Matrix Metalloproteinase Activity and Inhibition in Articular Cartilage: Effects on Composition and Biophysical Properties and Relevance to Osteoarthritis

by

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Abstract

Matrix metalloproteinases (MMPs), such as stromelysin, have been linked the degradation of articular cartilage in normal and pathologic tissue. Elevated levels of MMP production and mRNA expression have been detected in patients with rheumatoid arthritis, osteoarthritis and traumatic joint injury as well as in developing tissue and normal growth plate cartilage. While MMPs have been shown to degrade purified matrix macromolecules *in vitro*, their action against the matrix of intact cartilage has not been well established. The objectives of this thesis were threefold: (1) to characterize the ability of MMPs to degrade cartilage matrix constituents of intact cartilage; (2) to quantify the effects of this degradation on the functional biophysical properties of cartilage; and (3) to evaluate the ability of synthetic and natural MMP inhibitors to modulate these changes in composition and biophysical properties.

Bovine cartilage explants were treated with recombinant human stromelysin-1 and changes in biochemical, biomechanical, and physicochemical properties as well as histological appearance were assessed. Stromelysin treatment resulted in a time and dose dependent loss of proteoglycan fragments from the explants, increased degradation and loss of type IX collagen, and a concomitant swelling of the tissue. Histological examination demonstrated that proteoglycan loss proceeded inward from the exposed tissue surfaces, with a marked boundary between degraded and undegraded tissue. Stromelysin treatment resulted in marked decreases in equilibrium modulus and dynamic stiffness and a substantial increase in hydraulic permeability after three days in culture. Measurement of dynamic streaming potential showed that changes due to stromelysin treatment were strongly dependent on compression frequency, with dramatic decreases seen at high frequency prior to changes in mechanical properties, and little initial change seen at low frequency.

Cartilage from rabbit stifle joints which were exposed to stromelysin for 1 hour *in vivo* showed little difference in proteoglycan content from cartilage from contralateral

control joints, while synovial fluid proteoglycan content from stromelysin treated joints was significantly higher than that of controls. Histological examination revealed that loss of proteoglycan, as indicated by both toluidine blue and FVDIPEN antibody staining, occurred primarily in a narrow region of the tissue near the articular surface. Consequently, the mechanical properties, such as equilibrium modulus and dynamic stiffness, of cartilage samples from treated joints did not differ significantly from those of control joints, while electromechanical properties, such as streaming potential and electrokinetic coupling coefficient, of cartilage from treated joints were degraded significantly compared to controls. Systemic administration of a synthetic MMP inhibitor signficantly decreased release of proteoglycan fragments to synovial fluid, inhibited loss of proteoglycan as indicated by toluidine blue and FVDIPEN antibody staining, and prevented changes in cartilage electromechanical properties due to intraarticular injection of stromelysin.

The degradation of cartilage matrix by endogenous MMPs was characterized by treatment of explants with 4-aminophenylmercuric acetate (APMA) to activate native MMPs or by treatment with interleukin-1β (IL-1β) or retinoic acid (RA), which upregulate production of MMPs by chondrocytes, and may increase levels of other endogenous proteinases. Treatment with APMA resulted in significant loss of proteoglycan by 1 day in culture and in significant swelling response by 3 days in culture. Matrix degradation was manifest in changes in sample mechanical and electromechanical properties. Both a synthetic MMP inhibitor and the native MMP inhibitor, TIMP, significantly inhibited proteoglycan loss, swelling response, and changes in physical properties due to APMA treatment. Treatment with IL-1β and RA induced significant loss of proteoglycan after 4 days in culture. Treatment with IL-1β resulted in an increased swelling response after 8 days in culture, while RA treatment did not. Both IL-1β and RA treatments resulted in significant changes in tissue mechanical and electromechanical properties which were partially modulated by addition of TIMP to the culture media. Use of synthetic inhibitors also partially modulated proteoglycan loss from IL-1β and RA treated tissue. Analysis of fragments in the culture media from these samples indicated that cleavage of proteoglycan did not occur at the VDIPEN-FFGV site associated with MMP activity, but rather at the NITEGE-ARGSVL site on the aggrecan core protein. This was the only proteinase activity observed against aggrecan in this system, and it was partially inhibited by the addition of a synthetic MMP inhibitor to the culture media.

Thesis Supervisor:

Alan J. Grodzinsky, Professor of Electrical, Mechanical, and Bioengineering, MIT

Acknowledgments

I was fortunate enough to attend the Bioengineering Gordon Conference this summer, and at one point during the meeting I was pulled aside by Klaus Kuettner, who told me that I did not realize how lucky I was to have Alan Grodzinsky as my thesis supervisor. While I do know that I have been very fortunate, I do think there is some truth in this, and that I will appreciate this more and more in due time. Al has been a true mentor in every sense of the word. His dedication to teaching, research, and most of all people are testament to his consummate professionalism. Much of what I have learned over the past five years has been from Al, and he has made my time in graduate school as enjoyable as possible. My most sincere thanks go to Al.

If it were not for our collaboration with Vern Moore and Mike Lark, most of the pages in this thesis would be blank. Their material and intellectual support have been critical to all aspects of this work. Their contributions mean a lot to me on both a personal and professional level. I am very glad I had the chance to interact with them on this project. Many of Mike's and Vern's colleagues at Merck Research Laboratories have also contributed to this work. I have had the good fortune to meet and work with Jack Schmidt, Ellen Bayne, Denise Visco, Rick Mumford, and Bill Hagmann on various aspects of this research, and their contributions to this thesis are significant.

John Sandy from Shriner's Hospital in Tampa has been an invaluable resource for our analysis of cartilage degradation products. His advice and discussions of data are sincerely appreciated. Our collaboration with David Eyre and James Wu from the University of Washington at Seattle has given us important insights on collagen degradation in our experiments. Additionally, Robin Poole from Shriner's Hospital in Montreal has contributed to our analysis of collagen degradation.

Now, as usual, it is time for Linda to step in and stop things from getting too serious. Linda is truly a unique and special person. She is an invaluable resource to everyone in the lab and has taken upon herself the unenviable task of making sure a bunch on MIT students don't work too hard or take themselves too seriously. I spent many a summer lunch break on the tennis court with Linda and although I may not be ready to play in the US Open, I will think of her when I sit in the stands or watch on TV.

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Minerva Garcia, Scott Berkenblit, and Tom Quinn have been my lab contemporaries. We have seen the graduations of Bob, Mike, and Young-Jo and seen a new generation of cartilage squeezers on the horizon. We also remember fondly the search for Al's coffee mug and advertisements for the rectomagnetograph. We had great times at Coop's and Check Point Charley - Bar and Laundromat and managed to survive all of Carl Flannery's excursions into the French Quarter. They are all fine scientists as well as good friends. I will miss the scientific discussions and the casual conversations. (Besides, I think Minerva still owes me cookies.)

There should be some special commendation for the people with whom I have shared and office. Ann, Dave, and Adi have over the years put up with my perpetually messy desk and have had to track me down countless times in the Athena cluster across the hall to answer phone calls. Thank you for your extreme patience, especially over the past few months.

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Thanks go to Rick, Fred, Jeff, and Rick (eh?). They have been great friends to me in my time here and many of my best memories of MIT involve them. As time goes on I am sure I will remember barbecues on Silvey Place and nights of sampling homebrews more than I will long nights spent in lab. Just remember – never own property, but if you do, never rent to female Harvard students who play musical instruments, especially if they have plants and vacuum at 3:00 in the morning.

Special thanks go to my family, Mom, Joseph, Carol, Ron, Heidi, Michael, Ryan, and Andrew. Without their support, I never would have been here, let alone made it through. I feel that after more than five years of listening to me complain, MIT should also award a degree to my sweetheart, Jane. She has heard more about cartilage than any person would want to know, and even listened to me babble on about stromelysin on Valentine's Day. Her enduring love, support, and patience are more than I could have ever hoped for or expected.

I truly have just one regret of my experience here, and that is that my father was unable to see me finish and read this thesis. Well, Dad, I made it. We miss you.

Lawrence J. Bonassar
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Chapter I

Introduction

1.1 Articular Cartilage

Articular cartilage is an avascular, aneural, alymphatic, connective tissue which covers the ends of bones in synovial joints. Its primary functions are to lubricate the surface of articulating joints and to distribute stresses generated by muscle contraction and body weight over bone surfaces. Articular cartilage is a porous, highly hydrated material, with water content of approximately 80% by volume. The solid component of cartilage consists of cells, called chondrocytes, and an extracellular matrix, with cell concentration being approximately 100×10^6 cells/ml [117]. The extracellular matrix is composed primarily of hyaluronic acid, various proteoglycans including aggrecan, decorin, and biglycan, and collagen types II, IX, and XI. A schematic diagram of cartilage structure is shown in figure 1.1 [57].

The mechanical properties of articular are related to its complex physical structure. The long, thin, rope like collagen fibrils present in the matrix serve to strengthen the tissue in tension and shear. The large proteoglycan, aggrecan, associates non-covalently with hyaluronan to form a network of large aggregates throughout the tissue. Branching off from the aggrecan core protein are many glycosaminoglycan (GAG) chains which contain sulfate and carboxylic acid charge moities, which endow the tissue with a significant charge density of approximately 0.1 to 0.2 moles of charge per liter of fluid [80]. Under physiological conditions all of these charge groups are effectively ionized. The attraction of positive counterions from the surrounding fluid to the negative charges fixed on the matrix creates an electrical double layer at the surface of the GAG chains. When compressive strains are applied to the tissue, the effective distance between double layers is decreased. As the double layers overlap, significant electrostatic repulsion develops which is translated into a marked increase in tissue stiffness [48]. It is estimated that as much as one half of

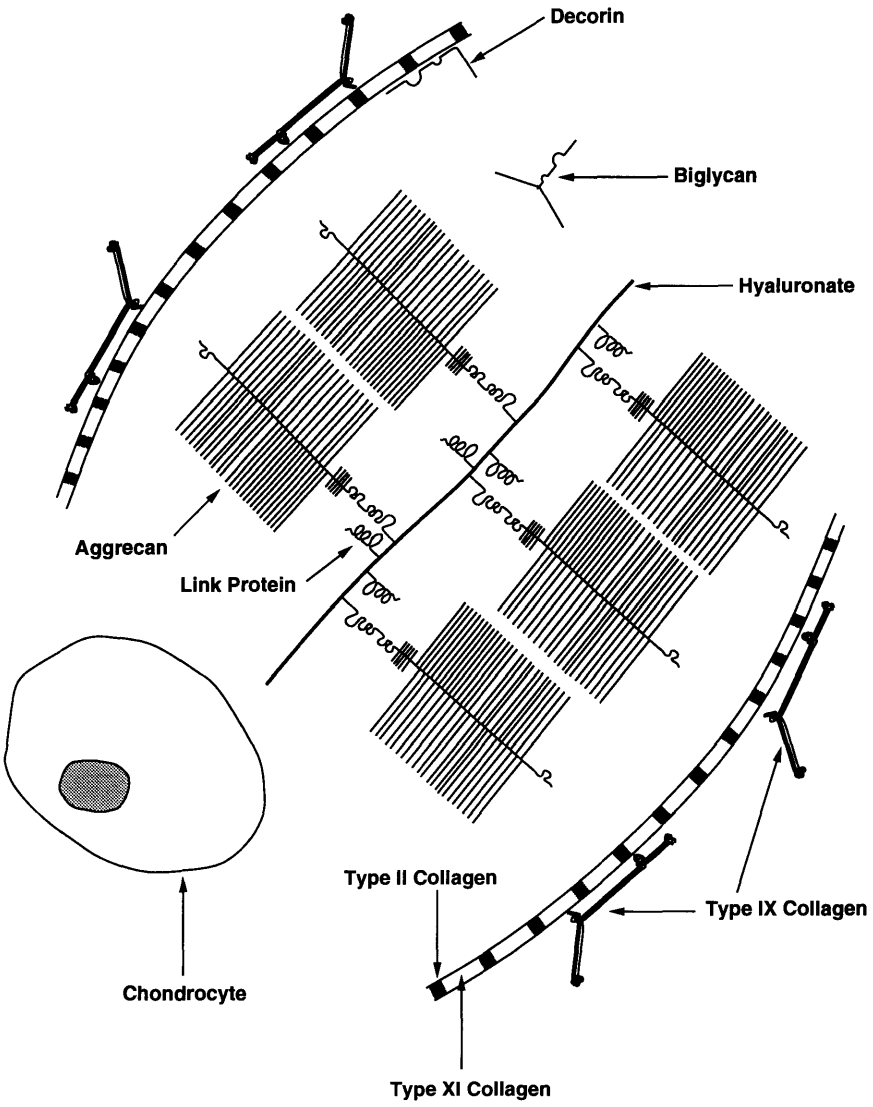

Figure 1.1: Schematic illustration of cartilage extracellular matrix

equilibrium tissue stiffness is attributed to electrostatic repulsion of GAG chains [39].

The fluid phase of the highly hydrated matrix also has a significant impact on the physical properties of articular cartilage. As the tissue is compressed, fluid leaves the tissue. The frictional forces which result from fluid movement relative to the solid phase tend to stiffen the tissue. As loading time is decreased (or loading frequency is increased), the velocity of the fluid relative to the matrix increases and thus frictional forces increase as well. This results in a dynamic tissue stiffness that is highly frequency dependent.

Fluid motion and the high charge density immobilized on the matrix combine to produce a phenomenon known as streaming potential. As previously stated, the charge groups fixed on the matrix cause a high density of mobile ion space charge to develop in the fluid surrounding the matrix in order to preserve electroneutrality (Donnan equilibrium). When strains are applied to the tissue, fluid motion thus separates the mobile fluid phase cations from the immobilized matrix anions. This charge separation results in the generation of local electric fields in the tissue. The sum of all the local electric fields across the tissue due to fluid streaming is called the streaming potential. Fig 1.2 illustrates the generation of a streaming potential in response to a uniaxially applied displacement [40].

1.2 Cartilage Biochemistry

The chemical constituents of the articular cartilage matrix are of extreme importance when discussing tissue function. The physical properties of the tissue are strongly dependent on the structure and organization of the macromolecules in the matrix [51]. These macromolecules include proteoglycans, collagens, and other proteins such as link protein, and hyaluronic acid. These matrix constituents are connected by chemical and mechanical cross links to form an effectively continuous three-dimensional network throughout the tissue. In addition, each type of matrix macromolecule has its own distinct chemical nature which contributes to the bulk properties of the material.

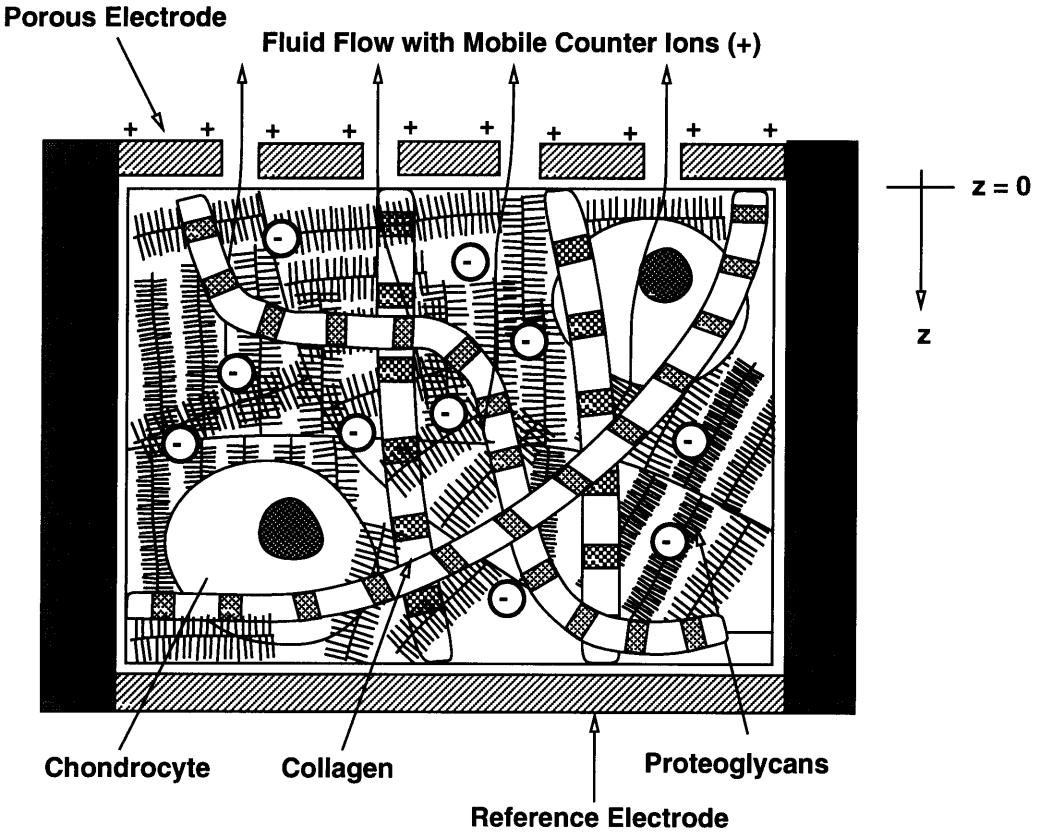

Figure 1.2: Illustration of streaming potential: mobile counterions present in the fluid phase are separated from charges bound to matrix when a mechanical stress or displacement is applied.

1.2.1 Proteoglycans

As a group, all proteoglycans in articular cartilage have some structural similarities. As the name implies, proteoglycans (PGs) are macromolecules composed of proteinous regions and glycosylated regions. Articular cartilage PGs consist of carbohydrate chains which are covalently bonded to a protein core. The nature of both the core protein and the chains varies with PG type. For the purposes of this discussion, cartilage PGs are divided into two types: large aggregating PGs (aggrecan); and small PGs (decorin, biglycan, and fibromodulin).

Aggrecan

The most abundant proteoglycan in articular cartilage by weight is the large aggregating proteoglycan, or aggrecan. Aggrecan is a macromolecule of considerable size, with total M_r of one to three million [56]. The core protein M_r is approximately 300,000 and extends to a length of approximately 300 nm [51]. In addition, there are approximately 100 chondroitin sulfate chains and 30 keratan sulfate chains bound to the protein core which provide the balance of molecular mass.

The aggrecan molecule is divided into five structural units, with three globular domains and two interglobular domains, as is shown in figure 1.3 [52]. The N-terminal domain on aggrecan, G1, associates with hyaluronan and link protein to form a link stabilized (non-covalent) aggregate. G1 is a globular domain with three folds. The N-terminal fold is an immunoglobulin fold (Ig fold) while the remaining two are a tandem repeat sequence characterized by a disulfide bond induced double loop [51]. The M_r of the G1 core is approximately 39 kDa and it is thought to bind a small number of keratan sulfate chains.

Progressing towards the C-terminal end, there is a short extended domain called the interglobular domain. This domain is approximately 21 nm long as has an M_r of 12 kDa. It is the site of attachment for several keratan sulfate chains and serves to link G1 to the second globular domain, G2. G2 is similar in structure to G1, although the Ig fold is omitted, leaving G2 with 2 folds. The G2 M_r is approximately 25 kDa and G2 contains slightly more keratan sulfate than does G1.

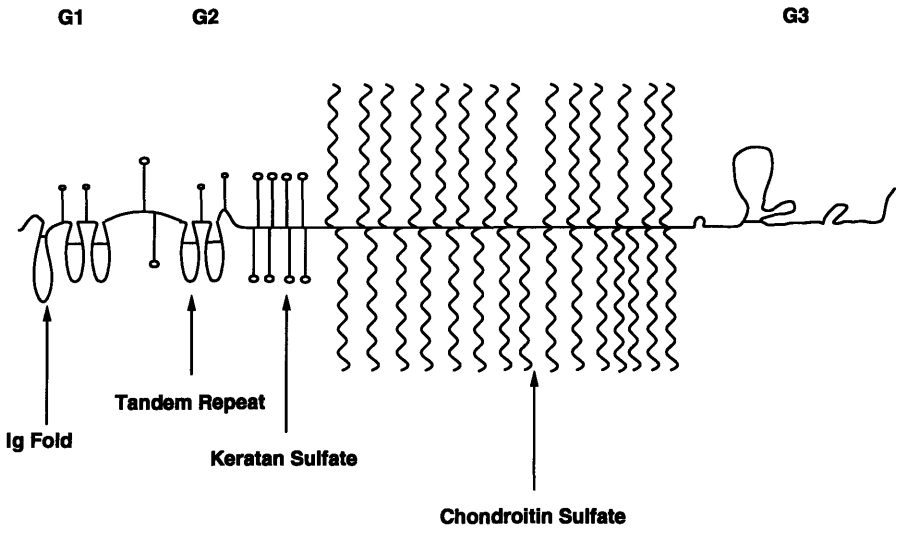

Figure 1.3: Schematic diagram of aggrecan structure [16].

The largest domain in aggrecan is the chondroitin sulfate bearing domain which extends from the C-terminal side of G2. The length of this domain is approximately 260 nm with a core protein M_r of 195 kDa. Adjacent to the G2 domain is a small 15 kDa region in which keratan sulfate chains attach to the core, and this is followed by a long 180 kDa region where chondroitin sulfate chains are attached. The C-terminal group is the globular domain G3, which bears little resemblance to G1 or G2. There appears to be only one loop in G3 which has an M_r of 25 kDa. G3 shows some weak carbohydrate affinity and it is thus postulated that it may play a role in matrix organization.

Small Proteoglycans

The small proteoglycans of primary interest in articular cartilage are decorin, biglycan, and fibromodulin. Both decorin and biglycan contain dermatan sulfate GAG chains as well as the chondroitin sulfate and keratan sulfate chains common to aggrecan. The GAG chain binding region lies in the N-terminal section of both molecules. Decorin and biglycan have

similar, but non-identical structures. Both have a core protein M_r of 40 kDa. Decorin, with a single CS chain has a total M_r of approximately 85 kDa, while biglycan, with a 2 CS chains has a total M_r of 130 kDa [56,57]. Decorin is known to bind to type IX collagen fibrils during formation and may inhibit the production of such fibrils. Though structurally similar, biglycan appears to have no such function. Additionally, it has been observed that both decorin and biglycan have the ability to immobilize certain growth factors. Thus, it is possible that these molecules play a key role in regulating matrix growth [57].

1.2.2 Collagens

As may be expected, collagens play a key role in the matrix structure of articular cartilage. Collagen fibers themselves are very strong in tension and in large part their function is to resist the osmotic swelling pressure created by the highly charged proteoglycans. This structure allows the collagen to provide strength when the tissue is exposed to tensile and shear forces. The collagens most prevalent in articular cartilage are types II, IX, and XI. Together, these three types make up 99% by weight of all the collagen present in articular cartilage [33]. Of the three, type II is by far the most common, constituting 90-95% of all the collagen in the tissue.

Type II Collagen

Type II collagen is found throughout the articular cartilage matrix and it forms the framework for the entire tissue. The type II network serves to entrap proteoglycans within the tissue and restrain tissue swelling due to osmotic pressure of the charged proteoglycans. The type II molecule is composed of three identical α1(II) chains wound together. The type II collagen molecule consists of a single continuous triple helical domain, with with unwound telopeptide regions on both the C- and N-termini. Type II molecules assemble into fibrils by means of cross-linking hydroxylysyl pyridinoline residues in telopeptide regions. This structure gives type II collagen a distinctive banded structure which repeats every 67 nm [33]. The continuous assembly of type II collagen molecules into fibrils results in an

increase in fibrillar diameter with age, from less than 20 nm in fetal tissue and 50 to 100 nm in adult tissue. The type II fibril orientation varies with location in the tissue. In the uppermost regions of the tissue, near the articular surface, the fibrils are oriented tangential to the surface, while deeper in the tissue, the fibrils are essentially oriented randomly.

Type XI Collagen

Type XI collagen constitutes approximately 3% by weight of all the collagen in the articular cartilage matrix [33]. Biochemical and immunochemical data suggest that the primary function of type XI collagen is to act as the core for type II fibrils. Unlike type II, type XI collagen is heterogeneous trimer, composed of three chains α1(XI), α2(XI), and α3(XI). The α3(XI) chain is indistinguishable from α1(II), which may aid in its function as the core molecule of type II collagen. The α1(XI) and α2(XI) chains are composed of separate and distinct amino acid sequences [33].

An interesting feature of type XI collagen is that the molecule retains the N-terminal propeptide during normal function. The propeptide segment is beyond the N-terminal non-collagenous region and contains a short collagenous segment followed by a globular region. The function of this region is unclear, but it has the effect of preventing further lateral expansion of the fibrils. This N-terminal segment is also the region in which interfibrillar cross-linking takes place, with the attachment occurring by means of a hydroxylysyl pyridinoline residue.

Type IX Collagen

Type IX collagen represents 1% of the collagenous protein in mature articular cartilage. There is evidence that as much as 10% of the collagen in fetal articular cartilage is type IX [32], implying that it may play an important role in matrix assembly. Type IX collagen is a heterotrimer of chains α1(IX), α2(IX), and α3(IX). The molecule consists of three collagenous domains, COL1, COL2, and COL3, which are woven in a triple helix, and four globular domains, NC1, NC2, NC3, and NC4, which are located in between the collagenous

domains and at the ends of the molecule.

The type IX molecule itself is both collagen and proteoglycan. In the NC3 domain there is a site of attachment for a chondroitin sulfate chain which has ionizable charge groups. The N-terminal region of the COL2 domain play an additional role in the function of type IX collagen. This region appears to contain the sites of attachment which cross link type IX collagen to type II collagen. All three type IX chains, α1(IX), α2(IX), and α3(IX), contain the hydroxylysine residues which covalently bind to the α1(II) chain on the type II molecule [31,134]. This method of attachment results in the type II and type IX molecules being oriented in an antiparallel fashion. Such attachment has been observed by electron microscopy [124].

1.2.3 Hyaluronic Acid

Hyaluronan is the long chain backbone macromolecule to which aggrecan molecules attach to form aggregates. Hyaluronate molecules are extremely large with M_r up to 5 to 6 $\times 10^6$ and a length of up to 10 μm. Typical sizes for hyaluronan are an M_r of 3 to 6 $\times 10^5$ and a length of .5 to 1 μm [52]. As expected, the size of the hyaluronic acid chain determines how many proteoglycans can bind to the molecule. Since GAG chains typically extend from the core proteins of proteoglycans, steric hindrance is an important determinant of molecular packing. It is estimated that these hindrances allow for one aggrecan molecule per 7 kDa of hyaluronate chain [52]. As such, the largest of the hyaluronate molecules can bind from 400 to 800 aggrecan molecules. The M_r of such aggregates can be as much as 100 $\times$ 10^6 kDa [56].

As previously mentioned, proteoglycans are held in the matrix by means of an attachment between the G1 domain of aggrecan and the hyaluronate backbone. This attachment is further enhanced by a small polypeptide known as link protein which binds to both the G1 domain of aggrecan and to hyaluronate. Link protein is similar in size and composition to the G1 domain of aggrecan. Like the G1 domain, it consists of an Ig fold and a protein tandem repeat sequence. It is postulated that the primary role of link protein

may be to increase the stability of aggrecan/hyaluronan complexes in the first stages of aggregation, when the binding of proteoglycan to hyaluronate may be incomplete.

1.3 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disorder which affects a significant portion of our population. In 1980 it was estimated that 61 million Americans had some degree of arthritis in the hands or knees as detected by x-ray[59]. Approximately one third (23 million) of such cases are severe enough that they require medical attention or inhibit normal lifestyle. Among those of age 55 to 64, 63% of all men and 75% of all women have evidence of OA, and of those over 75 frequency rises to 80% of all men and almost 90% of all women. From 15% to 30% of all visits to general practitioners may be attributed to loss of mobility cause by OA. In 1980 the annual cost of treatment for OA was estimated at $8.6 billion. For most patients, treatment of OA typically involves the use of nonsteroidal antiinflammatory drugs (NSAIDS) which tend to reduce joint pain and swelling [11]. NSAIDS are effective at treating the symptoms of OA, but do nothing to alter the course of the disease [126]. At the present time, there is no effective way to stop the process of degeneration of OA.

The biochemical events which contribute to OA are not understood completely. There are a large number of enzymes, hormones, growth factors, cytokines, and mediators as well as mechanical factors which govern the synthesis and breakdown of the matrix. In OA, cartilage matrix composition is altered substantially, weakening the tissue considerably, to the extent that mechanical wear from joint motion can result in erosion of cartilage down to the bone surface [14]. In its degenerated state, OA cartilage exhibits dramatically different biochemical, physicochemical, mechanical, and electromechanical properties from normal tissue. OA cartilage shows a distinct decrease in proteoglycan content [19,83], marked collagen network fibrillation [33,44], increases in tissue hydration [3,78], and loss of compressive stiffness [3,58,102]. The role of enzymatic degradation in OA has been studied, but is not completely understood. In normally functioning tissue, there is a balance between levels of endogenous proteinases and inhibitors, while in OA, proteinases are

in excess compared to levels of inhibitors. It has been suggested [27] that families of enzymes, including metalloproteinases [20,92,96,100], serine proteases [66], and a novel "aggrecanase" [110] contribute to cartilage degradation in OA. Given that various enzymes have specific expression patterns [23,76], and some act as activators of other enzymes [16,119,120], it seems likely that the enzymatic component of OA involves a "cascade" of activities which work in tandem to degrade the tissue.

1.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are are a family of at least 12 enzymes which are divided into three main classes: collagenases, gelatinases, and stromelysins. Gelatinases are not thought to play a major role in degradation of cartilage matrix, however, interstitial collagenase (MMP-1) is known to degrade collagen types II and X [127], while stromelysin-1 (MMP-3) is known to degrade aggrecan [35], link protein [92], and collagen types II, IX, X, and XI [134]. Both collagenase and stromelysin are produced by chondrocytes and synovial fibroblasts [23,76,96]. These MMPs are present in elevated levels in cartilage and synovial fluid of patients with degenerative OA [74,96,98], as well as in animal models [20,81,100]. Elevated levels have likewise been detected in cases of knee joint injury [74] and heat shock [123]. There is also evidence of MMP gene expression in early stages of soft tissue development [12] as well as immunolocalization of MMPs in cartilage growth plate [13].

Members of the MMP family are synthesized and secreted in a latent zymogen form which requires activation by proteolytic cleavage of a peptide sequence at the N-terminus of the molecule [127]. This activation can take place from cleavage by plasmin, trypsin, or organo-mercurial compounds such as 4-aminophenylmercuric acetate (APMA). Nagase *et al* demonstrated that in the presence of APMA, native prostromelysin or procollagenase will undergo a conformational change which results in the production of an intermediate which is then self-catalyzed to produce an active form of the enzyme [89]. Previously, APMA has been used to activate latent MMPs produced by cartilage after treatment with

IL-1β [100] or in animal models of arthritis [99].

1.4.1 Stromelysin

Stromelysin, also known as matrix metalloproteinase 3 (MMP-3), collagenase activator protein (CAP), proteoglycanase, and transin, is a neutral metalloprotease produced by chondrocytes and synovial fibroblasts [24,35,76]. It is produced in a latent proenzyme form which has a molecular weight of 55 kD and can be activated by plasmin cleavage to produce 45 kD, 22 kD, and 19 kD active forms. These active forms have been shown to be effective at cleaving several different matrix components in cartilage, including aggrecan [23,92,100], link protein [92], and collagen types II, IX, X, and XI [134].

Stromelysin has been shown to be present in degraded tissue and has been implicated in normal tissue turnover, as well as remodeling and development. It has been found in elevated levels in cartilage and synovial fluid in patients with arthritis [24,75,82], as well as in animal models [95,96]. Elevated protein and mRNA expression levels have likewise been detected in cases of mechanical injury [74,101] and heat shock [123]. There is also evidence of stromelysin gene expression in early stages of soft tissue development [12] as well as immunolocalization of stromelysin in cartilage growth plate [13].

The manner in which stromelysin degrades articular cartilage matrix constituents has been researched extensively. In studies conducted on human articular cartilage, it has been shown that that stromelysin cleaves the Asn^{341}-Phe^{342} bond in the interglobular domain and the Asp-Iso bond at several sites in the chondroitin sulfate-bearing region of aggrecan [35]. This has the effect of liberating the G1 domain from the rest of the aggrecan molecule. In addition, stromelysin has been shown to cleave the histidine-isoleucine bond in human link protein 3 (LP3) [92]. The combination of these two cleavages has the effect of liberating the GAG-bearing domains of aggrecan from the domains of the molecule that are bound to hyaluronate. This action effectively mobilizes the charge groups which were previously immobilized by their attachment to the matrix.

In addition to aggrecan and link protein, stromelysin has been shown to be effective

at degrading the three major types of collagen which are present in articular cartilage matrix. Experiments conducted on bovine epiphysieal cartilage have shown that stromelysin removed short telopeptide sequences from the α1(II) chain as well as effecting the cross links between chains of type II collagen molecules [134]. Stromelysin cleaves the Ala-Gln and Val-Met bonds of the cross-linking hydroxylysine segment, effectively eliminating the link. Stromelysin has similar action against type XI collagen, where it has been seen to cleave the α1(XI) chain in the cross-linking hydroxylysine residue. The effects of stromelysin on type IX collagen, however, are far more pronounced. Stromelysin separates the COL1 domain from the COL2 and COL3 domains by means of a cleavage in the NC2 region [134]. This cleavage takes place on the α1(IX), α2(IX), and α3(IX) chains, completely severing the COL1 domain. In addition, it was seen that stromelysin liberated a large globular segment from the α1(IX) chain of the NC4 domain. The action of stromelysin on the cross-linking hydroxylysine segments of type II collagen also has the effect of decoupling the type IX and type II molecules [134].

1.4.2 Collagenase

Interstitial collagenase, often called tissue collagenase or MMP-1, is neutral metalloproteinase produced by chondrocytes and synovial fibroblasts [23]. The zymogen form of the enzyme has an M_r or 52 kD which is reduced to 41 kD upon activation. The activate form degrades matrix collagen types II and X as well as aggrecan [90]. Collagenase is the only proteinase known to degrade type II collagen in the triple helical domain, and this cleavage results in significant unwinding of the remaining collagenous protein [60]. The degradation of aggrecan by collagenase occurs at the Asn^{341}-Phe^{342} bond in the interglobular domain of the core protein. This site is identical to the cleavage site of stromelysin on aggrecan.

Collagenase expression in chondrocytes is elevated in osteoarthritis [23,96]. Likewise, increased levels of collagenase protein are found in cartilage [90], synovial fluid and synovial tissue [127] from patients with rheumatoid arthritis and osteoarthritis. Stimulation of cartilage explants by IL-1β results in increased production of collagenase by

chondrocytes [90].

1.4.3 Inhibition of Metalloproteinases

There are several naturally occurring inhibitors of MMP activity, including α_2-macroglobulin (α_2-M), and tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2). α_2-M is an extremely large molecule (~750 kDa) produced by the liver and found in synovial fluid. It inhibits MMPs by presenting a peptide sequence which is cleaved by the enzyme, then engulfing the enzyme and restricting its access to other substrates. Both TIMPs are also large (~28 and 22 kDa, respectively) and are produced by chondrocytes and synovial fibroblasts. The TIMPs inhibit MMPs by binding tightly to the MMP active site. In healthy joints, there is as much as a two-fold molar excess of TIMP compared to stromelysin and collagenase [74], while in cases of OA enzyme concentrations have been seen to exceed levels of native TIMPs ([74,81]. Efforts to use TIMP to inhibit degradation proteoglycan induced by IL-1α in cartilage explants have not been successful, presumably due to the inability such a large protein to penetrate the dense cartilage matrix.

Although α_2-M and TIMP effectively inhibit metalloproteinases, their size brings into question their suitability as therapeutic agents. In a tissue explant environment, based on the partitioning of similarly sized proteins into cartilage [79], the amount of TIMP or α_2-M which penetrates into the tissue may be as little as 1% or 0.01%, respectively, of that which is added to the culture media. Consequently, there has been a significant effort to develop low molecular weight synthetic MMP inhibitors which should penetrate the cartilage matrix more easily. Several types of synthetic MMP inhibitors have been developed, including those based hydroxamate compounds [1,113] and others based on peptide sequences [22,38].

1.5 Models of Cartilage Degradation

Interleukin-1 (IL-1) is an 18 kD cytokine produced by several cell types including endothelial cell, synoviocytes and chondrocytes [114]. Although typically associated with

inflammatory diseases such as rheumatoid arthritis [24,82], both the α and β forms of IL-1 have been shown to be relevant to degradation in osteoarthritis as well [94,101]. IL-1 interacts with chondrocytes by means of a receptor on the cell surface [21]. Stimulation of cartilage explants with IL-1 has been shown to result in widespread matrix degradation, including loss of tissue PGs [4,16,61] and collagens [16]. IL-1 treatment directly influences cell metabolism, decreasing synthesis of PGs [4,16,85,121] and collagens [46,47,122] while decreasing chondrocyte proliferation [43]. Additionally, IL-1 induces production and mRNA expression of MMPs in chondrocytes [64], while modulating production of TIMP [114]. Although tissue treated with IL-1 contains an excess of MMPs, the PG degradation products which result are not those typically associated with MMP cleavage [108]. These fragments, however, are similar to those found in human OA patients [111]. Despite this apparent discrepancy, MMP inhibitors have been shown to be effective at inhibiting cartilage matrix degradation induced by IL-1 [16,87,113].

