 was upregulated significantly at 2 and 8 hours with a peak of 1.6-fold, but by 24 hours until 48 hours was significantly decreasingly down-regulated to a minimum point at 48 hours, loosing greater than 50% expression compared to control. When combining compression and IGF-1 treatment, all time points were lower than either compression or IGF-1 treatment alone, and 24-48 hour were decreasingly down-regulated to a significant level, loosing greater than 60% expression compared to control at 48 hours.

2.5 Discussion

To understand the transcriptional responses under different conditions, the 23 genes observed were clustered according to IGF-1 treatment alone, 50% static compression alone, and the combination of static compression and IGF-1 treatment. Lastly, to understand a more global view of transcriptional activity, all conditions and time points were clustered together. The groupings assigned by clustering analysis can be seen in Table 2.2A. Looking at the groupings under IGF-1 treatment alone, Link, type II collagen, and aggrecan which were significantly upregulated, were grouped together, while Txnip, the only molecule that was significantly down regulated by IGF-1 treatment for multiple time points is uniquely grouped (Table 2.2B). Groupings of compression alone isolate IL-6, highly non-responsive to compression over the time course measured, in its own group. Transcription factors, highly initially upregulated genes, were grouped together while transiently upregulated proteinases were allocated to a different grouped, both of which support previous findings for compression (Table 2.2C) [19]. Examining the combination of the treatments, Fibronectin was partitioned to a unique group for its seemingly non-responsive behavior to compression and IGF-1 treatment. The majority of proteinases were also grouped together (Table 2.2D). Looking at all three sets of clusters, of note, MMP-1 and IGF-2 were grouped together under the three different conditions, as well as link protein, and the components of the AP-1 complex, c-Fos and c-Jun. Type II collagen and IGF-1, TIMP-3 and MMP-3, Sox-9 and TIMP-1 were allocated in the same group for all three conditions. Compiling all conditions observed into an expression vector, cluster analysis revealed that all cytokines were grouped together, and were significantly upregulated under IGF-1 treatment alone compared to

controls. Under compression alone and IGF-1 and compression experiments this extensive upregulation was not observed, suggesting that IGF-1 has a stimulatory effect on cytokines which is mitigated when compression is present. Also of note, Aggrecan, type II collagen, and link protein were all in separate groupings, suggesting that there may be different mechanisms involved in the activation of these molecules. Of the strongly upregulated group, transcription factors, growth factors, protease inhibitors, and proteinases were grouped together, namely c-Fos, c-Jun, TGF-β, TIMP-3, MMP-3, MMP-13, and ADAMTS-5.

With the 4 different sets of data clustered, (compression, IGF-1, compression + IGF-1, all conditions) three interesting pairings were always present: MMP-1 and IGF-2, TIMP-3 and ADAMTS-5, type II collagen and IGF-1. MMP-1 or collagenase-1 is shown to cleave key ECM molecules including collagen I, collagen II, aggrecan (at the MMP cleavage site in the interglobular domain), fibronectin, and link protein [25]. MMP-1 has been shown to play a role in the regulation of paracrine signals, through the degradation of cytokines such as IL-1β [26]. Collagenase-1 has also been shown to degrade insulin-like growth factor binding proteins (IGFBP) 3 and 5, which indirectly increases presence of free (unbound) IGF [27, 28]. IGF-1 and IGF-2 are known to bind IGFBP-3, the most abundant IGF binding protein in human serum [29]. IGF-2 is known to stimulate DNA and proteoglycan synthesis in chondrocytes [30] and has been shown to act in an autocrine fashion [31]. IGF-2 has also been shown to stimulate type 1 IGF receptor, a key receptor for IGF-1 and also IGF-2 with lesser affinity [32, 33]. The co-expression of MMP-1 and IGF-2 suggests that the two contain each other through their anabolic and

catabolic activities and have the capability of a strong anabolic response, due to the stimulatory secondary effects of MMP-1.

TIMPs act in a stochiometric fashion to reversibly inhibit metallo-proteinases [34]. Of the four known TIMPs (1-4), TIMP-3 has been shown to be a strong inhibitor of Aggrecanase-1 (ADAM-TS4) and Aggrecanase-2 (ADAM-TS5) with K*i* values in the subnanomolar range [35, 36]. When added exogenously to bovine nasal and porcine articular cartilage, TIMP-3 retains the ability to inhibit aggrecanase activity induced by catabolic factors [37]. In the current study, TIMP-3 is shown to be significantly upregulated with the addition of IGF-1, and acts in a compression independent manner (Figure 2.3). Although Aggrecan can be degraded by multiple members of the matrix metalloproteinase family, it has recently been shown that ADAMTS-5 is the primary aggrecanase responsible for aggrecan degradation in a murine model of osteoarthritis [38]. The constant grouping based on expression profile of TIMP-3 and ADAMTS-5 suggests a biological control may present to ensure the turn over of aggrecan and the regulation of anabolic and catabolic factors. Supporting these ideas, past clustering analysis with cartilage under multiple conditions also grouped ADAMTS-5 and TIMP-3 together [19].

Type II Collagen, a key matrix protein, adds structure and strength to articular cartilage. IGF-1 has been shown to elevate levels of type II collagen under a number of different conditions [3, 10]. The co-expression of type II collagen and IGF-1 under compressive and/or IGF-1 treated conditions suggests a positive feedback loop between IGF-1 and type II collagen. Under IGF-1 treatment both are upregulated, while under compression, both are down regulated. This supports previous data suggesting that IGF-1

acts in an autocrine fashion [1, 32]. Further studies using promoter analysis must be performed to confirm if these pairings are co-expressed or if these results are an artifact of the selected genes measured.

In contrast with previously published reports [3, 10, 11], the current results show a slight upregulation of type II collagen in response to IGF-1 alone, compared to a significant upregulation (Figure 2.2H). A possible explanation for the discrepancy in the magnitude of type II collagen expression induced by IGF-1 was the 0% compression control present in the current study, where free swell conditions were used in previously published studies. Aggrecan was previously shown to have no significant change in the first 48 hours [3], which this study supports (Figure 2.2F). Previously published data show that MMP-1, MMP-3, and MMP-13 were unaffected by the addition of IGF-1 after 48 hours, while the current data suggests a significant increase for 4 out of the 5 time points in MMP-1 and MMP-13 (Figure 2.2I, 2.2B). Again, reasons for this discrepancy may be due to the 0% static compression present during IGF-1 treatment. IGF-1 mRNA levels were maximum at 32 hours (Appendix A), where previous studies reported them to peak at 24 hours [1]. Nixon et al. examined time points at 0, 4, 14, 24, 48, and 72 hours, thus finding a maximum of 32 hours is not in direct contradiction to the previously published finding. Sox-9 expression was shown to be significantly upregulated by IGF-1 at 4 of the 5 measured time points (2, 8, 32, 48), which previously was shown to be unresponsive to IGF-1 at 72 hours (Appendix A). Many differences in the two experiments exist possibly explaining the difference in IGF-1 response, i.e., bovine vs. human, explant tissue vs. monolayer, 300 ng/mL IGF-1 vs. 100 ng/mL IGF-1.

In agreement with previously published data [13, 15, 18, 19], under static compression, type II collagen and aggrecan were initially upregulated and then down regulated to nearly control levels (Figure 2.2H, 2.2G).

Examining the transcriptional effects of IGF-1 on aggrecan and link, IGF-1 appears to act via a compression-dependent manner (Figure 2.3A, 2.3C), whereas the transcriptional effects of IGF-1 on collagen II and fibronectin appear to be independent of compression (Figure 2.3F, Appendix B). This phenomenon could easily be explained by the compacted ECM present under compression, dramatically restricting transport of IGF-1 to chondrocytes. To test this idea, experiments were performed allowing IGF-1 to be incubated for 24 hours (transport studies performed by Bonassar *et al.* [5]), to allow IGF-1 to diffuse completely into the cartilage explant. Results of this experiment replicate the trends seen in Figure 2.3A, 2.3C, suggesting that aggrecan and link act in a compression-dependent manner when treated with IGF-1. Whether this change is due to decreased receptor-ligand affinity, or to mechanotransduction intracellular signaling interference, remains to be determined.

At the transcriptional level, the current study points out the anabolic response due to the treatment of IGF-1 under 0% compression loading, and the mixed anabolic and catabolic signals under 50% static compression coupled with IGF-1. To further investigate the transcriptional response to the protein synthesis under IGF-1 treatment and compression, Figure 2.6 shows the levels of aggrecan protein synthesis, normalized to 0% compression 0 IGF-1 compared to gene expression of aggrecan. Aggrecan gene expression under 0% compression, 300 ng/ml IGF-1 is upregulated compared to control

for all 5 time points measured (Figure 2.6B). Examining the corresponding aggrecan protein level at 0% compression, 300 ng/ml IGF-1, there is a strong increase in aggrecan synthesis at 8, 24, and 48 hours (Figure 2.6A). These data suggest that aggrecan synthesis is transcriptionally regulated by IGF-1. Examining the gene expression of aggrecan under 50% compression 0 IGF-1, there is an initial bolus of transcript at early time points, followed by a return to control levels (Figure 2.6B). Examining the protein synthesis levels of aggrecan, at the same early time points, aggrecan synthesis is reduced below control and this reduction is sustained for all time points measured, suggesting that the inhibition of aggrecan synthesis by compression, with no IGF-1, is not transcriptionally regulated (Figure 2.6A). Thus, in attempts to reconcile the responsiveness of aggrecan under 50% static compression at the translational level with the unresponsiveness of aggrecan at the transcriptional level, the authors support the hypothesis [4] that under static compression, proteoglycan synthesis occurs through post-transcriptional machinery.

2.6 Conclusion

Experiments have been performed to assess expression levels of a range of ECM related genes in response to static loading in the presence and absence of IGF-1. Through k-means clustering analysis, major co-expression trends were elucidated, grouping genes into highly responsive, non-responsive, and differentially active gene profile groups. The gene pairs MMP-1 and IGF-2, TIMP-3 and ADAMTS-5, and type II collagen and IGF-1 were consistently co-expressed in multiple clustering conditions, suggesting the possibility of strong regulation and control relationships between members of each pair.

Aggrecan and link protein responded to IGF-1 in a compression-dependent manner, whereas type II collagen and fibronectin appeared to respond to IGF-1 in a manner independent of compression. While aggrecan transcripts were significantly upregulated with the addition of IGF-1 under 0% compression, IGF-1 was unable to upregulate aggrecan when the cartilage explants were statically compressed to 50% strain. In comparing aggrecan gene expression to aggrecan synthesis, these data suggest that aggrecan synthesis is transcriptionally regulated by IGF-1 while the inhibition of aggrecan synthesis by compression is not transcriptionally regulated. However, more studies are needed to elucidate the specific stimulatory mechanism(s) induced by IGF-1 and the post-translational inhibitory effects of compression.

2.7 References

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Figure 2.1. A schematic of the four conditions measured. 5 plugs were punched for each time point and matched for time. IGF-1 treatment and static compression were applied at time 0, and plugs were flash frozen at 2, 8, 24, 32, and 48 hours.