The vitamin A derivative all-trans retinoic acid (RA) has also been shown to induce widespread matrix degradation in cartilage explants. [16,86]. RA stimulation involves interaction with a nuclear receptor, directly altering DNA transcription [93]. On the tissue levels, RA treatment results in loss of tissue PGs and collagens [16,17,86] decreased synthesis of PG [86] and collagens [26,136] by chondrocytes. RA has also been shown to induce expression and production of MMPs [16], although degradation products from such systems are not those typically associated with MMP activity (JD Sandy, unpublished results). This matrix degradation inhibited in the presence of MMP inhibitors, but not in the presence of inhibitors of cathepsin B [16].

1.6 Overview and Objectives

Many studies have investigated the action of MMPs at either the organ or molecular level. By comparison, there has been less effort to understand these phenomena on a *tissue* level. While is it clear that MMPs are present in cartilage under both normal [12,13] and pathological [74,96,98] conditions and are quite capable of degrading matrix components

in solution [35,90,92,134], their action against intact tissue is unknown. Factors such as transport through the cartilage matrix and effects of intratissue chemical environment are capable of affecting the way in which these enzymes act on their appropriate substrates. In addition, the effects of this degradation on the functional physical properties of the tissue are unknown.

In Chapter II, the effects of degradation by one particular enzyme, stromelsyin, were characterized. Alterations in tissue composition as assessed by biochemical assays and histology were correlated with changes in physicochemical, mechanical, and electromechanical behavior. Effects on tissue proteoglycans were assessed by measurements of tissue GAG content and characterization of the size and nature of the degradation fragments in culture media. Spatial distribution of proteoglycan loss was determined by histology. Damage to matrix collagens was assessed by the use of antibodies to collagen types II and IX to characterize the size and amount of degradation products. Swelling studies were performed to assess the ability of the damaged collagen network to resist osmotic pressure. Uniaxial confined compression studies were used to characterize changes in mechanical and electromechanical behavior due to stromelysin treatment. Therefore, the objectives of this thesis are to characterize the effects of MMP activity on cartilage at the tissue level and to quantify the effects of this degradation on the physical properties of cartilage.

Chapter III investigated the effects of stromelysin treatment on intact joint cartilage *in vivo* and the modulation of these effects by the systemic administration of an MMP inhibitor. Rabbit joints were exposed to stromelysin, while changes in cartilage and synovial fluid proteoglycan content were observed. Cartilage samples removed from these joints were tested in uniaxial confined compression characterize changes in mechanical and electromechanical behavior due to stromelysin treatment. In addition, groups of animals which received this treatment also received intravenous injection of a synthetic MMP inhibitor developed at Merck Research Laboratories, in an attempt in evaluate its ability to inhibit stromelysin in an *in vivo* environment.

While the work of Chapters II and III detailed the effects of addition of exogenous

stromelysin on cartilage, Chapter IV focused on the degradative capacity of MMPs native to the tissue, using APMA to activate endogenous enzymes. The resulting degradation was characterized by assessing tissue proteoglycan content, and correlating biochemical changes with alterations in tissue swelling properties as well as mechanical and electromechanical behavior. In addition, both TIMP, an MMP inhibitor native to cartilage, and MC1, a synthetic *N*-carboxyalkyl peptide inhibitor, were assessed for their ability to inhibit biochemical and physicochemical changes induced by APMA treatment. The disparity in size between TIMP (25 kDa) and MC1 (500 Da) allowed for assessment of the importance of transport through the cartilage matrix to overall inhibitor efficacy in intact tissue.

Many investigators have characterized the the cell-mediated degradation induced by treatment with IL-1β or RA. Given that both are known to upregulate production of MMPs, attempts have been made to inhibit degradation in these systems using synthetic metalloproteinase inhibitors. Chapter V details attempts to compare effects of IL-1β and RA treatment to effects of APMA treatment on cartilage proteoglycan content as well as changes in swelling properties, mechanical and electromechanical behavior. This framework allowed for the evaluation of effects of transport on matrix degradation, given that IL-1β is a lerge protein (18 kDa) while RA is much smaller (400 Da). In addition, the ability of TIMP to inhibit biochemical and physical property changes which resulted from IL-1β and RA treatment was assessed.

The subsequent data and analyses in the appendices describe additional work and methods used to characterize MMP-related cartilage degradation. Appendix A describes the optimization of tissue treatment and culture parameters for degradation studies. Appendix B is a survey of physicochemical measurements recorded in an attempt to determine which were most reflective of changes in swelling behavior. Appendix C describes stromelysin degradation of cartilage in light of Michaelis-Menten enzyme reaction kinetics. Appendix D contains an evaluation of several MMP inhibitors in terms of their ability to prevent degradation induced by stromelysin, APMA, IL-1β, and RA. Both broad spectrum inhibitors and those with specific target enzymes were used in an attempt to characterize the roles of

the given enzymes in the observed degradation. Appendix E describes fabrication and use of a plane-ended indenter for the evaluation of electromechanical properties of cartilage on intact guineau pig joints. Appendix F is a summary of work characterizing the effects of *in vivo* IL-1β treatment on the physical properties of rabbit cartilage.

Chapter II

Changes in Cartilage Composition and Physical Properties Due to Stromelysin Degradation

2.1 Abstract

Bovine cartilage explants were treated with recombinant human stromelysin-1 (SLN) in concentrations ranging from 1-500 μg/ml (20 nM-10 μM) and changes in biochemical, biomechanical, and physicochemical properties as well as histological appearance were assessed. SLN treatment resulted in a dose-dependent loss of aggrecan fragments from the explants, with ~40% loss of glycosaminoglycan (GAG) content after 72 hours at a concentration of 200 nM. N-terminal sequencing of degradation products confirmed that SLN was responsible for proteoglycan cleavage. Further analysis showed the presence of elevated levels of type IX collagen fragments within treated explants and in the incubation media. Histological examination demonstrated that proteoglycan loss proceeded inward from the exposed tissue surfaces, with a marked boundary between degraded and undegraded tissue. Physicochemical studies revealed that SLN treatment resulted in tissue swelling, while treatment with chondroitinase ABC or trypsin did not. SLN treated tissue exhibited marked decreases in equilibrium modulus and dynamic stiffness and a substantial increase in hydraulic permeability by 72 hours, with similar kinetics to the loss of GAG. The significant decreases in equilibrium modulus and dynamic stiffness, together with swelling and the loss of type IX collagen fragments suggest that the structural integrity of the collagen network had been compromised. Measurement of dynamic streaming potential showed that changes due to SLN treatment were strongly dependent in compression frequency, with dramatic changes seen at high frequency prior to changes in mechanical properties, and little initial change seen at low frequency. Streaming potential, an electrokinetic phenomenon, was therefore a more sensitive indicator of initial, focal loss of GAG from the tissue due to SLN treatment than purely mechanical measurements such as dynamic stiffness.

2.2 Introduction

Articular cartilage is an avascular, aneural, alymphatic, connective tissue which covers the ends of bones in synovial joints. Its primary functions are to distribute loads over bone surfaces and provide a low friction surface over which bones can move. Articular cartilage is a porous, highly hydrated material, with water content of 70-80% by volume [88]. The solid component of cartilage consists of an extracellular matrix and a sparse population of chondrocytes, present in a concentration of approximately 10-100 $\times$ 10^6 cells/ml [117]. The cartilage matrix is composed primarily of hydrated collagen fibrils, highly charged proteoglycan molecules and other glycoproteins [73,130]. The osmotically swollen matrix and the high water content are mainly responsible for the complex mechanical behavior that characterizes the response of the tissue to physiologic loads [65].

In osteoarthritis (OA), the combination of altered cartilage matrix composition and mechanical wear from joint motion can result in erosion of cartilage down to the bone surface [14]. In its degenerated state, OA cartilage exhibits dramatically different biochemical, physicochemical, mechanical, and electromechanical properties from normal tissue. OA cartilage shows a distinct loss of proteoglycan [19,83], marked collagen network fibrillation [44,102], increases in tissue hydration [3,78], and loss of compressive stiffness [3,58]. The role of enzymatic degradation in OA has been studied, but is not completely understood. It has been suggested [27] that families of enzymes, including metalloproteinases [20,92,96,100], serine proteases [66], and a novel "aggrecanase" [110] contribute to cartilage degradation in OA. Given that various enzymes have specific expression patterns [23,76], and some act as activators of other enzymes [16,119,120], it seems likely that OA involves a "cascade" of enzymatic activities which work in tandem to degrade the tissue.

Alterations of biochemical composition and matrix structure which result from enzyme activity are manifest in changes in cartilage material properties and physical behavior. Enzymatic degradation has been shown to result in decreased compressive stiffness [53], changes in tensile stiffness, strength, and fracture strain [66,112], decreased shear modulus [55], changes in creep behavior [66,112], and increased indentation displacement [53]. In

addition, marked decreases in compression-induced streaming potential have been shown to be particularly sensitive indicators of alterations in matrix proteoglycan composition associated with enzymatic degradation [41].

Stromelysin-1, also known as matrix metalloproteinase 3 (MMP-3), is a neutral metalloproteinase produced by chondrocytes and synovial fibroblasts [23,76,96]. Prostromelysin has a molecular weight of 55 kD, which can be cleaved by plasmin, trypsin, or organomercurials to produce 45, 22, and 19 kD active forms which cleave several different cartilage matrix components, including aggrecan [35,92], link protein [92], and collagen types II, IX, X, and XI [134].

Stromelysin has been shown to be present in arthritic tissue and has been implicated in normal matrix turnover, as well as remodeling and development. It has been found in elevated levels in cartilage and synovial fluid in patients with degenerative OA [74,96], as well as in animal models of OA [20,100]. Elevated levels have likewise been detected in cases of knee joint injury [74] and heat shock [123]. There is also evidence of stromelysin gene expression in early stages of soft tissue development [12] as well as immunolocalization of stromelysin in cartilage growth plate [13].

The manner in which stromelysin degrades articular cartilage ECM constituents has been studied extensively using solutions of isolated matrix molecules *in vitro*. It has been shown that stromelysin cleaves the Asn^{341}-Phe^{342} bond in the interglobular domain of purified human aggrecan, generating a large chondroitin sulfate-bearing product with the N-terminal sequence ^{342}Phe-Phe-Gly-Val, and liberating the G1 domain, with the C-terminal sequence Ileu-Pro-Glu-Asn^{341}, from the remainder of the molecule [35,37]. Significantly, this form of the G1 domain is also present in human articular cartilage, providing evidence for stromelysin activity *in situ* [35]. Likewise, stromelysin cleaves the His^{16}-$Ileu^{17}$ bond of link protein, and this product has also been detected in human cartilage [91].

In addition to aggrecan and link protein, stromelysin has been shown to be effective at degrading the three major types of collagen (II, IX, and XI) present in articular cartilage. Stromelysin removes short telopeptide sequences from the α1(II) chain, and also cleaves

the cross-linking hydroxylysine segments, effectively eliminating this link between α1(II) chains [134]. Stromelysin separates the COL1 domain from the COL2 and COL3 domains of type IX collagen by means of a cleavage of the α1(IX), α2(IX), and α3(IX) chains in the NC2 region [134]. In addition, it was seen that stromelysin could liberate a large globular segment from the α1(IX) chain of the NC4 domain. These combined actions have the effect of decoupling the type IX–type II collagen complex [134].

Studies on the effects of stromelysin on cartilage matrix constituents have thus far focused on degradation of isolated matrix constituents in solution. However, the effects of stromelysin on the composition and physical properties of intact cartilage have received less attention. The primary objectives of this work were to (1) determine the effects of stromelysin treatment on the biomechanical and electromechanical properties of cartilage explants and (2) to correlate the extent and kinetics of changes in these material properties to changes in certain biochemical, histological and physicochemical characteristics of the tissue.

2.3 Materials and Methods

2.3.1 Cartilage Explant and Culture

Saddle sections of 1-2 week old calves were obtained from a local abbatoir (A. Arena, Hopkington, MA) within four hours of slaughter. The femoropatellar groove was isolated and 3 mm diameter $\times$ 1 mm thick cartilage disks were harvested using a dermal punch (Miltex Instruments, Lake Success, NY) and a sledge microtome (Model 860, American Optical, Buffalo, NY) as described previously [105]. Recombinant human prostromelysin (200 μg/ml) in 25 mM Tris HCl, 10 mM $CaCl_2$, 0.05% Brij 35 and 0.01% NaN_3 [129] was activated with trypsin as described previously [71]. The enzyme had specific activity as native human gingival fibroblast stromelysin using ^{3}H-transferrin as a substrate [71]. The enzyme was concentrated to $\sim$2 mg/ml using a Centricon 10 ultrafilatration membrane (Amicon, Inc., Beverly, MA) and diluted into media for tissue culture experiments. Cartilage

disks were incubated in groups of 4 in 24-well culture dishes (Costar, Cambridge, MA) in 1 ml DMEM (Gibco, Grand Island, NY) containing 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco) for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere. Groups of 4 disks were then placed in 1 ml DMEM containing graded levels of enzymes, including recombinant human stromelysin-1 (SLN), trypsin (Type III from bovine pancreas, Sigma, St. Louis, MO), or chondroitinase ABC (protease free, Seikagaku America, Rockville, MD), and incubated for times up to 72 hours. Upon removal from culture groups of plugs were allocated for biochemical, histological, physicochemical and biomechanical analyses.

2.3.2 Biochemical Analysis

Disks allocated for biochemical analysis were frozen at -20 °C, lyophilized, and digested with 1 ml of 125 μg/ml papain digestion solution (Sigma) as described previously [105]. 20 μl portions of digests and culture media were assayed for sulfated GAG by reaction with 2 ml of dimethylmethylene blue dye solution in polystyrene cuvets (VWR, Boston, MA) and spectrophotometry (Model λ3B, Perkin Elmer, Norwalk, CT) [34] using whale/shark chondroitin sulfate (Sigma) as the standard.

Aggrecan fragments released into medium during stromelysin digestion were purified into the D1 fraction of a CsCl gradient. Core proteins were prepared by enzymic deglycosylation, Superose 12 chromatography and desalting, and the amino-termini were determined as previously described [110]. In addition, portions of medium containing 100 μg GAG were analyzed by chromatography on Sepharose CL-2B in the presence of 4% (w/w) link protein and 4% (w/w) hyaluronan, and fractions were assayed for sulfated GAG.

Degradation products of type IX collagen were detected in the stromelysin-treated disks and culture media by western blot analysis. Rabbit antiserum against purified bovine type IX collagen containing structurally intact COL1, COL2, COL3, NC2, and NC3 domains was produced as described previously [25]. The antiserum reacted specifically with type IX collagen and did not cross-react with any of the noncollagenous matrix proteins of

cartilage or with collagen types I, II, III, V, VI, or XI. Tissue slices (14 mg) from stromelysin treated bovine plugs were extracted with 200 μl of a 1% (w/v) sodium dodecyl sulfate (SDS) solution containing 0.05 M dithiothreitol (DTT) to cleave disulfide bonds. Extracts were fractionated on SDS-PAGE and electroblotted onto a PVDF membrane for western blot detection of collagen IX degradation products. The concentration of type IX collagen fragments in culture medium was also quantified by competitive enzyme linked immunosorbent assay (ELISA) on a microtiter plate using this antiserum.

2.3.3 Histology

Cartilage disks were fixed with ruthenium hexxaammine trichloride (RHT) and stained with Toluidine Blue 0 as described by Hunziker *et al* [62]. Each disk was fixed in a 2% (v/v) glutaraldehyde (Polysciences, Warrington, PA) solution buffered with 0.05 M sodium cacodylate (Bio-Rad, Richmond, CA) and containing 0.15 M NaCl (pH adjusted to 7.4), with 0.7% (w/v) RHT powder (Polysciences) added 10 minutes prior to fixation. Samples were fixed at room temperature for 4 hours and an additional 16 hours at 4°C. Samples were then washed three times for ten minutes in a solution containing 0.075 M NaCl and 0.1 M sodium cacodylate (pH 7.4) at room temperature and stored in 1 ml 70% ethanol solution at 4°C. Tissue was embedded in paraffin and thick (5 ± 1 μm) sections were stained with Toluidine Blue 0 for analysis in the light microscope.

2.3.4 Swelling Studies

The protocol for swelling studies was motivated by the technique used by Maroudas [78] to compare the swelling of normal and osteoarthritic cartilage. Disks removed from culture medium were padded briefly with a paper towel to remove surface water, and wet weights were measured (AE 163 Balance, Mettler Instrument Corp, Hightstown, NJ). Disks were then twice reequilibrated in a hypotonic saline solution (0.01 M NaCl at pH 7.0) for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were

recorded.

2.3.5 Biomechanical and Electromechanical Evaluation

Cartilage disks were placed in an electrically insulating poly(methylmethacrylate) cylindrical confining chamber, similar to that used by Frank *et al* [41]. The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA) which was interfaced to a computer and frequency generator (Model 5100, Rockland Systems, West Nyack, NY). Samples were equilibrated in 0.15 M phosphate buffered saline (PBS)(Gibco) at pH 7.4 containing 100 U/ml penicillin G and 100 μg/ml streptomycin at room temperature. Disks were subjected to confined compression between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted at the base of the chamber. An identical electrode was mounted in the surrounding PBS bath.

Disks were first compressed by sequential increments of 0.5-1.5% strain, up to a maximum of 20% total strain. After stress relaxation, the equilibrium stress corresponding to each increment of static strain was detected with the load cell of the Dynastat and recorded on the computer. The resultant equilibrium stress was plotted against applied strain and the slope of the best fit linear regression curve gave the equilibrium confined compression modulus. At a given static offset strain, sinusoidal strains of $<1\%$ amplitude were superimposed on the static strain at frequencies ranging from 0.01 Hz to 1 Hz. The resultant oscillatory load was detected by the load cell and the oscillatory streaming potential was detected by the chamber electrodes which were connected to a high-impedance Universal Amplifier, Model 11-4113-02 (Gould, Inc., Cleveland, OH). Load and streaming potential were simultaneously recorded by the computer. The amplitude of the dynamic load was normalized to disk area and to the amplitude of the applied strain to give dynamic stiffness, while the amplitude of the streaming potential was normalized to the amplitude of the applied strain [39,40]. Equilibrium modulus and dynamic stiffness data were used in combination with the method of Frank and Grodzinsky [40] to calculate the effective hydraulic permeability of the tissue specimen. In all tests the amplitude of the applied

strain was chosen to be small enough to elicit a linear mechanical response from the sample [39,40] (i. e. the total harmonic distortion in the measured load was <10%). The porous platen remained in contact with the sample at all times during the testing cycle.

2.4 Results

2.4.1 Biochemical Analysis

Treatment with SLN induced both a time and dose dependent loss of proteoglycan constituents from bovine cartilage explants (Fig 2.1). The data are reported as GAG remaining in the tissue normalized to the total GAG (tissue + media GAG) at that time point, versus time in culture for disks incubated in media containing SLN concentrations ranging from 1 to 500 μg/ml (20 nM to 10μM). Control tissue at t = 0 contained 270 $\pm$31 μg GAG/mg dry weight. For comparison between SLN concentrations, the time course of GAG loss was fit to an exponential of the form $e^{-t/\tau}$, giving decay times ranging from 344 hr at 1 μg/ml to 6.6 hr at 500μg/ml SLN. GAG loss clearly increased with SLN dose, with maximal loss achieved in 72 hours for concentrations exceeding 100 μg/ml. Control disks incubated in DMEM alone showed little GAG loss. By comparison, disks incubated in media containing 100 μg/ml trypsin demonstrated maximal GAG loss in approximately 3 hours, while disks incubated with 100 μg/ml SLN and a two-fold molar excess of tissue inhibitor of metalloproteinases (TIMP) showed GAG loss that was indistinguishable from controls.

To examine the effect of SLN treatment on the structure of the released aggrecan, media from the disks exposed to 100 μg/ml SLN for 72 hours was first evaluated for aggregability with hyaluronan on associative Sepharose CL-2B. The profile (Fig 2.2A) showed that the fragments eluted as a broad peak over the K_{av} range from 0.3 to 0.7 and greater than 95% were non-aggregating. This indicated that stromelysin had cleaved the aggrecan to separate the chondroitin sulfate-rich region from the hyaluronan binding region, and additionally had cleaved the chondroitin sulfate-rich region in several places. This interpretation was supported by N-terminal sequence analysis of the aggrecan fragments

which gave evidence for at least two cleavage products (Fig 2.2B). One sequence, detected at 5 pmol was FFGVXGXE and this is consistent with the known interglobular cleavage of the Asn^{341}-Phe^{342} bond in human aggrecan [35,37]. Another sequence, detected at about 10 pmol, was IXGLP, and this is consistent with cleavage between aspartic acid and isoleucine residues in the sequence GVEDISGLP, which is present in the chondroitin sulfate-rich region of the bovine aggrecan core protein [2]. N-terminal sequencing showed no evidence of cleavage products which could not be attributed to SLN.

In addition to aggrecan degradation, SLN treatment resulted in degradation of type IX collagen, as evidenced by the presence of type IX collagen fragments in the culture media of disks treated with 100 μg/ml SLN for 72 hours (Fig 2.3A). The ELISA showed that the level of immunoreactive type IX collagen fragments in the culture media of treated tissue was three times higher than that of controls. Western blot analysis (Fig 2.3B) indicated that more of the immunoreactive type IX collagen fragments were extracted from SLN-treated cartilage disks as compared to that of non-treated controls. The identity of each type IX fragment and the sites of SLN cleavage remain to be determined. However, the fragments are large and do not appear to be equivalent to those produced *in vitro* from monomeric type IX collagen molecules by SLN [134]. Similar analyses using anti-type II collagen antibodies also revealed the presences of elevated levels of type II collagen fragments in extracts of treated explants and in the incubation media (data not shown).

2.4.2 Histology

The loss of proteoglycan constituents from SLN treated disks as determined by biochemical analysis was reflected in the absence of toluidine blue staining of SLN-treated disks (Fig 2.4B) compared to that of untreated disks (Fig 2.4A). While control disks showed toluidine blue throughout the tissue, those treated with SLN showed an absence of stain most pronounced in the surface region at early times in culture, consistent with a substantial loss of GAG. Thus by 24 hours of treatment with 100 μg/ml SLN, the loss of toluidine blue stained GAG had progressed into the upper $\sim$100 μm of the tissue, with a marked boundary

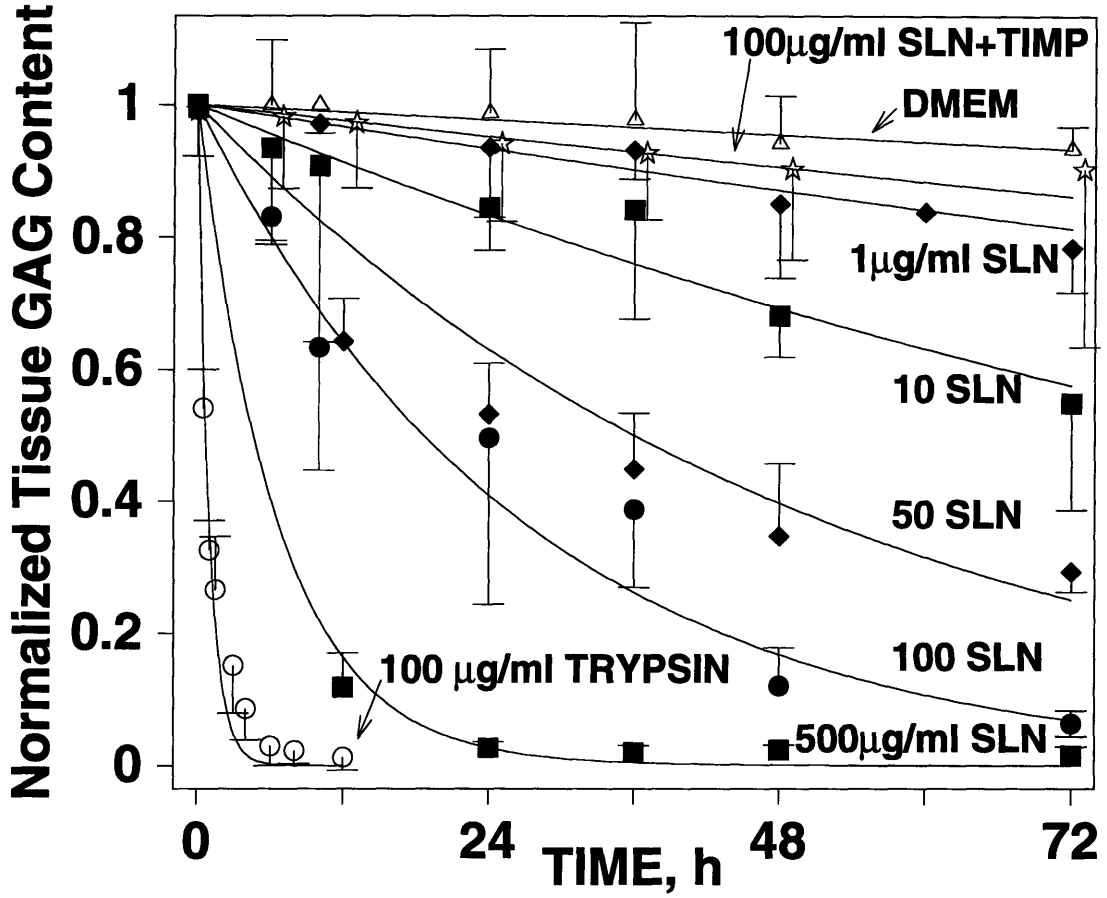

Figure 2.1: Normalized GAG loss from cartilage disks incubated in DMEM with graded levels of recombinant human stromelysin-1 (SLN), 100 μg/ml SLN + 100 μg/ml TIMP, or 100 μg/ml trypsin. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point. Solid lines are best fit singles exponential decay curves of the form $1e^{-t/\tau}$. The best fit decay times are $\tau = 344$, 130, 52, 26, and 6.6 hr for respective SLN concentrations of 1, 10, 50, 100, and 500 μg/ml; $\tau = 2.8$ hr for 100 μg/ml trypsin, $\tau = 624$ hr for 100 μg/ml SLN + 100 μg/ml TIMP and $\tau = 1019$ hr for DMEM alone.

between degraded and undegraded regions. The section shown in Fig 2.4 is a magnified view which shows only a portion of the disk; full cross-section views at 24, 48, and 72 hours [97] indicate that GAG loss had started at the periphery and progressed into the plug from all surfaces exposed to SLN.

2.4.3 Swelling Studies

Swelling of tissue incubated in the presence of 100 μg/ml SLN, 50 μg/ml trypsin, or 0.1 Units/ml chondroitinase ABC was quantified as the ratio of plug wet weight in 0.01 M NaCl to wet weight in DMEM (Fig 2.5). Untreated plugs swelled by 6-7% in hypotonic saline at all time points, while those treated with 100 μg/ml SLN showed increased swelling with time up to a maximum of 25% after 72 hours in culture. Plugs treated with 50 μg/ml trypsin or 0.1 Units/ml chondroitinase ABC showed swelling that was indistinguishable from controls. Trypsin treatment resulted in nearly complete GAG loss by 4 hours, while Chondroitinase ABC treatment resulted in 50% GAG loss by 72 hours (data not shown), with GAG loss proceeding from the surfaces into the tissue in a front like manner (similar to that in Fig 2.4), as described previously [41]. Given that both trypsin and chondroitinase ABC treatment induced significant GAG loss, it seems likely that the increased swelling of SLN-treated disks is not related to loss of proteoglycan constituents.

2.4.4 Biomechanical and Electromechanical Properties

Treatment with 100 μg/ml SLN resulted in a dramatic decrease in the equilibrium confined compression modulus, H_A (Fig 2.6A). The modulus of control disks was 0.89 ± 0.25 MPa at time $t=0$ and remained essentially constant over 72 hours in culture. In contrast the modulus of disks incubated in the presence on 100 μg/ml SLN decreased to 10% of the initial value by 72 hours (Fig 2.6A), with kinetics similar to those of GAG loss for a 100 μg/ml SLN dose (see Fig 2.1). The hydraulic permeability, k, of these same disks (Fig 2.6B) was calculated from the measured equilibrium modulus (Fig 2.6A) and the measured dynamic stiffness (Fig 2.7A), as previously described [40]. The hydraulic permeability

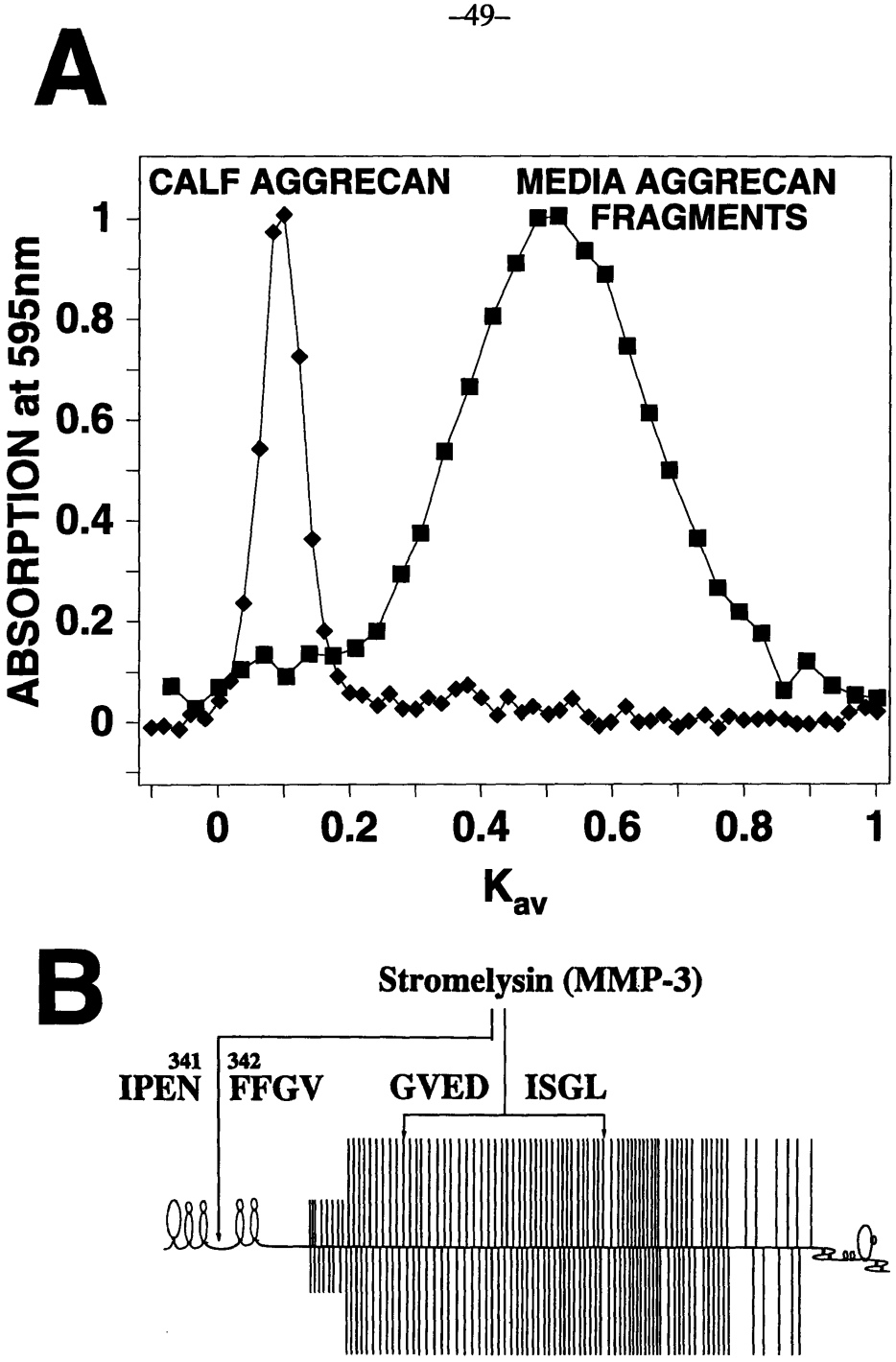

Figure 2.2: **A:** Sepharose CL-2B fractionation of culture media. A portion of medium from disks treated with 100 μg/ml SLN for 72 hours containing 100 μg GAG was mixed with 4% (w/w) hyaluronan and 4% (w/w) link protein and fractionated under associative conditions. Also shown is the elution profile of calf aggrecan (A1D1) fractionated under the same conditions. **B:** Schematic diagram showing suggested sites of stromelysin cleavage of bovine aggrecan. The sequences and residue numbers shown are based on bovine data obtained in the present work and the original human cDNA sequence.

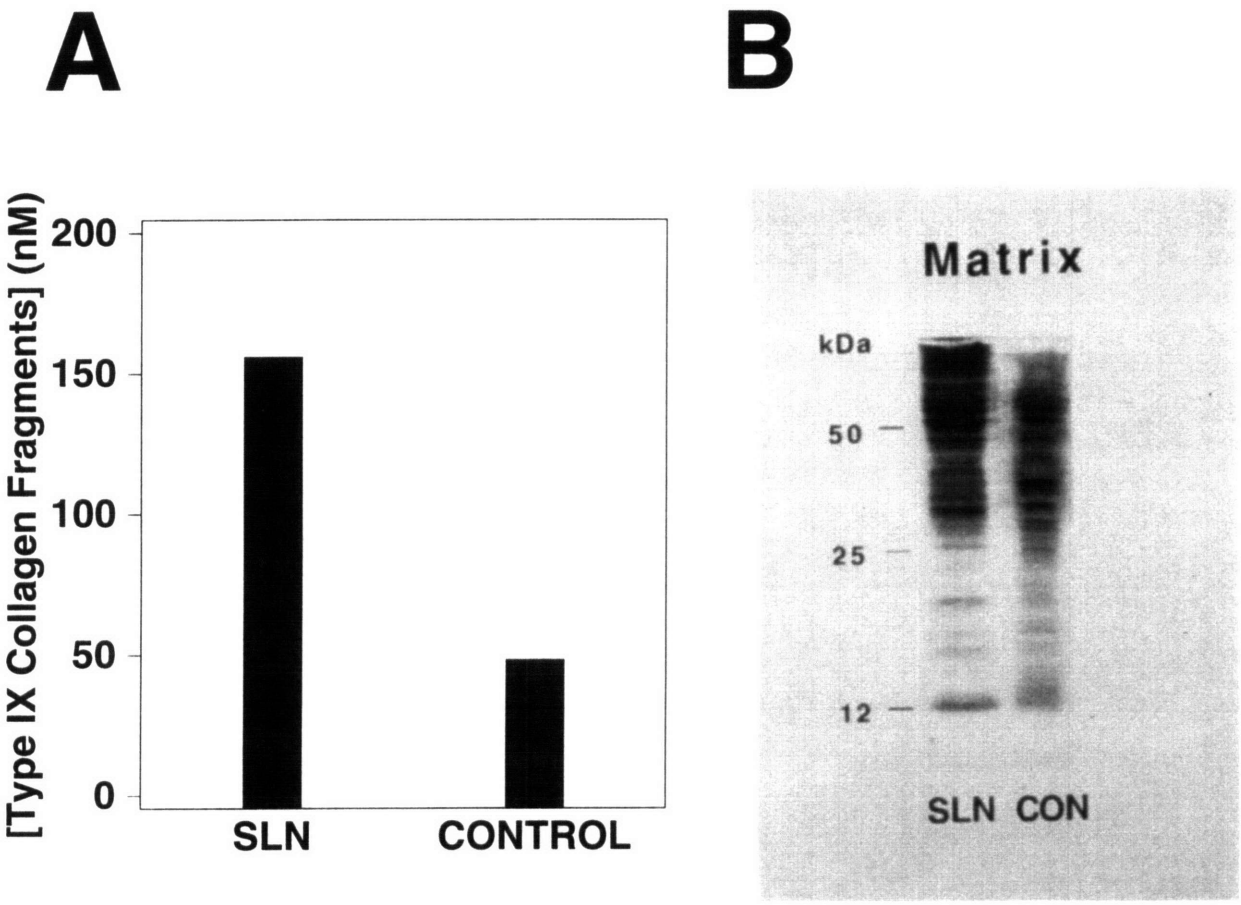

Figure 2.3: **A:** ELISA of immunoreactive type IX collagen fragments in the culture media from disks treated with 100 μg/ml SLN for 72 hours, using anti-bovine type IX collagen antibodies. **B:** Western blot analysis of immunoreactive type IX collagen fragments from the extracts of tissue slices (1% SDS, 0.05 M dithithreitol) taken from disks treated with 100 μg/ml SLN for 72 hours.

of control disks was $1.08 \pm 0.19 \times 10^{-15}$ $m^2(Pa \cdot s)^{-1}$ at time t = 0 and remained constant over three days in culture. However, the permeability of disks incubated in the presence of 100 μg/ml SLN markedly increased with time to 15 times that of control disks at 72 hours.

Similar to equilibrium modulus, magnitude of dynamic stiffness decreased dramatically after treatment with 100 μg/ml SLN (Fig 2.7A). Applying constant amplitude sinusoidal strains in displacement control, the measured dynamic stiffness and streaming potential (Fig 2.7B) increased monotonically with frequency, as described previously [39,40]. In the present experiments, the dynamic stiffness of control disks at t = 0 was 4.40 ± 0.84 MPa at 0.01 Hz and 11.56 ± 1.67 MPa at 1 Hz, and did not change significantly over 72 hours. In contrast, the dynamic stiffness of disks incubated in 100 μg/ml SLN decreased to $\sim$10% of initial values after 3 days in culture. As with equilibrium modulus, the kinetics of changes in dynamic stiffness were similar to those of GAG loss for a 100 μg/ml SLN dose (see Fig 2.1). The relative decrease in dynamic stiffness with time in culture was independent of frequency in the 0.01 to 1 Hz range.

Electromechanical measurements revealed that streaming potential magnitude also decreased dramatically for disks treated with 100 μg/ml SLN (Fig 2.7B). Initial values of streaming potential ranged from 0.10 ± 0.02 mV/% at 0.01 Hz to 0.33 ± 0.05 mV/% at 1 Hz. The streaming potential of control disks did not vary significantly during time in culture at any frequency, while that of treated plugs decreased to 30% and 10% of initial values at 0.01 Hz and 1 Hz respectively after 3 days. In marked contrast to dynamic stiffness, the amount by which the streaming potential of disks treated with 100 μg/ml SLN decreased with time showed a marked frequency dependence. At high frequency (1 Hz), the streaming potential decreased quickly with time in culture while at low frequency (0.01 Hz) the decrease in streaming potential was less rapid.

2.5 Discussion

The combined results of Figs 1 and 2 strongly suggest that SLN is responsible for the proteoglycan loss seen in our studies. The dose dependence of GAG loss and the absence

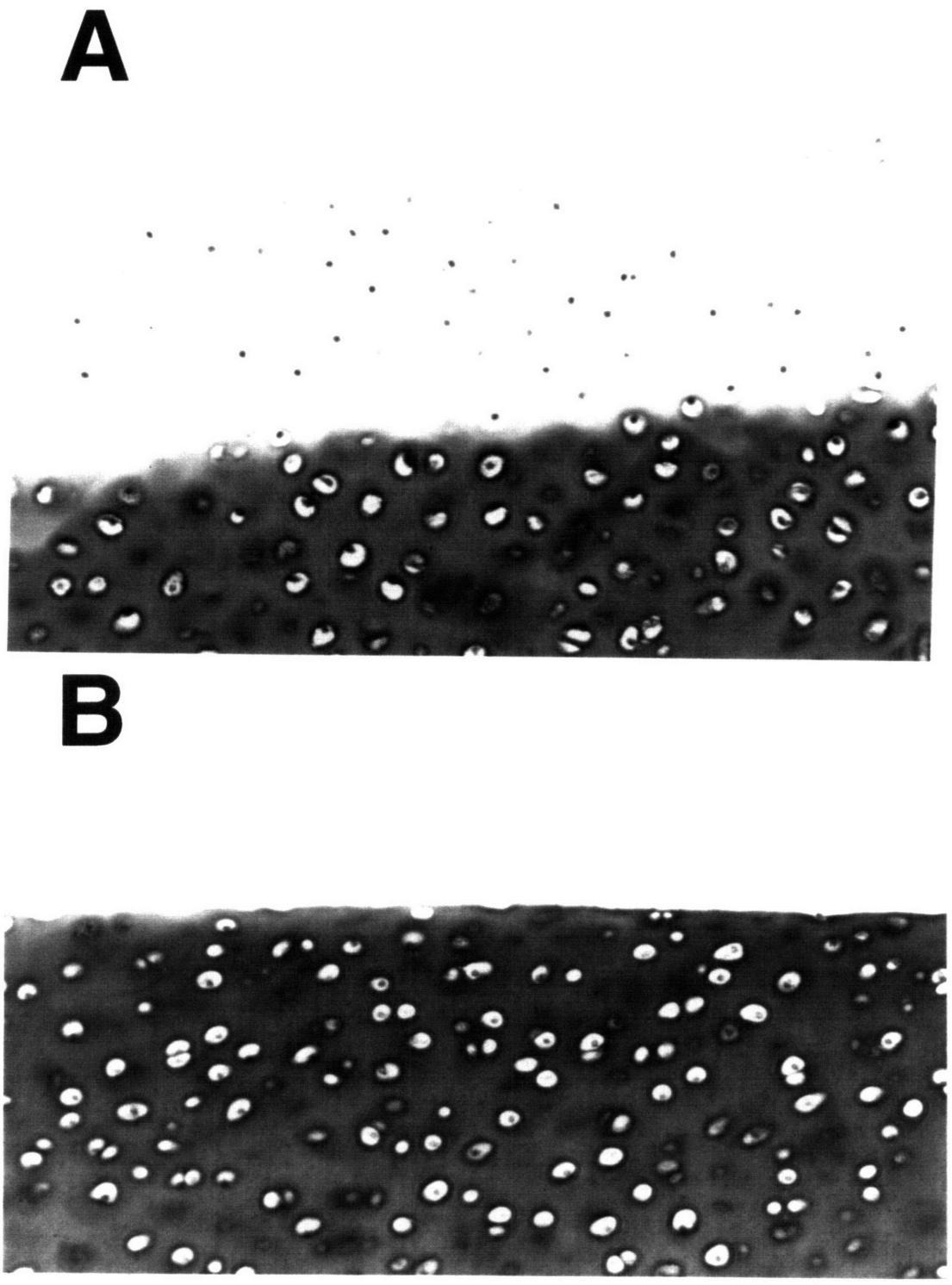

Figure 2.4: Light micrographs of cartilage disks incubated in DMEM alone for 72 hours (**A**) and DMEM with 100 μg/ml SLN for 24 hours (**B**), represented at 100×. SLN treatment resulted in an apparent front of enzymatic digestion moving inward from all exposed specimen surfaces, shown by the absence of toluidine blue stain for GAG. (5 μm section; bar = 100 μm; ▼: tissue surface.)

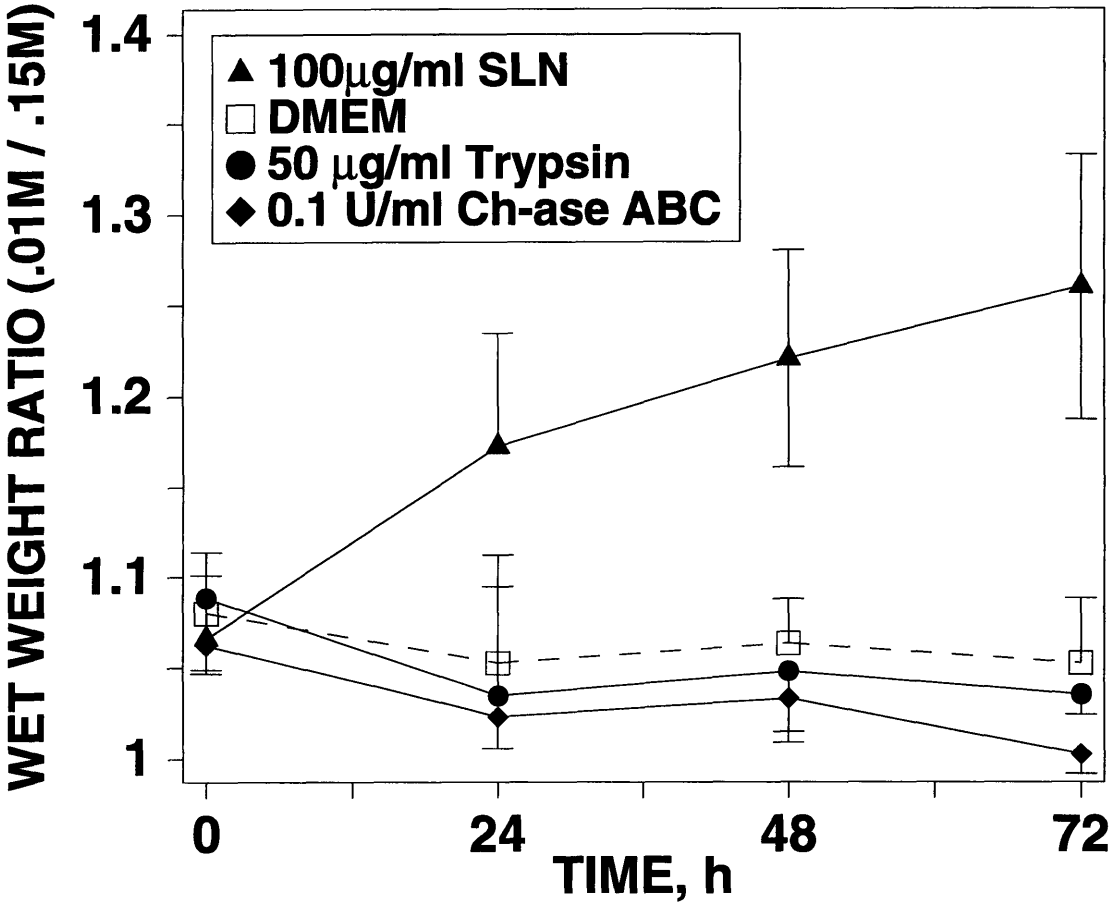

Figure 2.5: Ratio of wet weight in 0.01 M NaCl to wet weight in DMEM for samples incubated in DMEM alone, DMEM with 100 μg/ml SLN, DMEM with 50 μg/ml trypsin or DMEM with 0.1 Units/ml chondroitinase ABC. Data are mean ± SD.

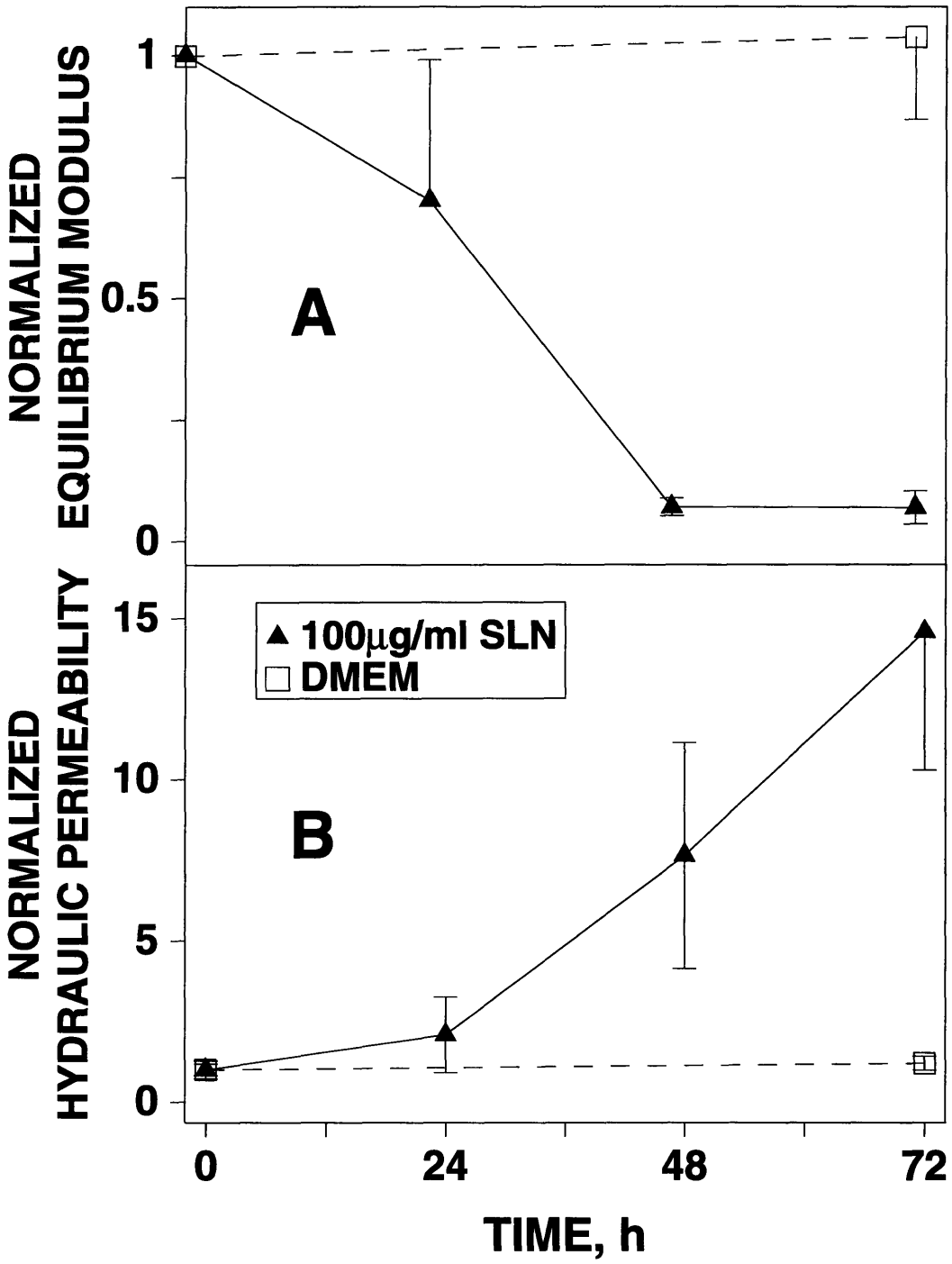

Figure 2.6: Equilibrium modulus (**A**) and hydraulic permeability (**B**) of cartilage disks as a function of time in culture. Disks were incubated in DMEM alone (dotted line) or in DMEM with 100 μg/ml SLN (solid line). Data are mean $\pm$ SD (n = 4); mean values are normalized to the value at t = 0.

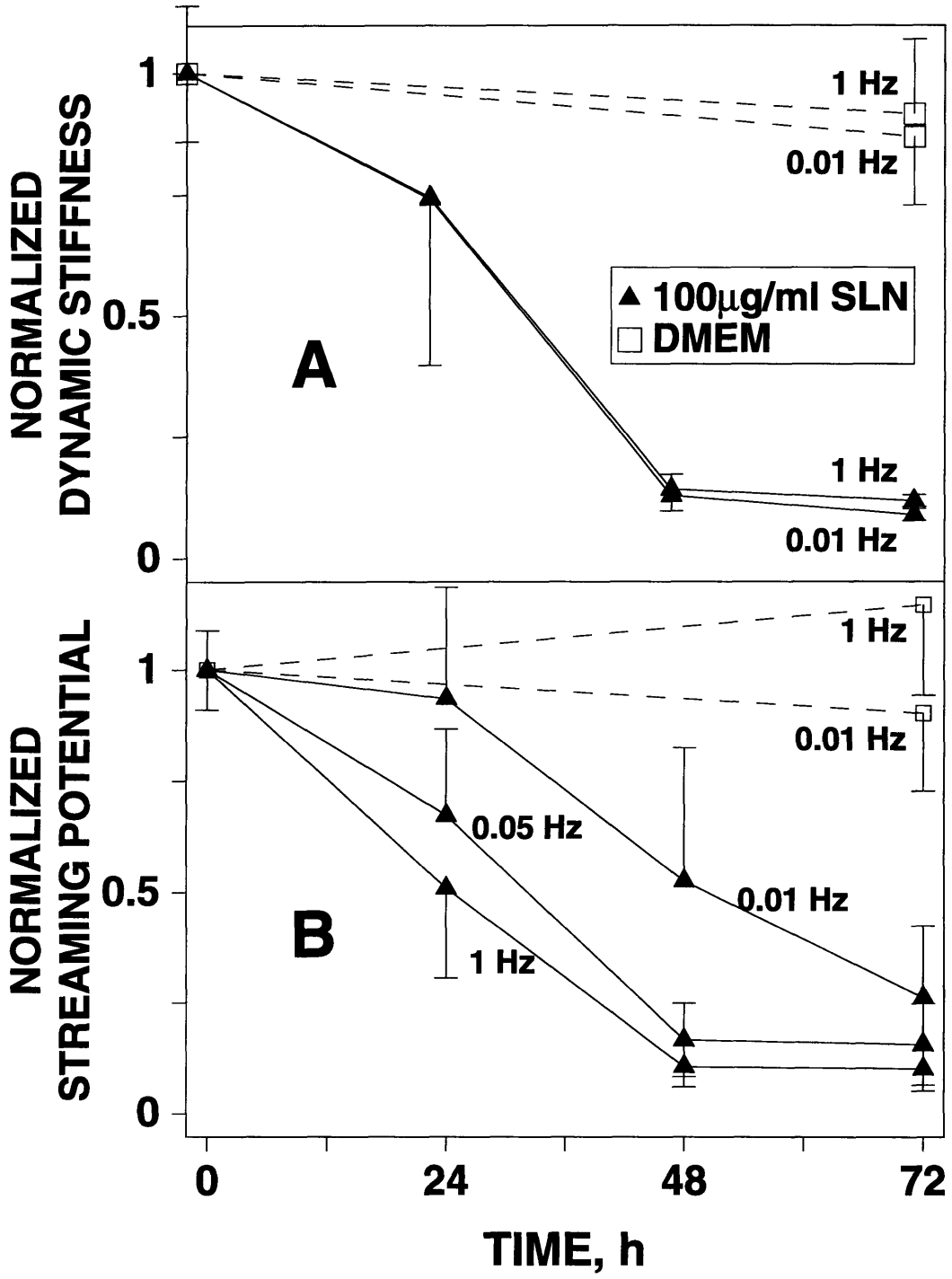

Figure 2.7: Dynamic stiffness (**A**) and streaming potential (**B**) of cartilage disks as a function of time in culture. Data are mean ± SD (n = 4); mean values are normalized at each frequency to the value at t = 0.

of loss seen when TIMP was added simultaneously with SLN demonstrate that the GAG loss was induced by the addition of SLN. Moreover, the presence of specific SLN-generated aggrecan cleavage products in culture media and the lack of detectable cleavage products generated by other proteinases further suggests that direct SLN cleavage of aggrecan occurred within the tissue during culture. This is of special interest, given that recent work has shown that total levels of SLN (active plus inactive) in synovial fluid of patients with OA are as high as 300 nM (15 μg/ml) [74], which is within the concentration range tested here. Given that matrix degradation in OA may progress gradually over many years, it is interesting that the concentrations of active SLN in the 15 μg/ml range appear quite capable of causing significant cartilage matrix damage in a much shorter time frame (Fig 2.1). A likely explanation for this is that a majority of the SLN in OA synovial fluid is most probably inactive, being either in the proenzyme form or bound to endogenous inhibitors [74,128].

Histological examination (Fig 2.4) showed that *initial* loss of GAG occurred primarily in the surface regions of the disks, leaving interior regions relatively undegraded. This observation of a moving front of enzymatic degradation progressing inward from exposed tissue surfaces has been documented previously for the cases of degradation of PG constituents by Chondroitinase ABC [41] and *Streptomyces* hyaluronidase [49]. Together, these results suggest that such high molecular weight enzymes ($M_r \sim$ 20-150 kD), having limited numbers of spatially accessible cleavage sites within a dense matrix, do not simply diffuse rapidly into the tissue and degrade it uniformly; rather, they slowly digest their way into the tissue from the surface with diffusion-reaction, substrate-limited kinetics. *In vivo*, it is conceivable that the degradative enzymes in synovial fluid or those produced by chondrocytes in the superficial zone must first degrade the matrix in the superficial zone before they can gain access to the interior regions of the tissue. Indeed, Okada *et al* [95] showed immunolocalization of SLN in chondrocytes mainly in the superficial and transition zones of 90% of the human osteoarthritic samples analyzed, in regions where there was depletion of PG, as revealed in toluidine blue stained sections.

The results shown in Figs 3, 5, and 6 also suggest that SLN has caused significant

damage to other constituents of the cartilage matrix besides the proteoglycans. Data in Fig 2.3 show that the addition of SLN to the cartilage culture media causes an increase in release of type IX collagen fragments into the culture media, as well as an increase in residual type IX collagen fragments within the tissue. It can be seen (Fig 2.3B) that the extracts show a broad spectrum of immunoreactive bands with a greater proportion of higher molecular weight components in the SLN-treated disk extract. In interpreting this finding it is necessary to consider that type IX collagen molecules are heavily cross-linked to type II collagen and to each other [135]. Thus, because stromelsyin cleaves the type IX molecule only at very specific sites, it is expected that a ladder of polymeric fragments will result from the degradation of the cross-linked matrix protein. Since type IX collagen has been implicated as a possible crosslinking agent between type II fibrils [30], studies on cartilage swelling were specifically designed to assess the integrity of the collagen network during and after SLN treatment. As described by Maroudas [78], the swelling state of cartilage is determined by a balance between the osmotic pressure of the proteoglycans which tends to swell the tissue and the restraining tensile forces of the collagen network, which tend to limit tissue swelling. If the collagen network is compromised, as in OA [78], it is less able to counteract the osmotic pressure, and the tissue swells.

The addition of SLN to culture media caused an increase in swelling induced by equilibration in 0.01 M NaCl, up to 25% after three days. The addition of trypsin or chondroitinase ABC, enzymes known to cause loss of GAG from cartilage, did not alter swelling compared to untreated controls (Fig 2.5). Taken together these results strongly suggest that SLN treatment reduced the ability of the collagen network to restrain the residual swelling pressure of the remaining PG constituents. The data of Fig 2.3 motivate the hypothesis that degradation of type IX collagen and damage to the type II/type IX complex contribute to this swelling. Although there is no direct evidence of cleavage by proteinases other than stromelysin, it should be noted that the possibility of some degradation by endogenous proteinases, in addition to and perhaps activated by the exogenous stromelysin, cannot be ruled out when interpreting the results.

Additional evidence of damage to the cartilage matrix beyond removal of proteoglycans is seen in the results of mechanical compression experiments (Fig 2.6A). Previous studies have shown that neutralization or shielding of proteoglycan fixed charge groups (by pH or ionic strength alteration) or removal of charge bearing GAG chains by enzymatic treatment could decrease the modulus or dynamic stiffness of cartilage disks by approximately 50% [28,41,42]. The results in Fig 2.6A show that exposure to SLN resulted in a 90% loss in equilibrium modulus, a significantly greater loss than that associated with enzymes which degrade or remove only proteoglycan constituents. The substantial decrease in equilibrium modulus suggests that degradation of the cartilage matrix occurred beyond loss of proteoglycans, possibly including damage to the collagen network.

Dynamic compression studies also showed a large increase in hydraulic permeability (Fig 2.6B) with time in culture after treatment with 100 μg/ml SLN. At the same time the GAG content had decreased to 10% of its original value (Fig 2.1). This increase in hydraulic permeability which accompanied GAG loss is consistent with the previous work of Maroudas *et al*, who demonstrated that proteoglycans are the primary source of resistance to fluid flow in the cartilage matrix [79].

The streaming potential and its dependence on frequency (Fig 2.7B) are of particular interest regarding the sensitivity of electromechanical measurements to the spatial distribution of enzymatic degradation (Fig 4). The fluid flow that occurs within the cartilage disk during sinusoidal compression not only contributes to dynamic stiffness (Fig 7A), but also tends to separate counterions in the interstitial fluid from proteoglycan negative fixed charge groups [39,40]. The voltage which results from this charge separation is known as the streaming potential. The measured streaming potential of Fig 2.7B is the total potential drop across the disk. This potential can be viewed as the the sum of the local potential drops across incremental layers of the tissue.

At each depth, the local potential drop is proportional to the proteoglycan charge density and the local fluid velocity relative to the matrix [42]. At high frequency, the fluid velocity profile is steep, and fluid flow occurs predominantly in a thin region near

the surface of the disk. At lower frequencies, the velocity profile is less steep and extends further into the tissue [40]. Thus, most of the streaming potential drop at 1 Hz will occur within the top most 50-100 μm of the 1 mm thick disks and the potential is therefore most sensitive to the proteoglycan content in the top most layer. At frequencies $\leq$0.01 Hz, however, the potential drops across a much thicker portion of the disk and therefore reflects the proteoglycan density of deeper as well as surface regions.

Interpreted in this context, the significant decrease in dynamic stiffness and streaming potential with increasing incubation time in SLN is an expected consequence of the loss of GAG (Fig 2.1). These results are consistent with findings of previous studies of the effects of enzymatic degradation on cartilage electromechanical behavior [41]. However, the time dependence of the decrease in potential is highly dependent on frequency, while the decrease in stiffness is independent of frequency. At 24 hours, the tissue has experienced approximately 50% loss in GAG content (Fig 1), primarily confined to the outer 100-150 μm tissue layer (Fig 2.4B). The mean stiffness at 24 hours decreased by 25% at all frequencies, but was not significantly different from controls. The streaming potential frequency response was quite different, with negligible loss by 24 hours at 0.01 Hz, but a significant loss at 1 Hz. This decrease in potential at 1 Hz but not at 0.01 Hz is consistent with the sensitivity of the streaming potential at higher frequencies to the focal loss of GAG from the surface region of the disk. Given that the dynamic stiffness had no such frequency dependence, it is apparent that streaming potential is more sensitive to the initial, focal loss of proteoglycans from cartilage that results from SLN treatment.

Chapter III

In Vivo Effects of Stromelysin on the Composition and Physical Properties of Rabbit Articular Cartilage in the Presence and Absence of a Synthetic Inhibitor

3.1 Abstract

Objectives. To characterize the effects of intraarticular injection of recombinant human stromelysin (SLN) on the matrix composition and physical properties of cartilage from lapine stifle joints and modulation of these effects by the systemic administration of an n-carboxyalkyl synthetic matrix metalloproteinase inhibitor (MC1).

Methods. Female 6–8 week old New Zealand white rabbits received intraarticular injection of 100 μg activated SLN in one stifle joint and buffer in the contralateral control knee and were sacrificed after one hour. A separate group of animals received intravenous injection of either 30 mg/kg MC1 or buffer prior to intraarticular injection of SLN. Joints were dissected and analyzed for proteoglycan (PG) loss into joint fluid, tissue biochemical composition and histological analysis by toluidine blue or anti-VDIPEN antibody staining, or frozen for physical property analysis. Disks of femoropatellar groove cartilage were also harvested from the stifle joint and tested in uniaxially confined compression for determination of electromechanical and mechanical properties.

Results. Lapine stifle joints which received injection of SLN without systemic administration of MC1 showed a 13-fold increase in proteoglycan (PG) in synovial fluid. Cartilage from these joints showed significant decreases in streaming potential at 1 Hz and electrokinetic coupling coefficient, but no change in equilibrium modulus, dynamic stiffness, and hydraulic permeability. Treatment with MC1 resulted in a significant decrease in loss of PG into joint fluid and elimination of changes in cartilage high frequency streaming potential and coupling coefficient in joints which received intraarticular injection of SLN.

Conclusions. The one hour exposure to SLN *in vivo* resulted in loss of PG and exposure of the VDIPEN epitope of the aggrecan core protein in a small region of the tissue near the articular surface. This highly localized degradation resulted in changes in the electromechanical behavior, but little or no change in mechanical properties. Systemic administration of MC1 was able to significantly decrease loss of PG into synovial fluid and prevent even the highly localized tissue degradation and the resultant changes in electromechanical behavior caused by intraarticular SLN injection.

3.2 Introduction

In healthy joints, articular cartilage serves to distribute loads over bone surfaces and provide a low friction surface over which bones can move. The porous and highly hydrated matrix has a sparse population of chondrocytes of approximately 10-100 $\times$ 10^6 cells/ml [117] responsible for regulating matrix composition and preserving tissue integrity. The cartilage matrix is composed primarily of hydrated collagen fibrils, highly charged proteoglycan molecules and other glycoproteins [73,130]. The high tissue water content and electrostatic interactions between proteoglycan fixed charge groups are mainly responsible for the complex mechanical behavior that characterizes the response of the tissue to physiologic compressive loads [65].

In osteoarthritis (OA), the combination of altered cartilage matrix composition and mechanical wear from joint motion can result in erosion of cartilage down to the bone surface [14]. In its degenerated state, OA cartilage exhibits dramatically different biochemical, physicochemical, mechanical, and electromechanical properties from normal tissue, including a distinct loss of proteoglycan [19,74,83], marked collagen network fibrillation [44,102], increases in tissue hydration [3,78], and loss of compressive stiffness [3,58]. The role of enzymatic degradation in OA has been studied extensively, but is not completely understood. It has been suggested [27] that families of enzymes, including matrix metalloproteinases [20,92,96,100], serine proteases [66], and a novel "aggrecanase" [110] contribute to cartilage degradation in OA. Given that there are coordinated patterns

of enzyme expression [23,76], and that many enzymes are synthesized in zymogen form and require activation by other enzymes [16,119,120], it seems likely that the enzymatic component of OA involves a "cascade" of activities which work in tandem to degrade the tissue.

Alterations of biochemical composition and matrix structure which result from enzyme activity are manifest in changes in cartilage material properties and physical behavior. Enzymatic degradation has been shown to result in decreased compressive stiffness [53], changes in tensile stiffness, strength, and fracture strain [66,112], decreased shear modulus [55], changes in creep behavior [66,112], and increased indentation displacement [53,66]. In addition, marked decreases in compression-induced streaming potential have been shown to be particularly sensitive indicators of alterations in matrix proteoglycan composition associated with enzymatic degradation [41], and in characterizing the spatial distribution patterns of degradation caused by enzymes such as stromelysin [8].

Stromelysin-1, also known as matrix metalloproteinase 3 (MMP-3), is a neutral metalloproteinase produced by chondrocytes and synovial fibroblasts [23,76,96], is present in significant quantities in arthritic tissue and has been implicated in normal matrix turnover, as well as remodeling and development. Stromelysin degrades several cartilage matrix components including aggrecan [35,92], link protein [92], and collagen types II, IX, and XI [134]. It has been found in elevated levels in cartilage and synovial fluid in patients with degenerative OA [74,96], as well as in animal models of OA [20,100]. Elevated levels of mRNA expression have likewise been detected in patients with knee joint injury [74] and heat shock [123], respectively. There is also evidence of elevated stromelysin gene expression in early stages of soft tissue development [12] as well as immunolocalization of stromelysin in cartilage growth plate [13]. In addition, stromelysin is capable of radically altering the composition and physical properties of cartilage explants [8].

Although studies have focused on the effects of stromelysin on isolated cartilage matrix constituents or on cartilage explants *in vitro*, less attention has been given to the action of stromelysin on intact cartilage *in vivo*. Therefore, the primary objectives of this

work were (1) to determine the effects of intraarticular injection of stromelysin on the composition of rabbit stifle joint cartilage, (2) to correlate alterations in cartilage matrix composition with changes in physical properties, and (3) to evaluate modulation of these effects by systemic administration of a synthetic matrix metalloproteinase inhibitor.

3.3 Materials and Methods

3.3.1 In Vivo Treatment

In one series of experiments, 6 female New Zealand white rabbits from 6–8 weeks in age (2–3 kg) received intraarticular (IA) injection into one stifle joint of 100 μg recombinant human stromelysin (SLN) in 1.0 ml phosphate buffered saline (PBS), while the contralateral joint was injected with PBS alone. In another series of experiments a group of 8 rabbits received intravenous (IV) injection of 30 mg/kg of a $\sim$500 Da synthetic matrix metalloproteinase inhibitor (MC1) N-[1(R)-carboxy-ethyl]-α-(S)-(2-phenyl-ethyl)glycine-(L)-leucine, N-phenylamide [22], in a buffer composed of 70% PBS, 15% dimethylsulfoxide and 15% Cremaphore EL (polyethylene glycerol tricinoleate, BASF, Parsippany, NJ) 30 minutes prior to IA injection of SLN. A second group of 8 animals received IV injection of buffer alone, 30 minutes prior to IA injection of SLN. One hour after injection with SLN, all rabbits were sacrificed and both joints were lavaged with two 1.0 ml aliquots of of PBS. After sacrifice, all groups of joints were frozen and stored for characterization of joint cartilage physical properties. Separately, animals were treated identically to the 3 treatment groups described above and their joints were subsequently harvested for histological examination. In a separate series of experiments, 7 groups of 4 animals received IV injection of 10 mg/kg MC1. Animals were sacrificed at selected times after IV injection and joint fluids were lavaged and assayed for MC1 content.

3.3.2 Histology

Freshly dissected samples of cartilage were embedded in O. C. T. compound (Tissue-Tek) and frozen in liquid nitrogen. Six μm cryosections were cut through the cartilage samples, fixed in Nakane fixative [84], and permeabilized with 0.1% Triton X-100 in PBS (Gibco). To assess GAG content, sections were stained using toluidine blue [45]. To monitor accumulation of the rabbit aggrecan neoepitope FMDIPEN [6], and anti-VDIPEN antibody was used [72]. This antibody specifically recognizes the C-terminal sequence (FVDIPEN^{3}41) of the stromelysin-generated hyaluronan binding region of human aggrecan [72]. It has also been shown to react with the corresponding neoepitope in rabbit aggrecan, FMDIPEN [6]. Bound anti-VDIPEN IgG was detected by immunoperoxidase microscopy using the ABC technique (Elite kit: Vector Laboratories, Inc., Burlingame, CA). The peroxidase reaction product was developed using a glucose oxidase/DAB/nickel method [115].

3.3.3 Physical Properties

Intact lapine stifle joint were isolated after sacrifice and stored at -70 °C for up to 3 months. For physical property tests. The stifle joints were dissected to expose the femoropatellar groove, and up to 3 bone/cartilage cylinders were harvested from each joint using a 3 mm dermal punch (Miltex Instruments, Lake Success, NY). Cartilage was separated from the bone immediately prior to mechanical compression, using a scalpel to cut approximately plane parallel cartilage disks. Each disk was immediately mounted in an electrically insulating cylindrical confining chamber described previously [41]. This chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA) interfaced to a computer and frequency generator (Model 5100, Rockland Systems, West Nyack, NY). Cartilage samples were equilibrated in 0.15 M phosphate buffered saline (PBS)(Gibco, Grand Island, NY) at pH 7.4 containing 100 U/ml penicillen G and 100 μg/ml streptomycin at room temperature. Samples were compressed between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted in the

base of the chamber. An identical electrode was mounted in the surrounding PBS bath as a reference electrode. Cartilage disks were mounted in the chamber with the top of the disk (the articular surface) in contact with the porous platen and bottom of the plug (the cartilage/bone interface) in contact with the chamber electrode, preserving the anatomical orientation of the tissue relative to the testing chamber.

After mounting each disk in the confined compression chamber, individually, the distance between the porous platen and bottom electrode was decreased until a (~5-10 g) signal was detected by the load cell, and this distance was taken to be the sample thickness. Subsequently, each cartilage disk was compressed by sequential increments of 0.5–1.0% static strain to a maximum of 20% total strain. After stress relaxation, the equilibrium stress resulting from each increment in imposed static strain in the 15-20% range was detected by the Dynastat load cell and recorded by the computer. The equilibrium confined compression modulus was obtained by a best fit linear regression of these equilibrium stress-strain data. At a given static offset strain, sinusoidal strains of <1% amplitude were superimposed the stating strain in frequencies ranging from 0.01 Hz to 1 Hz. The resultant oscillatory loads were detected by the Dynastat load cell and the oscillatory streaming potentials were measured by the chamber electrodes which were connected to a high-impedance Universal Amplifier, Model 11-4113-02 (Gould, Inc., Cleveland OH). The load and streaming potential signals were recorded simultaneously by the computer. The total time for equilibration and testing one sample by this procedure was <1 hour. The dynamic stiffness was obtained by normalizing the measured load to the applied strain and sample area, while the amplitude of the streaming potential was normalized to the applied dynamic strain. Dynamic stiffness, streaming potential, and equilibrium modulus data were used with the method of Frank and Grodzinsky [40] to calculate an effective electrokinetic coupling coefficient (a parameter which relates the streaming potential to load developed in the tissue) and an effective hydraulic permeability (a parameter which indicates the ease with which fluid moves through the cartilage matrix). In all tests, the amplitude of the applied strain was chosen to be small enough to elicit a linear response from the sample

[39,40] and the total harmonic distortion in the measured load was <10% for all samples tested. The porous platen remained in contact with the samples at all times during the testing cycle.