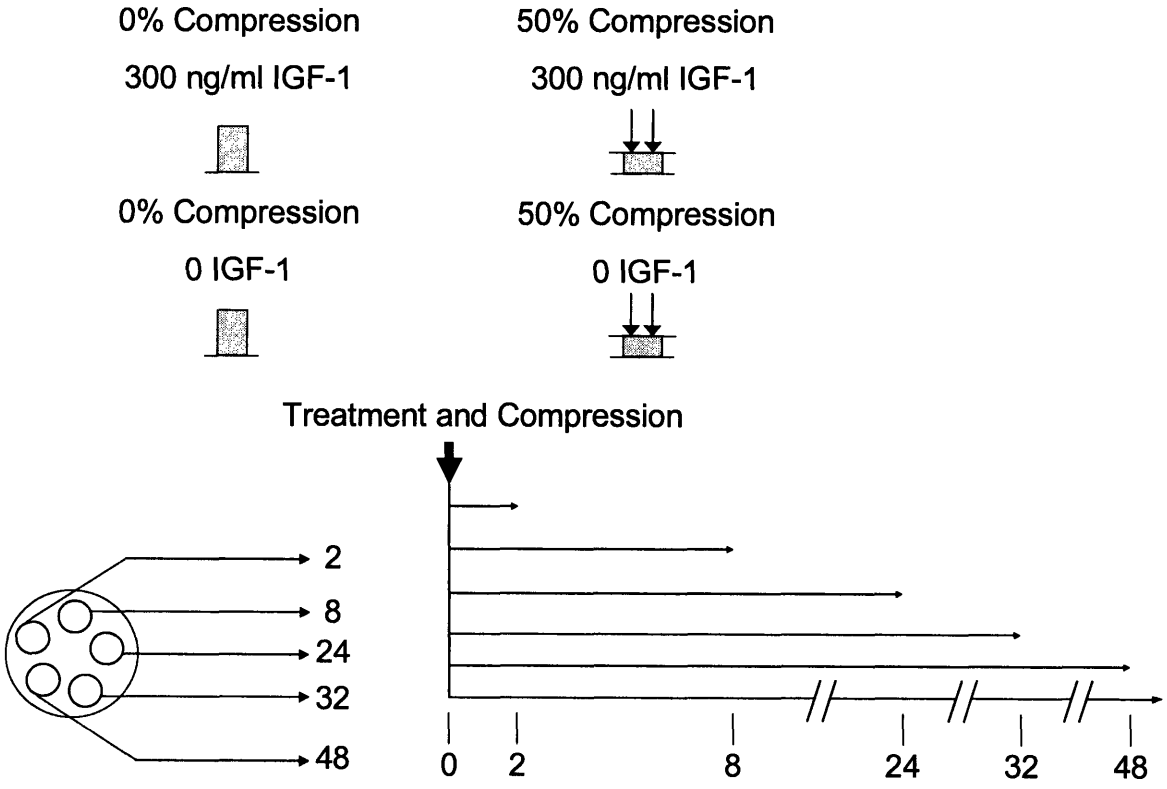


Figure 2.2. Gene expression of proteinases, growth factors, and ECM molecules. 8 cartilage disks were pooled for each time point for each experiment. All genes were normalized to 18s and plotted relative to 0% compression 0 IGF-1. Significance was measured by the Wilcoxon sign ranked test compared to 0% compression 0 IGF-1 (* p-value <0.07). Mean ± SE (n=4)

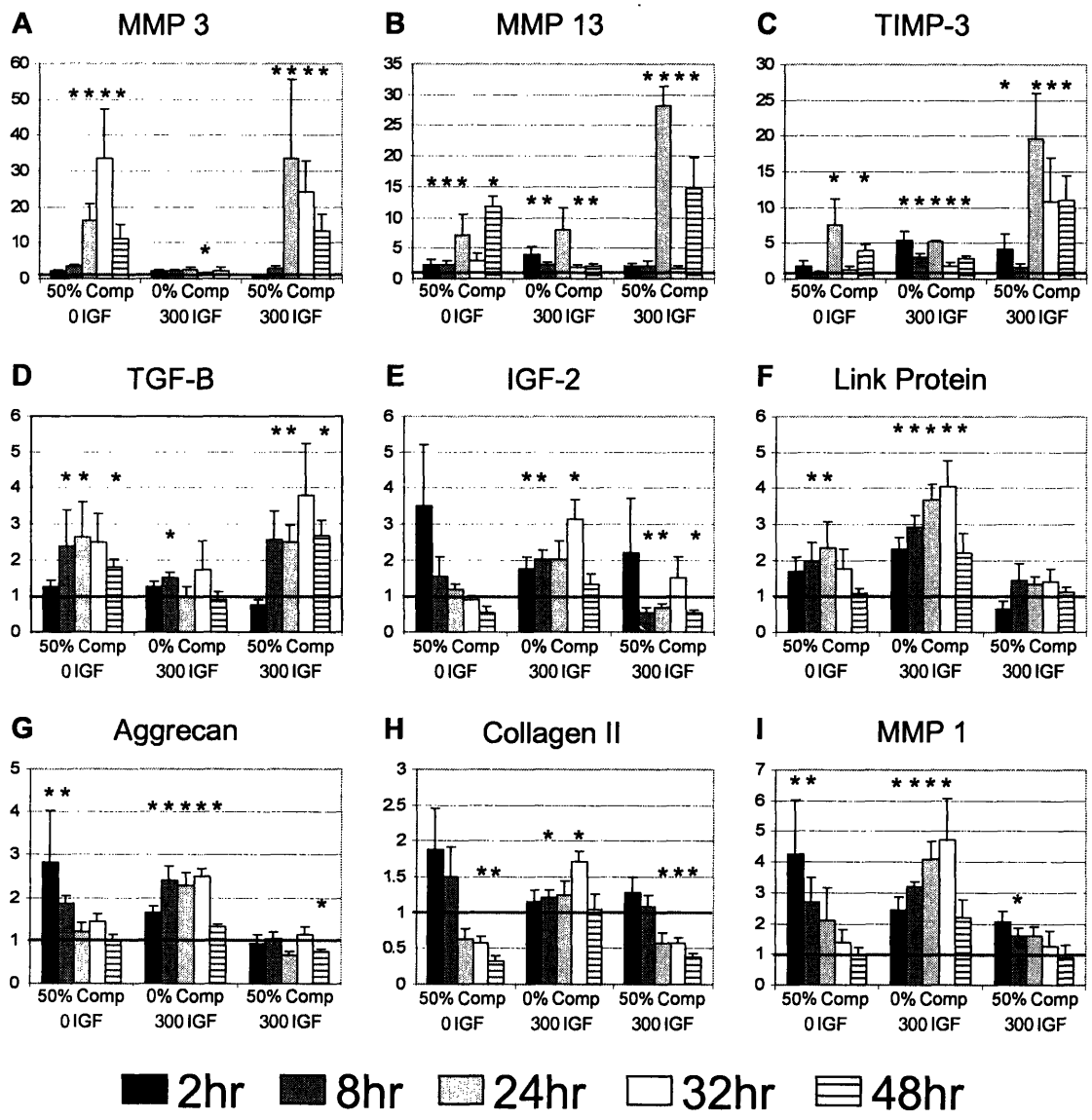


Figure 2.3. Effects of IGF-1. Gene expression was plotted normalized to like loading condition in order to elucidate the effects of IGF-1 under compression or non-compression. Aggrecan (A) and Link (C) respond to IGF-1 in a compression dependent manner, while TIMP-3 (D) and Collagen II (F) respond to IGF-1 in a compression independent manner. Significance was measured by the Wilcoxon sign ranked test compared to like compression 0 IGF-1. (* p-value <0.07). Mean ± SE (n=4)

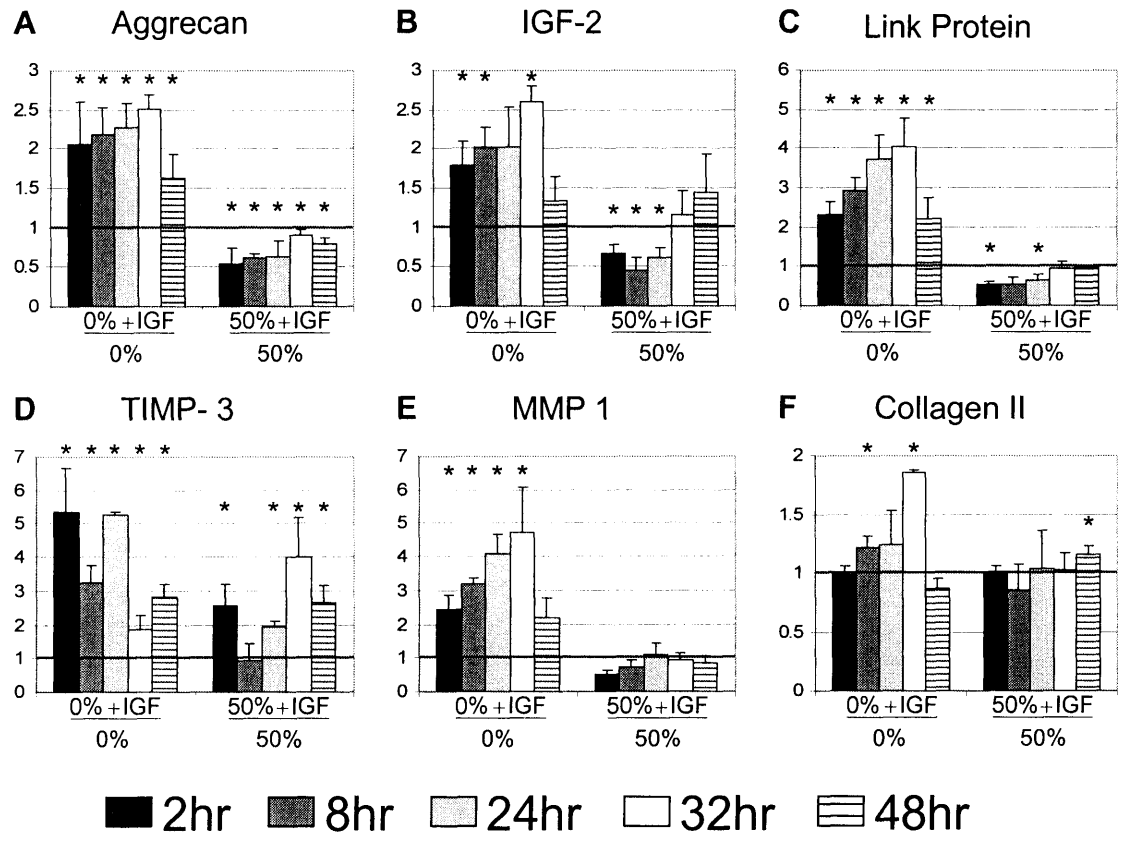


Figure 2.4. Standardized gene expression visualized in principle component space. Principle component 1, 2, and 3 represent 80% of the variance in the data. Genes were allocated to one of five distinct groups by way of k-means clustering. Large solid black circles denote the centroid of the corresponding group.