3.3.4 Biochemical Analyses

After physical property tests, cartilage disks were frozen at -20 °C, lyophilized, and digested with 1 ml of 125 μg/ml papain digestion solution (Sigma, St. Louis, MO) as described previously [105]. Small (20 μl) portions of the digests from each disk were assayed for sulfated GAG content by quantification of their reaction with 2 ml of dimethylmethylene blue (DMB) dye solution in polystyrene cuvets (VWR, Boston, MA) by spectrophotometric measurements (Model 3λB, Perkin Elmer, Norwalk, CT) [34], using whale/shark chondroitin sulfate (Sigma) as the standard.

Synovial fluid lavages taken immediately after sacrifice were assayed for MC1 content by mass spectrometry and for GAG content by the DMB dye binding assay as previously described [34].

3.4 Results

3.4.1 Biochemical Analysis

Analysis of joint fluid lavages revealed that synovial fluid MC1 content peaked at >6 μg at 30 minutes after IV injection of 10 mg/kg MC1 and remained at levels >0.3 μg for 5 hours after IV injection (Fig 3.1). IA injection of 2.0 ml of PBS yields 2.3 ± 0.3 ml of fluid, implying a joint fluid volume of approximately 300 μl. Without IV MC1, average GAG content of disks from SLN treated joints was 88% that of disks from control, which had 0.053 ± 0.010 (g GAG)/(g tissue wet weight). With IV MC1, GAG content of treated disks was 90% of disks from control joints (Fig 3.2). In neither case was the difference statistically significant. Measured levels of GAG in joint fluids from SLN treated joints (with no IV injection) were 280 ± 15 μg GAG/joint, with approximately 4 mg of GAG from

harvested joint cartilage. In the absence of IV MC1, joint fluid PG levels from SLN treated joints were 13 times higher than contralateral controls, and with IV injection of MC1, there was a 70% reduction in joint fluid PG levels (Fig 3.2).

3.4.2 Histology

Examination of sections of femoropatellar groove cartilage revealed a marked of decrease in Toluidine Blue staining (Fig 3.3A) and pronounced staining of anti-VDIPEN (Fig 3.3D) in regions of the tissue near the articular surface in joints injected with 100 μg/ml SLN in the absence of IV MC1. Control joints showed minimal or no loss in Toluidine Blue staining (Fig 3.3B) and little presence of anti-VDIPEN antibody (Fig 3.3E). Cartilage from rabbits which received IV injections of 30 mg/kg MC1 showed a marked decrease in loss of Toluidine Blue staining (Fig 3.3C) and presence of anti-VDIPEN antibody (Fig 3.3F) in SLN injected joints, as compared to cartilage from animals which received blank IV injections.

3.4.3 Physical Properties

Using the Dynastat load cell to ensure that both platen surfaces were in contact with the specimen, the effective thickness of cartilage disks harvested from rabbit joints was determined to be 486 $\pm$ 62 μm and 491 $\pm$ 106 μm for control and SLN-treated joints, respectively. Treatment with IV MC1 did not significantly alter the measured disk thickness. For rabbits which did not receive IV injection, the streaming potential ranged from 7.8 μV/% at 0.01 Hz to 21.8 μV/% at 1 Hz for cartilage from control joints and from 4.0 μV/% at 0.01 Hz to 5.5 μV/% at 1 Hz from cartilage from joints which received IA injection of 100 μg SLN (Fig 3.4A). Values of dynamic stiffness ranged from 4.4 MPa at 0.01 Hz to 1.38 MPa at 1 Hz and from 3.9 MPa at 0.01 Hz to 1.24 MPa at 1 Hz for cartilage disks from control and SLN treated joints respectively (Fig 3.4B).

From stress-strain data measured in the range of 15–20% static strain, the equilibrium confined compression modulus of cartilage disks from control joints was 219 kPa.

Using modulus, streaming potential, and dynamic stiffness data, the hydraulic permeability and electrokinetic coupling coefficient were calculated to be 1.36×10^{-14} m^2/Pa·s and 0.98×10^{-9} V/Pa respectively. By comparison, indentation studies performed on femoropatellar cartilage from skeletally mature New Zealand White rabbits [5] gave values of 510 kPa for equilibrium modulus and 3.38×10^{-15} m^2/Pa·s for hydraulic permeability, with cartilage thickness of 200 μm. The material properties of cartilage disks from control joints in animals which received either IV injection of buffer did not differ from those which received MC1 and were not significantly different from those of samples from animals which did not receive IV injections.

The measured values of streaming potential and dynamic stiffness at 1 Hz, coupling coefficient, equilibrium modulus, and hydraulic permeability were normalized to values for control disks obtained from animals given no IV injection (Fig 3.5) and IV injection of buffer (Fig 6A) or 30 mg/kg MC1 (Fig 3.6B). For animals given no IV injection, dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability of cartilage from SLN injected joints did not differ significantly from controls, while streaming potential at 1 Hz was 25% of control and coupling coefficient was 51% of control ($p < 0.02$ and 0.05 respectively (Fig 3.5)). For SLN treated cartilage, streaming potential was significantly different ($p < 0.05$) from control at all frequencies ≥ 0.1 Hz. Results from experiments with rabbits given IV buffer (Fig 3.6A) were similar to those with no IV (Fig 3.5), with no observed difference in dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability between control and SLN injected joints, but significant differences in streaming potential at 1 Hz and coupling coefficient. The streaming potential of cartilage from SLN treated joints was significantly less that from contralateral controls for all testing frequencies ≥ 0.1 Hz (Fig 3.4). For animals which received 30 mg/kg MC1, there was again no difference in dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability. In addition, streaming potential at 1 Hz and coupling coefficient of treated cartilage were 91% and 84% of control values, and this difference was not statistically significant (Fig 3.6B).

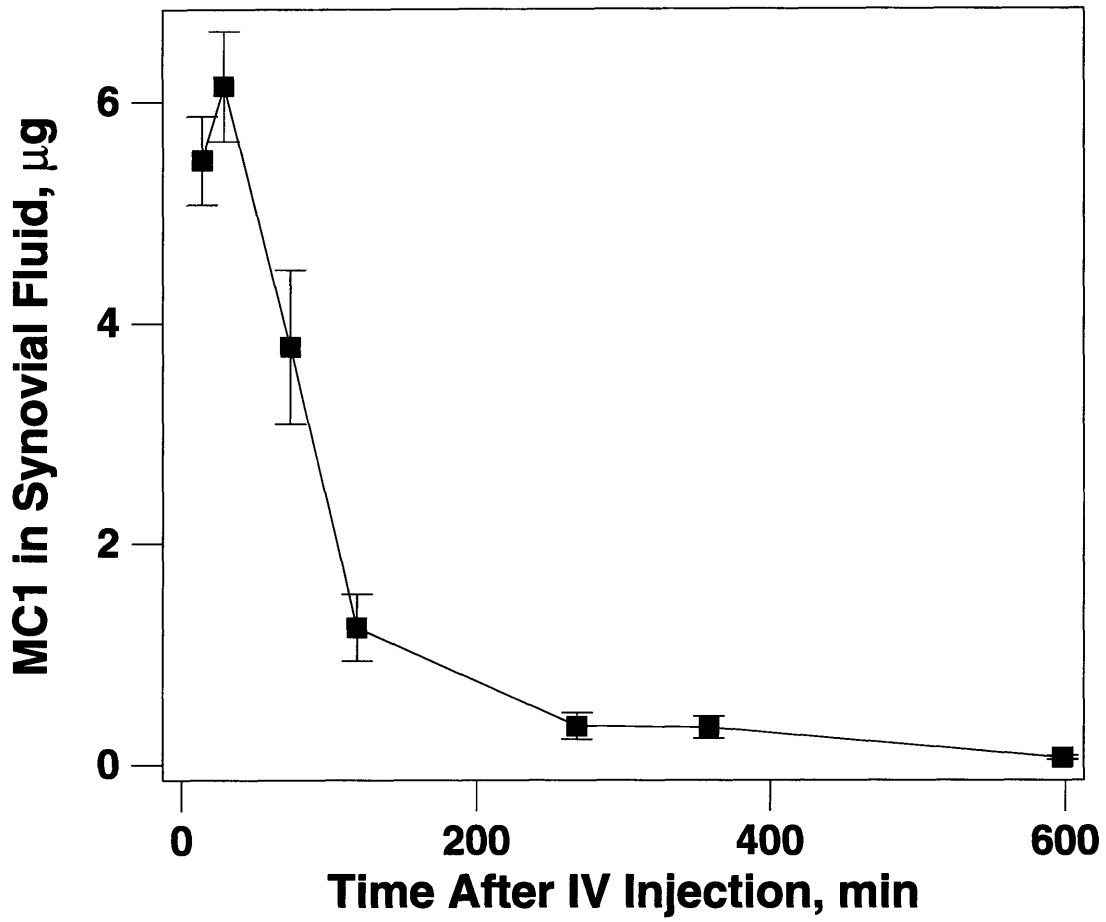

Figure 3.1: Amount of synthetic matrix metalloproteinase inhibitor (MC1) in synovial fluid lavage vs. time after intravenous injection of 10 mg/kg MC1 (n=4 ± SEM). For 2 × 1 ml injections of PBS, 2.3 ± 0.3 ml was recovered, giving an average joint fluid volume of 0.3 ml.

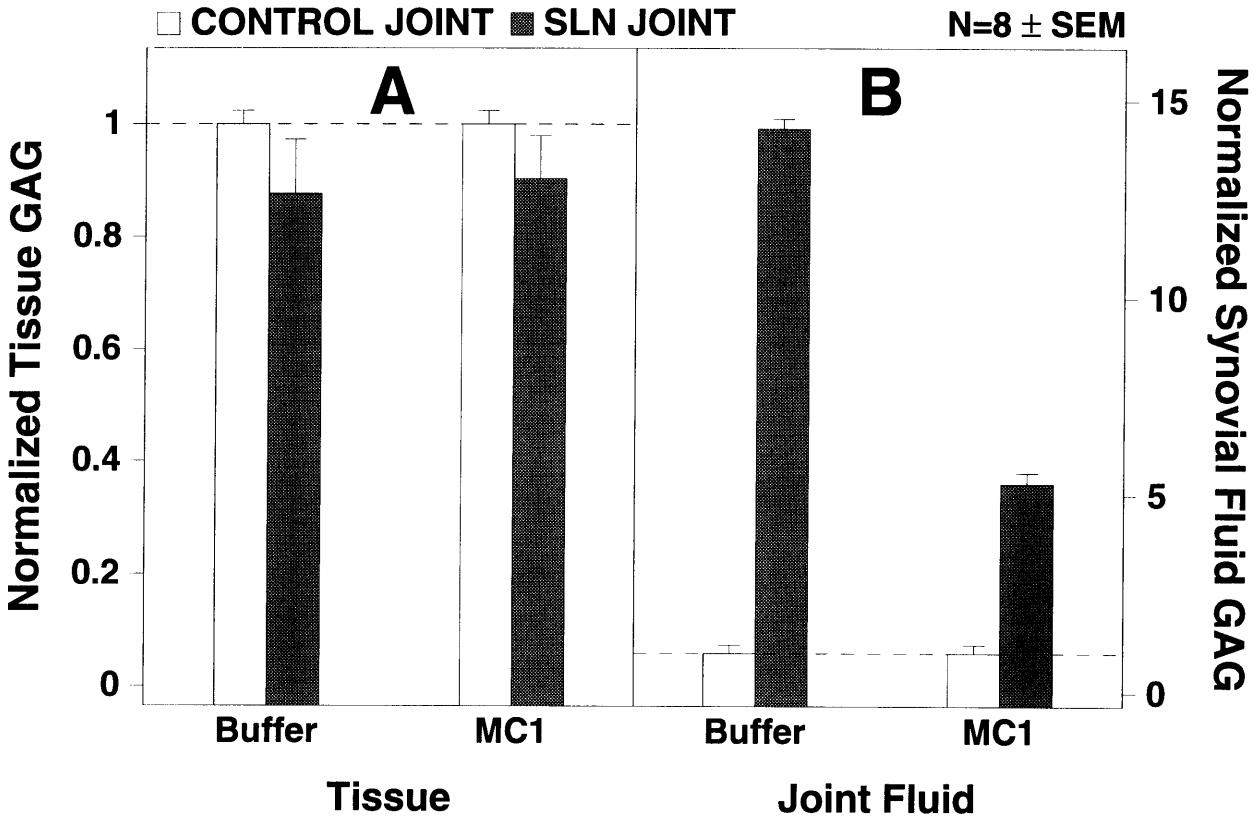

Figure 3.2: **A:** GAG content of mechanically tested cartilage disks from the femoropatellar grooves of 8 rabbits which received intravenous injection of 30 mg/kg MC1 and 8 rabbits which received IV injections of buffer with no MC1. Disk GAG concentrations (per tissue wet weight) from SLN-injected joints were normalized to GAG content of disks from contralateral control joint. **B:** Normalized GAG content of joint fluid lavages from lapine stifle joints from animals which received intravenous injection of 30 mg/kg MC1 or buffer alone.

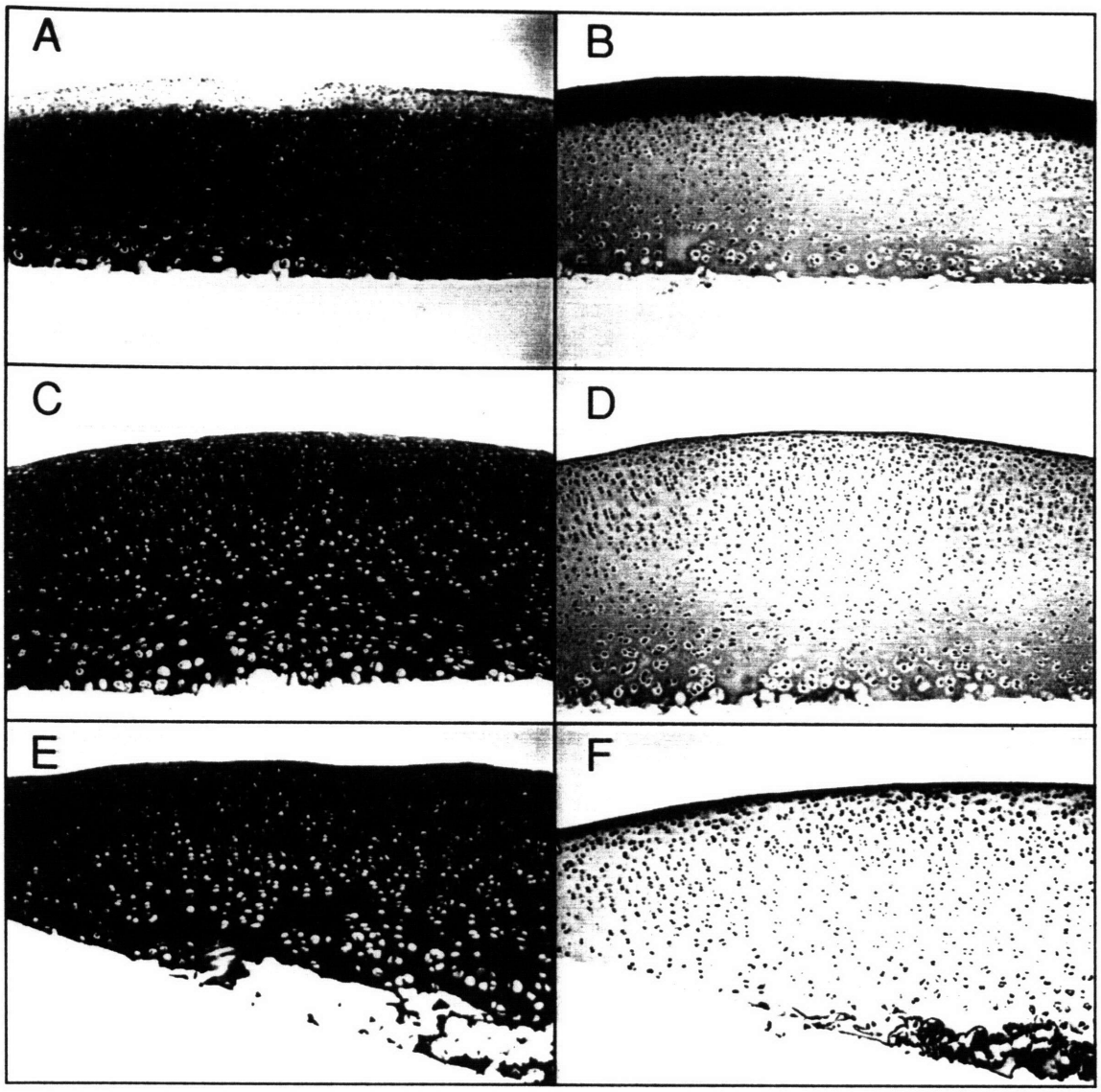

Figure 3.3: Toluidine blue staining of femoropatellar groove cartilage from stifle joints which received intraarticular injection of SLN (A), buffer (C), or SLN with intravenous MC1 (E). Immunostaining of FMDIPEN neoepitope in cartilage from SLN injected joints (B), buffer injected joints (D), or SLN injected joints from rabbits that received an intravenous injection of MC1 (F).

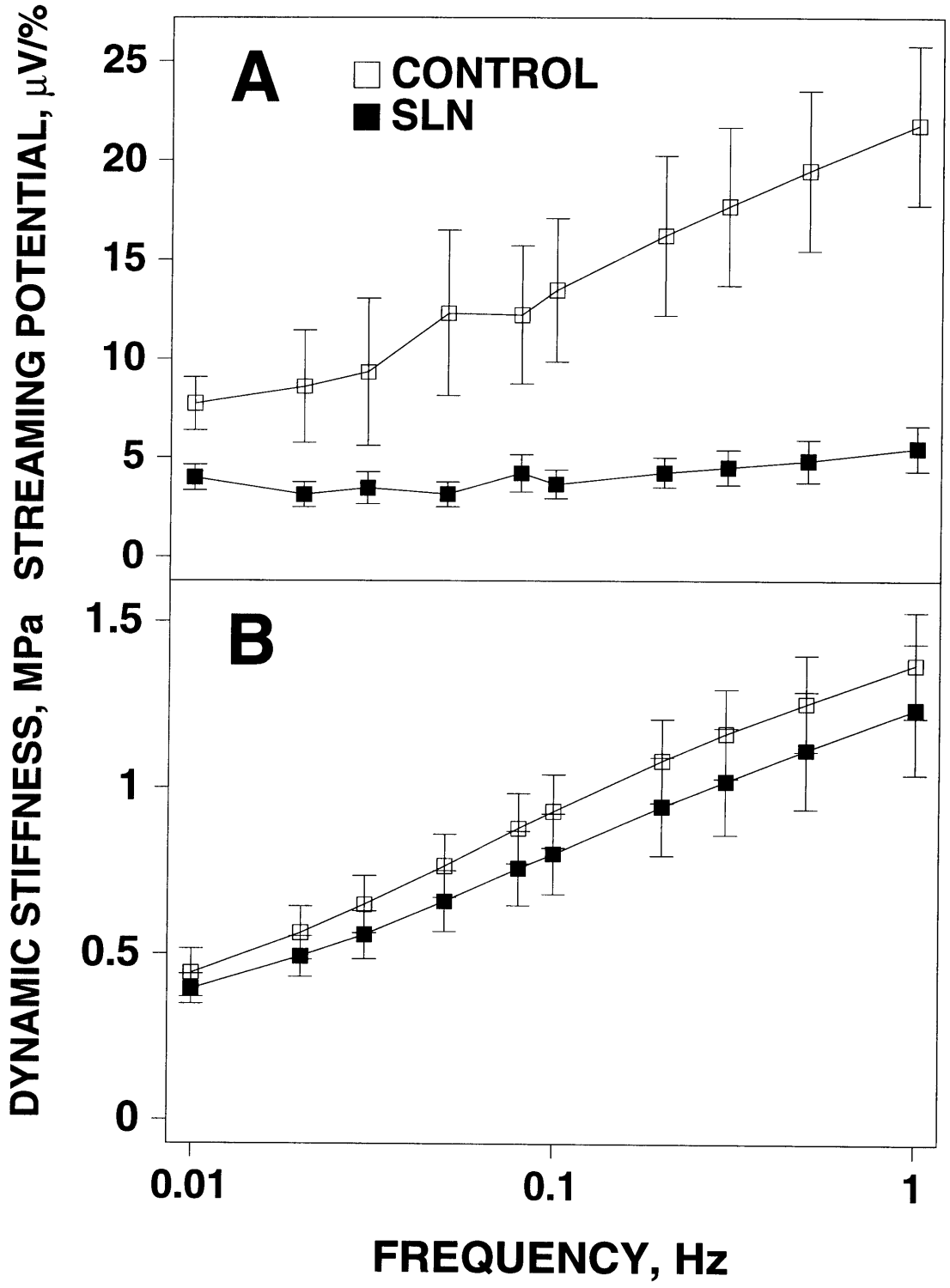

Figure 3.4: Frequency response of measured streaming potential (**A**) and dynamic stiffness (**B**) of cartilage disks from a group of 6 lapine stifle joints which received intraarticular injection of 100 μg SLN and contralateral control joints (n = 6 $\pm$ SEM).

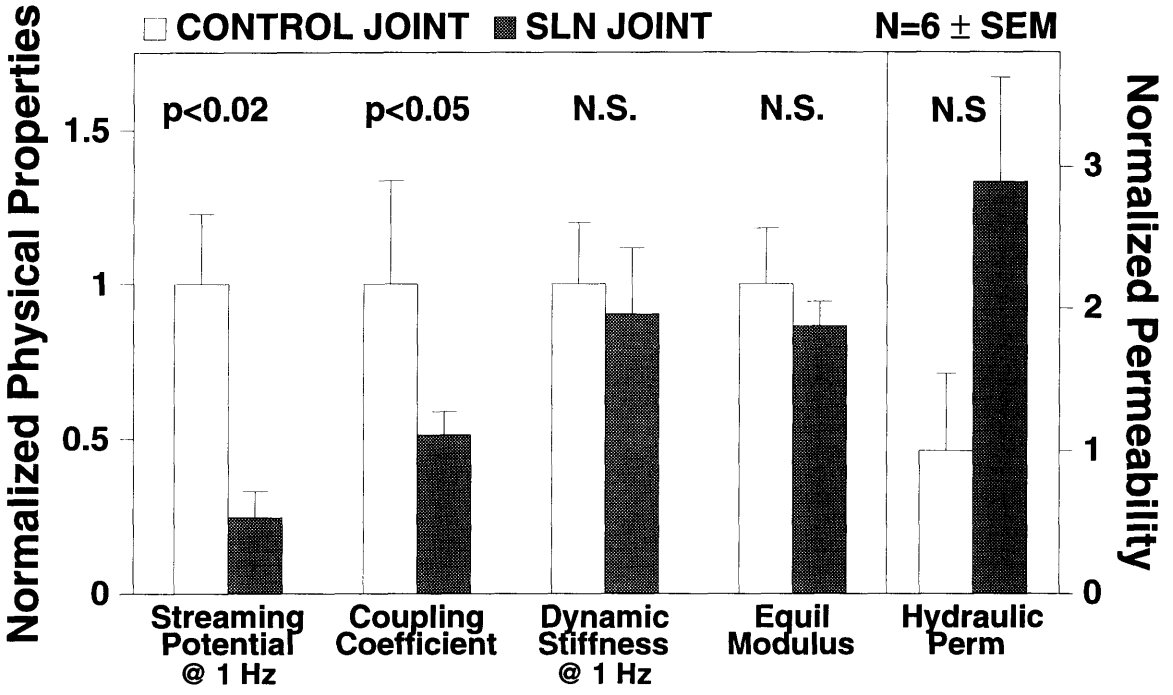

Figure 3.5: Normalized streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability of cartilage disks from lapine stifle joints which received intraarticular injection of 100 μg SLN and from contralateral control joints. (The dynamic properties of these disks are shown in Fig 3.4.)

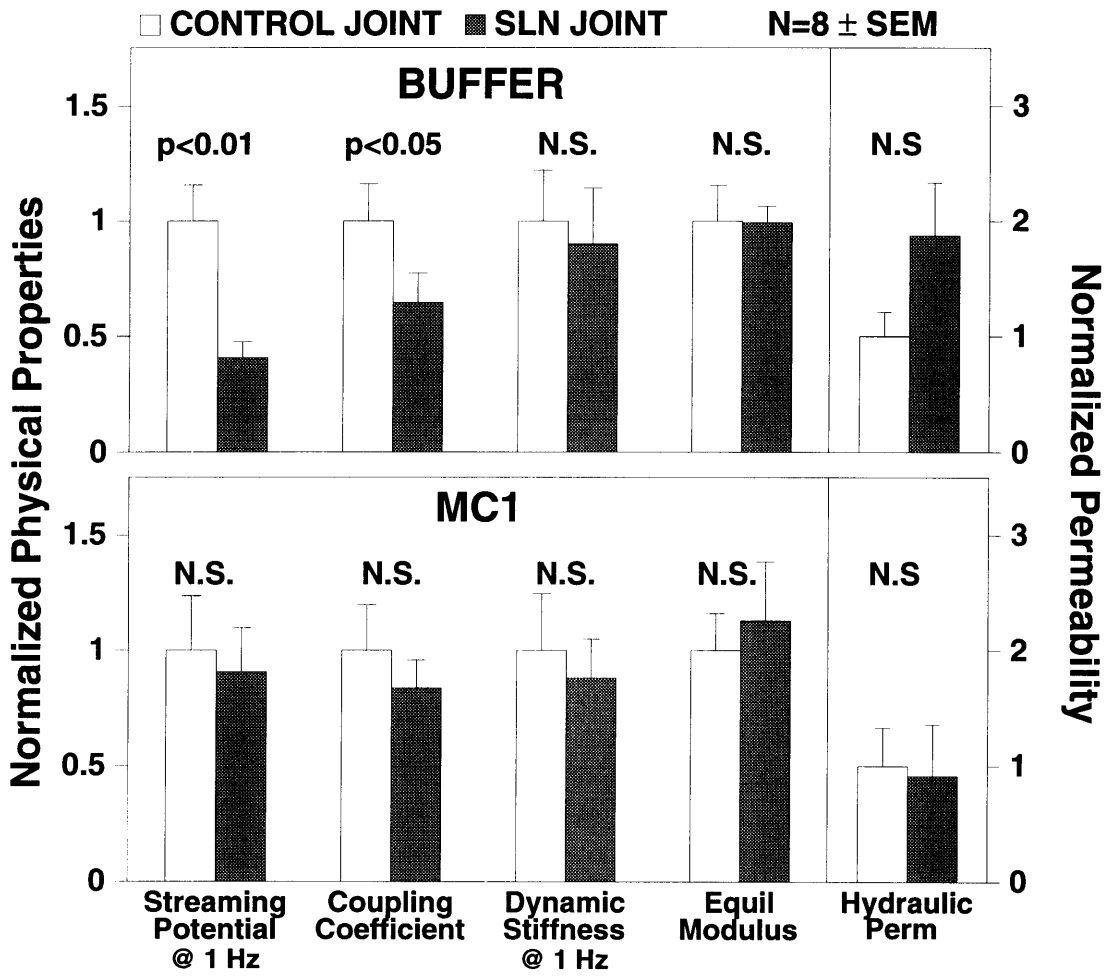

Figure 3.6: **A:** Normalized streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability of cartilage disks from a group of 8 animals which received intravenous injection of buffer. Stifle joints which received intraarticular injection of 100 μg SLN are compared to contralateral control joints. **B:** Normalized streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability of cartilage disks from a group of 8 animals which received intravenous injection of MC1. Stifle joints which received intraarticular injection of 100 μg SLN are compared to contralateral control joints. (The GAG content of cartilage and synovial fluid from these joints is shown in Fig 3.2.)

3.5 Discussion

All indicators of cartilage degradation induced by IA injection of 100 μg SLN were significantly inhibited by IV treatment with 30 mg/kg MC1. The loss of PG fragments from cartilage to the synovial fluid was significantly decreased by the administration of MC1 prior to IA injection of SLN (Fig 3.2). The loss of Toluidine Blue staining (Fig 3.3A) and the presence of anti-VDIPEN antibody (Fig 3.3D) near the articular surface which resulted from IA injection of SLN did not occur following administration of IV MC1 (Fig 3.3C,3F). High frequency streaming potential and electrokinetic coupling coefficient, which were both dramatically decreased (Fig 3.6A) were similar to control values with IV injection of MC1 prior to IA injection of SLN (Fig 3.6B). Taken together these data seem to indicate that MC1 was quite effective at inhibiting SLN inside the joint. While it is unclear if inhibition occurred in the tissue itself, it seems likely that MC1 was able to inhibit SLN while it was still in the synovial fluid, and therefore prevent degradation and the resultant changes in the physical properties of the joint cartilage.

For the 1 hour duration of the *in vivo* experiments, levels of MC1 in synovial fluids were seen to be in relative abundance compared to levels of IA injected SLN. At 75 minutes after IV injection of 10 mg/kg MC1, which corresponds to the time at which the rabbits were sacrificed, levels of MC1 in synovial fluid were in excess of 3.75 μg (Fig 3.1). Given that the volume of joint fluid was measured to be $\sim$300 μl, the effective MC1 concentration was approximately 25 μM. Similarly, injection of 100 μg of SLN ($\sim$50 kDa) into the joint gave an effective SLN concentration of approximately 6.6 μM, and thus a 4-fold molar excess of MC1. For experiments involving IV a 30 mg/kg dose of MC1 (Fig 3.6B), it likely that the molar ratio of MC1 to SLN was even greater than 4 to 1.

Measurements of GAG content of joint fluid and tissue indicate that while there was loss of GAG to the synovial fluid due to IA injection of SLN, the majority of the GAG remains within the cartilage (Fig 3.2). Comparison of SLN treated and untreated joints shows that GAG content of mechanically characterized cartilage disks from SLN treated joints was $\sim$90% of controls. This is consistent with joint fluid data which show a loss of

290 μg of PG from SLN treated joints, corresponding to <10% of the 4 mg of PG measured in the joint cartilage. The relatively small amount of PG loss seen here is consistent with the 1 hour duration of the experiment. *In vitro* studies [8] have also demonstrated that the time required to remove most of the PG in the joint cartilage would be on the order of days for the SLN concentrations used here.

Histological examination revealed the marked loss of toluidine blue staining near the articular surface (Fig 3.3A), and intense staining for VDIPEN neoepitope in the same region (Fig 3.3D), suggesting that the surface loss of PG from the cartilage was due to cleavage of aggrecan by SLN. This is also similar to observations *in vitro* which indicated that cartilage explants cultured in media containing SLN [8] or other enzymes [41,49] were initially degraded at the tissue surface when the relatively high molecular weight enzymes had a limited number of spatially accessible cleavage sites and were not able to penetrate the dense cartilage matrix until it had been degraded. Thus, for the short times ($\sim$1 hour) relevant to these experiments, SLN injected into the joint did not appear to have access to deeper tissue regions and preferentially degraded a thin layer of cartilage near the articular surface. This is of particular interest, given that other studies have shown that SLN production may be localized to the superficial zones of cartilage [96] or the synovium [20] in osteoarthritis.

The value of equilibrium modulus reported here (219 kPa) is 2-3 times lower and hydraulic permeability (1.36×10^{-14} m^2/Pa·s) and measured cartilage thickness (486 μm) are 2-3 times higher than data reported by Athanasiou *et al* [5]. Given that the cartilage from the skeletally mature animals examined by Athanasiou *et al* is likely to be stiffer, less thick, and denser (and consequently less permeable) than the cartilage from the 6-8 week old rabbits used in this study, these differences seem reasonable.

The frequency response of the streaming potential and dynamic stiffness (Fig 3.4) are typical of the poroelastic material behavior exhibited by articular cartilage [40]. The frequency behavior of the streaming potential and dynamic stiffness of cartilage disks from SLN injected joints are also consistent with the biochemical (Fig 3.2) and histological data

(Fig 3.4). Since the degradation caused by injection of SLN is confined to regions near the articular surface, the bulk of the tissue remained undegraded. Thus, the mechanical integrity of bulk of the tissue, as reflected in properties such as equilibrium and dynamic stiffness, was not appreciably altered. However, the streaming potential, which is highly dependent on localized fluid flows [8,40], is much more sensitive to such focal degradation. In high frequency tests, fluid flow occurs mainly in a narrow region near the tissue surface, and thus the streaming potential is significantly decreased by the loss of PG near the tissue surface [8,41]. At lower testing frequencies, when fluid exchange occurs throughout a greater portion of the tissue, the streaming potential reflects the bulk composition of the tissue which has not change significantly. Thus, the difference in streaming potential between cartilage from SLN treated and control joints is greatest at high frequency and decreases as the frequency is lowered (Fig 3.4).

When analyzed together, the spectrum of properties measured suggest that the electromechanical measurements are more sensitive than the purely mechanical measurements to the focal degradation caused by IA injection of SLN with either no IV injection (Fig 3.5) or IV injection of control buffer (Fig 3.6A). These electromechanical properties (streaming potential and electrokinetic coupling coefficient) which reflect the streaming potential behavior across all frequencies, are affected by local distributions of fixed charge, and therefore PG, while the mechanical properties (dynamic stiffness, equilibrium modulus and hydraulic permeability) are less sensitive to surface alterations in matrix composition. These data are also consistent with studies on degradation of cartilage explants by SLN *in vitro* [8] in which short term exposure resulted in significant changes in streaming potential at high frequency, while mechanical properties such as dynamic stiffness were not affected.

Chapter IV

Activation and Inhibition of Endogenous Matrix Metalloproteinases in Articular Cartilage: Effects on Composition and Physical Properties

4.1 Abstract

Bovine cartilage explants were cultured in the presence of 1 mM 4-aminophenylmercuric acetate (APMA) to activate endogenous matrix metalloproteinases (MMPs) and changes in biochemical, biomechanical, and physicochemical properties were assessed. Additionally, graded levels of either recombinant human tissue inhibitor of metalloproteinases-1 (TIMP) or a synthetic metalloproteinase inhibitor (MC1) were used to inhibit degradation induced by APMA. Treatment with APMA resulted in as much as 80% loss in tissue proteoglycan content and a >3-fold increase in the presence of denatured type II collagen as determined by the presence of CB11B peptide after 3 days in culture. Physicochemical studies revealed that APMA treatment resulted in a significant increase in tissue swelling, consistent with damage to the collagen network. Activation of MMPs by APMA also resulted in >80% decrease in equilibrium modulus, dynamic stiffness, and streaming potential and >50% decrease in electrokinetic coupling coefficient. The addition of 4 μM, 400 nM and 40 nM TIMP inhibited PG loss by 95%, 50%, and 20%, respectively and all doses effectively inhibited swelling response. The addition of 4 μM and 400 nM MC1 inhibited PG loss by 95% while 40 nM MC1 inhibited PG loss by 60%, and all doses effectively inhibited tissue swelling. Cartilage disks treated with 4 μM TIMP and 1 mM APMA had significantly greater streaming potential, electrokinetic coupling coefficient, dynamic stiffness and equilibrium modulus than disks treated with APMA alone.

4.2 Introduction

Degradation of cartilage extracellular matrix by proteinases is a prominent feature of osteoarthritis. Although several classes of proteinases are found in mammalian cells, recently much attention has been paid to the role of matrix metalloproteinases (MMPs) in cartilage destruction in the process of OA [90,127].

MMPs are are a family of at least 12 enzymes which are divided into three main classes: collagenases, gelatinases, and stromelysins. Gelatinases are not thought to play a major role in degradation of cartilage matrix; however, interstitial collagenase (MMP-1) is known to degrade collagen types II and X [127], while stromelysin-1 (MMP-3) is known to degrade aggrecan [35], link protein [92], and collagen types II, IX, X, and XI [134]. Both collagenase and stromelysin are produced by chondrocytes and synovial fibroblasts [23,76,96]. These MMPs are present in elevated levels in cartilage and synovial fluid of patients with degenerative OA [74,96,98], as well as in animal models [20,81,100]. Elevated levels have likewise been detected in cases of knee joint injury [74] and heat shock [123]. There is also evidence of MMP gene expression in early stages of soft tissue development [12] as well as immunolocalization of MMPs in cartilage growth plate [13].

Members of the MMP family are synthesized and secreted in a latent zymogen form which requires activation by proteolytic cleavage of a peptide sequence at the N-terminus of the molecule [127]. This activation can take place from cleavage by plasmin, trypsin, or organo-mercurial compounds such as 4-aminophenylmercuric acetate (APMA). Nagase *et al* demonstrated that in the presence of APMA, native prostromelysin or procollagenase will undergo a conformational change which results in the production of an intermediate which is then self-catalyzed to produce an active form of the enzyme [89]. Previously, APMA has been used to activate latent MMPs produced by cartilage after treatment with IL-1β [100] or in animal models of arthritis [99].

There are several naturally occurring inhibitors of MMP activity including α_2-macroglobulin (α_2-M) and tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2). α_2-M is a large molecule ($\sim$750 kDa) produced by the liver and found in synovial

fluid. It inhibits MMPs by presenting a peptide sequence which is cleaved by the enzyme, then engulfing the enzyme and restricting its access to other substrates. Both TIMPs are also large (~28 and 22 kDa, respectively) and are produced by chondrocytes and synovial fibroblasts. The TIMPs inhibit MMPs by binding tightly to the MMP active site. In healthy joints, there is as much as a two-fold molar excess of TIMP compared to stromelysin and collagenase in synovial fluid [74], while in cases of OA enzyme concentrations have been seen to exceed levels of native TIMPs [74,81]. Efforts to use TIMP to inhibit degradation proteoglycan induced by IL-1α in cartilage explants have not been successful [1], presumably due to the inability such a large protein to penetrate the dense cartilage matrix.

Alterations of biochemical composition and matrix structure which result from enzyme activity are manifest in changes in cartilage material properties and physical behavior. Enzymatic degradation has been shown to result in decreased compressive stiffness [53], changes in tensile stiffness, strength, and fracture strain [66,112], decreased shear modulus [55], changes in creep behavior [66,112], and increased indentation displacement [53,66]. In addition, marked decreases in compression-induced streaming potential have been shown to be particularly sensitive indicators of alterations in matrix proteoglycan composition associated with enzymatic degradation [41], and in characterizing the spatial distribution patterns of degradation caused by large enzymes such as stromelysin [7–9].

Previous studies have established the effects of MMPs on isolated cartilage matrix components in solution. In addition, there have been many studies on degradation of intact cartilage induced by IL-1 and retinoic acid, but the mechanism by which the degradation occurs in these systems is not completely resolved. Given that significant quantities of MMPs have been detected even in undegraded tissue, activation of these native enzymes has the potential to dramatically alter the cartilage matrix. Therefore, the primary objectives of this study were to (1) determine the effects of APMA-activation of endogenous MMPs on the biochemical, physicochemical, and physical properties of cartilage explants and (2) evaluate the ability of TIMP-1 and a low molecular weight MMP inhibitor to prevent

APMA-induced cartilage degradation.

4.3 Materials and Methods

4.3.1 Cartilage Explant and Culture

Saddle sections of 1-2 week old calves were obtained from a local abbatoir (A. Arena, Hopkington, MA) within four hours of slaughter. The femoropatellar groove was isolated, and 1 mm thick cartilage × 3 mm diameter disks were harvested using a a sledge microtome (Model 860, American Optical, Buffalo, NY) and dermal punch (Miltex Instruments, Lake Success, NY) as described previously [105]. Cartilage disks were incubated in groups of 4 in 24-well culture dishes (Costar, Cambridge, MA) in 1 ml DMEM (Gibco, Grand Island, NY) containing 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco) for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere.

Groups of 4 disks were then placed in 1 ml DMEM in the presence or absence of 1 mM 4-aminophenylmercuric acetate (APMA) (Sigma, St. Louis, MO) to activate endogenous MMPs, and removed from culture at times up to 72 hours. In addition, selected groups of samples which received APMA treatment were also cultured in the presence of MMP inhibitors or a cocktail of proteinase inhibitors which did not inhibit MMPs. Separate groups of plugs were treated with 100 μg/ml recombinant human stromelysin as described previously [8]. Recombinant human tissue inhibitor of metalloproteinases-1 in 25 mM Tris HCl, 10 mM $CaCl_2$, and 0.01% NaN_3 was concentrated to ~300 μg/ml using a Centricon 10 ultrafiltration membrane (Amicon, Inc., Beverly, MA) as described previously [70] and diluted into media for tissue culture experiments.

To study inhibition of matrix degradation, plugs were incubated with recombinant human tissue inhibitor of metalloproteinases-1 (TIMP) [70] or a synthetic MMP inhibitor N-[1(R)-carboxy-ethyl]-α-(S)-(2-phenyl-ethyl)glycine-(L)-leucine, N-phenylamide (MC1) [22] in concentrations ranging from 40 nM to 4 μM, simultaneous to APMA treatment. To prevent degradation from other matrix proteinases, the following cocktail was

used: 10 mM benzamidine hydrochloride (Sigma) to inhibit trypsin-like activity, 10 mM N-ethylmaleimide (Sigma) to inhibit sulfylhydry-dependent proteinases, 1 mM phenylmethanesulfonyl fluoride (Sigma) to inhibit serine proteinases such as cathepsin B, 1 μM pepstatin (Calbiochem) to inhibit acid proteinases including cathepsin D. Upon removal from culture, plugs were allocated for biochemical, physicochemical and biomechanical analyses. Separate groups of plugs were cultured in DMEM which contained 10 μCi/ml [^{35}S]sulfate and [^{3}H]proline (New England Nuclear, Boston, MA) for 24 hours, then washed 3 times in phosphate buffered saline at 4 °C to assess levels of tissue biosynthetic activity.

4.3.2 Biochemical Analyses

Disks allocated for biochemical analysis were frozen at -20 °C, lyophilized, and digested with 1 ml of 125 μg/ml papain digestion solution (Sigma) as described previously [105]. 20 μl portions of digests and culture media were assayed for sulfated GAG by reaction with 2 ml of dimethylmethylene blue dye solution in polystyrene cuvets (VWR, Boston, MA) and spectrophotometry (Model λ3B, Perkin Elmer, Norwalk, CT) [34] using whale/shark chondroitin sulfate (Sigma) as the standard.

In addition, 100 μl portions of the digest were analyzed for radioactivity by mixing with 2 ml Ecolume scintillation fluid (ICN Biochemicals, Irvine, CA) and measuring ^{3}H-cpm and ^{35}S-cpm in a liquid scintillation counter (RackBeta 1211, LKB, Turku, Finland), with corrections for spillover and dilution quenching. Radiolabel incorporation was normalized to sample DNA content as measured by reaction of 100 μl of digest with 2 ml Hoechst 33258 dye solution (Hoechst-Celanese, Summit, NJ) in an acrylic cuvet (VWR) and fluorometry (SPF-500C Spectrofluorometer, SLN Instruments, Dowington, PA), using calf thymus DNA as a standard [69].

Portions of tissue digests were assayed for the presence of cyanogen bromide peptide 11 of the α(II) chain (α1(II)-CB11B peptide) of type II collagen in the laboratory of Dr. A Robin Poole. Mouse antiserum purified against α1(II)-CB11B peptide was produced as described previously [60]. Serum samples were tested for reactivity against heat denatured

type II collagen, but showed no reactivity against hydroxylysine residues. The amount of α1(II)-CB11B epitope in tissue digests was quantified by competitive enzyme linked immunosorpent assay (ELISA) on a microtiter plate using this antiserum. Hydroxyproline content of portions of tissue digests was evaluated by colorimetric assay as described previously [116].

4.3.3 Swelling Studies

The protocol for swelling studies was motivated by the technique used by Maroudas [78] to compare the swelling of normal and osteoarthritic cartilage. Disks removed from culture medium were patted briefly with a paper towel to remove surface water, and wet weights were measured (AE 163 Balance, Mettler Instrument Corp, Hightstown, NJ). Disks were then twice reequilibrated in 1 ml of hypotonic saline solution (0.01 M NaCl at pH 7.0) for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded. Previously, it has been shown that this method is sensitive to damage to the cartilage matrix collagens, but is not sensitive to changes in tissue proteoglycan content [8].

4.3.4 Biomechanical and Electromechanical Evaluation

Cartilage disks were placed in an electrically insulating poly(methylmethacrylate) cylindrical confining chamber, similar to that used by Frank *et al* [41]. The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA) which was interfaced to a computer and frequency generator (Model 5100, Rockland Systems, West Nyack, NY). Samples were equilibrated in 0.15 M phosphate buffered saline (PBS)(Gibco) at pH 7.4 containing 100 U/ml penicillin G and 100 μg/ml streptomycin at room temperature. Disks were subjected to confined compression between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted at the base of the chamber. An identical electrode was mounted in the surrounding PBS bath.

Disks were first compressed by sequential increments of 0.5-1.5% strain, up to a

maximum of 20% total strain. After stress relaxation, the equilibrium stress corresponding to each increment of static strain was detected with the load cell of the Dynastat and recorded on the computer. The resultant equilibrium stress was plotted against applied strain and the slope of the best fit linear regression curve gave the equilibrium confined compression modulus. At a given static offset strain, sinusoidal strains of $<1\%$ amplitude were superimposed on the static strain at frequencies ranging from 0.01 Hz to 1 Hz. The resultant oscillatory load was detected by the load cell and the oscillatory streaming potential was detected by the chamber electrodes which were connected to a high-impedance Universal Amplifier, Model 11-4113-02 (Gould, Inc., Cleveland, OH). Load and streaming potential were simultaneously recorded by the computer. The amplitude of the dynamic load was normalized to disk area and to the amplitude of the applied strain to give dynamic stiffness, while the amplitude of the streaming potential was normalized to the amplitude of the applied strain [39,40]. Equilibrium modulus and dynamic stiffness data were used in combination with the method of Frank and Grodzinsky [40] to calculate the effective hydraulic permeability of the tissue specimen. In all tests the amplitude of the applied strain was chosen to be small enough to elicit a linear mechanical response from the sample [39,40] (i. e. the total harmonic distortion in the measured load was $<10\%$). The porous platen remained in contact with the sample at all times during the testing cycle.

4.4 Results

4.4.1 Biochemical Analyses

Treatment with 1 mM APMA resulted in significant loss of proteoglycan (PG) fragments from cartilage disks after 12 hours, with losses ranging from $>50\%$ (Fig 4.1) to $>80\%$ (Fig 4.2) after 3 days in culture. GAG content of control samples at the beginning of the culture was 652 ± 86 μg GAG/cartilage disk. Control samples or those treated only with 4 μM TIMP showed $<10\%$ PG loss after 72 hours of treatment. Disks treated with 1 mM APMA with a cocktail of non-MMP proteinase inhibitors showed loss of PG similar to that of

disks treated with APMA alone (Fig 4.1). Exposure of cartilage disks to 1 mM APMA with simultaneous treatment with graded levels of TIMP resulted in a dose dependent inhibition of APMA-induced PG loss (Fig 4.2A). A 4 μM dose of TIMP resulted in >90% inhibition of PG loss, while 400 nM and 40 nM doses yielded ~50% and ~20% inhibition respectively. Similar treatment with MC1 resulted in >95% inhibition of PG loss for both the 4 μM and 400 nM doses of MC1 and >60% inhibition at 40 nM (Fig 4.2B).

Incorporation of radiolabeled macromolecules containing [^{35}S]sulfate and [^{3}H]proline into the cartilage matrix matrix was significantly suppressed by the addition of APMA into the culture media. Control disks incorporated 4063 $\pm$ 548 [^{3}H] cpm/μg DNA compared to 79 $\pm$ 15[^{3}H] cpm/μg DNA for disks which were cultured with 1 mM APMA for 24 hours. In addition during 1 day in culture control disks incorporated 5677 $\pm$ 472 [^{35}S] cpm/μg DNA, while disks treated with APMA incorporated 57 $\pm$ 7.6 [^{35}S] cpm/μg DNA. Both [^{3}H]proline and [^{35}S]sulfate data indicate that APMA-treated cartilage disks were biosynthetically inactive. Data are given as mean $\pm$ standard deviation for n = 4 disks.

APMA treatment also resulted in increased unwinding of type II collagen (Fig 4.3), as determined by the appearance of the collagen CB11B epitope. The amount of type II collagen with accessible CB11B peptide was normalized to total collagen content to give the percentage of denatured type II collagen. In control samples after 3 days in culture, ~2% of the type II collagen was denatured, as compared to ~7% in samples treated with 1 mM APMA. The amount of denatured collagen in disks treated with 100 μg/ml SLN was similar to that of control disks.

4.4.2 Swelling Studies

Swelling of tissue incubated in the presence of 1 mM APMA, 1 mM APMA with 40 nM TIMP, and 1 mM APMA with 40 nM MC1 was quantified as the ratio of plug wet weight in 0.01 M NaCl to wet weight in DMEM (Fig 4.4). Untreated plugs swelled by 2-3% in hypotonic saline at all time points, while those treated with 1 mM APMA showed increased swelling with time up to a maximum of 18% after 72 hours in culture. Samples treated with

1 mM APMA + 40 nM TIMP or 1 mM APMA + 40 nM MC1 both showed little increase in swelling ratio after three days in culture, and the difference between these treatments and controls was not statistically significant. Higher doses (400 nM and 40 μM) of TIMP and MC1 were also tested, and plugs which received these treatments demonstrated swelling behavior similar to that of control plugs after three days in culture (data not shown).

4.4.3 Biomechanical and Electromechanical Evaluation

Treatment with 1 mM APMA resulted in dramatic decreases in streaming potential (Fig 4.5A) and electrokinetic coupling coefficient (Fig 4.5B) in cartilage disks after 1 day in culture [97]. Measured values of streaming potential ranged from 33.5 μV/% at 0.01 Hz to 133.8 μV/% at 1 Hz and the electrokinetic coupling coefficient was calculated to be 1.92×10^{-9} V/Pa for control plugs at the start of the experiment and did not differ significantly over 3 days in culture. Although values of streaming potential did vary with frequency of testing as expected [40], the relative changes in streaming potential with time were independent of frequency (Fig 4.5).

Treatment with 1 mM APMA also resulted in dramatic decreases in dynamic stiffness (Fig 4.6A) and equilibrium confined compression modulus (Fig 6B) in cartilage disks after 3 days in culture. Measured values of dynamic stiffness ranged from 3.88 MPa at 0.01 Hz to 10.13 MPa at 1 Hz and equilibrium modulus was 1.47 MPa for control plugs at the start of the experiment and did not differ significantly over 3 days in culture. Similar to the streaming potential, the relative decreases in dynamic stiffness were independent of frequency.

The addition of 4 μM TIMP to the culture media affected changes in the physical properties of cartilage disks treated with APMA (Fig 4.7). In particular, the electromechanical properties, streaming potential and electrokinetic coupling coefficient of disks treated with 1 mM APMA were $<$20% and $\sim$50% respectively of the values of disks which received 1 mM APMA with 4 μM TIMP after three days in culture. The purely mechanical properties of APMA-treated disks, equilibrium modulus and dynamic stiffness, were $\sim$50%

and ~60% respectively, of disks which were cultured with both 1 mM APMA and 4 μM TIMP for three days.

4.5 Discussion

4.5.1 Biochemical Analyses

The specificity of matrix degradation is demonstrated in Figure 4.1. Treatment with 1 mM APMA resulted in significant GAG loss which was inhibited completely by simultaneous treatment with the MMP inhibitor, TIMP. Furthermore, the use of non-MMP proteinase inhibitors during APMA treatment did not significantly alter GAG loss, implying that MMP are responsible for the PG degradation seen in these experiments. This is of special concern, given that radiolabel incorporation data showed that the tissue did not synthesize [^{35}S]sulfate or [^{3}H]proline labeled molecules, suggesting that the chondrocytes were non-viable. In such circumstances, cell death might result in release of a variety of proteinases capable of degrading the tissue matrix. However, this does not appear to be the case in this system, where MMPs specifically appear to be the cause of GAG loss. In addition, the lack of biosynthetic activity by the chondrocytes indicates that MMPs are not being produced during the course of the culture, and that the degradation which occurs results from activity of enzymes which are present in the tissue at the start of culture.

The amount of PG degradation which resulted from APMA treatment showed some variation between experiments (Figs 4.1 and 4.2). This is not unexpected, since the observed GAG loss will be a function of the amount of endogenous MMP and MMP inhibitors present in the tissue, which is likely to vary greatly between individual animals. In both cases presented here, the activation of the MMPs native to the tissue resulted in significant loss of PG, up to 80% after 3 days.

This degradation by endogenous MMPs was completely inhibited by the presence of 4 μM TIMP in the culture media (Fig 4.2A). This indicates that the TIMP in the culture media was able to penetrate the cartilage matrix. This is in contrast with previous studies

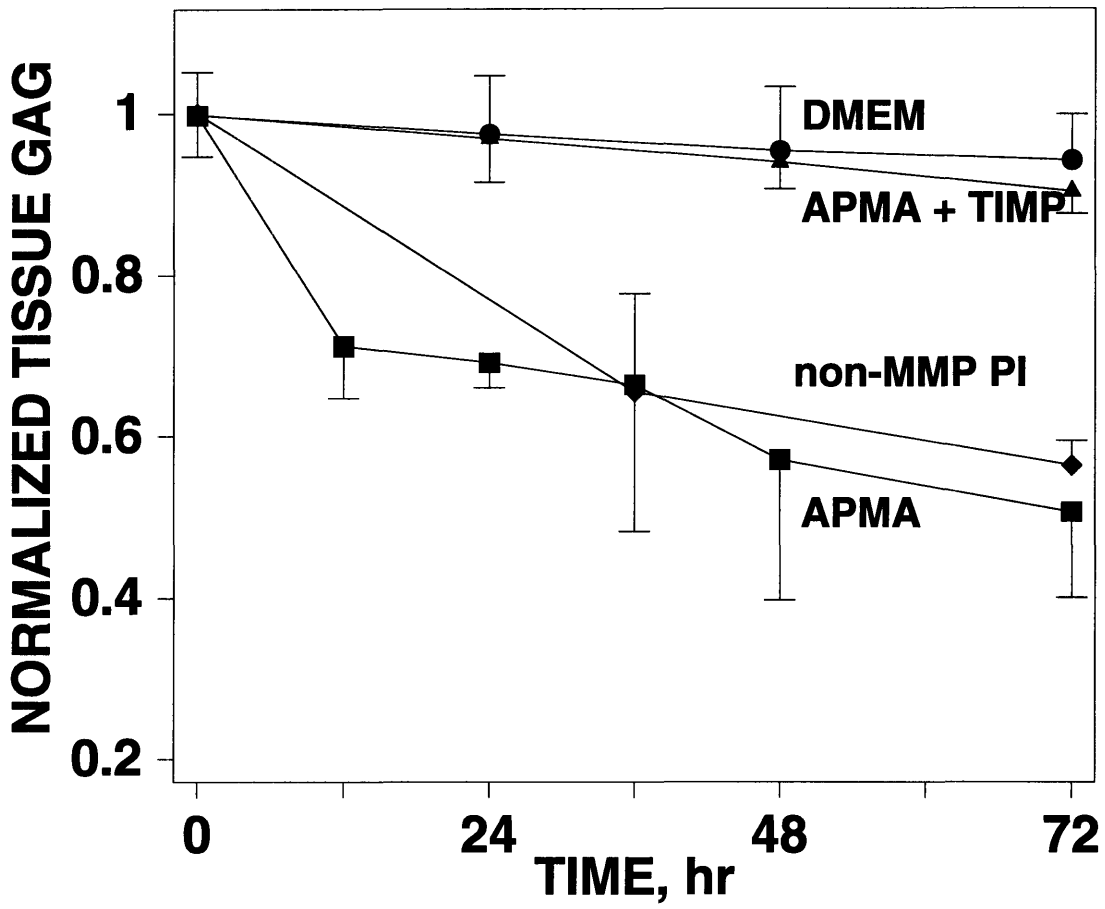

Figure 4.1: Normalized GAG loss from cartilage disks incubated in DMEM alone or DMEM with 1 mM APMA, 1 mM APMA + 4 μM TIMP, or 1 mM APMA + a proteinase inhibitor cocktail without a metalloproteinase inhibitor. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

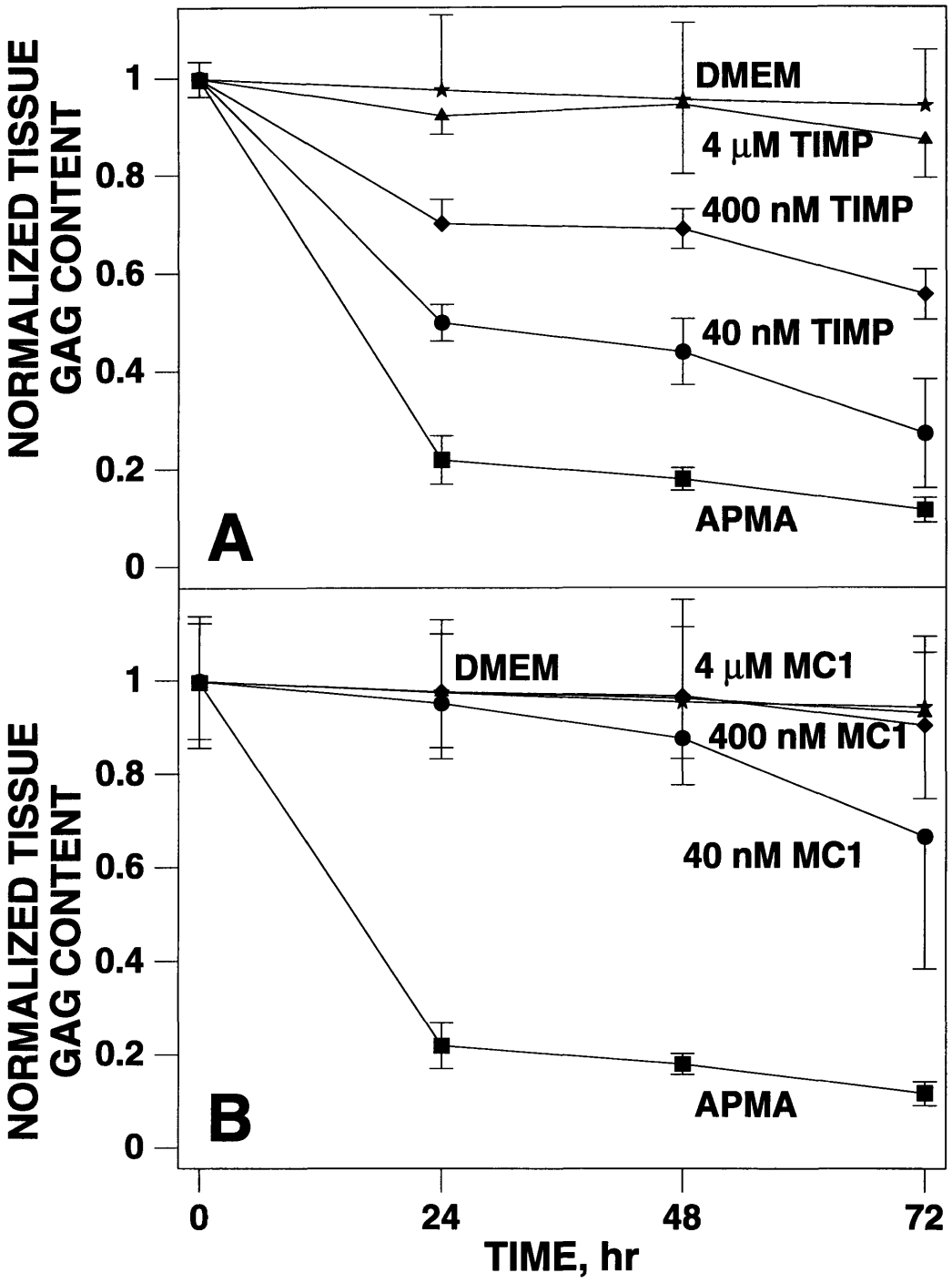

Figure 4.2: **A:** Normalized GAG loss from cartilage disks incubated in DMEM alone or DMEM with 1 mM APMA + graded levels of tissue inhibitor of metalloproteinases (TIMP) ranging from 40 nM to 4 μM. **B:** Normalized GAG loss from cartilage disks incubated in DMEM alone or DMEM with 1 mM APMA + graded levels of a synthetic metalloproteinase inhibitor (MC1) ranging from 40 nM to 4 μM. Data are mean $\pm$ SD (n=4).

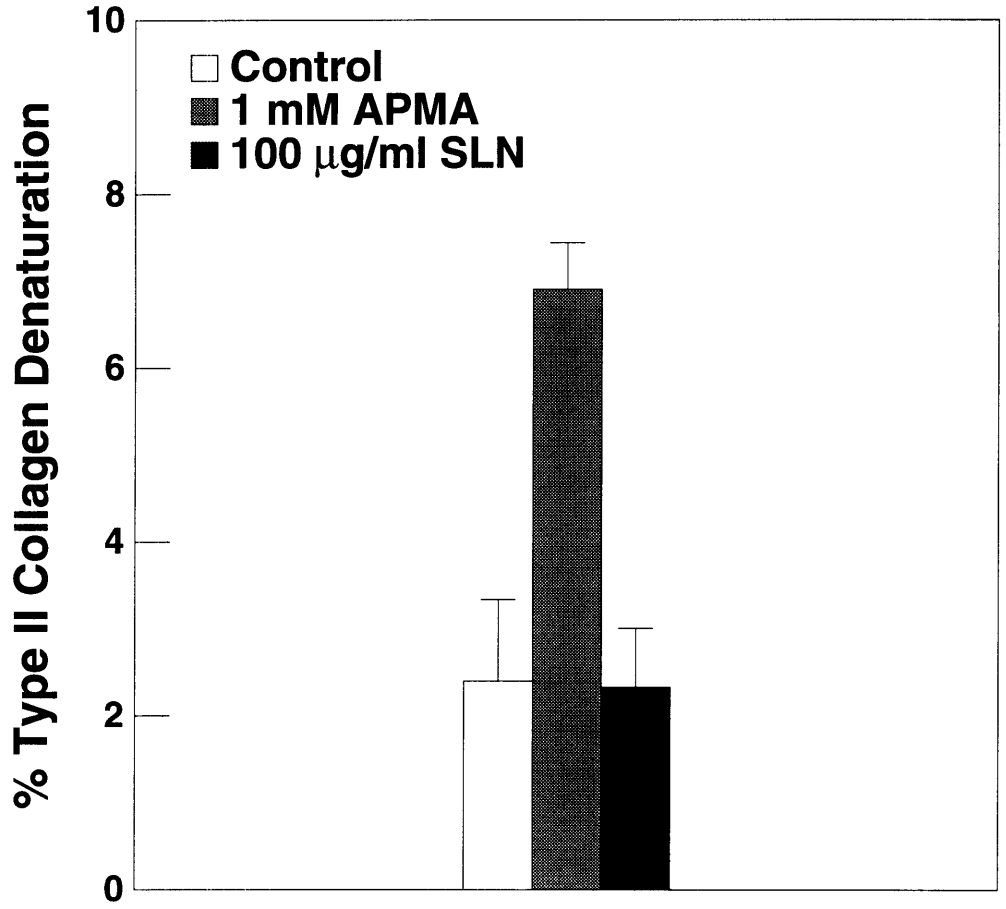

Figure 4.3: Type II collagen denaturation as determined by ELISA for α(II)-CB11B peptide in cartilage disks cultured in DMEM alone, DMEM with 1 mM APMA, or DMEM with 100 μg/ml recombinant human stromelysin for 72 hours. The amount of denatured type II collagen was normalized to total amount of type II collagen to give a percentage denaturation. Data are mean $\pm$ SD (n=3).

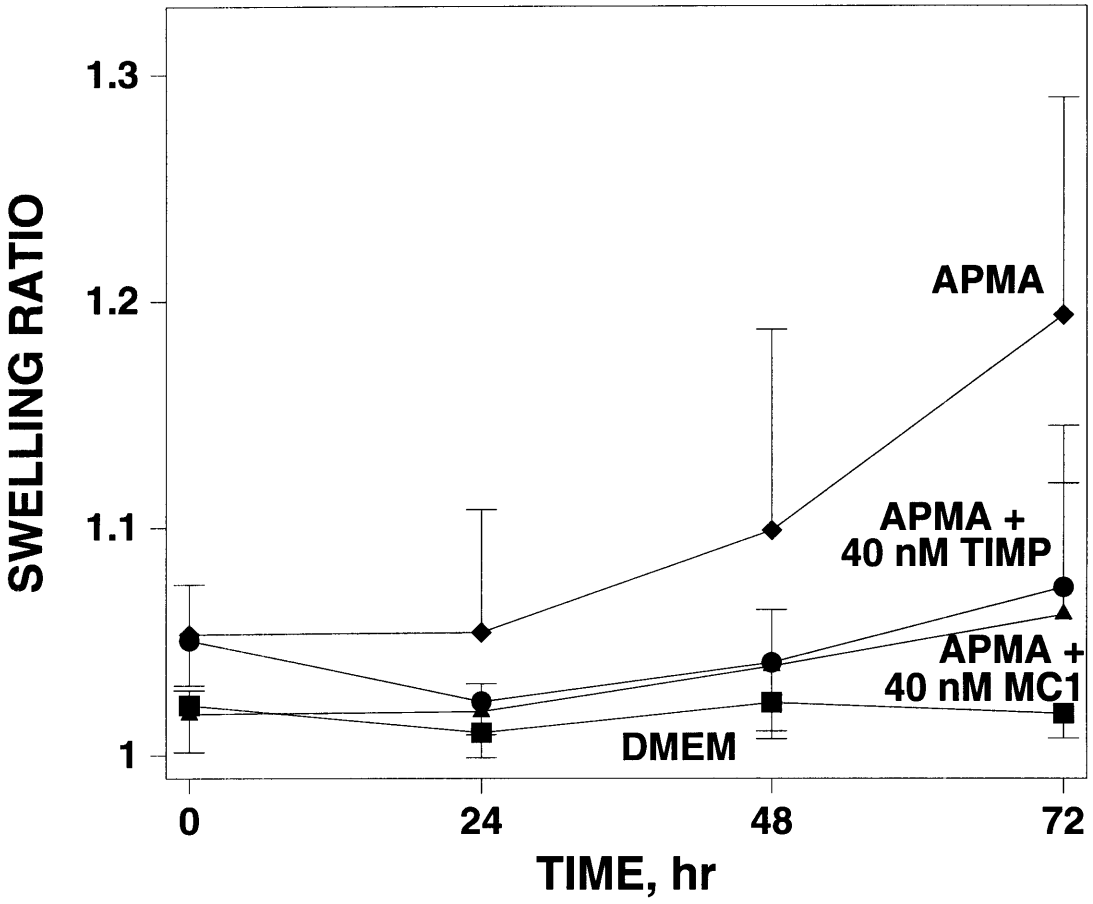

Figure 4.4: Swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) of cartilage disks cultured in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA + 40 nM TIMP, or DMEM with 1 mM APMA + 40 nM MC1. Data are mean ± SD (n=4).

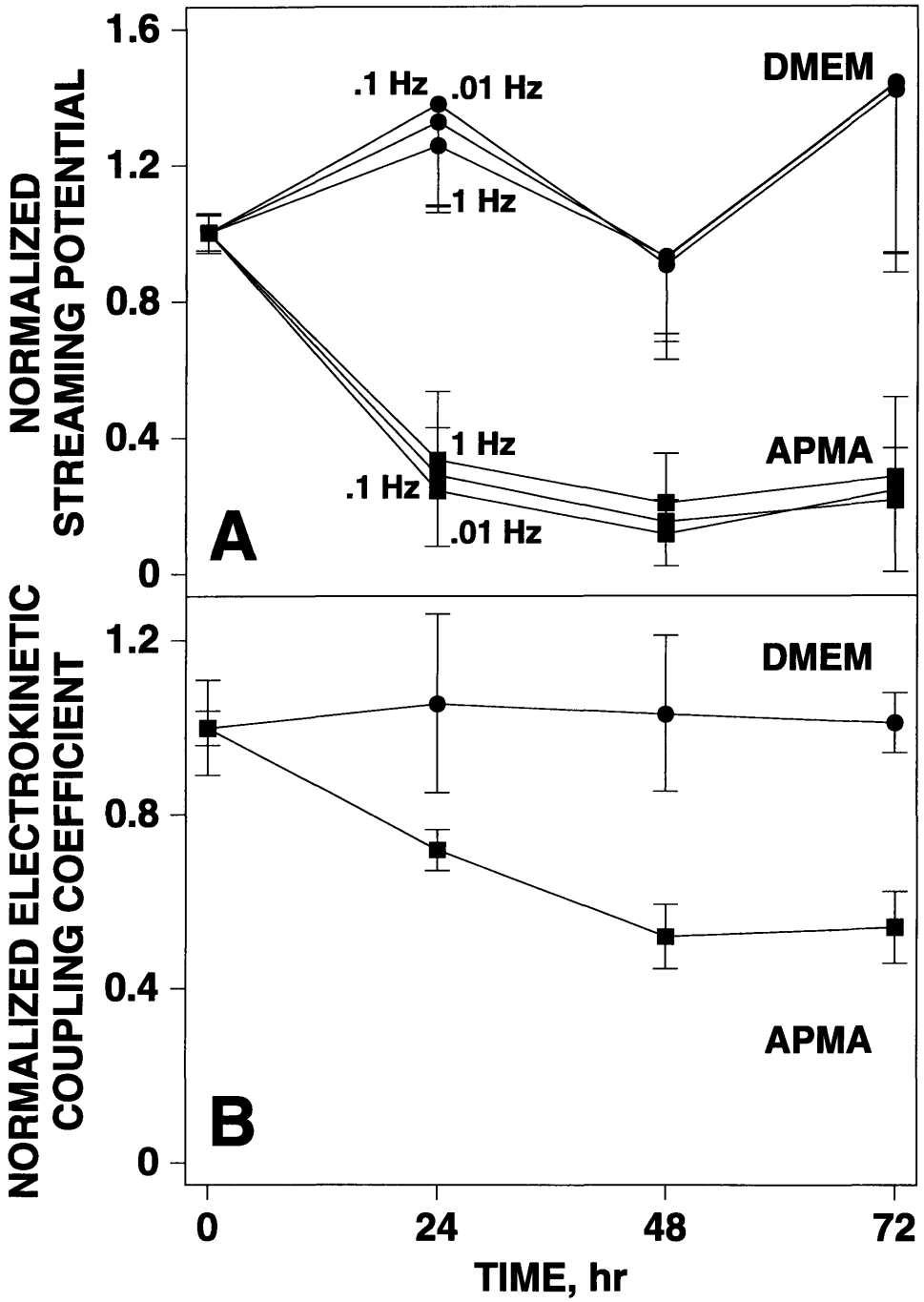

Figure 4.5: **A:** Streaming potential of cartilage disks of cultured in DMEM alone or DMEM with 1 mM APMA as a function of time in culture. Data are mean ± SD (n = 4); mean values are normalized at each frequency to the value at t = 0. **B:** Electrokinetic coupling coefficient of disks of cultured in DMEM alone or DMEM with 1 mM APMA as a function of time in culture. Data are mean ± SD (n = 4); mean values are normalized to the value at t = 0.

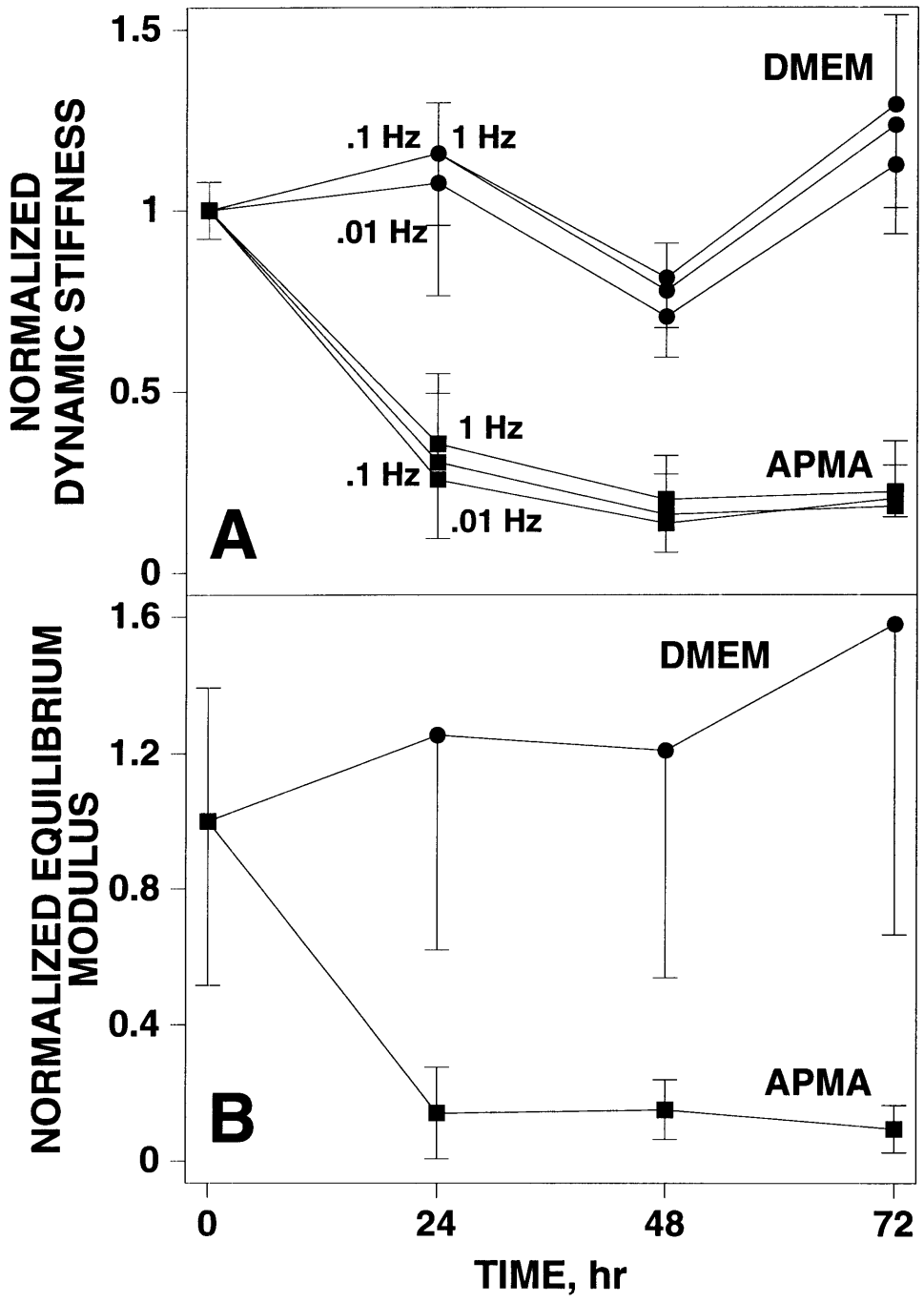

Figure 4.6: **A:** Dynamic stiffness of cartilage disks of cultured in DMEM alone or DMEM with 1 mM APMA as a function of time in culture. Data are mean ± SD (n = 4); mean values are normalized at each frequency to the value at t = 0. **B:** Equilibrium modulus of disks of cultured in DMEM alone or DMEM with 1 mM APMA as a function of time in culture. Data are mean ± SD (n = 4); mean values are normalized to the value at t = 0.