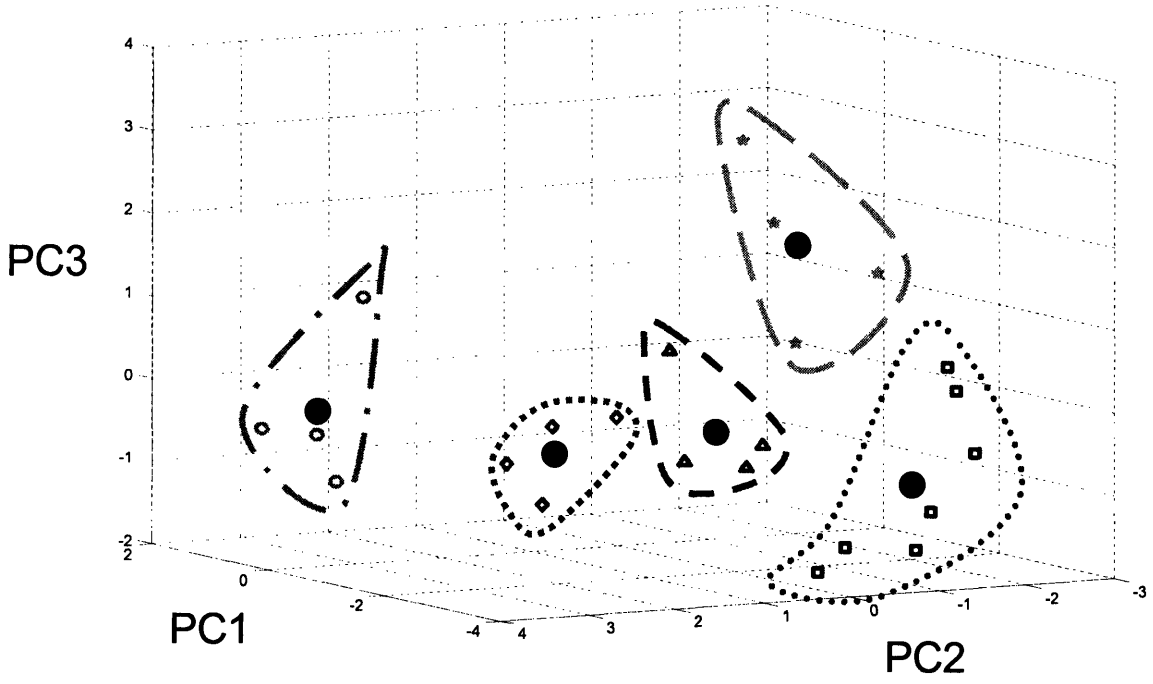


Figure 2.5. Five expression profiles represent the combination of 0% compression 300 ng/ml of IGF-1, 50% compression 0 ng/ml of IGF-1, and 50% compression 300 ng/ml of IGF-1. Centroid profiles were calculated through the average projection coordinates of genes in each group, and transformed from principle component space through use of the calculated principle components. Mean ± SE (n varies based on group component number)

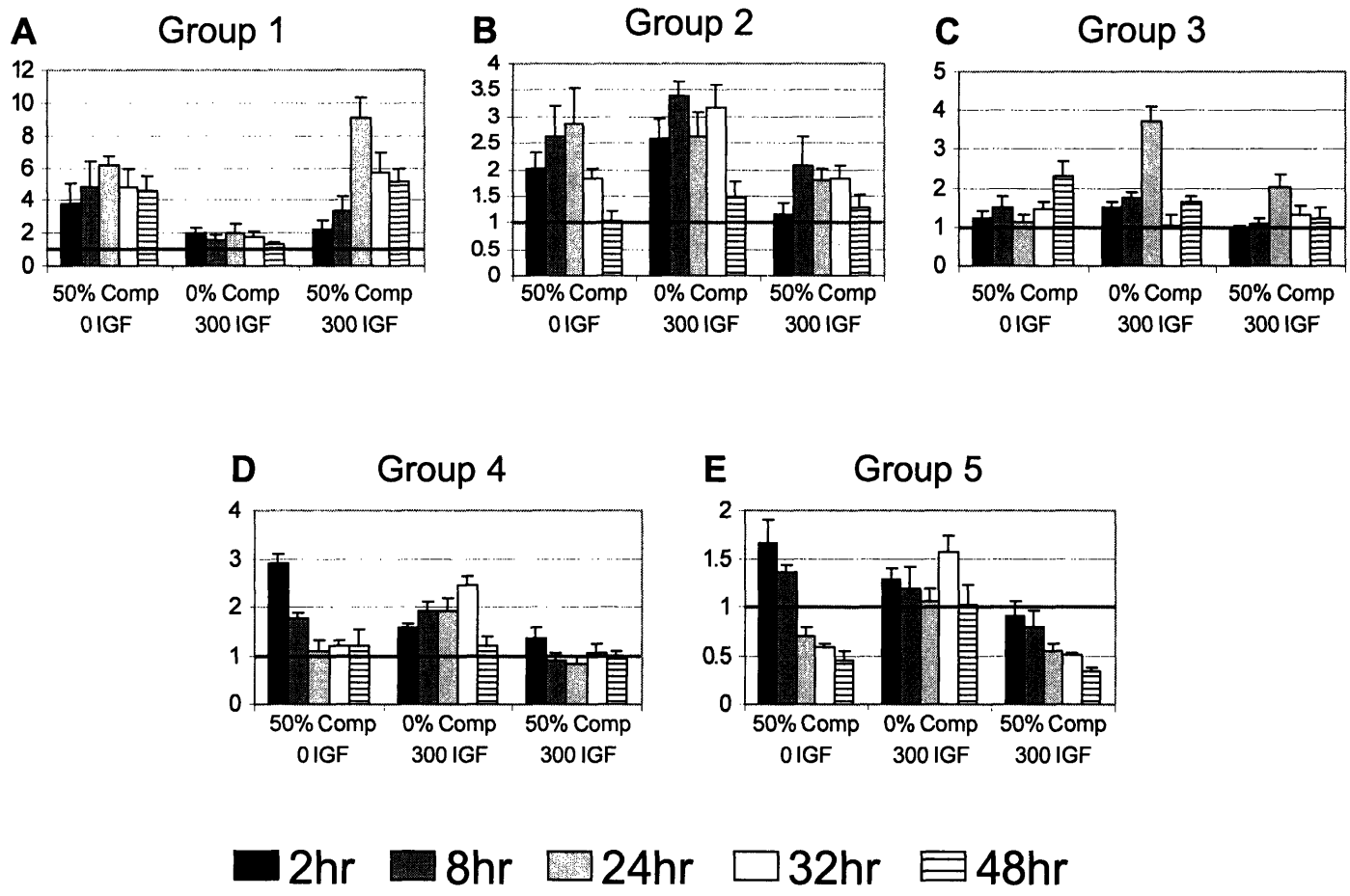


Figure 2.6. Aggrecan Protein Synthesis compared to Aggrecan Gene Expression. (A) Aggrecan protein synthesis as measured by 35S radiolabel incorporation normalized to 0% compression 0 IGF-1 adapted from Bonassar et al [5]. Mean plotted. (B) Aggrecan gene expression normalized to 18s and plotted relative to 0% compression 0 IGF-1. Significance was measured by the Wilcoxon sign ranked test compared to like compression 0 IGF-1. (* p-value <0.07).

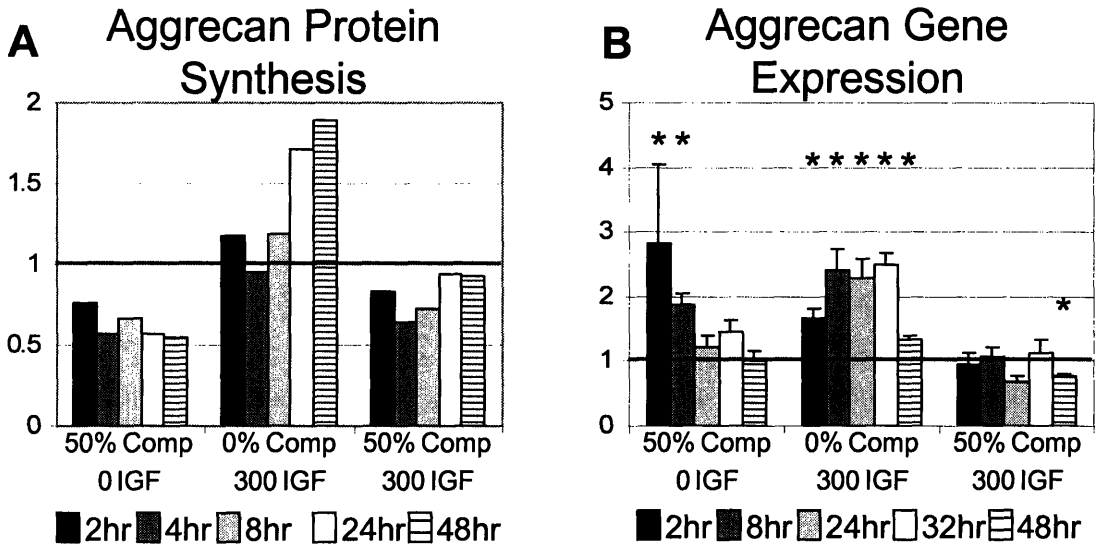


Table 2.1. 24 Cartilage Relevant Genes. Primers were designed Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi). Standard dilutions were used to calculate relative mRNA copy number.

Matrix Molecules	Proteases	Protease inhibitors	Cytokines	Growth Factors	Transcription Factors	Stress Activated Genes	Housekeeping Gene
Type II Collagen	MMP1	Timp-1	TNF-a	IGF-1	c-Jun	HSP90	18s
Aggrecan	MMP3	Timp-2	IL-1	IGF-2	c-Fos	Txnip	
Link Protein	MMP13	Timp-3	IL-4	TGF-B	Sox-9		
Fibronectin	ADAMTS-5		IL-6				

Table 2.2. Gene clustering groupings. Resulted gene sorting according to extent and kinetics of expression. Specific gene allocation and centroid coordinates when all data are clustered (A), 0% compression 300 ng/ml IGF-1 data clustered (B), 50% compression 0 IGF-1 data clustered (C), and 50% compression 300 ng/ml IGF-1 data clustered (D).

A

Group	Genes	Centroid Coordinates (PC1, PC2, PC3)
1	TGF-B, c-Fos, c-Jun, Timp-3, ADAMTS-5, MMP13, MMP3	(-2.46, 2.37, -0.33)
2	Sox-9, HSP90, Timp-1, Link	(-3.10, 0.95, -0.07)
3	TNF-a, IL-1, IL-4, IL-6	(-2.78, -1.50, -0.94)
4	IGF-2, MMP1, Fibronectin, Aggrecan	(-2.71, -0.24, 2.03)
5	IGF-1, Txnip, Timp-2, Collagen II	(0.32, 3.21, -0.19)

B

Group	Genes	Centroid Coordinates (PC1, PC2, PC3)
1	TGF-B, Sox-9, Timp-2, Timp-1, Fibronectin	(-1.81, 0.93, 0.43)
2	Txnip	(1.11, 0.91, -1.55)
3	IGF-2, IGF-1, c-Jun, c-Fos, ADAMTS-5, MMP1, Link, Collagen II, Aggrecan	(-2.05, 0.39, -0.25)
4	TNF-a, IL-6, IL-4, MMP13	(-1.48, -1.58, -0.22)
5	IL-1, HSP90, Timp-3, MMP3	(-2.03, -0.57, -0.05)

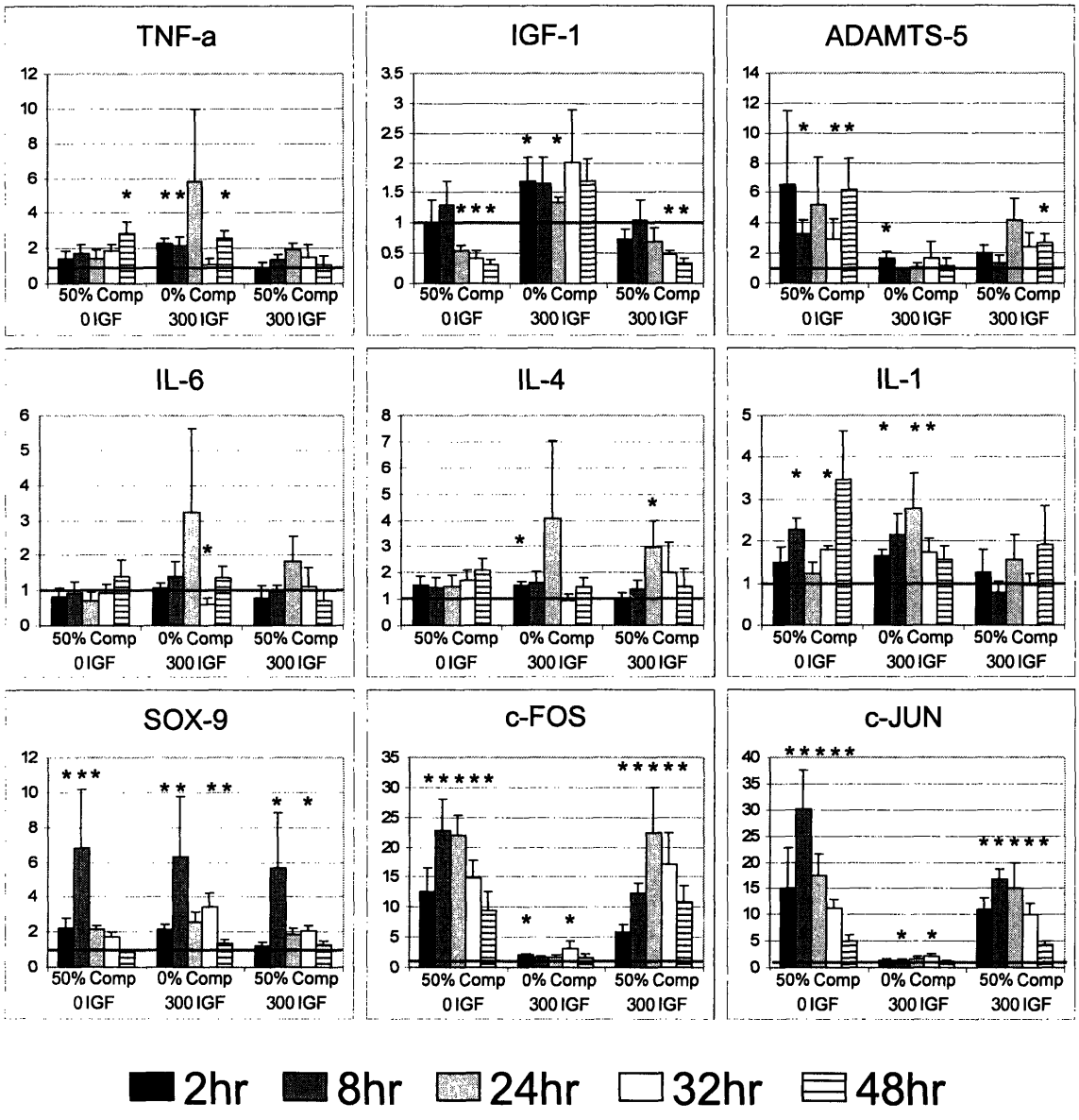
C

Group	Genes	Centroid Coordinates (PC1, PC2, PC3)
1	IGF-1, Timp-2, Collagen II	(0.44, 2.03, -0.13)
2	IGF-2, Txnip, MMP1, Fibronectin, Aggrecan	(-1.41, 1.42, -0.48)
3	TNF-a, IL-4, IL-1, Timp-3, ADAMTS-5, MMP13, MMP3	(-1.92, 0.16, 0.56)
4	TGF-B, c-Jun, c-Fos, Sox-9, HSP-90, Timp-1, Link	(-1.73, -0.86, -0.37)
5	IL-6	(0.59, -1.18, -1.76)

D

Group	Genes	Centroid Coordinates (PC1, PC2, PC3)
1	IGF-2, MMP1	(-0.52, 0.62, -1.65)
2	IL-6, IL-4, IL-1, Timp-3, ADAMTS-5, MMP13, MMP3	(-1.88, -0.49, 0.14)
3	TGF-B, TNF-a, c-Jun, c-Fos, Sox-9, HSP90, Timp-1, Link	(-1.88, 0.62, 0.05)
4	Fibronectin	(1.34, -1.62, -0.59)
5	IGF-1, Txnip, TIMP-2, Collagen II, Aggrecan	(1.77, 0.70, 0.14)

Appendix A


TNF-a
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ADAMTS-5
IL-6
IL-4
IL-1
SOX-9
c-FOS
c-JUN
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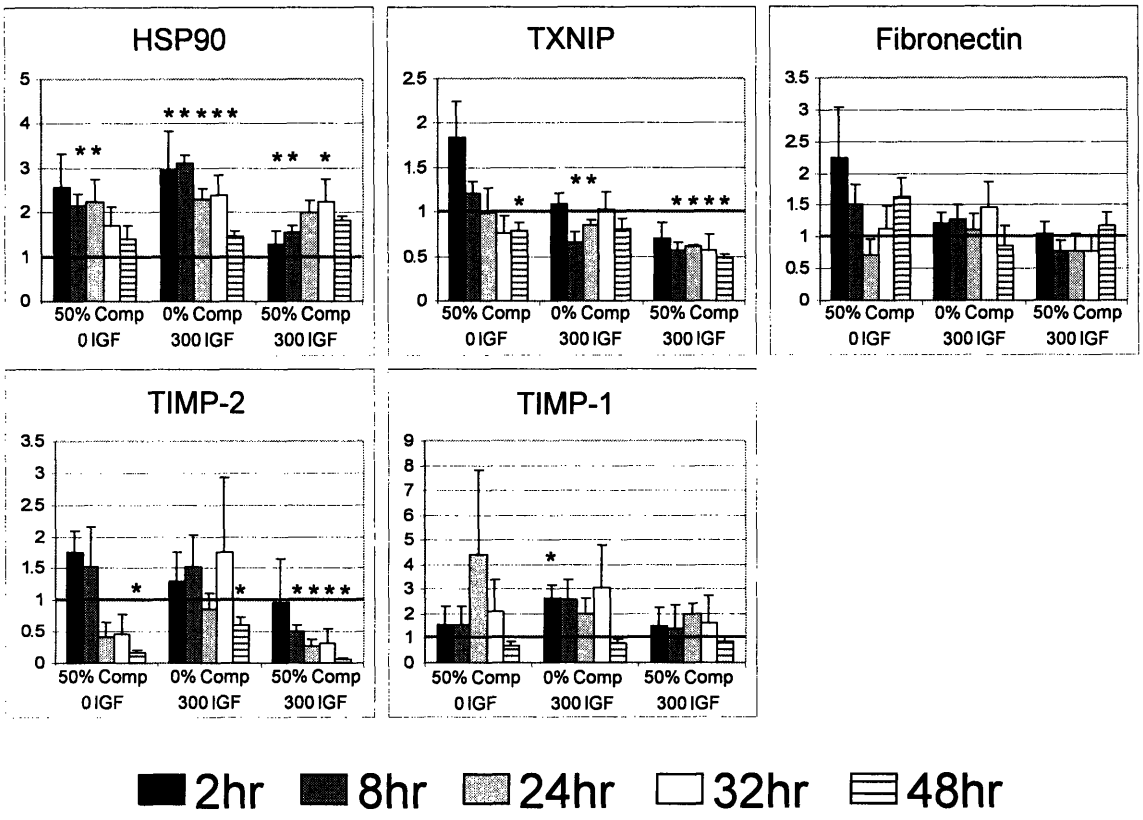
0% Comp 300 IGF
50% Comp 300 IGF
2hr
8hr
24hr
32hr
48hr