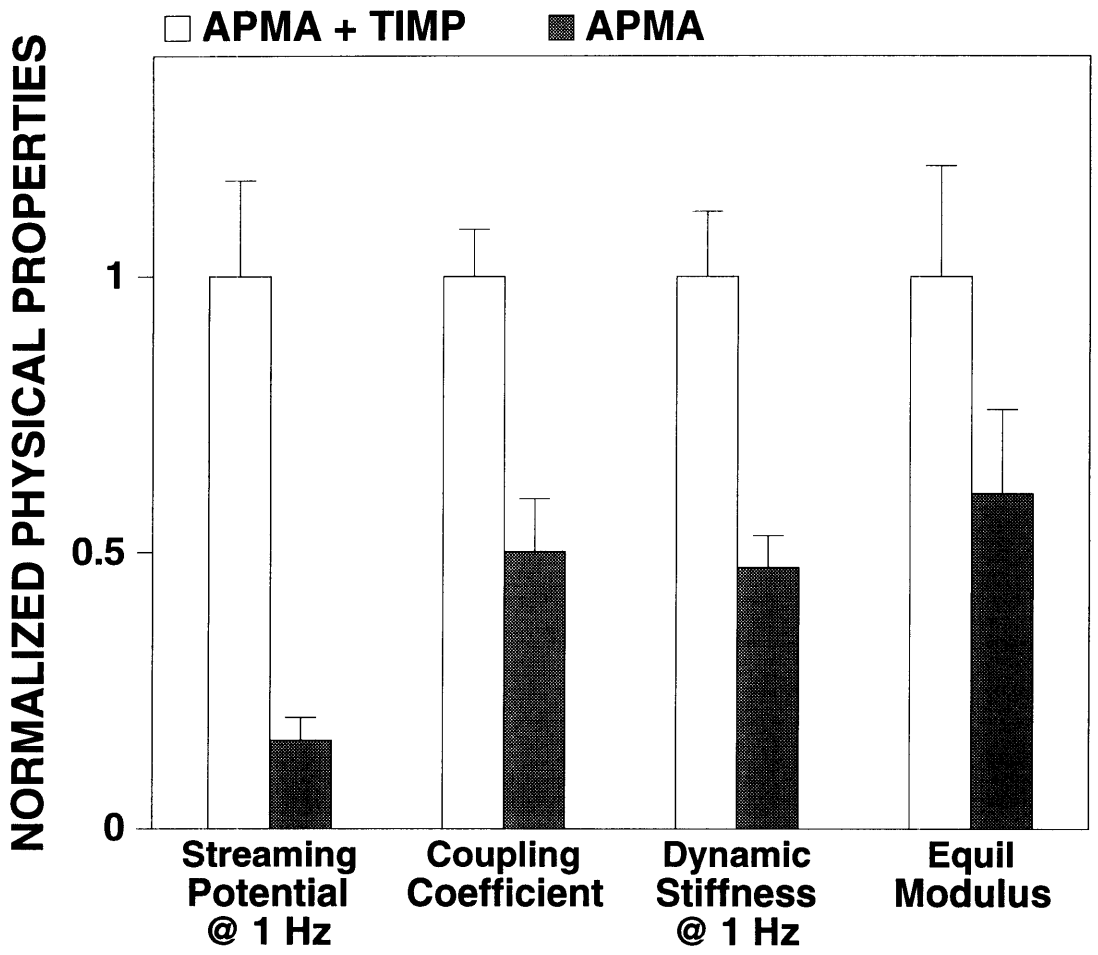

Figure 4.7: Streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, and equilibrium modulus of cartilage disks cultured in the presence of 1 mM APMA or 1 mM APMA + 4 μM TIMP for 72 hours. Data are mean $\pm$ SD (n = 4); mean values for each property were normalized to the mean value of disks which received treatment with 1 mM APMA + 4 μM TIMP.

in which lower doses of TIMP (~50 nM) were unable to inhibit PG loss induced by IL-1α treatment, presumably due to transport restrictions [1]. The fact that addition of TIMP to the culture media was not as effective at inhibiting degradation as MC1 is interesting, in that in studies on digestion of peptide substrates in solution TIMP is a far more potent inhibitor of MMP than MC1 (k_i of TIMP against stromelysin: <1 nM [70] vs. k_i of MC1 against stromelysin: ~300 nM [22]). In the present study, however, there is the added complication that any inhibitor present in the culture media must penetrate into the tissue to inhibit the endogenous MMP activated by APMA. Since TIMP is quite large (~25 kDa), it is likely that its transport through the cartilage matrix is hindered. Based on the partitioning of similarly sized proteins into cartilage [79], the amount of TIMP which penetrates into the tissue may be as little as 1% of that which is added to the culture media; thus, a 4 μM culture media concentration could result in an intratissue concentration as small as ~40 nM. This is in contrast to MC1, which is quite small (~500 Da) and consequently not hindered in its movement into the tissue. Thus, characteristics of transport through the cartilage matrix seem to have an important impact on the overall efficacy of treatment with MMP inhibitors.

The results of the ELISA for presence of the α1(II)-CB11B peptide sequence revealed that APMA treatment caused significant damage to the collagen network as well as damage to PG network. The appearance of this epitope indicates that type II collagen is in an unwound or denatured state. The presence of APMA in the culture media for 3 days resulted in denaturing of ~7% of the type II collagen in cartilage explants, compared to ~2% in control tissue. Hollander *et al* showed that exposure of type II collagen to either bacterial or recombinant human collagenase resulted in collagen denaturation and exposure of the α1(II)-CB11B epitope. It seems likely that the type II collagen denaturation seen here is the result of collagenase activity, given that exposure of explants to active stromelysin did not increase the presence of the α1(II)-CB11B epitope. This is inherently interesting in that active stromelysin has been shown to activate procollagenase [119,120] in solution, yet there is no evidence of this occurring in these cartilage explants (Fig 4.3).

4.5.2 Swelling Studies

The damage to matrix collagens in consistent with the increased swelling response of cartilage disks treated with APMA (Fig 4.4). As described by Maroudas [78], the swelling state of cartilage is determined by a balance between the osmotic pressure of the proteoglycans which tends to swell the tissue and the restraining tensile forces of the collagen network, which tend to limit tissue swelling. If the collagen network is compromised, as in OA [78], it is less able to counteract the osmotic swelling forces, and the tissue swells. The unwinding of type II collagen fibrils caused by APMA treatment weakens the cartilage matrix, making it less able to counteract osmotic swelling pressure. Previously it was shown that stromelysin treatment resulted in an increased swelling response, presumably due to damage to type IX collagen [8]. Given that APMA is likely activating prostromelysin as well as procollagenase, it is possible that type IX collagen has also been degraded, further contributing to the swelling response.

Addition of 40 nM TIMP or MC1 to the culture with APMA resulted in a swelling response which was indistinguishable from controls. Presumably, this indicates that the inhibitors are preventing the damage to the collagen network which causes the swelling response. It is interesting that at 40 nM, TIMP and MC1 did not inhibit GAG loss completely, but appeared to prevent collagen damage. This is consistent with studies of IL-1α degradation of cartilage, which have shown that collagen damage, as indicated by hydroxyproline release, could be completely inhibited by doses TIMP or synthetic inhibitors which did not completely prevent PG degradation [1,29]. It is possible that this is due to restricted transport of enzymes through the cartilage matrix. Degradation can only take place when collagenases have access to tissue collagens. With the PG network intact, collagenase transport is restricted. Thus, it may be necessary for the PG to be cleared out before the collagen network is significantly damaged.

4.5.3 Biomechanical and Electromechanical Evaluation

The large decreases in electromechanical properties of the tissue are consistent with severe degradation of matrix PG (Fig 4.5). As in previous studies, the magnitude and kinetics of streaming potential are similar to changes in tissue GAG content [8,41], since loss of GAG reduces tissue fixed charge density, thus lowering the charge separation which occurs due to fluid flow. The changes in streaming potential with time were similar at all frequencies tested. This is in contrast to previous studies of stromelysin degradation of cartilage [8], where the decrease in streaming potential was highly frequency dependent. In such studies, the degradation of cartilage explants was initially localized at the tissue surface, due to the restriction of transport of the large enzyme into the tissue. APMA (~500 Da), however, is much smaller than stromelysin (~50 kDa) and can activate MMP throughout the tissue, causing a more spatially uniform degradation of this tissue. As a result, at any time during the course of the experiment the fixed charge density of degraded samples is similar through all sections of the tissue and relative changes in streaming potential are similar at all frequencies.

The electrokinetic coupling coefficient is a material property which reflects the amount of potential developed due a given stress. Thus, while the streaming potential decreases (as described above), the stress developed in the tissue, reflected in the dynamic stiffness (Fig 4.6A), also decreases. As a result, there is a net decrease in the coupling coefficient (~50%) but it is not as dramatic as the changes in streaming potential.

Changes in dynamic stiffness and equilibrium modulus of cartilage disks are due to degradation of both PG and collagen matrix constituents (Fig 4.6). Electrostatic interactions between charge groups on GAG chains serve to stiffen cartilage in compression. Previous studies have shown that neutralization or shielding of proteoglycan fixed charge groups (by pH or ionic strength alteration) or removal of charge bearing GAG chains by enzymatic treatment could decrease the modulus or dynamic stiffness of cartilage disks by approximately 50% [28,41,42]. Given that the dynamic stiffness and equilibrium modulus decrease to ~20% of their initial value and that swelling studies and ELISA revealed

damage to the collagen network, it seems likely that collagen damage contributed to the changes in mechanical properties as well.

As detailed previously, addition of 4 μM TIMP to the culture media effectively inhibited the degradation of cartilage matrix components induced by APMA, and this is reflected in the study of physical properties as well (Fig 4.7). After three days in culture, cartilage disks cultured with APMA but not TIMP had values for coupling coefficient, dynamic stiffness, and equilibrium modulus which were ~50% and streaming potential which were ~20% of those which received treatment with TIMP as well as APMA. This is consistent with previous studies [8,28,41,42,65] which relate enzymatic cartilage matrix with changes in physical properties and indicates that it is possible to prevent these changes by inhibiting the enzymatic degradation.

Chapter V

Inhibition of Cartilage Degradation and Changes in Physical Properties Induced by IL-1β, Retinoic Acid, and APMA.

5.1 Abstract

Bovine cartilage explants were treated with 1 mM 4-aminophenylmercuric acetate (APMA), 100 ng/ml recombinant human interleukin-1β (IL-1β) or 1 μM all-trans retinoic acid (RA) and changes in biochemical, biomechanical, and physicochemical properties were assessed. Additionally, samples cultured with APMA, IL-1β, or RA were treated with 4 μM recombinant human tissue inhibitor of metalloproteinases-1 (TIMP) to inhibit this degradation. Cartilage explants treated with APMA lost 60% of tissue GAG after 3 days in culture, while treatment with IL-1β or RA each resulted in >90% GAG loss after 8 days in culture. IL-1β induced a significant increase in swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) after 8 days in culture, while swelling behavior of RA treated tissue was indistinguishable from controls at all time points. Addition of TIMP to the culture media inhibited loss of tissue GAG by >95%, 40%, and 35% respectively from samples treated with APMA, IL-1β, or RA. Measurements of equilibrium modulus and streaming potential of tissue treated with APMA plus TIMP were significantly higher than APMA samples which were not exposed to TIMP. Streaming potential and equilibrium modulus of IL-1β treated samples both decreased to 10% of their initial values after 8 days in culture, while those treated with IL-1β and TIMP showed decreased by only 50% and 10% respectively. Measurement of dynamic streaming potential showed that changes due to treatment with IL-1β alone were strongly dependent in compression frequency, with dramatic changes seen at high frequency prior to changes in mechanical properties, and little initial change seen at low frequency. Streaming potential and equilibrium modulus of RA treated samples

both decreased to 10% of their initial values after 8 days in culture, while those treated with RA and TIMP showed decreased by only 60% and 10% respectively.

5.2 Introduction

Both interleukin-1 and retinoic acid have been commonly used to induce cell-mediated degradation of cartilage explants. Interleukin-1 (IL-1) is an 18 kD cytokine produced by several cell types including endothelial cell, synoviocytes and chondrocytes [114]. Although typically associated with inflammatory diseases such as rheumatoid arthritis [24,82], both the α and β forms of IL-1 have been shown to be relevant to degradation in osteoarthritis as well [94,101]. IL-1 interacts with chondrocytes by means of a receptor on the cell surface [21]. Stimulation of cartilage explants with IL-1 has been shown to result in widespread matrix degradation, including loss of tissue PGs [4,16,61] and collagens [16]. IL-1 treatment directly influences cell metabolism, decreasing synthesis of PGs [4,16,85,121] and collagens [46,47,122] while decreasing chondrocyte proliferation [43]. Additionally, IL-1 induces production and mRNA expression of MMPs in chondrocytes [63,64], while modulating production of TIMP [114]. Although tissue treated with IL-1 contains an excess of MMPs, the PG degradation products which result are not those typically associated with MMP cleavage [108]. These fragments, however, are similar to those found in human OA patients [111]. Despite this apparent discrepancy, MMP inhibitors have been shown to be effective at inhibiting cartilage matrix degradation induced by IL-1 [16,87,113].

The vitamin A derivative all-trans retinoic acid (RA) has also been shown to induce widespread matrix degradation in cartilage explants. [16,86]. RA stimulation involves interaction with a nuclear receptor, directly altering DNA transcription [93]. On the tissue levels, RA treatment results in loss of tissue PGs and collagens [16,17,86] decreased synthesis of PG [86] and collagens [26,136] by chondrocytes. RA has also been shown to induce expression and production of MMPs [16], although degradation products from such systems are not those typically associated with MMP activity (JD Sandy, unpublished results). This matrix degradation inhibited in the presence of MMP inhibitors, but not in

the presence of inhibitors of cathepsin B [16].

While the degradative action of IL-1 and RA have been well documented, the effects of this degradation on the physical properties of cartilage explants have not been characterized. In addition, the use of synthetic MMP inhibitors to prevent degradation induced by IL-1 or RA has been demonstrated [16,87,113], while the inhibition of PG loss by TIMP in these systems has not been documented. The cartilage degradation induced by the presence of APMA provides a valuable comparison for these systems, given that the degradation in the APMA system is known to be caused by the action of MMPs [10] and chondrocytes are known to be metabolically inactive. Therefore, the objectives of this study were (1) to quantify the effects of IL-1β and RA treatment on the physical properties and proteoglycan loss of cartilage explants, (2) to characterize the modulation of this degradation by tissue inhibitor of metalloproteinases (TIMP) and (3) to compare these results to the effects of activation of endogenous MMPs by APMA and inhibition of this degradation by TIMP.

5.3 Methods

5.3.1 In Vitro Culture

Saddle sections of 1-2 week old calves were obtained from a local abbatoir (A. Arena, Hopkington, MA) within four hours of slaughter. The femoropatellar groove was isolated and 3 mm diameter $\times$ 1 mm thick cartilage disks were harvested using a dermal punch (Miltex Instruments, Lake Success, NY) and a sledge microtome (Model 860, American Optical, Buffalo, NY) as described previously [105]. Groups of 4 plugs were maintained in culture at 37 °C in a 5% CO_2 atmosphere for up to 8 days in 1 ml DMEM containing one of the following treatments: (a) 1 mM 4-aminophenylmercuric acetate (APMA) (Sigma, St. Louis, MO), (b) 100 ng/ml recombinant human IL-1β, or (c) 1 μM retinoic acid (RA) (Sigma) in 0.1% dimethylsulfoxide (Sigma) in the presence or absence of 4 μM recombinant human TIMP-1. Media for IL-1β or RA experiments also contained 0.1% bovine serum

albumin (Sigma) and was changed every two days.

The protocol for swelling studies was motivated by the technique used by Maroudas [78] to compare the swelling of normal and cartilage. Upon removal from culture disks were removed from culture medium and patted briefly with a paper towel to remove surface water, and wet weights were measured (AE 163 Balance, Mettler Instrument Corp, Hightstown, NJ). Disks were then twice reequilibrated in 1 ml of hypotonic saline solution (0.01 M NaCl at pH 7.0) for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded. Previously, it has been shown that this method is sensitive to damage to the cartilage matrix collagens, but is not sensitive to changes in tissue proteoglycan content [8]. After swelling measurements, lyophilized samples were digested with 1 ml of 125 μg/ml papain digestion solution (Sigma) and assayed for sulfated GAG by reaction with 2 ml of dimethylmethylene blue dye solution in polystyrene cuvets (VWR, Boston, MA) and spectrophotometry (Model λ3B, Perkin Elmer, Norwalk, CT) [34] using whale/shark chondroitin sulfate (Sigma) as the standard.

5.3.2 Physical Properties

Cartilage disks were placed in an electrically insulating poly(methylmethacrylate) cylindrical confining chamber, similar to that used by Frank *et al* [41]. The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA) which was interfaced to a computer and frequency generator (Model 5100, Rockland Systems, West Nyack, NY). Samples were equilibrated in 0.15 M phosphate buffered saline (PBS)(Gibco) at pH 7.4 containing 100 U/ml penicillin G and 100 μg/ml streptomycin at room temperature. Disks were subjected to confined compression between a porous polyethylene platen and a 6.35 mm Ag/AgCl pellet electrode (Annex Research, Costa Mesa, CA) mounted at the base of the chamber. An identical electrode was mounted in the surrounding PBS bath.

Disks were first compressed by sequential increments of 0.5-1.5% strain, up to a maximum of 20% total strain. After stress relaxation, the equilibrium stress correspond-

ing to each increment of static strain was detected with the load cell of the Dynastat and recorded on the computer. The resultant equilibrium stress was plotted against applied strain and the slope of the best fit linear regression curve gave the equilibrium confined compression modulus. At a given static offset strain, sinusoidal strains of $<1\%$ amplitude were superimposed on the static strain at frequencies ranging from 0.01 Hz to 1 Hz. The resultant oscillatory load was detected by the load cell and the oscillatory streaming potential was detected by the chamber electrodes which were connected to a high-impedance Universal Amplifier, Model 11-4113-02 (Gould, Inc., Cleveland, OH). Load and streaming potential were simultaneously recorded by the computer. The amplitude of the dynamic load was normalized to disk area and to the amplitude of the applied strain to give dynamic stiffness, while the amplitude of the streaming potential was normalized to the amplitude of the applied strain [39,40]. Equilibrium modulus and dynamic stiffness data were used in combination with the method of Frank and Grodzinsky [40] to calculate the effective hydraulic permeability of the tissue specimen. In all tests the amplitude of the applied strain was chosen to be small enough to elicit a linear mechanical response from the sample [39,40] (i. e. the total harmonic distortion in the measured load was $<10\%$). The porous platen remained in contact with the sample at all times during the testing cycle.

5.4 Results

5.4.1 In Vitro Culture

Incubation of cartilage explants in the presence of 1 mM APMA resulted in $\sim 75\%$ loss of tissue GAG after three days in culture, which was completely inhibited by the presence of 4 μM TIMP in culture media (Fig 5.1). Treatment with 100 ng/ml IL-1β resulted in $>90\%$ loss of tissue GAG after 6 days in culture, compared to $\sim 60\%$ loss from samples exposed to 4 μM TIMP and 100 ng/ml IL-1β, and $\sim 15\%$ loss in controls samples incubated in DMEM alone (Fig 5.2A). Additionally, samples treated with 100 ng/ml IL-1β had a swelling ratio of 1.07 after 8 days in culture, compared to 1.03 for samples treated with 1 μM RA and

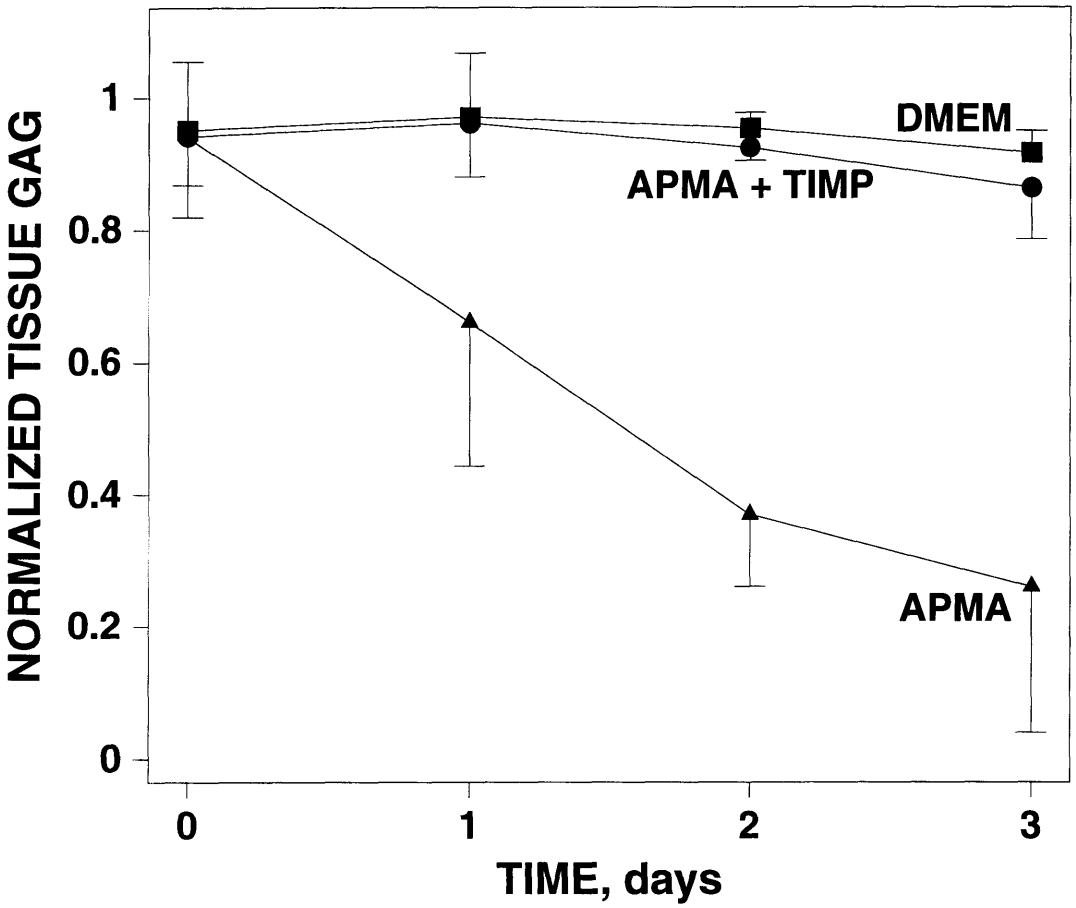

Figure 5.1: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA plus 4 μM TIMP. Data are plotted as mean $\pm$ SD (n=4).

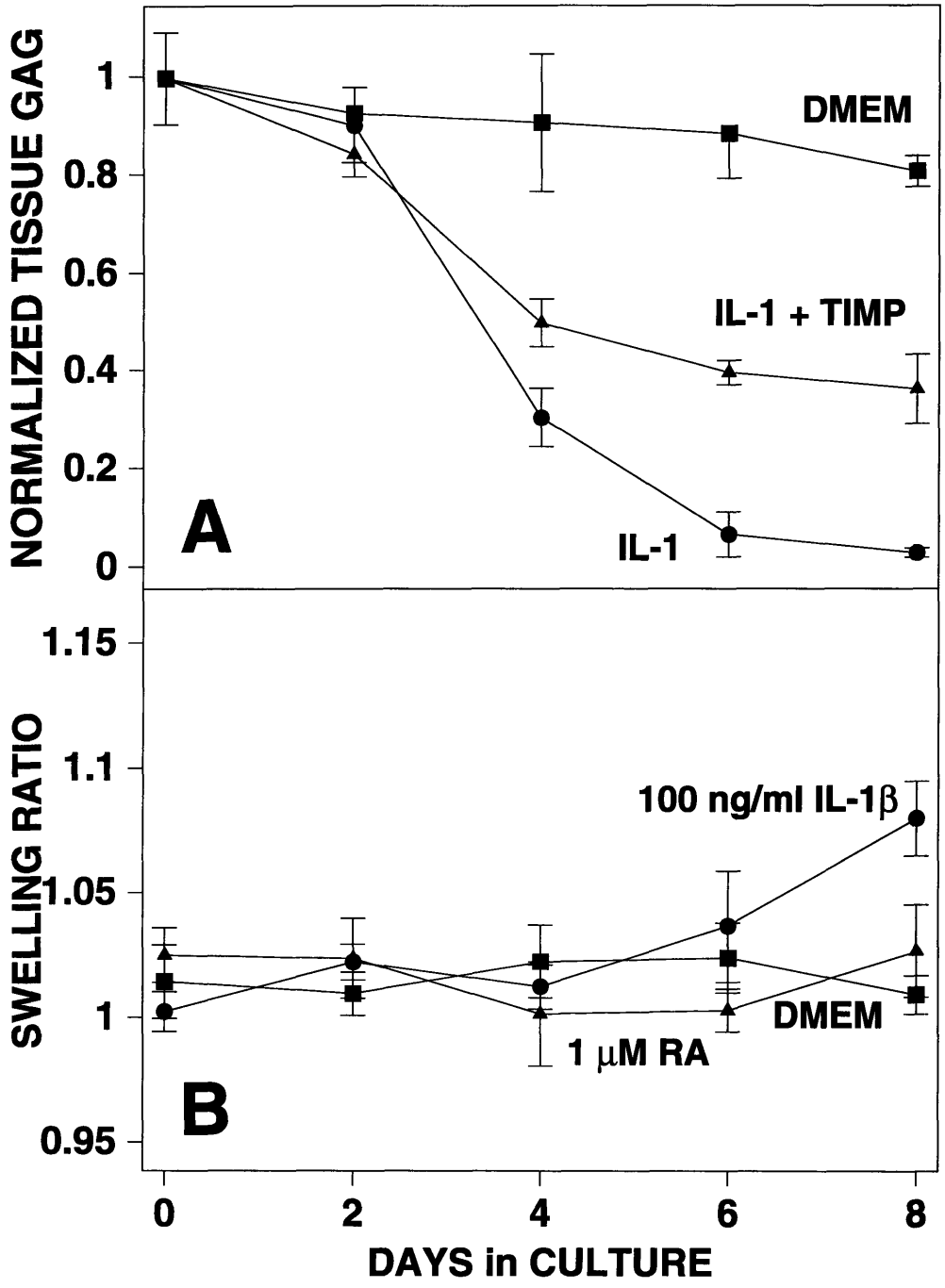

Figure 5.2: **A:** Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 100 ng/ml IL-1β, DMEM with 100 ng/ml IL-1β plus 4 μM TIMP **B:** Swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) of cartilage disks incubated in DMEM alone, DMEM with 100 ng/ml IL-1β, or DMEM with 1 μM retinoic acid. Data are plotted as mean $\pm$ SD (n=4).

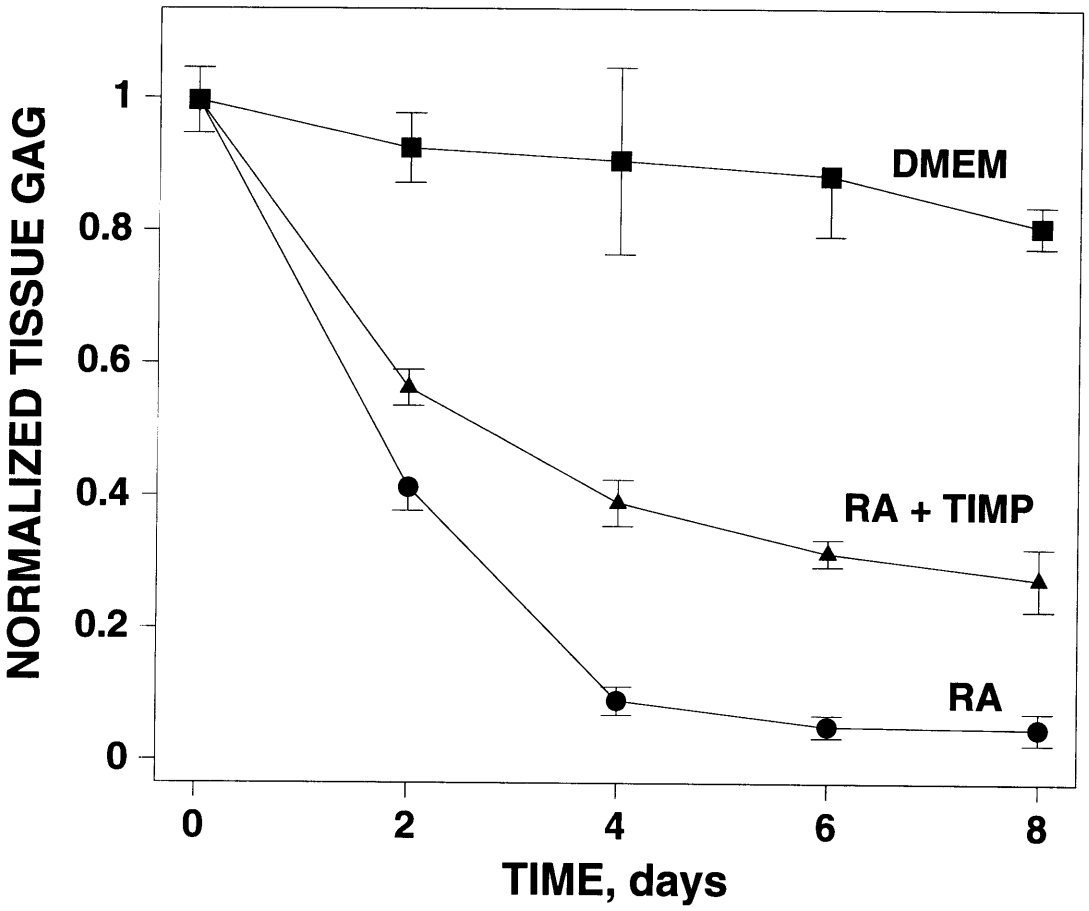

Figure 5.3: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 μM RA, DMEM with 1 μM RA plus 4 μM TIMP. Data are plotted as mean $\pm$ SD (n=4).

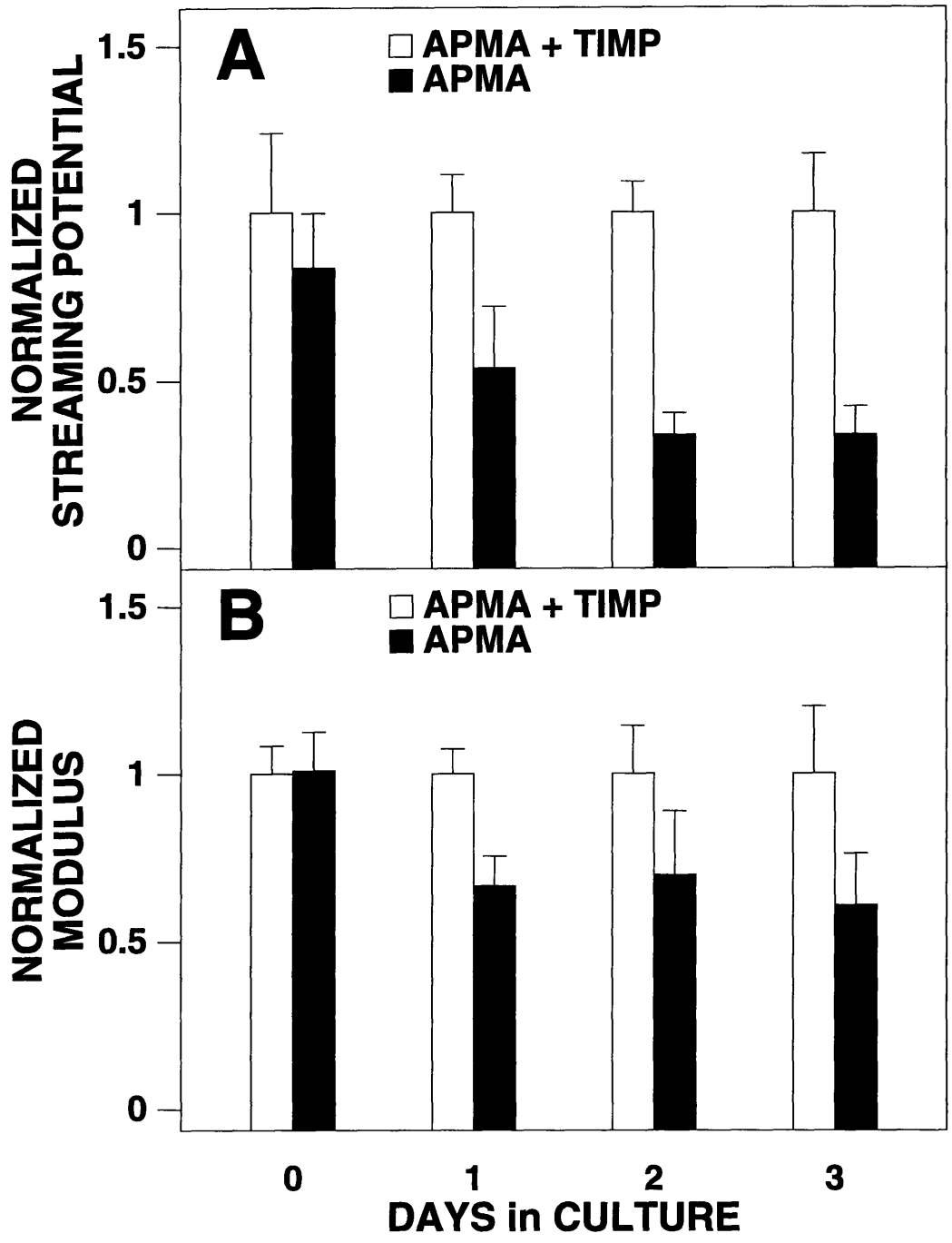

Figure 5.4: **A:** Time course of streaming potential for cartilage disks incubated in DMEM with 1 mM APMA or DMEM with 1 mM APMA plus 4 μM TIMP. Data are normalized to values of disks treated with APMA plus TIMP at each time point. **B:** Time course of equilibrium modulus for cartilage disks incubated in DMEM with 1 mM APMA or DMEM with 1 mM APMA plus 4 μM TIMP. Data are normalized to values of disks treated with APMA plus TIMP at each time point. Data are plotted as mean $\pm$ SD (n=4).