Appendix A


HSP90
TXNIP
Fibronectin
TIMP-2
TIMP-1
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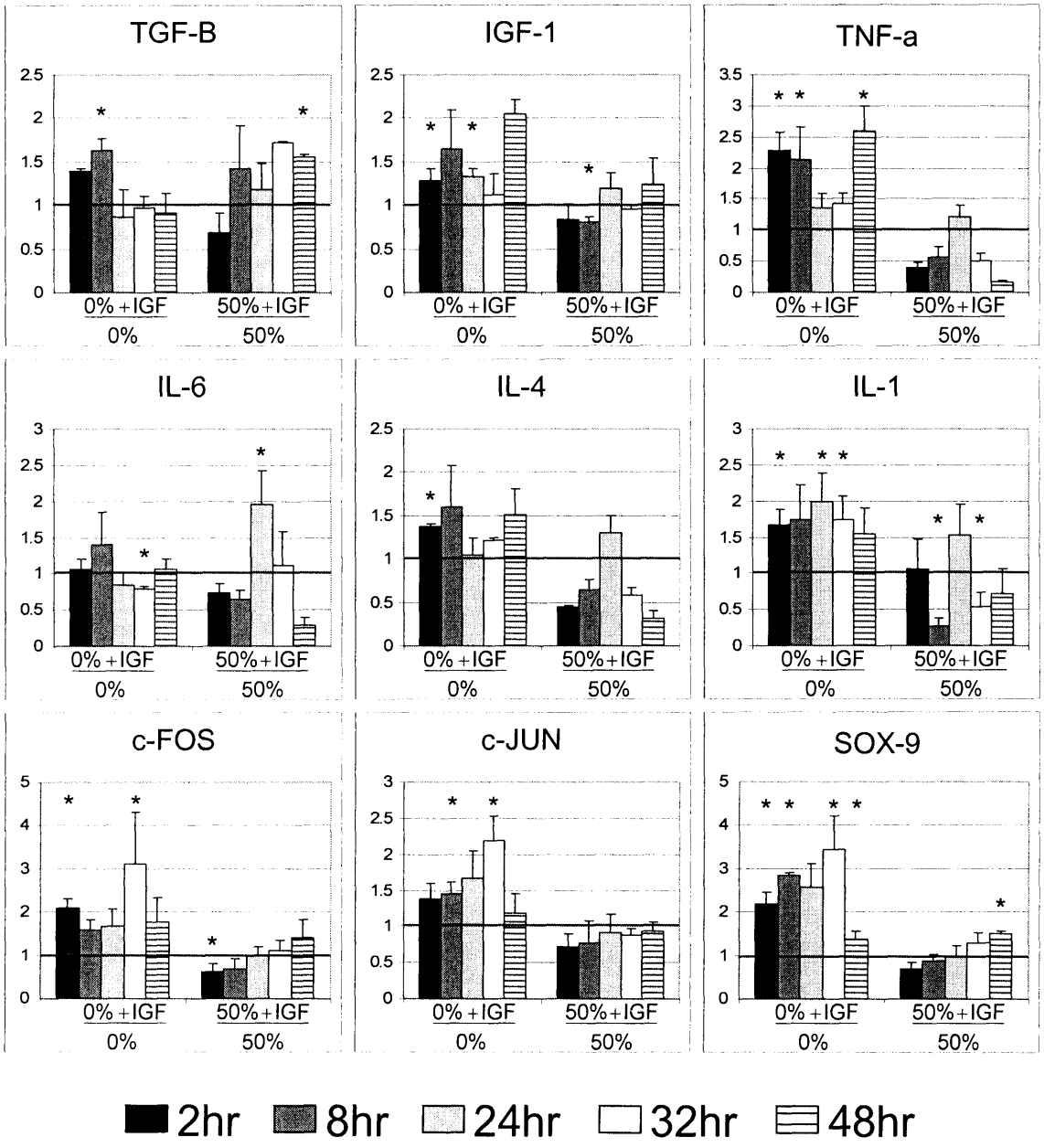
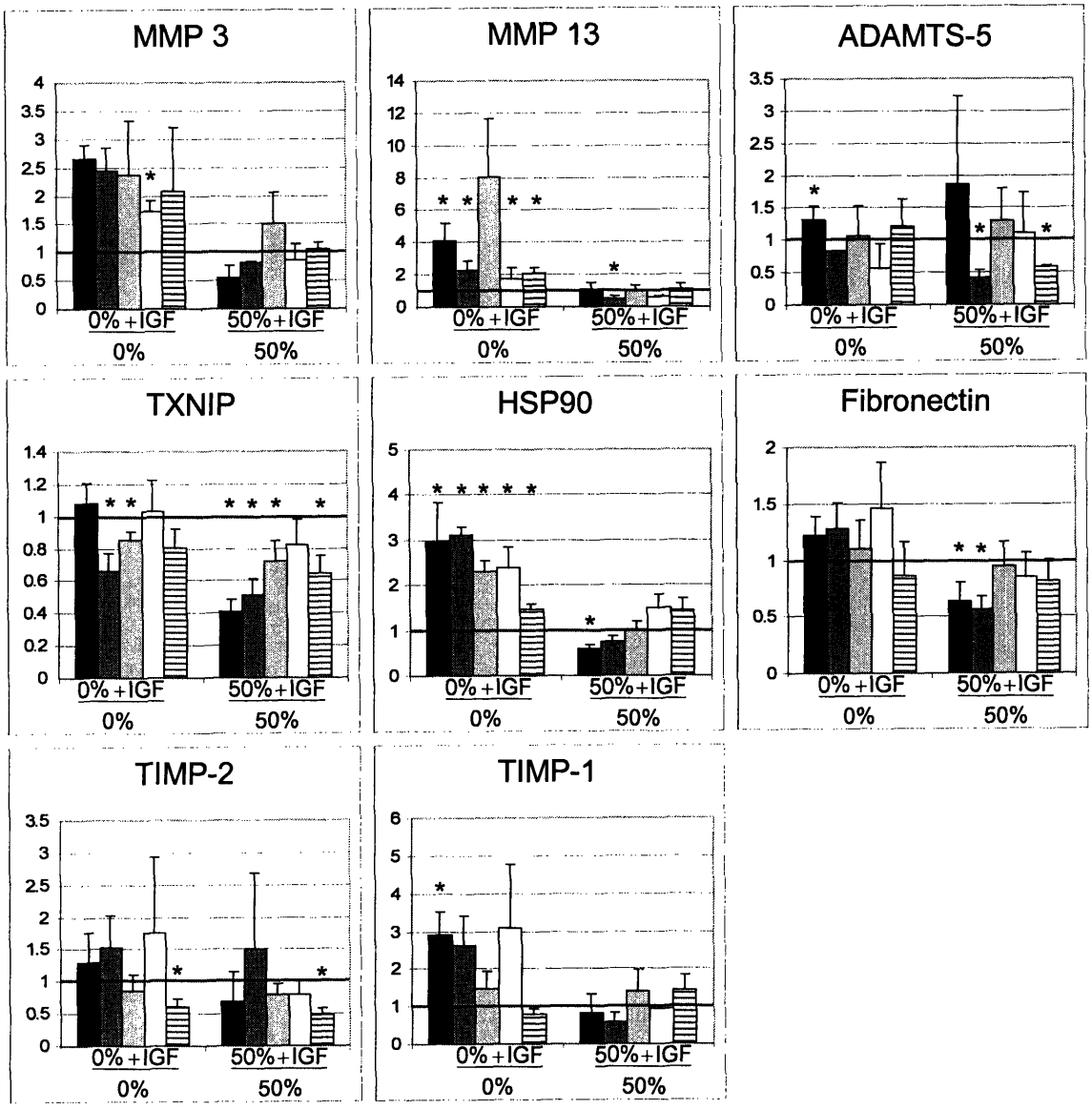
0% Comp 300 IGF
50% Comp 300 IGF
2hr
8hr
24hr
32hr
48hr

Appendix B


TGF-B
IGF-1
TNF-a
IL-6
IL-4
IL-1
c-FOS
c-JUN
SOX-9
0% +IGF
50%+IGF
0%
50%
2hr
8hr
24hr
32hr
48hr

Appendix B


MMP 3
MMP 13
ADAMTS-5
TXNIP
HSP90
Fibronectin
TIMP-2
TIMP-1
0% +IGF
50%+IGF
0%
50%
2hr
8hr
24hr
32hr
48hr

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Cellular Events Leading to Chondrocyte Death After Cartilage Impact Injury

D. M. Green,[1] P. C. Noble,[2] J. S. Ahuero,[1] and H. H. Birdsall[3]

Objective. **We undertook this study to test our postulate that leukocytes extend the zone of injury in cartilage after acute mechanical trauma.**

Methods. **Fresh cadaveric canine femoral condyles were subjected to 20–25-MPa impact injury. Condyle explants or dispersed chondrocytes were cultured with autologous blood mononuclear leukocytes (MNLs). Viability of chondrocytes at varying distances from the impact site was assessed by trypan blue exclusion.**

Results. **Mechanical injury caused a significant loss of viable chondrocytes over 7 days, even in cartilage >10 mm from the impact site. After biomechanical stress, death of cells within 10 mm of the impact could be largely prevented by addition of N^G-monomethyl-L-arginine to inhibit nitric oxide (NO) generation. Chondrocytes within 10 mm of the impact were also susceptible to killing by living MNLs, but not by incubation with the supernatants of endotoxin-activated MNLs. Chondrocytes in this vulnerable zone expressed intercellular adhesion molecule 1 (ICAM-1) (CD54), facilitating attachment of MNLs that localized adjacent to the chondrocytes. Leukocytes killed dispersed chondrocytes harvested from the impact zone by generation of reactive oxygen species. Leukocyte-mediated killing could be blocked by desferoxamine or by antibodies to CD18, which prevent attachment of leukocytes to ICAM-1–expressing chondrocytes.**

Conclusion. **Our data suggest that after mechanical injury, chondrocytes distant from the site may be killed through the generation of NO. Inflammatory leukocytes further extend the zone of chondrocyte death by adhering to chondrocytes expressing ICAM-1 and by inducing the accumulation of free oxygen radicals in the chondrocyte cytoplasm. Patients may benefit from therapies that reduce infiltration of inflammatory leukocytes into acutely injured cartilage.**

In vivo, human articular cartilage is repeatedly subjected to peak stresses of up to 15–20 MPa (1). After exposure to mechanical injury exceeding 20 MPa, up to 50% of chondrocytes die within 6–96 hours, principally by apoptosis (2,3). Proteoglycans are also degraded in the zone of injury (1,4), releasing glycosaminoglycans into synovial fluids (5). Although it is well appreciated that acute impact loading causes degeneration of articular cartilage via intracellular and extracellular processes, the role of the inflammatory system in the response of articular cartilage to mechanical trauma has yet to be fully elucidated. Traumatic and ischemic injury to parenchymal tissues typically stimulates an influx of leukocytes (6). Even though their products may initially exacerbate tissue injury, the infiltrating leukocytes ultimately promote tissue regeneration and healing (7). The situation may be different in cartilage. Considering the limited regenerative potential of cartilage, tissue injury caused by leukocytes might outweigh any reparative phase they might direct.

When activated in vitro, cultured chondrocytes release chemoattractants such as interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) that direct the migration of leukocytes (8,9). Leukocytes, in turn, are a source of proinflammatory cytokines, such as IL-1 and tumor necrosis factor α (TNFα), that induce apoptosis in both hypertrophic and nonhypertrophic chondrocytes (10,11). IL-1 can also induce chondrocytes to produce sufficient nitric oxide (NO) to cause cell

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[1]D. M. Green, MD, J. S. Ahuero, MD: Baylor College of Medicine, Houston, Texas; [2]P. C. Noble, PhD: Baylor College of Medicine and Methodist Hospital, Houston, Texas; [3]H. H. Birdsall, MD, PhD: Baylor College of Medicine and Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas.

Address correspondence and reprint requests to H. H. Birdsall, MD, PhD, Research Office, MS 151, Michael E. DeBakey Veterans Affairs Medical Center, 2002 Holcombe, Houston, TX 77030. E-mail: birdsall@bcm.tmc.edu.

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death (11). These proinflammatory cytokines upregulate the expression of intercellular adhesion molecule 1 (ICAM-1) on chondrocytes, which allows the attachment of leukocytes, and facilitate the accumulation of toxic agents within the chondrocyte cytoplasm (12). In addition, these cytokines stimulate the release of metalloproteinases by injured chondrocytes and leukocytes, and these metalloproteinases promote the degradation of the extracellular matrix (10,13–18).

While it is clear that mediators released by activated leukocytes can injure cultured and/or activated chondrocytes in vitro (10,15–18), few studies have examined the effects of inflammatory cells on chondrocytes embedded in their native matrix and activated by mechanical injury. Moreover, the mechanisms by which inflammatory cells cause localized destruction of articular cartilage through direct interaction with chondrocytes are not fully understood. Using in vivo and in vitro models, this study is the first to examine the mechanisms of chondrocyte death after impact injury and the role of leukocytes in extending the zone of injury to chondrocytes still resident in the articular matrix.

MATERIALS AND METHODS

Mechanical injury of condyles. Canine hind knee joints were collected within 1 hour after death from 24 skeletally mature young adult animals and sectioned, separating the medial and lateral condyles. Each condyle was stored in 40 cc of Dulbecco's phosphate buffered saline (DPBS) for no more than 1 hour before being subjected to impact injury. Each specimen was mounted beneath a drop weight impactor modified from the design of Thompson et al (19). An indentor, consisting of a stainless steel rod of 6 mm diameter with a 0.5-mm rounded edge, was positioned perpendicular to the surface of the articular cartilage. A 2-mm–thick piece of butyl rubber was placed between the cartilage and the metallic tip to distribute impact and prevent punch-out injury to the cartilage. A sliding weight was used to strike the indentor, generating a peak compressive stress of 20–25 MPa over a period of ~10 msec. This stress has been determined by others to cause reproducible cartilage injury (20). This system has also been tested in an in vivo model and has been shown by magnetic resonance imaging evaluation to create predictable osteochondrial injury (21).

Prior to impact injury, each condyle was mounted on a metal plate at 30 degrees to the horizontal, and the indenting rod was placed on a flat portion of the articular surface and clamped in place. Three impacts were then delivered in rapid succession. Because of the curvature of the condyle, the 30-degree angle allowed 2 sites to be impacted perpendicular to the articular surface with minimal shear. After impacting 1 side of the condyle, the specimen was turned 180 degrees and subjected to another series of 3 impacts. The condyle was then divided in half between the impact sites and placed into fresh DPBS. The left condyle remained uninjured and served as a control, and was likewise divided into medial and lateral halves. The impacted and control specimens were kept moist during the experimental procedures, received the same length of exposure on the test platform, and underwent the same processing except for impact injury.

Culture of condyle explants. The condyle halves were washed 3 times in 40 ml of DPBS, and the basal side was irrigated to remove all macroscopic evidence of marrow. The condyles were placed in 40 ml of RPMI 1640 with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in a T-75 flask. This volume of medium was sufficient to completely submerge the tissue and maintain the explants without acidification of the buffer. Samples were cultured in 5% CO_2 and room air at 37°C. In some cases, condyles were cultured either with autologous mononuclear leukocytes (MNLs) at a final concentration of 1×10^5/ml or with the supernatant of endotoxin-stimulated MNLs (20% [volume/volume]). In some experiments, N^G-methyl-L-arginine (L-NMA; Sigma, St. Louis, MO) was added at a dose of 1 m*M*, which has been shown to inhibit NO synthase in chondrocytes (22,23). Condyle explants cultured with MNLs were gently rocked on a daily basis to resuspend the MNLs.

Analysis of chondrocytes. After various intervals of 12 hours to 7 days, depending upon the experimental protocol, specimens were removed from culture, and full-thickness cartilage samples were harvested with a 4-mm biopsy punch from 1) the area immediately contacted by the indentor (the impact zone), 2) a site 6–9 mm from the edge of the impact zone, and 3) a site at the periphery of the articular surface >10 mm from the impact zone. Samples were processed for histologic analysis by frozen section or digested to harvest individual chondrocytes. To disperse chondrocytes, cartilage was digested in collagenase (2 mg/ml; Sigma) in complete medium (RPMI 1640 with 10% FCS and 1% penicillin/streptomycin) for 10 hours at 37°C in 5% CO_2. Chondrocytes were harvested by centrifugation at 1,100 revolutions per minute for 5 minutes and transferred into complete medium. Cell viability was assessed using trypan blue exclusion; a minimum of 100 cells were counted in duplicate from each sample. Chondrocytes were readily distinguished from leukocytes on the basis of size. Each punch biopsy specimen yielded, on average, $5.62 \pm 0.15 \times 10^5$ chondrocytes (mean ± SEM of 56 specimens), and there were no significant differences in the total numbers of chondrocytes recovered from biopsy samples obtained from the different sites in the impacted condyles or from the sham-treated condyles ($P = 0.37$ by Kendall's test).

Preparation of MNLs. Autologous blood was collected into preservative-free heparin. MNLs, which include lymphocytes and monocytes, were isolated by density-gradient centrifugation over Ficoll-Hypaque (Sigma) (24). Isolated MNLs were washed in Ca^{2+}-free and Mg^{2+}-free Hanks' buffered saline (Life Technologies, Gaithersburg, MD) and then resuspended in complete medium at a concentration of 4×10^5/ml. MNL viability was >95%. All reagents were nonreactive for endotoxin by the *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA), which will detect as little as 0.03 endotoxin units/ml. MNLs were not activated by this isolation protocol, since they have the same expression of L-selectin, CD11b/CD18, and CD11a/CD18 as unseparated MNLs in whole blood. MNLs were cultured in polypropylene tubes to minimize subsequent monocyte isolation and/or loss through

adherence to the culture vessel walls. Some MNLs were stimulated with 1 μg/ml of *Escherichia coli* endotoxin (Sigma) for 12 hours, and the supernatant, which contained the proinflammatory cytokines they released, was collected by centrifugation.

Flow cytometric detection of adhesion molecules on chondrocytes. Chondrocytes were suspended in complete medium at 5×10^5/ml and incubated with Cell Tracker Green (Molecular Probes, Eugene, OR) for 20 minutes at 37°C to identify viable cells (24). These cells were then washed with DPBS, stained with mouse monoclonal antibody to canine ICAM-1 (clone G-5; Santa Cruz Biotechnology, Santa Cruz, CA) at 10 ng/sample for 30 minutes at 4°C, washed, and stained with a phycoerythrin-conjugated goat anti-mouse antibody (Dako, Carpinteria, CA) for 30 minutes at 4°C. Cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry to assess the percent of viable chondrocytes expressing surface ICAM-1 (25). Delimiters on the flow cytometer were set using cells stained in the same manner but with nonspecific mouse immunoglobulin in lieu of anti–ICAM-1.

Immunohistochemical detection of adhesion molecules on chondrocytes. For immunohistochemistry, tissues were mounted in OCT embedding compound and quick-frozen. Five-micron sections were mounted on slides, fixed in acetone for 4 minutes, then rinsed in water and PBS. Endogenous peroxides were blocked with 3% H_2O_2 for 30 minutes. The sections were then rinsed and treated with 1% bovine serum albumin for 15 minutes to block nonspecific staining. Slides were stained with monoclonal anti–ICAM-1 (anti-CD54) followed by biotinylated secondary rabbit anti-mouse IgG and developed with avidin–biotin complex, diaminobenzidine, and H_2O_2. Slides were then counterstained with hematoxylin to visualize nuclei.

Interactions of MNLs with chondrocytes. The condylar explants were immersed in a suspension of MNLs at a final concentration of 1×10^5/ml in complete medium. To aid in identification, MNLs were prestained with a fluorescent green dye (Cell Tracker Green) as previously described (24). After 12 hours, explants were dipped in medium to remove nonadherent leukocytes. Punch biopsy samples were obtained and sectioned perpendicular to the cartilage surface. Sections were examined with an inverted fluorescence microscope to identify the location of MNLs that had adhered to the articular cartilage.

Detection of oxidative species transferred to chondrocytes. Chondrocytes were released from collagenase-digested cartilage biopsy samples 12 hours after injury and were then incubated with 5- (and 6-)dichlorodihydrofluoresceindiacetate (DCFDA; Molecular Probes) at a final concentration of 100 μM for 40 minutes at 37°C (26), washed twice, and suspended in complete medium. DCFDA-labeled chondrocytes were mixed with an equal number of MNLs (5×10^4 of each) in a final volume of 200 μl in a 96-well plate and incubated at 37°C. Conversion of DCFDA to its oxidized fluorescent product was detected with a fluorescence plate reader at 6 hours. In other experiments, the incubation with leukocytes was extended to 72 hours, and viability was assessed by trypan blue exclusion. To inhibit oxidative radicals, we used superoxide dismutase (final concentration 200 units/ml; Sigma) or desferoxamine (final concentration 0.1 m*M*; Sigma). Desferoxamine chelates the iron that catalyzes the formation of hydroxyl radicals from O_2^- and H_2O_2. Superoxide dismutase inhibits the formation of hydroxyl radicals by degrading O_2^-. When added to culture medium, desferoxamine can diffuse into cells, but superoxide dismutase cannot. To inhibit adhesion of leukocytes, we used murine monoclonal anti-CD18 (R15.7; final concentration 50 μg/ml) (a gift from Dr. Wayne Smith, Baylor College of Medicine). This antibody was incubated with MNLs for 30 minutes prior to their addition to chondrocytes.

Statistical analysis. Values are presented as the mean ± SEM. Due to the small sample size, statistical significance of differences between variables was calculated using the nonparametric Mann-Whitney U test.

RESULTS

Viability of chondrocytes after mechanical injury. Only 45 ± 3% of the chondrocytes from the impacted zone were viable after 7 days in culture ($P = 0.03$; n = 4 dogs), compared with 92 ± 1% of chondrocytes from the uninjured controls (Figure 1). Chondrocyte viability was also significantly decreased in samples taken 6–9 mm from the impact site (44 ± 2%) ($P = 0.03$) and even in samples taken >10 mm from the impact site (60 ± 4%) ($P = 0.03$). The addition of L-NMA to inhibit production of NO partially inhibited chondrocyte death in the impact zone ($P = 0.03$) and

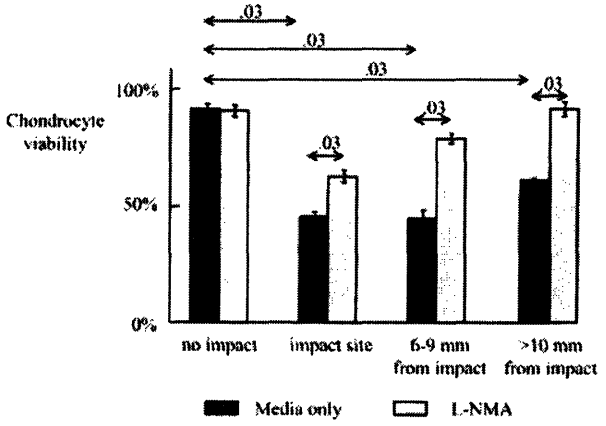

Figure 1. Effect of mechanical injury on cartilage. Explants from impact-injured and control uninjured condyles were cultured in medium alone or in the presence of 1 m*M* N^G-methyl-L-arginine (L-NMA). After 7 days, core biopsy samples were taken from the uninjured condyle, at the impact site, within 6–9 mm of the impact, and at a distant site >10 mm from the impact zone. Chondrocytes in the biopsy samples were released by collagenase treatment, and their viability was measured. Values are the mean ± SEM of data from 4 dogs. Impact injury led to significantly increased chondrocyte death in all sampled sites, and this could be blocked to varying degrees by the addition of L-NMA.

completely prevented the death of cells >10 mm from the impact site ($P = 0.03$) (Figure 1).