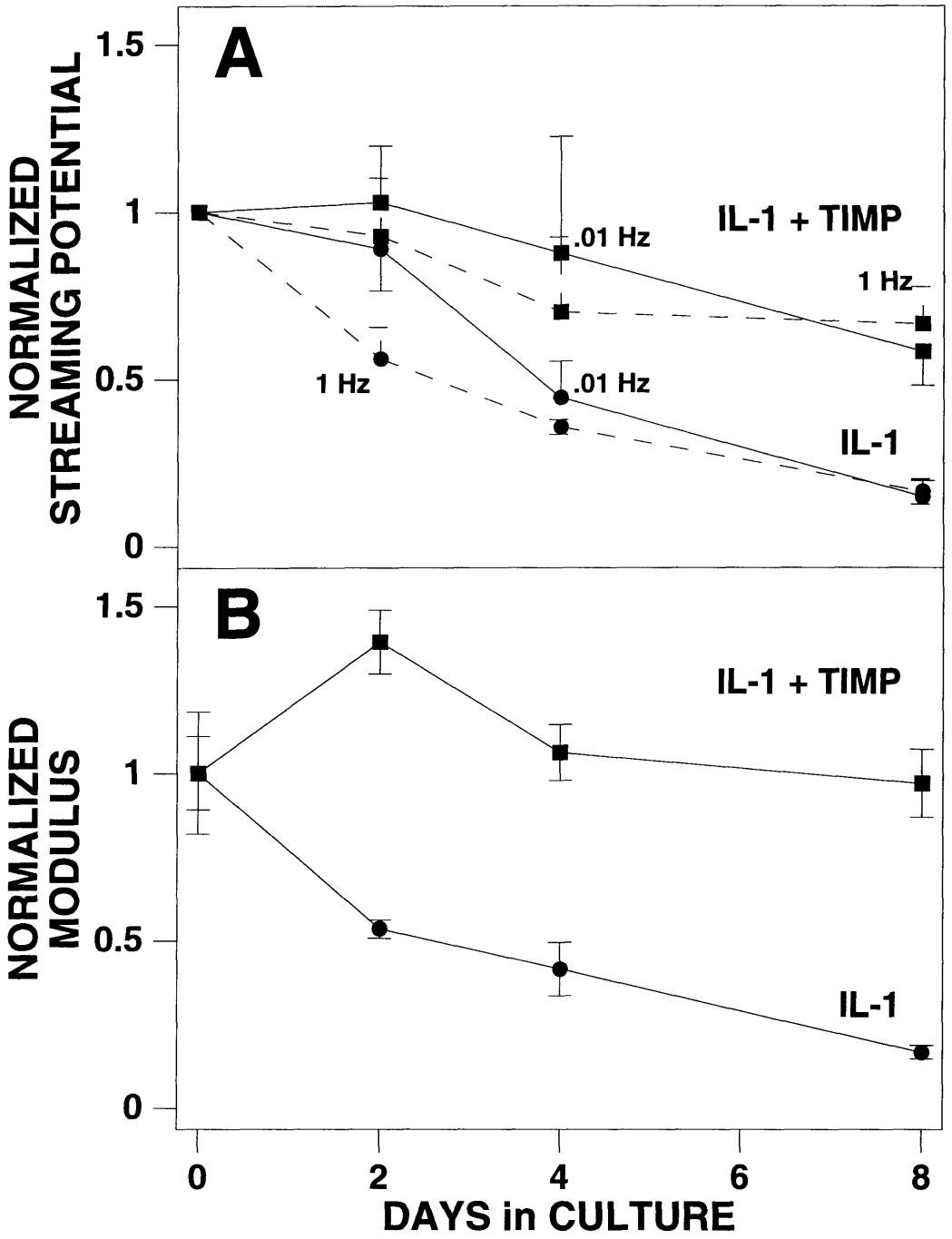

Figure 5.5: **A:** Time course of streaming potential for cartilage disks incubated in DMEM with 100 ng/ml IL-1β, DMEM with 100 ng/ml IL-1β 4 μM TIMP. Data for each treatment group are normalized to values of disks on day 0. **B:** Time course of equilibrium modulus for cartilage disks incubated in DMEM with 100 ng/ml IL-1β, DMEM with 100 ng/ml IL-1β 4 μM TIMP. Data for each treatment group are normalized to values of disks on day 0. Data are plotted as mean $\pm$ SD (n=4).

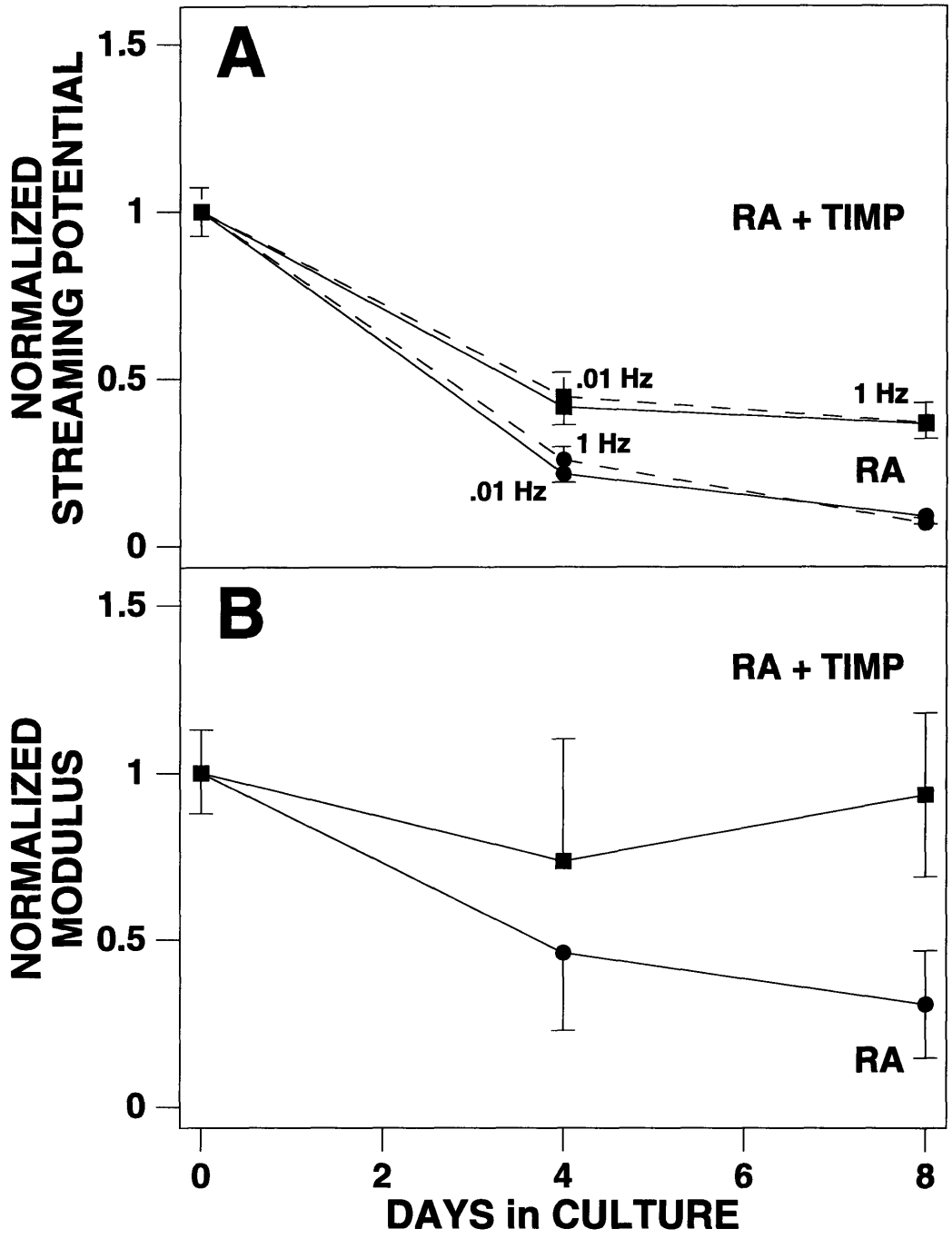

Figure 5.6: **A:** Time course of streaming potential for cartilage disks incubated in DMEM with 1 μM RA, DMEM with 1 μM RA plus 4 μM TIMP. Data for each treatment group are normalized to values of disks on day 0. **B:** Time course of equilibrium modulus for cartilage disks incubated in DMEM with 1 μM RA, DMEM with 1 μM RA plus 4 μM TIMP. Data for each treatment group are normalized to values of disks on day 0. Data are plotted as mean $\pm$ SD (n=4).

1.02 for controls (Fig 5.2.B). RA treatment resulted in >90% loss in tissue GAG after 8 days in culture, compared to ~15% for control samples, while tissue treated with 1 μM RA plus 4 μM TIMP lost ~70% of tissue GAG over this time period (Fig 5.3).

5.4.2 Physical Properties

The streaming potential and equilibrium modulus of tissue treated with 1 mM APMA or 1 mM APMA + 4 μM TIMP was normalized to the values of the samples treated with APMA plus TIMP at each time point (Fig 5.4). Values for streaming potential ranged from 65.1 ± 13.1 μV/% at 0.01 Hz to 392 ± 37.1 μV/% at 1 Hz, and the equilibrium modulus was measured to be 1.19 ± 0.22 MPa at the start of the culture. The streaming potential at 1 and 0.01 Hz and the equilibrium modulus of APMA treated samples decreased to <10% and ~60%, respectively, of those of samples treated with APMA plus TIMP after 3 days in culture, and both differed significantly from these samples after 1 day in culture.

Treatment with IL-1β resulted in decreases in streaming potential and modulus to ~15% of initial values after 8 days in culture (Fig 5.5). Streaming potential at 1 Hz decreased by ~50% after only 2 days in treatment, while streaming potential at 0.01 Hz decreased more slowly, showing only a 10% decrease after 2 days in culture. Addition of TIMP to the culture media inhibited the degradation of proteoglycans and the reduction in physical properties. Loss of streaming potential at 1 and 0.01 Hz was inhibited by 40% and loss in modulus by 100% compared to controls after 8 days in culture. Similarly, treatment with RA resulted in a decrease in streaming potential at all frequencies and in equilibrium modulus to <10% and ~30% of initial values after 8 days of treatment (Fig 5.6). With TIMP added to the RA culture media, the losses in streaming potential at all frequencies and modulus were inhibited by 40% and 90% respectively, compared to controls.

5.5 Discussion

In all experiments, the addition of TIMP to the culture media significantly inhibited degradation of cartilage proteoglycans and changes in physical properties. Inhibition of GAG loss

induced by exposure to APMA is consistent with previous studies which have demonstrated that matrix degradation is due to the activation of native latent MMPs [9]. N-terminal analysis has shown that proteoglycan fragments lost to media after incubation with APMA are predominantly due to cleavage at the VDIPEN-FFGVG site associated with MMP activity [109]. Given that MMPs appear to be the only cause of degradation in this system, the inhibition of MMPs by TIMP prevents matrix degradation (Fig 5.1) and prevents changes in tissue physical properties (Fig 5.4).

The time course of loss of GAG from samples treated with IL-1β or RA was similar to previous studies [16,87,113]. IL-1β treatment resulted in negligible GAG loss after two days in culture, followed by loss of >90% of tissue GAG in the four days which followed. This two day lag in loss of GAG may be a result of the time necessary for IL-1β to diffuse through the cartilage matrix and affect chondrocyte metabolism. In contrast, response to RA treatment was more rapid, with >60% loss of GAG after only two days in culture, consistent with the fact that RA is much smaller than IL-1β and would likely diffuse more easily through the cartilage matrix.

In addition, a swelling response was induced by treatment with IL-1β, but not RA (Fig 5.2B). This may indicate that the mechanisms for degradation of matrix collagens initiated by IL-1β and RA differ significantly. This swelling data is particularly interesting in that RA appeared to degrade matrix proteoglycans more quickly than IL-1β, but did not appear to have as much of an effect on matrix collagens as IL-1β.

The addition of 4 μM TIMP to the culture media significantly inhibited GAG loss induced by exposure to IL-1β (Fig 5.2) or RA (Fig 5.3). The inhibition in the IL-1β and RA systems reveals that portions of the associated degradative pathways were at least partially blocked, suggesting that MMPs play a role in the mechanisms of degradation. Given that both IL-1β and RA are known to upregulate MMP production [15,64] as well as downregulate production of native MMP inhibitors [114], it is possible that the amount of TIMP which was sufficient to inhibit the endogenous MMPs activated by APMA is not sufficient to inhibit the additional MMPs produced in the tissue due to stimulation by

100 ng/ml IL-1β or 1 μM RA. It is also possible that the TIMP added to the culture media has inhibited all MMPs in the system and that the remaining degradation has occurred through pathways which are independent of MMPs.

On a more basic level, these results also indicate that TIMP is indeed able to penetrate the dense cartilage matrix when added to the culture media. This is in contrast to previous studies in which TIMP treatment did not alter GAG loss from tissue treated with IL-1α [1], perhaps due to insufficient *intratissue* TIMP concentration. Based on the partitioning of similarly sized proteins into cartilage [79], the amount of TIMP which penetrates into the tissue may be as little as 1% of that which is added to the culture media; thus, a 4 μM culture media concentration could result in an intratissue concentration as small as ~40 nM. It is therefore necessary to have a concentration of TIMP in the culture media which is far in excess of MMP levels expected inside the tissue, since only a small fraction will penetrate into the tissue.

The more rapid loss of high frequency streaming potential at early time points in IL-1β treated samples is similar to that seen for samples treated with activated stromelysin [8], and suggests that at early times, degradation is localized to regions near the tissue surface. It is also of interest that the equilibrium modulus did not decrease as much or as quickly as the streaming potential in either the IL-1β or RA systems, and was more easily preserved by the addition of TIMP to the culture media. This is consistent with previous observations that streaming potential is more sensitive to loss of proteoglycans than measurements such as equilibrium modulus [39], which reflect the contributions of other matrix components such as collagen, which may be degraded more slowly than proteoglycans [1].

Chapter VI

Summary and Future Work

6.1 Summary

6.1.1 Stromelysin In Vitro Model

On the most basic level, *in vitro* studies revealed that activated stromelysin is capable of degrading several types of cartilage matrix molecules in intact tissue. Additionally, levels of stromelysin which are thought to be physiologic [74], caused matrix degradation on a short time scale, with ~40% loss of glycosaminoglycan (GAG) content after 72 hours at a concentration of 200 nM. N-terminal sequencing of degradation products confirmed that SLN was responsible for proteoglycan cleavage. Further analysis showed the presence of elevated levels of type IX collagen fragments within treated explants and in the incubation media. Histological examination demonstrated that proteoglycan loss proceeded inward from the exposed tissue surfaces, with a marked boundary between degraded and undegraded tissue. Physicochemical studies revealed that stromelysin treatment resulted in tissue swelling, while enzymes known to remove proteoglycans, but not affect matrix collagens, did not. Stromelysin treated tissue exhibited marked decreases in equilibrium modulus and dynamic stiffness and a substantial increase in hydraulic permeability by 72 hours, with similar kinetics to the loss of GAG. The significant decreases in equilibrium modulus and dynamic stiffness, together with swelling and the loss of type IX collagen fragments suggest that the structural integrity of the collagen network had been compromised. Measurement of dynamic streaming potential showed that changes due to stromelysin treatment were strongly dependent in compression frequency, with dramatic changes seen at high frequency prior to changes in mechanical properties, and little initial change seen at low frequency. Streaming potential, an electrokinetic phenomenon, was therefore a more sensitive indicator of initial, focal loss of GAG from the tissue due to stromelysin treatment than purely mechanical measurements such as dynamic stiffness.

6.1.2 Stromelysin In Vivo Model

Results from experiments involving intraarticular injection of stromelysin into rabbit stifle joints are consistent with data from studies of stromelysin degradation of cartilage explants *in vitro*. After one hour of exposure to stromelysin, significant amount of proteoglycan was lost to synovial fluid. Histology indicated that during this short time exposure, loss of PGs from joint cartilage occurred in a small region near the tissue surface. Toluidine Blue staining for GAG and immunoperoxidase staining of VDIPEN antibodies showed that the loss of GAG in these experiments was due to stromelysin activity. Assays for PG content of full thickness disks removed from the femoropatellar groove showed that tissue samples from treated and untreated joints had similar amounts of GAG, indicating that depth of penetration of matrix degradation was relatively small. With the bulk of the matrix intact, mechanical measurements of dynamic stiffness and equilibrium modulus were similar in samples of cartilage from treated and control joints. High frequency streaming potential measurements of cartilage disks from treated joints were significantly less than those from control joints, demonstrating the sensitivity of such measurements to focal changes in GAG content. Systemic administration of a MMP inhibitor (MC1) prior in intraarticular injection of stromelysin significantly modulated all indicators of cartilage degradation. Loss of PG fragments were significantly inhibited, appearance of VDIPEN epitope and loss of Toluidine Blue staining for GAG was significantly decreased, and all physical property measurements, including high frequency streaming potential, were similar between treated and control joints for animals which received an intravenous dose of MC1. Due to transport restrictions, it is likely that stromelysin did not penetrate the cartilage matrix and that the majority of the enzyme was present in the synovial fluid. In animals which received intravenous injection of MC1, it is likely that the synovial fluid also contained high levels of MC1, which inhibited stromelysin before it could degrade the joint cartilage.

6.1.3 Activation of Metalloproteinases by APMA

Observations from APMA experiments indicated that under normal physiologic conditions, cartilage contains significant amount of latent MMPs, which are capable of causing widespread matrix degradation upon activation. Treatment with APMA resulted in as much as 80% loss in tissue proteoglycan content and a >3-fold increase in the presence of denatured type II collagen as determined by the presence of CB11B peptide after 3 days in culture. This underscores the role of chondrocytes in maintaining matrix homeostasis. With such a high basal level of tissue proteases, activation mechanisms must be tightly controlled to avoid uncontrolled enzyme activity and unwanted matrix destruction. Assessment of physical behavior indicated that matrix degradation had a profound effect on tissue functional properties. Physicochemical studies revealed that APMA treatment resulted in a significant increase in tissue swelling response, consistent with damage to the collagen network.

Activation of MMPs by APMA also resulted in >80% decrease in equilibrium modulus, dynamic stiffness, and streaming potential and >50% decrease in electrokinetic coupling coefficient. The addition of 4 μM, 400 nM and 40 nM TIMP inhibited PG loss by 95%, 50%, and 20%, respectively and all doses effectively inhibited swelling response. The addition of 4 μM and 400 nM MC1 inhibited PG loss by 95% while 40 nM MC1 inhibited PG loss by 60%, and all doses effectively inhibited swelling response. Cartilage disks treated with 4 μM TIMP and 1 mM APMA had significantly greater streaming potential, electrokinetic coupling coefficient, dynamic stiffness and equilibrium modulus than disks treated with APMA.

6.1.4 Degradation Induced by IL-1β and Retinoic Acid

Similar to several previous studies [1,87,113], addition of IL-1β or RA to culture media resulted in >90% loss of proteoglycans after 6 days in culture. Additionally, tissue samples treated with IL-1β showed a significant increase in tissue swelling response after 8 days in culture, while those treated with RA showed no changes in swelling behavior. Cartilage explants treated with APMA showed significant loss of GAG after 3 days in culture, similar

to previous studies (Chapter IV).

Cartilage disks cultured in the presence of IL-1β showed a significant decreases in equilibrium modulus and streaming potential after 8 days in culture. Loss of streaming potential showed a marked frequency dependence, with high frequency measurements decreasing more quickly than low frequency measurements. This is consistent with degradation which is localized to regions near the sample surface, due to the restricted transport of the large cytokine (18 kDa) through the cartilage matrix. The pattern of degradation and changes in electromechanical behavior to those induced by treatment with active stromelysin (Chapter II). Degradation induced by treatment with RA resulted in significant decreases in equilibrium modulus and streaming potential measurements. In contrast to IL-1β experiments, the changes in streaming potential were independent of frequency. This is presumably due to a more uniform pattern of matrix degradation, since RA is relatively small (400 Da) and its transport through the cartilage matrix should not be hindered by size considerations. This is similar to degradation induced by APMA activation of endogeonous MMPs.

Addition of TIMP to IL-1β, RA, or APMA cultures significantly inhibited matrix degradation. Loss of matrix PG was inhibited by 40%, 35%, or 95% in IL-1β, RA, or APMA systems, respectively. Inhibition of matrix degradation also modulated changes in physical properties due to IL-1β, RA, or APMA treatment. Equilibrium modulus and streaming potential of samples treated with APMA and TIMP were significantly higher than those treated with APMA alone. Decreases in streaming potential and modulus related to IL-1β or RA treatment were also significantly inhibited by addition of TIMP to culture media. Previous studies were unable to verify that TIMP could inhibit IL-1β-induced PG degradation [1], presumably due to its inability to penetrate into the cartilage matrix. These results clearly show that TIMP was able to penetrate into cartilage and prevent PG and concomitant changes in physical properties due to activation or production of endogenous MMPs.

6.2 Future Work

6.2.1 Stromelysin In Vitro Model

Previous studies suggest that the pH for maximal stromelysin activity is in the range of 5.0–5.5 [131], much more acidic than the neutral conditions once presumed to be optimal. The idea that more acidic conditions could increase stromelysin activity and possibly affect the manner in which it cleaves the aggrecan core protein warrants further investigation. Given that the internal pH of cartilage is likely to be less than that of the culture media due to Donnan partitioning, the activity of stromelysin as a function of pH is an area of concern. Experiments involving culture of explants at lower pH could at least address the question of substrate specificity and could also give insight as to mechanisms of cartilage degradation.

6.2.2 IL-1β and Retinoic Acid Stimulation

The results of Chapter V motivate additional investigation of inhibition of degradation of IL-1β and RA treated explants. While studies have demonstrated that such treatments result in production of "aggrecanase" generated fragments [108] and that this degradation can be inhibited by MMP inhibitors [16,87,113], it is unclear which enzymatic processes are being inhibited. The use of antibodies to cleavage fragments which are present with and without inhibitor treatment would give insight into which proteinases are relevant to particular degradative processes.

Additionally, the difference in swelling response between IL-1β and RA bears further investigation. Given that this swelling response is associated with damage to network collagens, it is worthwhile to take advantage of biochemical techniques to assay collagen damage. Antibodies to type IX collagen [134] and type II collagen [60] have been use to evaluate stromelysin or APMA treated explants and would be of great use here to determine if the difference in swelling response between IL-1β and RA treatment correlates with biochemical assays for collagen damage.

The results of Chapter III and appendix F motivate exploration of use of IL-1β in

an *in vivo* environment. The upregulation of MMP production induced by IL-1β and the use of a synthetic inhibitor to prevent stromelysin degradation *in vivo* as well as inhibition of IL-1β degradation *in vitro* combine to suggest experiments which assess the ability of MMP inhibitors to prevent IL-1β induced degradation *in vivo*. These experiments not only involve degradation which may involve other proteinases besides MMPs, but contain the added complication over stromelysin experiments in that they require inhibitors to penetrate the cartilage matrix to prevent degradation.

6.2.3 Mechanical Stimuli

Production and expression of MMPs are upregulated in animal models of experimentally induced arthritis [96,100]. These models typically involve surgical alteration of joints and result in abnormal joint loading. This appears to suggest a scheme of MMP regulation that is influenced by mechanical loading. It is possible to evaluate this hypothesis more rigorously by imposing deformations or loads in a more well-defined geometry. Several studies have demonstrated alterations in biosynthetic rates of cartilage explants due to the influence of mechanical stimuli [67,68,104–106]. In particular, it has been demonstrated that large amplitude compressive strains induce degradation of tissue proteoglycans and collagens [103]. The mechanism by which this degradation occurs is still unknown. Given the ability of MMPs to degrade cartilage matrix (Chapters II, III, and IV) and the degradative capacity of MMPs present in the tissue (Chapters IV and V), the hypothesis that MMPs could be involved in this degradation seems reasonable. The use of MMP antibodies [74], immunolocalization techniques [13,95], and analysis of degradation fragments [108] would prove very useful in assessing the role of MMPs in mechanically mediated tissue catabolism.

As with other experiments, the translation to an *in vivo* system seems to be a logical extension of these experiments. An animal model n place at Merck Research Laboratories involves cartilage degradation induced by a partial transection of the medial meniscus of guineau pigs. This model is known to result in degradation similar to arthritis. Measuring the electromechanical behavior of cartilage from these animals using a plane-ended indenter

(Appendix E) would give more insight into the degradative process resulting from abnormal joint loading. Additionally the use of MMP inhibitors to prevent this degradation could give valuable insight into mechanisms of joint deterioration in this model.

6.2.4 Tissue Regrowth

Several investigators have studied the metabolic and biochemical responses cartilage explants to degradation induced by enzymes such as chymopapain [125,132,133] or chondroitinase ABC [107]. In these studies, the cartilage explants responded to the proteoglycan depletion induced by enzymatic treatment by upregulating proteoglycan synthesis. Using stromelysin to degrade the matrix and evaluating the metabolic response would be interesting in that chondrocytes may respond differently to an enzyme which is native to the tissue. The mechanism by which the cartilage cells respond to this degradation are relevant to an *in vivo* disease state where a damaged matrix can regrow if further degradation can be prevented. In theory, this regrowth could be altered by the introduction of mechanical stimuli which have been shown to upregulate cartilage biosynthetic rates [103,105].

Appendix A

Control Studies

A.1 Culture Protocol

In developing an experimental system for the characterization of enzymatic degradation of cartilage, it is of extreme importance to develop a reliable and reproducible protocol for the culture of cartilage disks. The main requirement for such a system is that there should be negligible matrix degradation in control samples over the time course of the experiment. For studies involving treatment with exogenous enzymes or those designed to assess degradation of cartilage matrix by endogenous proteinases, it is desirable to have a closed system in terms of enzyme production. The implication is that amount of enzymes to which a sample is exposed should be either the amount which was added to the system (in the case of exogenous enzyme addition), or the amount which exists within the tissue initially (for activation of endogenous proteinases). This allows for the assessment of the effect of the particular treatment on the integrity of the matrix, without any mediation from the cells.

Since chondrocytes are responsible for the maintenence of the cartilage matrix, they contain several types of proteinases which are capable of altering matrix composition [90]. Indeed, this is quite necessary for the matrix turnover and remodeling which the chondrocytes modulate [18,50,54]. However, if the tissue were non-viable and the chondrocyte cell membranes were ruptured, the proteinases stored within the cells would be released and be free to degrade the matrix. This is clearly unacceptable if the system is to be well defined and reproducible.

A.2 Methods

Several culture protocols and systems were evaluated for suitability for use as controls for enzymatic degradation studies. The use of frozen cartilage has the potential advantage of experimental convenience, but the freezing process is likely to result in cell death and rupture of cell membranes. To assess the effects of the freezing process on matrix degradation, cartilage disks were harvested as described previously (Chapter 3) and frozen at -20°C for 24 hours. Disks were thawed, then incubated in groups of 4 in 24-well culture dishes in 1 ml phosphate buffered saline (PBS) at 37 °C in a 5% CO_2 atmosphere and removed from culture at times up to 48 hours. Groups of disks were incubated in PBS alone or in the presence of 100 μg/ml bovine pancreas trypsin, 1000 μg/ml trypsin or a cocktail of proteinase inhibitors. This inhibitor cocktail included 10 mM benzamidine hydrochloride to inhibit trypsin-like activity, 10 mM N-ethylmaleimide to inhibit sulfylhydry-dependent proteinases, 1 mM phenylmethanesulfonyl fluoride to inhibit serine proteinases such as cathepsin B, 1 μM pepstatin to inhibit acid proteinases including cathepsin D.

Additionally, groups of plugs were harvested and immediately incubated in groups of 4 in 1 ml Dulbecco's Modified Eagle Media (DMEM) containing 100 U/ml penicillin G and 100 μg/ml streptomycin for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere. Groups of disks were then incubated in PBS, DMEM, DMEM with 10 mM Na_2EDTA to inhibit metalloproteinase activity, or DMEM with 50 μg/ml trypsin and removed from culture at times up to 72 hours.

After removal from culture, cartilage disks were frozen at -20 °C, lyophilized, and digested with 1 ml of 125 μg/ml papain digestion solution as described previously [105]. 20 μl portions of digests and culture media were assayed for sulfated GAG by reaction with 2 ml of dimethylmethylene blue dye solution in polystyrene cuvets and spectrophotometry [34] using whale/shark chondroitin sulfate as the standard.

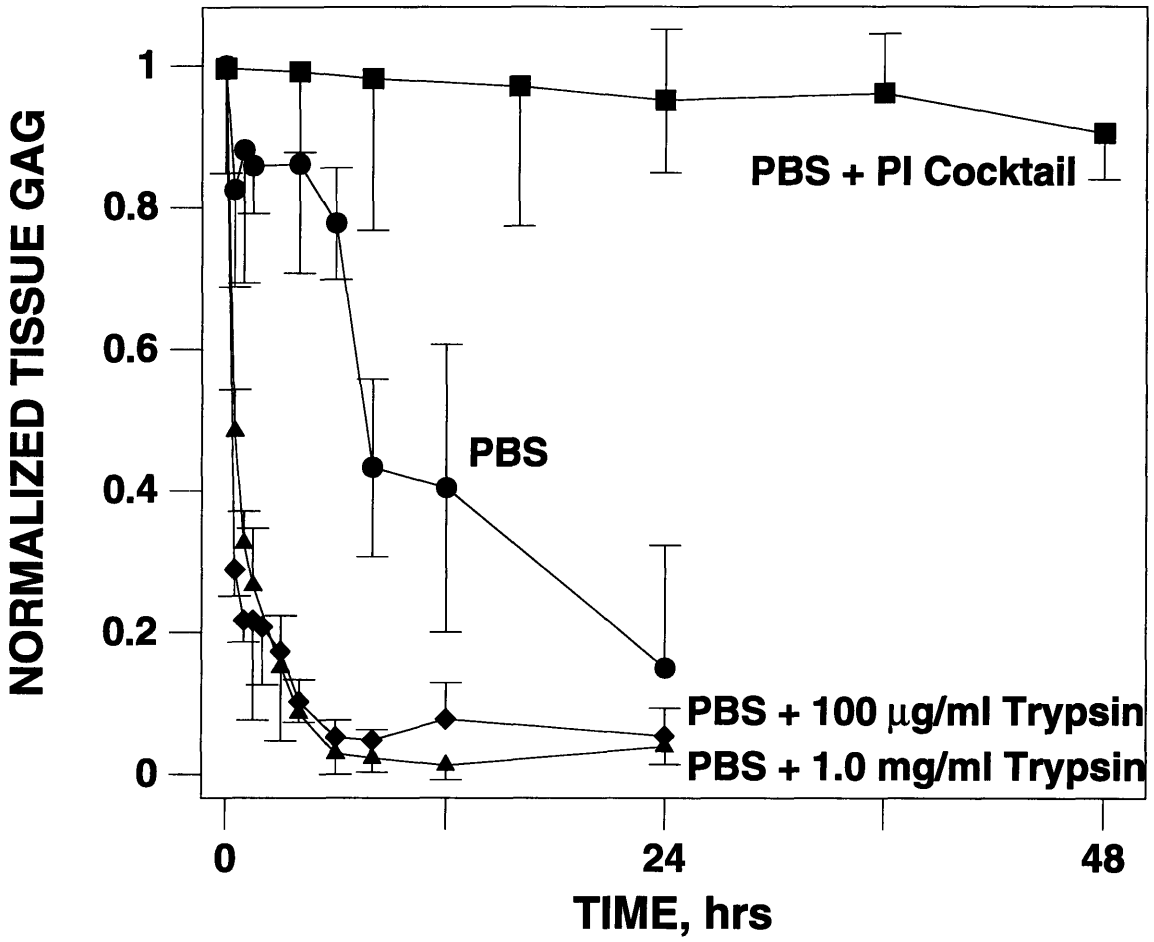

Figure A.1: Normalized GAG loss from previously frozen cartilage disks incubated in PBS alone or in the presence of 100 *mu*g/ml trypsin, 1000 *mu*g/ml trypsin or a cocktail of proteinase inhibitors including 10 mM benzamidine hydrochloride, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin. Data are mean $\pm$ SD (n=4).

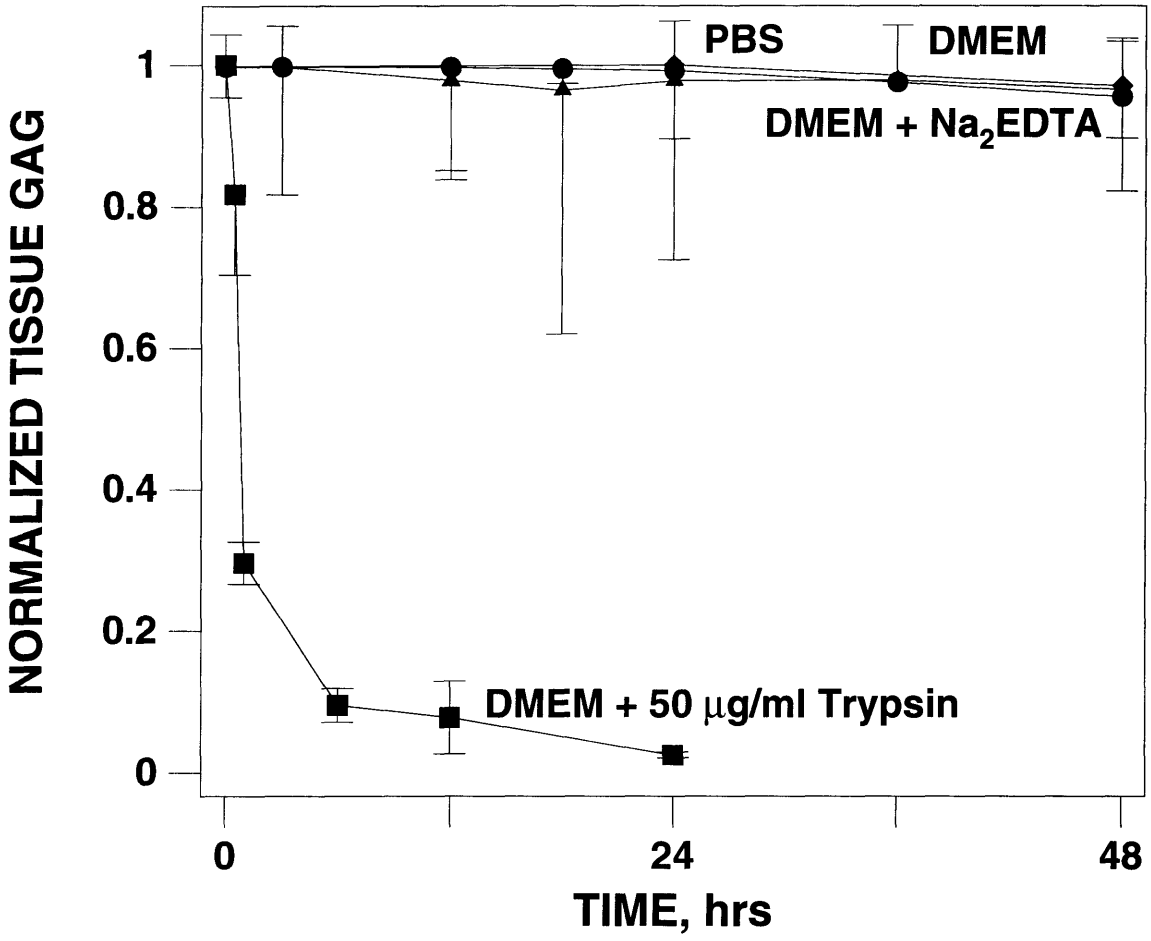

Figure A.2: Normalized GAG loss from fresh cartilage disks incubated in PBS, DMEM, DMEM with 10 mM Na_2EDTA, or DMEM with 50 μg/ml trypsin. Data are mean $\pm$ SD (n=4).

A.3 Results

Culture of frozen tissue in the absence of proteinase inhibitors resulted in >80% GAG loss after only 1 day in culture, while addition of the inhibitor cocktail prevented degradation to the extent that ~5% of GAGs were lost after 2 days in culture (Fig A.1). Addition of either 100 μg/ml or 1000 μg/ml trypsin to the culture media resulted in rapid matrix degradation, with >90% GAG loss after only 3 hours in culture. Cartilage disks from all conditions which were cultured immediately after explant showed little GAG loss in culture, with the exception of those samples incubated in the presence of 50 μg/ml trypsin (Fig A.2). Samples incubated in PBS, DMEM, or DMEM with Na_2EDTA lost <5% of tissue GAG after two days in cultured, while the addition of trypsin to the media yielded >90% GAG loss after 6 hours.

A.4 Summary and Conclusions

Given that, in the absence of proteinase inhibitors, previously frozen tissue lost significant amounts of GAG in less than 1 day, the use of such tissue for enzymatic degradation studies is undesirable. While addition of an inhibitor cocktail eliminates this loss, their use is not optimal since MMPs such as stromelysin are known to serve as activators for other enzymes [119,120], and use of broad spectrum of inhibitors would mask these "cascade" effects. Cartilage disks which were cultured immediately after harvest in either PBS or DMEM showed negligible degradation without proteinase inhibitors after 48 hours in culture. With longer media times, however, the use of DMEM would seem to be preferable to PBS, since tissue cultured in only saline would likely start to suffer from nutritional deprivation.