Effect of leukocytes on injured chondrocytes. To evaluate the ability of leukocytes to extend the zone of chondrocyte death after impact injury, we cultured autologous peripheral blood MNLs (e.g., monocytes and lymphocytes) with the cartilage explants for 7 days. In the impact zone, chondrocyte viability dropped from 55 ± 3% in the absence of MNLs to 33 ± 6% when cultured with MNLs ($P = 0.0002$; n = 8 dogs) (Figure 2). In the zone adjacent to the impact, chondrocyte viability dropped from 56 ± 4% in the absence of MNLs to 39 ± 8% when MNLs were present ($P = 0.001$). However, the viability of chondrocytes located >10 mm from the impact and that of chondrocytes from the unimpacted control condyle were unaffected by coculture with MNLs ($P = 0.8$ and $P = 0.22$, respectively). Chondrocyte killing appeared to require living MNLs, because incubation with the supernatants of endotoxin-activated MNLs, which contained elevated levels of TNF and IL-1, did not increase the numbers of nonviable chondrocytes from any of the samples above those seen with medium alone (data not shown).

MNL–chondrocyte interactions. To evaluate which chondrocytes in injured cartilage provide chemotactic stimuli and adhesive ligands for MNLs, we incubated the condyles with fluorescently tagged MNLs for 12 hours and examined thin cross-sections by fluorescence microscopy. MNLs adhered to cartilage explants that had received an impact injury (Figure 3), but not to uninjured cartilage explants (data not shown). The leukocytes were not randomly distributed across the cartilage; instead, each leukocyte was located immediately adjacent to a chondrocyte (Figure 3).

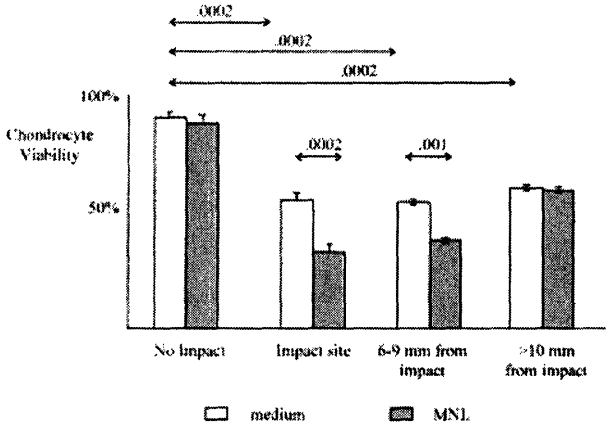

Figure 2. Effect of mononuclear leukocytes (MNLs) on injured cartilage. Explants from impact-injured and control uninjured condyles were cultured in medium alone or in the presence of autologous MNLs. After 7 days, core biopsy samples were taken from the uninjured condyle, at the impact site, within 6–9 mm of the impact, and at a distant site >10 mm from the impact. Chondrocytes in the biopsy samples were released by collagenase treatment, and their viability was measured. Values are the mean ± SEM of data from 8 dogs. Impact injury led to chondrocyte death in all sampled sites. Incubation with MNLs resulted in additional killing of chondrocytes at the site of impact and within 9 mm of the impact.

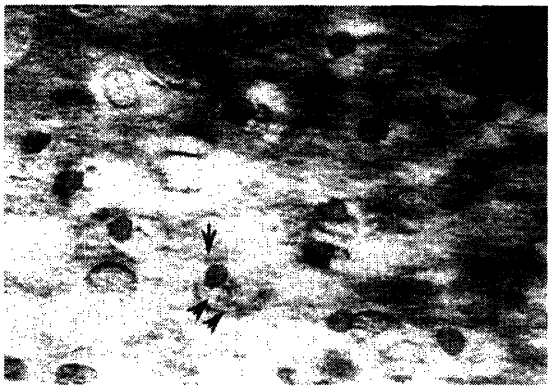

Figure 3. Adherence of mononuclear leukocytes (MNLs) to injured chondrocytes. Explants from impact-injured condyles were cultured for 12 hours with autologous MNLs that had previously been labeled with fluorescent green dye. Punch biopsy samples 4 mm in diameter were taken from the impact site, washed by immersion in Dulbecco's phosphate buffered saline to remove unattached leukocytes, and sectioned for examination by microscopy. The figure shows the fluorescent light image, to visualize the MNLs, superimposed on the photomicrograph taken with incandescent light, to show the location of the chondrocytes. The section is from the articular surface of the cartilage. MNLs (green, **single arrow**) tended to localize immediately adjacent to a chondrocyte (**double arrow**) (original magnification × 1,000).

Expression of ICAM-1 on chondrocytes. Parenchymal cell expression of ICAM-1 (CD54) mediates the attachment of leukocytes expressing counterreceptors such as CD11a/CD18 (lymphocyte function–associated antigen 1 [LFA-1]), CD11b/CD18, or CD11c/CD18. To evaluate whether stress-injured chondrocytes express ICAM-1, we harvested chondrocytes from explants after 24 hours in culture and used monoclonal antibodies to identify ICAM-1–positive cells by flow cytometry (Figure 4A). Uninjured chondrocytes did not display any ICAM-1. However, in the impact zone, 58 ± 7% of the chondrocytes expressed ICAM-1 ($P = 0.03$; n = 4 dogs). The fraction of chondrocytes expressing ICAM-1 decreased with distance from the impact site, with only 44 ± 4% ICAM-1–positive chondrocytes in the zone 6–9 mm from the impact site ($P = 0.03$ versus uninjured

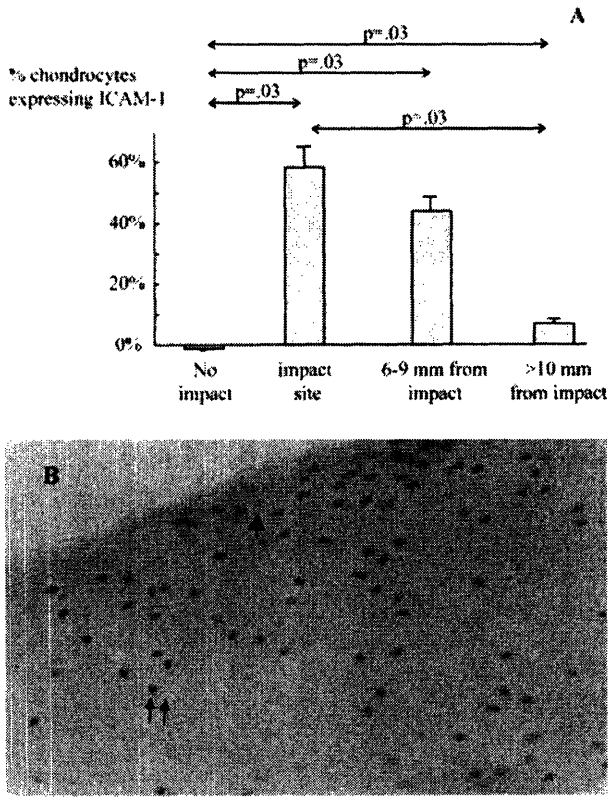

Figure 4. Expression of intercellular adhesion molecule 1 (ICAM-1) on injured chondrocytes. Condyles were subjected to an impact injury ex vivo; control condyles remained uninjured. **A,** Explants from the condyles were cultured for 24 hours, and then core biopsy samples were taken from the uninjured condyle, at the impact site, within 6–9 mm of the impact, and at a distant site >10 mm from the impact. Chondrocytes, released by collagenase, were assayed for expression of ICAM-1 by flow cytometry. Bars show the mean and SEM percent of viable chondrocytes that expressed ICAM-1. Experiments were performed in quadruplicate. **B,** Staining for ICAM-1 expression 12 hours after impact injury to articular cartilage. All cells were counterstained with hematoxylin to identify nuclei. Cells that are stained brown (**single arrow**) are ICAM-1 positive; blue cells (**double arrow**) are ICAM-1 negative. The majority of ICAM-1–positive cells are found in the top 25% of the articular cartilage (original magnification × 400).

chondrocytes). In samples taken >10 mm from the impact site, only 7 ± 1% of the chondrocytes expressed ICAM-1. This value was significantly higher than that in uninjured controls (P = 0.03), but also significantly lower than that in samples taken from zones closer to the site of impact (P = 0.03). To further identify the location within the cartilage of chondrocytes expressing ICAM-1, we examined sections by immunohistochemistry 12 hours after impact injury. The ICAM-1–expressing chondrocytes were predominantly located within the top 25% of the articular cartilage and were uncommon in the less superficial layers within the cartilage (Figure 4B).

Transmission of oxidative species from MNLs to chondrocytes. The fact that MNLs, but not the supernatant of activated MNLs, were able to kill injured chondrocytes suggested a mechanism involving direct transfer of toxic substances from the MNLs to the chondrocyte. To evaluate whether MNLs transfer oxidative radicals to the chondrocytes, we loaded the chondrocytes with a cytoplasmic dye, DCFDA, which fluoresces when oxidized. MNLs did not transfer oxidative radicals into control uninjured chondrocytes (P = 0.4) (Figure 5). However, if first injured by mechanical stress, DCFDA-labeled chondrocytes demonstrated a 1.6-fold increase in fluorescence, indicative of oxidative damage, when subsequently incubated with MNLs (P = 0.002; n = 6 dogs) (Figure 5). The increase in fluorescence was seen ~1–2 hours after addition of MNLs (data not shown) and peaked at 6 hours.

To determine whether reactive oxygen species (ROS) are transferred directly from the leukocyte to the chondrocyte or merely released into the microenvironment of the chondrocyte, we used a series of inhibitors that block generation of hydroxyl radicals. Superoxide dismutase, which cannot enter the cell cytoplasm, was unable to inhibit the increase of oxidative radicals in the chondrocytes (P = 0.59). Desferoxamine, which can enter the cytoplasm, blocked the increase of oxidative radicals in the chondrocytes (P = 0.002) (Figure 5). Addition of blocking antibodies to CD18, which prevent the binding of leukocytes to ICAM-1–expressing chondrocytes, also suppressed the increase of free oxygen radicals in the chondrocytes, by 70 ± 6% (P = 0.009) (Figure 5).

To evaluate whether the transmission of ROS was responsible for the leukocyte-mediated killing of chondrocytes, we investigated the effects of these same blocking agents on viability of chondrocytes from impacted cartilage. Impact injury alone caused 36 ± 2% of the chondrocytes to die within 3 days. Addition of leukocytes increased the fraction of nonviable chondrocytes to 70 ± 1%. Leukocyte-mediated injury was unaffected by superoxide dismutase (69 ± 1% nonviable chondrocytes) (P = 0.2). However, the additive effect of inflammatory leukocytes was fully blocked with desferoxamine (40 ± 1% nonviable chondrocytes) (P = 0.03) or L-NMA (42 ± 1% nonviable chondrocytes) (P = 0.03) and was partially prevented by anti-CD18 antibodies, which block adherence of MNLs to ICAM-1 (48 ± 1% nonviable chondrocytes) (P = 0.03) (Figure 6).

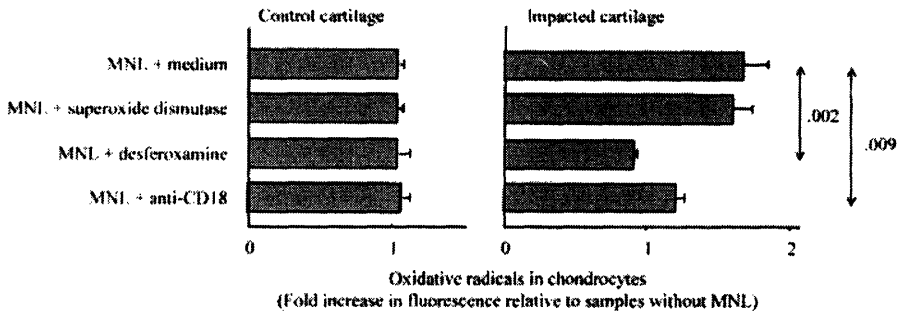

Figure 5. Transfer of oxidative radicals from mononuclear leukocytes (MNLs) to injured chondrocytes. Chondrocytes from the impact site or from uninjured condyles were released by collagenase treatment 12 hours after impact injury and loaded with an oxidation-sensitive fluorescent dye, 5- (and 6-)dichlorodihydrofluoresceindiacetate. Chondrocytes were incubated with MNLs or medium alone for 6 hours. Bars show the mean and SEM fold increase in fluorescence relative to chondrocytes incubated without MNLs (n = 6 dogs). The transfer of oxidative radicals could be inhibited with desferoxamine or with blocking antibodies to CD18, but not with superoxide dismutase (P = 0.59).

DISCUSSION

Our studies are the first to examine the fate of chondrocytes after they are exposed to mechanical stress while still resident within the cartilage. When articular cartilage was exposed to an acute impact, approximately half of the chondrocytes died within 7 days, whereas >90% of the chondrocytes survived in uninjured cartilage. A surprising finding was the reduced viability of chondrocytes located well outside the zone of direct impact. Chondrocyte death at all sites was blocked by L-NMA, an inhibitor of NO production (22,23). This finding is consistent with those of others who have demonstrated inhibition of apoptosis after NO blockade with L-NMA in an arthritis model (27). The directly impacted chondrocytes may have released NO, which triggered apoptosis of distant chondrocytes. In addition, the shock wave propagating from the epicenter of the impact may have activated distant chondrocytes and triggered production of NO.

Chondrocytes have shear stress response ele-

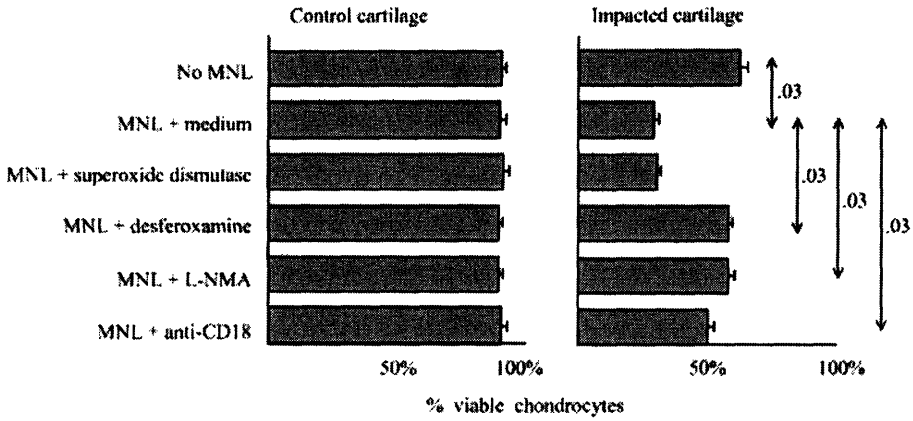

Figure 6. Effect of oxidative radicals from mononuclear leukocytes (MNLs) on chondrocyte viability. Chondrocytes from the impact-injured or uninjured condyles were released by collagenase treatment 12 hours after impact injury and incubated with medium or MNLs. After an additional 72 hours, chondrocyte viability was assayed by trypan blue exclusion. Values are the mean and SEM (n = 6 dogs). Viability of impact-injured chondrocytes was decreased in the presence of MNLs, and this could be prevented by the addition of desferoxamine, N^G-methyl-L-arginine (L-NMA), or anti-CD18, but not by the addition of superoxide dismutase (P = 0.2).

ments and are known to respond to biomechanical signals by up-regulating matrix metalloproteinases and proinflammatory cytokines (28,29). Clements et al demonstrated that cyclical loading of cartilage at physiologic peak stress levels causes apoptosis of cells both at the site of compression and in an area immediately around this site (30). In the present study, we examined cellular events following discrete traumatic impact of osteochondral specimens. The magnitude of the peak stresses generated in the present study was several times larger than that of those applied by Clements and coworkers. High-impact injury may have a different signaling cascade that makes chondrocytes susceptible to cell death from NO; this requires further study.

Chondrocytes injured by impact became vulnerable to further injury mediated by leukocytes. The vulnerability of chondrocytes to leukocyte-mediated injury was inversely proportional to the distance from the impact site. This outcome could be explained by differential rates of ICAM-1 expression, since chondrocytes within or immediately adjacent to the impact zone expressed ICAM-1, whereas those distant from the site of impact expressed very little ICAM-1, and no ICAM-1 was found on uninjured chondrocytes. ICAM-1 is an adhesion molecule that can be displayed by diverse somatic cells, particularly after injury or in the context of inflammation. Leukocytes express receptors, CD11a/CD18 and CD11b/CD18, which allow them to attach to target cells expressing ICAM-1. ICAM-1 was expressed primarily by chondrocytes in the upper layer of the cartilage. This is also where leukocytes adhered to the chondrocytes. The role of ICAM-1 in the interaction of leukocytes with injured chondrocytes was verified by showing that in the presence of blocking antibodies to CD18, the β-chain common to both leukocyte receptors, chondrocytes were no longer killed by leukocytes.

In this study, ICAM-1 expression was demonstrated on chondrocytes as early as 12 hours after injury. ICAM-1 has been demonstrated on isolated chondrocytes, cultured in vitro in the presence of proinflammatory cytokines (12). However, this is the first time up-regulation of ICAM-1 has been demonstrated after acute mechanical injury of articular cartilage. In our specimens, ICAM-1 was induced on injured chondrocytes even in the absence of MNLs, indicating that leukocyte-derived factors were not required. ICAM-1 was also found on chondrocytes that were not in the area of the direct impact.

There are several possible mechanisms for injury to chondrocytes distant from the direct site of impact. Previous studies have demonstrated that the ICAM-1 gene has a shear stress response element and that endothelial cells exposed to flow-mediated shear stress up-regulate their expression of ICAM-1 (31). During localized impact of a joint surface, a shear wave propagates through the cartilage and the underlying subchondral bone. We hypothesize that this perturbation triggers the shear stress response elements known to be present within chondrocytes (32), and this intercellular signaling event up-regulates ICAM-1. An alternative explanation is that chondrocytes at the impact site released soluble factors such as cytokines in response to mechanical injury, which then stimulated nearby chondrocytes to up-regulate ICAM-1. NO, for example, can initiate intracellular signaling cascades leading to up-regulation of cytokines (33). Basic fibroblast growth factor can also be released from extracellular stores by mechanical stress and activate the ERK signaling pathway, which leads to a variety of proinflammatory responses (34).

The preferential influx of MNLs into injured, as opposed to control, cartilage is probably mediated by chemotactic factors released by the chondrocytes. Previous studies of inflammatory arthropathies and of cartilage stimulated with IL-1 in vitro have demonstrated that 2 chemokines, MCP-1 and IL-8, are produced by stimulated chondrocytes (8,9). MCP-1 is a highly potent chemotactic factor for monocytes, and IL-8 is chemotactic for neutrophils. It is likely that leukocytes have access to impact-injured cartilage in vivo. We have found lymphocytes, monocytes, and neutrophils in the synovial fluid of dogs whose femoral condyles received an impact injury similar to that applied to the explants in these experiments (21). In the present study, we only evaluated the adhesion of MNLs to the chondrocytes. Other investigators have tested the ability of polymorphonuclear leukocytes to adhere to fragments of cartilage and have found evidence of surface macromolecules that block the adhesion of neutrophils (35).

Our studies show that leukocyte-mediated killing of impact-injured chondrocytes involves ROS and/or NO. The failure of superoxide dismutase, which remains extracellular, to inhibit the oxidation of an indicator dye in the chondrocyte cytoplasm indicates that the mechanism does not involve release of ROS into the extracellular milieu and subsequent uptake by the chondrocytes. Inhibition by desferoxamine, which enters cytoplasm, and by anti-CD18 antibodies, which block binding by leukocyte adhesion molecules, is most consistent with a model in which leukocytes adhere to chondrocytes and transfer ROS directly into their cytoplasm. Such a mechanism has been observed in other systems such as

leukocyte-mediated killing of injured myocytes (36). However, it is also possible that adherent leukocytes induced the chondrocytes to generate ROS. NO and ROS together can generate peroxynitrite (37), which is highly toxic for chondrocytes (38,39). In our experiments, cells were cultured in 5% CO_2 with room air, which may best model processes occurring on the cartilage surface near the synovial fluid. Mechanisms of injury for chondrocytes located more deeply in the cartilaginous matrix may differ due to the lower oxygen concentration in those sites (40–42).

Compared with medium alone, the supernatant of endotoxin-stimulated MNLs, which contained TNFα, IL-1, and IL-6, had no adverse effect on injured chondrocytes, suggesting that these cytokines are not responsible for killing in this model. Our results suggest that attachment to chondrocyte ICAM-1 mediated by leukocyte CD11a/CD18 (LFA-1) or CD11b/CD18 brings leukocytes into close apposition with the chondrocytes. Indeed, as we have shown, treatment of cultured chondrocytes with antibodies to CD18 decreased accumulation of free oxygen radicals in the chondrocytes by 70%. ICAM-1/LFA-1–mediated cell death of chondrocytes has been described previously (12), but this is the first time that mechanical injury has been shown to cause contact-mediated cell death of chondrocytes by inflammatory cells.

Our findings have possibly interesting clinical implications. First, we have demonstrated that acute mechanical injury of the articular surface causes death of chondrocytes located at a distance from the site of trauma. Second, up-regulation of adhesion molecules on the affected chondrocytes allows leukocytes to adhere and to extend the zone of injury beyond the impact site. Thus, the data in this study suggest that therapies to reduce acute influx of leukocytes into damaged cartilage should be considered in the future when treating osteochondral injuries.

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