Appendix B

Assessment of Cartilage Degradation

B.1 Physicochemical Measurements

The changes in cartilage composition which result from treatment with metalloproteinases are accompanied by alterations in tissue physicochemical properties. As described by Maroudas [77–79], the swelling state of cartilage is determined by a balance between the osmotic pressure of the proteoglycans, which tends to swell the tissue, and the restraining tensile forces of the collagen network, which tend to limit tissue swelling. Removal of fixed charge groups on the proteoglycans would result in a decrease in osmotic swelling pressure as well as in the solid mass of the tissue. Effects on the bulk wet weight are more difficult to predict, given that the solid components lost by the tissue may be replaced by water moving into the tissue, resulting in little change in net mass. Degradation of cartilage collagens results in a damaged collagen network which is less able to counteract osmotic swelling pressure. Maroudas [78] demonstrated that osteoarthritic cartilage swells by a greater amount than normal cartilage when equilibrated in hypotonic saline, due to collagen network damage. Given the multitude of physicochemical measurements which can be recorded, it is important to evaluate which measurements are sensitive to the various matrix degradation induced by different proteinases.

B.2 Methods

The femoropatellar groove of 1-2 week old calves was isolated and 3 mm diameter by 1 mm thick cartilage disks were harvested as described previously (Chap 2). Immediately after explant, disks were incubated in groups of 4 in 1 ml DMEM containing 100 U/ml penicillin G and 100 μg/ml streptomycin for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere. Groups of disks were incubated in DMEM alone or DMEM with 50 or

500 μg/ml recombinant human stromelysin (SLN), 100 μg/ml bovine pancreas trypsin, or 100 μg/ml recombinant human interstitial collagenase and removed from culture at times up to 72 hours. Cartilage disks were matched such that at each time point, tissue samples from all conditions originated from similar locations along the femoropatellar groove.

Upon removal from culture, groups of plugs were patted briefly with a paper towel to remove surface water, and wet weights were measured. Disks were then twice reequilibrated in 1 ml of hypotonic saline solution for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded. Values for tissue hydration were obtained by normalizing tissue water weight (wet weight - dry weight) to tissue dry weight. Samples were then frozen at -20°C, lyophilized and digested with papain.

B.3 Results

Cartilage samples incubated in the presence of 500 μg/ml SLN or 100 μg/ml trypsin showed significant loss of both tissue wet weight (Fig B.1) and dry weight (Fig B.2) as well as significant increase in tissue hydration (Fig B.3). After three days in culture, samples treated with 500 μg/ml SLN had average dry and wet weight values which were $\sim$50% and $\sim$75% of control values respectively, while those treated with 100 μg/ml trypsin were $\sim$50% and $\sim$70% of control values. Samples treated 50 μg/ml SLN did not have significantly different dry weight, wet weight, or hydration from controls after three days in culture.

Cartilage disks treated with 100 μg/ml recombinant human interstitial collagenase exhibited rapid GAG loss, with tissue GAG content decreasing to $<$5% of its initial value after only 48 hours in culture (Fig B.4). Additionally, collagenase treated samples had an increased swelling response, demonstrating significantly different swelling behavior from control samples after only 12 hours in culture (Fig B.5). After 3 days in culture, tissue treated with collagenase exhibited a swelling ratio of 1.1 ± 0.03, as compared to 1.02 ± 0.025 for controls.

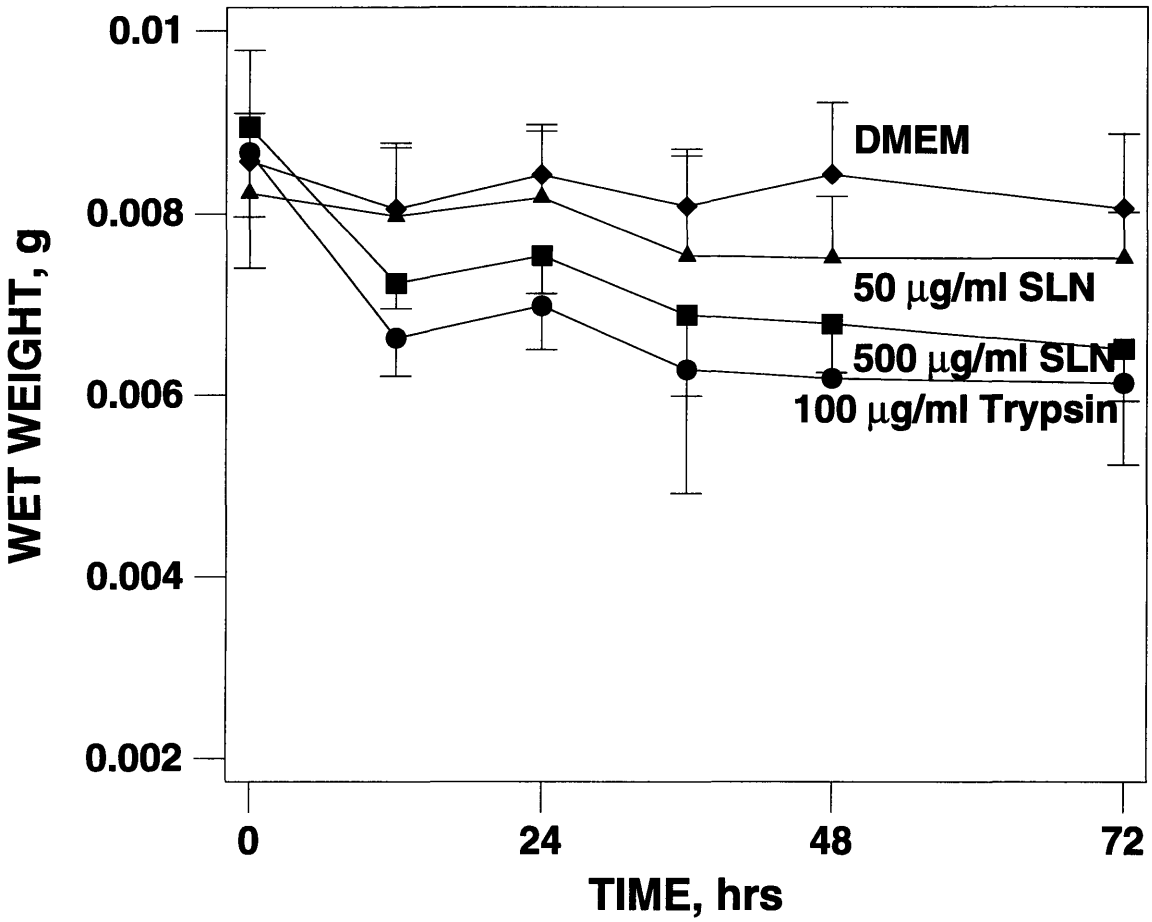

Figure B.1: Wet weights of cartilage disks incubated in DMEM alone or DMEM with 50 μg/ml recombinant human stromelysin (SLN), 500 μg/ml SLN, or 100 μg/ml bovine pancreas trypsin. Data are mean $\pm$ SD (n=4).

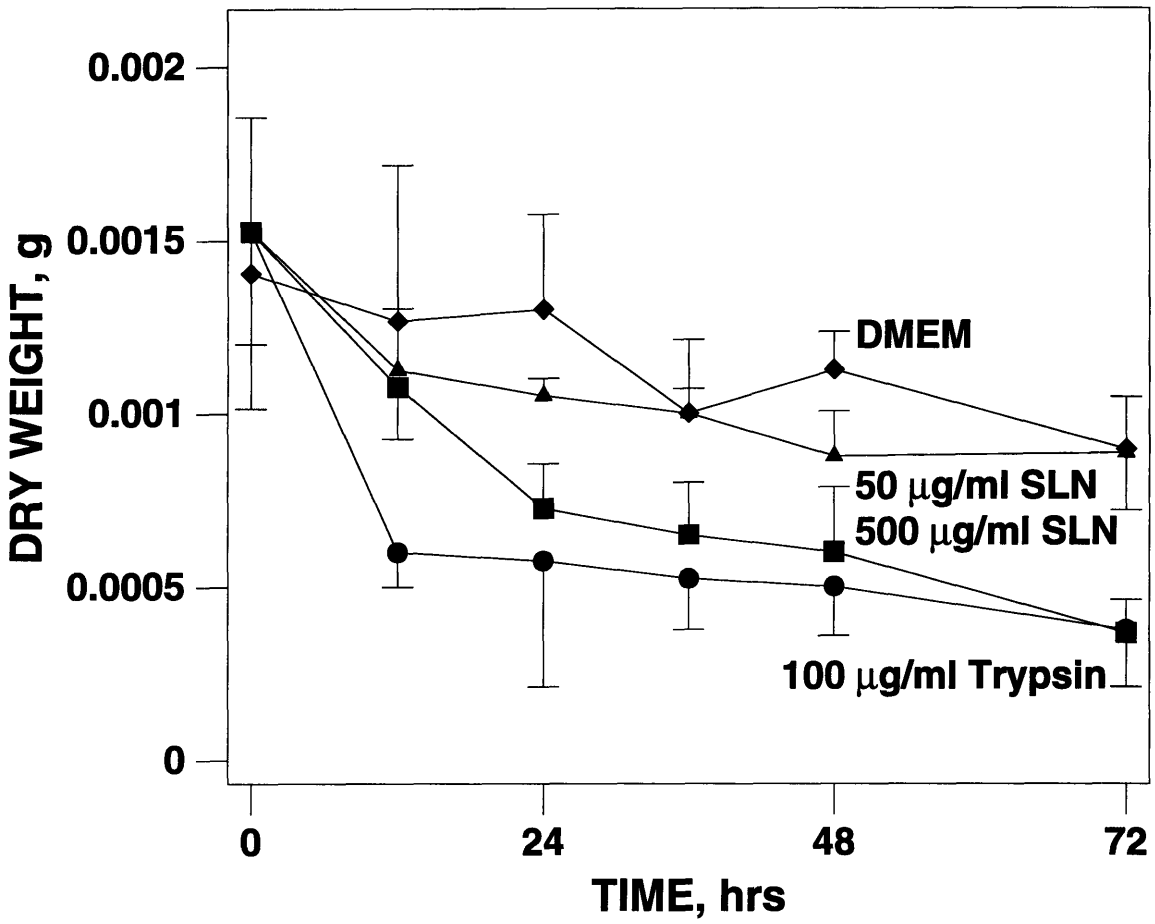

Figure B.2: Dry weights of cartilage disks incubated in DMEM alone or DMEM with 50 μg/ml recombinant human stromelysin (SLN), 500 μg/ml SLN, or 100 μg/ml bovine pancreas trypsin. Data are mean ± SD (n=4).

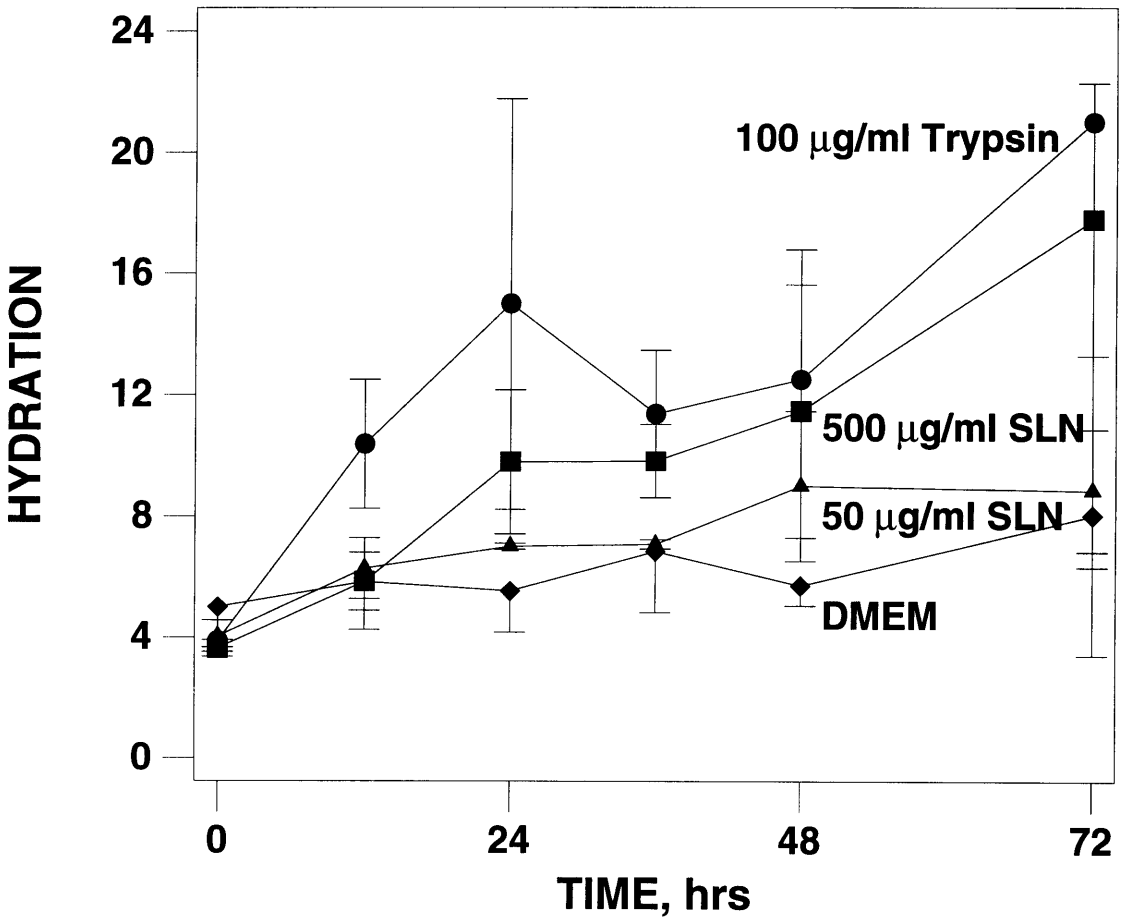

Figure B.3: Hydration values for cartilage disks incubated in DMEM alone or DMEM with 50 μg/ml recombinant human stromelysin (SLN), 500 μg/ml SLN, or 100 μg/ml bovine pancreas trypsin. Data are mean $\pm$ SD (n=4).

B.4 Summary and Conclusions

Measurements of both cartilage wet weights are dry weights from samples treated with either stromelysin or trypsin are consistent with the significant of the cartilage matrix. As these enzymes react with their respective substrates in the tissue, proteoglycans and other matrix constituents are lost from the samples, thus decreasing the measured mass. Both of these measurements, however, depend on the specimen size, which may vary from sample to sample. This is of particular concern, given that samples were experimentally matched across conditions at any given time point, but not necessarily matched across all times. This is particularly evident in dry weight measurements, were the mass of control samples in culture for 72 hours was different from those removed from culture at the start of the experiment (Fig B.3). The lack of consistent data for controls during the time of the experiment, makes interpretation of treatment groups more difficult. In theory, hydration measurements have the advantage of normalizing samples water content to tissue dry weight. However, in systems for which the the dry mass is not constant, this normalization becomes less advantageous. Consequently, the physical relevance of extremely large hydration values observed for tissue treated with large doses of either stromelysin or trypsin (Fig B.3) in unclear. An increase in hydration typically implies that the sample has taken up water, whereas in these cases it likely involves no water uptake, but rather loss of solid mass.

In separate experiments, control and collagenase-treated samples were weighed upon removal from culture, then reequilibrated in hypotonic saline and weighed again. The weight in hypotonic saline was then normalized to wight in culture media to give a swelling ratio (Fig B.5) which is independent of sample size. Indeed, control samples incubated in DMEM alone showed relatively constant swelling ratio over 3 days in culture (Figure B.4). An increase in swelling ratio implies damage to the tissue network collagens, which are unable to resist the swelling pressure of the proteoglycans. Treatment with 100 μg/ml recombinant human interstitial collagenase did result in an increase in swelling ratio, consistent with the known activity of this enzyme against type II collagen.

The large loss of GAG induced by treatment with interstitial collagenase is inherently

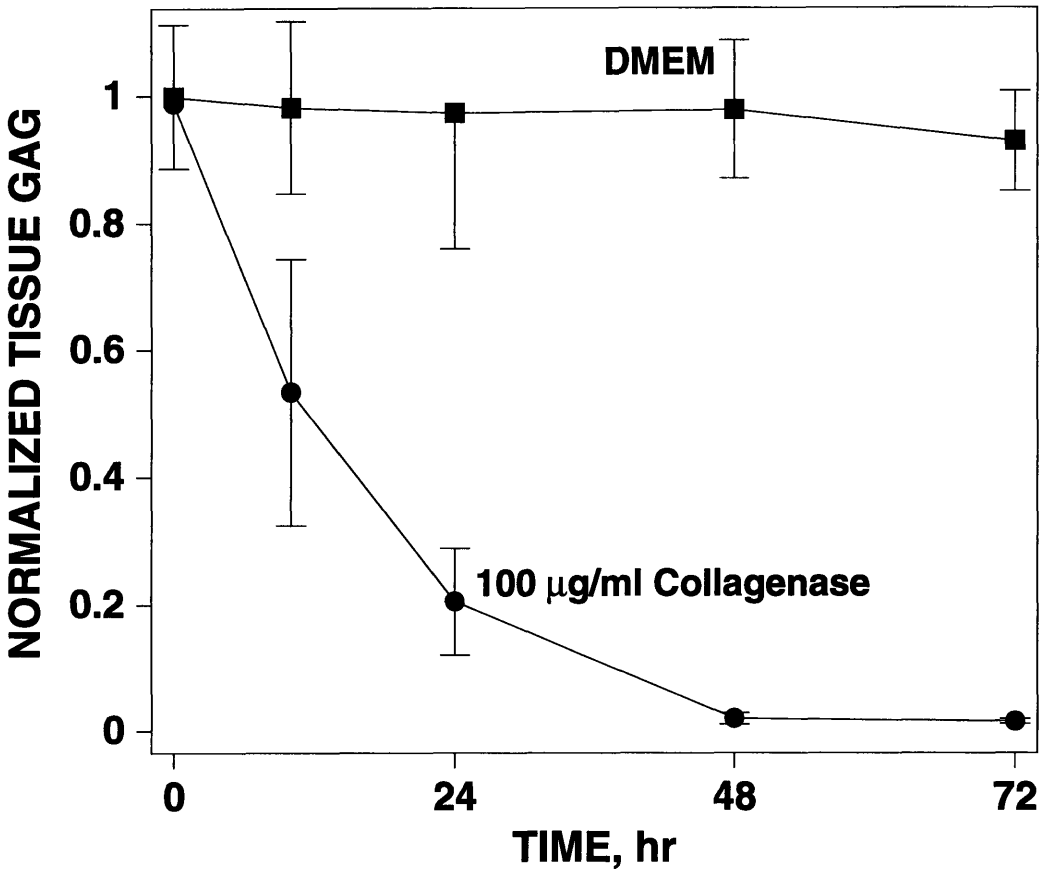

Figure B.4: Normalized GAG loss from cartilage disks incubated in DMEM alone or DMEM with 100 μg/ml recombinant human interstitial collagenase. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

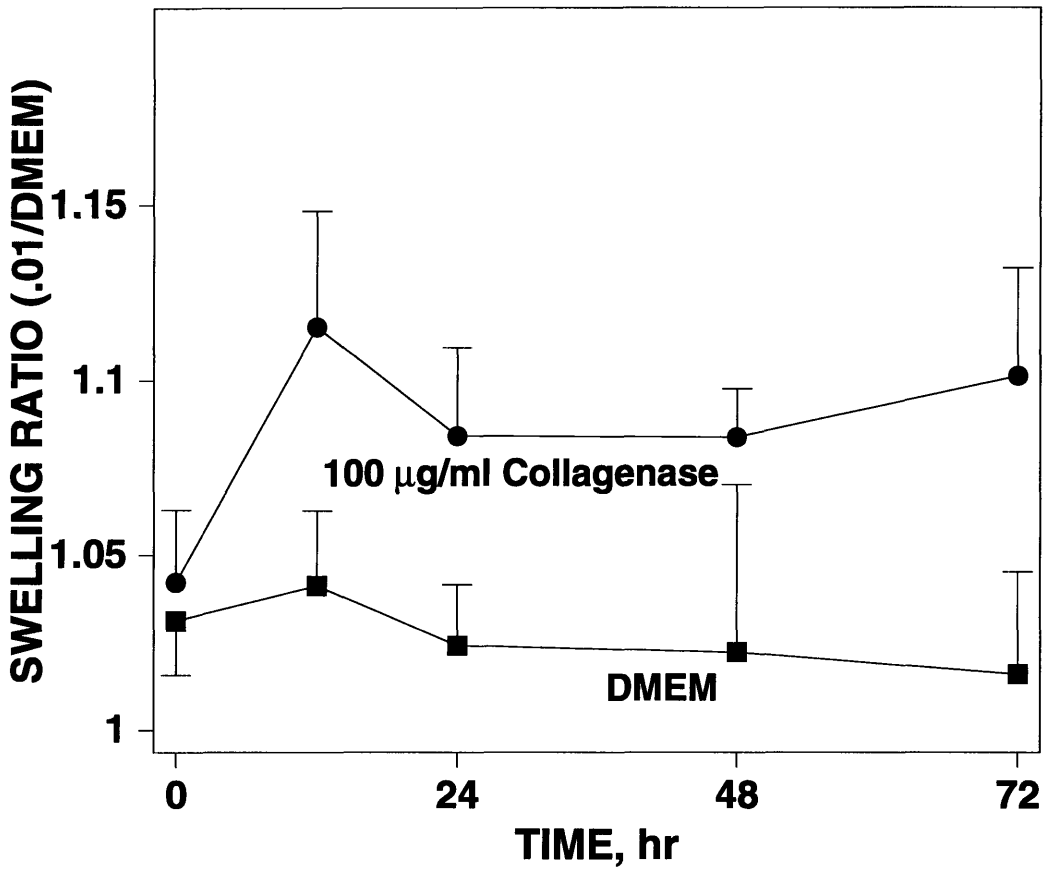

Figure B.5: Swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) of cartilage disks cultured in DMEM alone or DMEM with 100 μg/ml recombinant human interstitial collagenase. Data are mean ± SD (n=4).

interesting. Collagense is capable cleaving the aggrecan core protein in the interglobular domain, just as stromelysin. However, its primary degradative activity is thought to be against type II collagen. This aggressive degradation of proteoglycan then brings into question the stability of the enzyme. Upon further analysis, it was determined that the active collagenase had indeed auto-lysed, resulting in several smaller forms of the enzyme (M. W. Lark, personal communication). These smaller forms likely lose their specificity for type II collagen and exhibit normal MMP activity against proteoglycan, resulting in the significant loss of GAG seen here (Fig B.4).

Appendix C

Stromelysin Reaction Kinetics

C.1 Transport and Reaction

In characterizing the kinetics of loss of proteoglycan fragments from explants exposed to recombinant human stromelysin, there are a number of transport and reaction events which may be rate limiting. Given the sizes of the active enzyme (~46 kDa) and cleaved aggrecan fragments (>200 kDa), it is likely that their respective transport into and out of the intact cartilage matrix is hindered considerably. Consequently, upon initial exposure, stromelysin is unable to penetrate the dense matrix and is only able to degrade the matrix near the tissue surface. With time, degradation progresses from the tissue surface, and the enzyme is able to diffuse more easily through the degraded matrix and access substrate in deeper portions of the tissue. In this scheme, a front of degradation moves into the tissue from the surface, and the rate of loss of proteoglycan fragments to the culture media is determined by the kinetics of the reaction between the enzyme (stromelysin) and substrate (aggrecan) producing a product (proteoglycan fragment). The kinetics of such a reaction were described by Michaelis and Menten [118].

C.2 Michaelis-Menten Kinetics

In this treatment, the following components are considered: the enzyme (E), the substrate (S), the enzyme-substrate complex (ES) and the product (P).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \tag{C.1}$$

At steady state, there is no net accumulation or disappearance of enzyme-substrate complex, and thus:

$$k_1[E][S] = (k_2 + k_3)[ES] \tag{C.2}$$

Rearranging to obtain an expression for equilibrium:

$$[ES] = \frac{[E][S]}{k_M} \quad \text{where} \quad k_M = \frac{k_2 + k_3}{k_1} \tag{C.3}$$

Assuming reaction rates are relatively fast, then the number of enzyme-substrate complexes is relatively small compared to amounts of enzyme, substrate, or product, and thus at all times:

$$[S_0] = [S] + [P] \tag{C.4}$$

The rate of generation of product is then equal to the rate of disappearance of reactant.

$$\frac{d[P]}{dt} = -\frac{d[S]}{dt} = k_3 \frac{[E][S]}{k_M} \tag{C.5}$$

Separating variables gives

$$-\int_{[S_0]}^{[S]} \frac{d[S]}{[S]} = \int_0^t \frac{k_3[E]}{k_M} dt \tag{C.6}$$

and integration yields

$$-\ln \frac{[S]}{[S_0]} = \frac{k_3[E]}{k_M} t \tag{C.7}$$

thus

$$\frac{[S]}{[S_0]} = e^{t/\tau} \quad \text{where} \quad \frac{1}{\tau} = \frac{k_3[E]}{k_M} \tag{C.8}$$

The major consequence of such a development is that for a system which is dominated purely by the kinetics of an enzyme-substrate reaction, the rate of consumption of substrate, in this case cartilage matrix proteoglycan, will be exponential with time. Furthermore, the time constant, τ, for the degradation of matrix proteoglycan, is inversely proportional to the amount of enzyme to which the tissue is exposed.

The data in figure 2.1 show the loss of tissue proteoglycan with time in culture from samples treated with graded levels of active stromelysin. The data for each enzyme concentration are fit to a single exponential of the form $e^{-t/\tau}$. In figure A.1, the inverse time constant $(1/\tau)$ was plotted against stromelysin dose. All data points are N = 2, with the

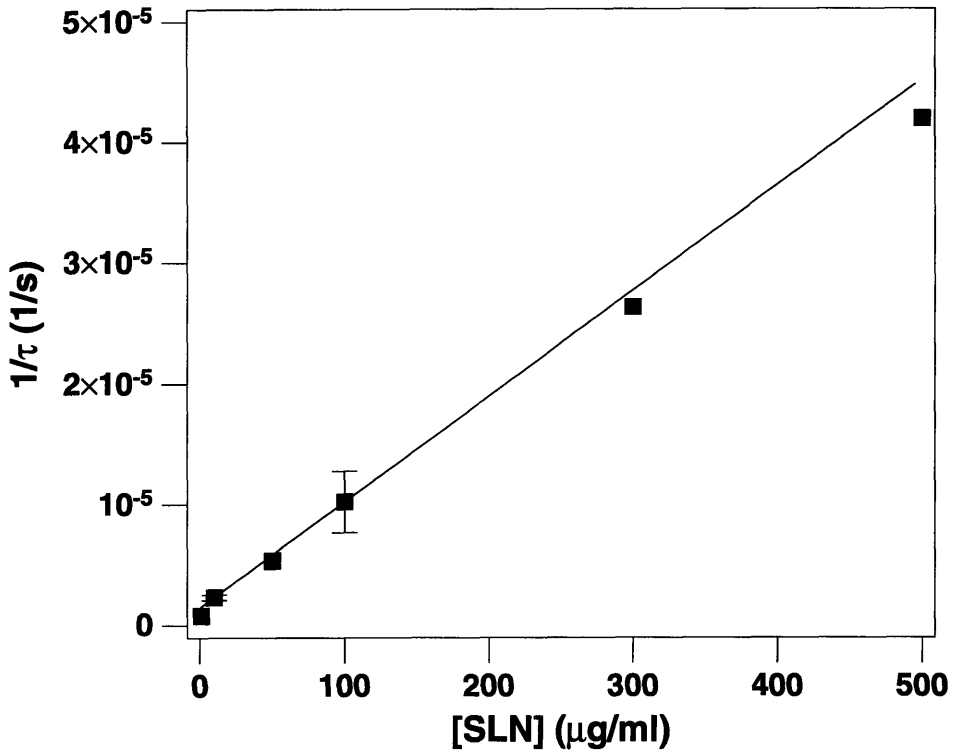

Figure C.1: Inverse time constant of proteoglycan loss plotted against concentration of stromelysin in culture media. Values of τ were obtained at each concentration by fitting the times course of proteoglycan loss to an exponential of the form $e^{-t/\tau}$ (See Fig 2.1).

exception of 100 (N = 5) and 10 μg/ml SLN (N = 3). The data were fit by linear regression and yielded a best fit slope of 4.37 $\frac{1}{\text{moles}\cdot\text{sec}}$, corresponding to the ratio k_3/k_M. Given that the data for matrix degradation match well with reaction kinetics, it seems likely that there is little or no penetration of the active enzyme into the undegraded matrix.

This is also consistent with histology performed on stromelysin-treated cartilage (Fig 2.3) which demonstrates a marked boundary between the degraded surface region and undegraded inner portion of the tissue. This correlation between histological evidence and GAG data is also quantitative, given that histology showed that proteoglycans were removed from a region which penetrated $\sim$150 μm into the sample on all sides of the 3 mm diameter, 1 mm thick disk, leaving a disk with a degraded surface region and an inner core 2.7 mm in diameter and 0.7 mm thick which is undegraded. A comparison of undegraded

volume (4.01 mm^3) to total volume (7.07 mm^3), shows a fractional GAG content of 56.7%, as compared to the measurement of 50.1% by the DMB dye binding assay.

Appendix D

Inhibitor Studies

D.1 Introduction

Inhibitors of MMPs which are native to cartilage, such as TIMP and α_2-M, are generally quite large. Consequently, the transport of such large proteins through the dense cartilage matrix is typically hindered significantly [79]. As a result, there has been a significant effort to design low molecular weight inhibitors which would have more favorable transport characteristics. Several classes of inhibitors have been developed, including those which bind to the enzyme active site [1] and other which attach to propetide regions [38]. In general, the efficacy of such inhibitors has been evaluated by characterizing their ability to inhibit the reaction of a given enzyme to an isolated substrate in solution. While this allows for very precise quantitation of reaction kinetics, it does not account for transport through the cartilage matrix, which can have a profound effect on inhibitor efficacy.

The addition of APMA to cartilage explants has been shown to result in significant degradation of matrix proteoglycans and collagens, due to activation of endogenous MMPs [9]. Since APMA is relatively small ($\sim$ 350 Da) its transport through cartilage is relatively unhindered and the resulting degradation should be distributed throughout the matrix. Such a culture system is well suited for evaluation of inhibitor efficacy, in that any such inhibitor would be required to penetrate the cartilage matrix to effectively inhibit the MMPs activated by APMA. Similarly, treatment with either IL-1β or RA results in the production of MMPs by chondrocytes, and inhibition of these enzymes would require diffusion of inhibitors from the culture media into the cartilage matrix.

Several *N*-carboxyalkyl peptide inhibitors [22] were evaluated for their ability to inhibit cartilage matrix degradation resulting from APMA activation of MMPs. These peptide inhibitors contain chelating groups and are designed to bind to the active site of an MMP and remove the Zn^{++} bound to the enzyme. The removal of the metal ion results

Inhibitor	k_i vs. Stromelysin	k_i vs. Collagenase	Molecular Weight
MC1	310 nM	750 nM	476
MC2	230 nM	470 nM	483
MC482	30 nM	5.7 μM	594
MC561	4.2 μM	660 nM	467
MC354	30 nM	300 nM	498
TIMP	<0.1 nM	<0.1 nM	28000

Table D.1: Coefficients of inhibition against stromelysin and collagenase and molecular weights of metalloproteinase inhibitors MC1, MC2, MC482, MC561, MC354, and TIMP. Values of k_i of synthetic inhibitors were measured for inhibition of reaction of MMPs against a peptide-modified substance P.

in a conformational change which renders the enzyme inactive [22]. (These inhibitors were graciously provided by Dr. William K. Hagmann, Merck Research Laboratories.) Four synthetic compounds were tested: L-696,418 (MC1), L-702,842 (MC2), L-744,482 (MC482), L-707,346 (MC562), L-712,354 (MC354) and compared to TIMP for their ability to inhibit matrix degradation induced by stromelysin, APMA, IL-1β or RA. MC1, MC2 and MC354 are broad spectrum MMP inhibitors. MC482 is primarily a stromelysin inhibitor, with low efficacy against collagenase. MC5621 is primarily a collagenase inhibitor, with low efficacy for stromelysin. Relative efficacy of compounds is determined by comparison of reaction constant (k_i) characterizing the inhibition of enzymatic cleavage of a peptide-modified substance P [22]. A lower value of k_i indicates that a smaller amount of an inhibitor is required to yield a given amount of inhibition. Therefore, inhibitors with the lowest values of k_i are the most potent inhibitors. Values of k_i for these inhibitor against MMPs are shown in table D.1.

D.2 Methods

The femoropatellar groove of 1-2 week old calves was isolated and 3 mm diameter by 1 mm thick cartilage disks were harvested as described previously (Chap 2). Immediately after explant, disks were incubated in groups of 4 in 1 ml DMEM containing 100 U/ml penicillin G and 100 μg/ml streptomycin for approximately 16 hours at 37 °C in a 5% CO_2 atmosphere. Groups of disks were incubated in DMEM with 100 μg/ml recombinant human stromelysin (SLN). After 12 hours in culture, 2 μg/ml MC1 was added to the culture media of half the groups. In separate experiments, groups of cartilage disks were incubated in DMEM alone, DMEM with 1 mM APMA, or DMEM with 1 mM APMA plus graded levels of MMP inhibitors MC1, MC2, MC482, MC561, or TIMP ranging from 40 nM to 4 μM. Cartilage disks were removed from culture at times up to 72 hours for biochemical analysis and swelling studies. In a third set of experiments, groups of plugs were treated with 100 ng/ml IL-1β or 1 μM RA in the presence and absence of synthetic inhibitors MC1 and MC354. Cartilage samples were incubated for up to 8 days, with media changes every 2 days.

For swelling studies, after removal from culture, groups of plugs were patted briefly with a paper towel to remove surface water, and wet weights were measured. Disks were then twice reequilibrated in 1 ml of hypotonic saline solution for 1 hour at room temperature. Surface water was again removed and wet weights were measured. Following wet weight measurements, disks were lyophilized, and dry weights were recorded. Values for tissue hydration were obtained by normalizing tissue water weight (wet weight - dry weight) to tissue dry weight. Samples were then frozen at -20°C, lyophilized and digested with papain.

D.3 Results

Addition of 2 μg/ml MC1 to culture media containing 100 μg/ml SLN after 12 hours of incubation prevented further loss of GAG from cartilage explants (Fig D.1). After 12 hours in culture, SLN treatment induced ($\sim$15%) loss of GAG from cartilage disks, and this loss

continued to the point where >70% of GAG was lost from samples which received no inhibitor. Plugs which received MC1 12 hours into culture showed no significant GAG loss after treatment.

Cartilage plugs incubated in the presence of 1 mM APMA in the absence of MMP inhibitor lost >60% of tissue GAG after 3 days in culture. Samples cultured with 2 μg/ml MC1, 2 μg/ml MC2, or 100 μg/ml TIMP in addition to 1 mM APMA did not show significant GAG loss after three days in culture (Fig D.2). Incubation of cartilage disks in 1 mM APMA plus 2 μg/ml MC482 or 2 μg/ml MC561 resulted in 20% and 50% loss of GAG, respectively, compared to 80% loss of GAG for samples treated with 1 mM APMA and no inhibitor (Fig D.3). Samples treated with APMA without inhibitor had a swelling ratio of 1.13 after three days in culture, compared to 1.10 and 1.05 for samples treated with APMA and MC561 or MC482 and 1.03 for controls in DMEM alone (Fig D.4).

Dose response experiments showed that 2, 0.2 and 0.02 μg/ml MC482 inhibited APMA-induced GAG loss by 85%, 55%, and 10% respectively (Fig D.5). Similar experiments showed that 2, 0.2 and 0.02 μg/ml MC561 inhibited APMA-induced GAG loss by 40%, <5%, and <5% respectively (Fig D.6). These data, combined with similar data for MC1 and TIMP (see chapter 4) are plotted as percentage inhibition of GAG loss at 72 hours in culture vs dose of inhibitor (Fig D.7). These data show that for all doses used, MC1 was the most effective inhibitor of APMA induced degradation. TIMP and MC482 showed similar efficacy across the range of doses used, while MC561 was the least effective inhibitor, showing only moderate efficacy at the highest dose used.

Cartilage disks cultured in the presence of 0.1% BSA with 2 μg/ml MC1 and 1 mM APMA lost ~30% of tissue GAG after three days in culture, compared to <5% for controls or disks cultured in 2 μg/ml MC1 and 1 mM APMA and 60% loss from samples cultured in 1 mM APMA (Fig D.8).

Cartilage disks cultured in the presence of 100 ng/ml IL-1β lost >90% of tissue GAG content after 8 days in culture, while samples treated with 100 ng/ml IL-1β plus 100 μM MC354 exhibited <40% loss of GAG, compared to ~20% for controls (Fig D.9).

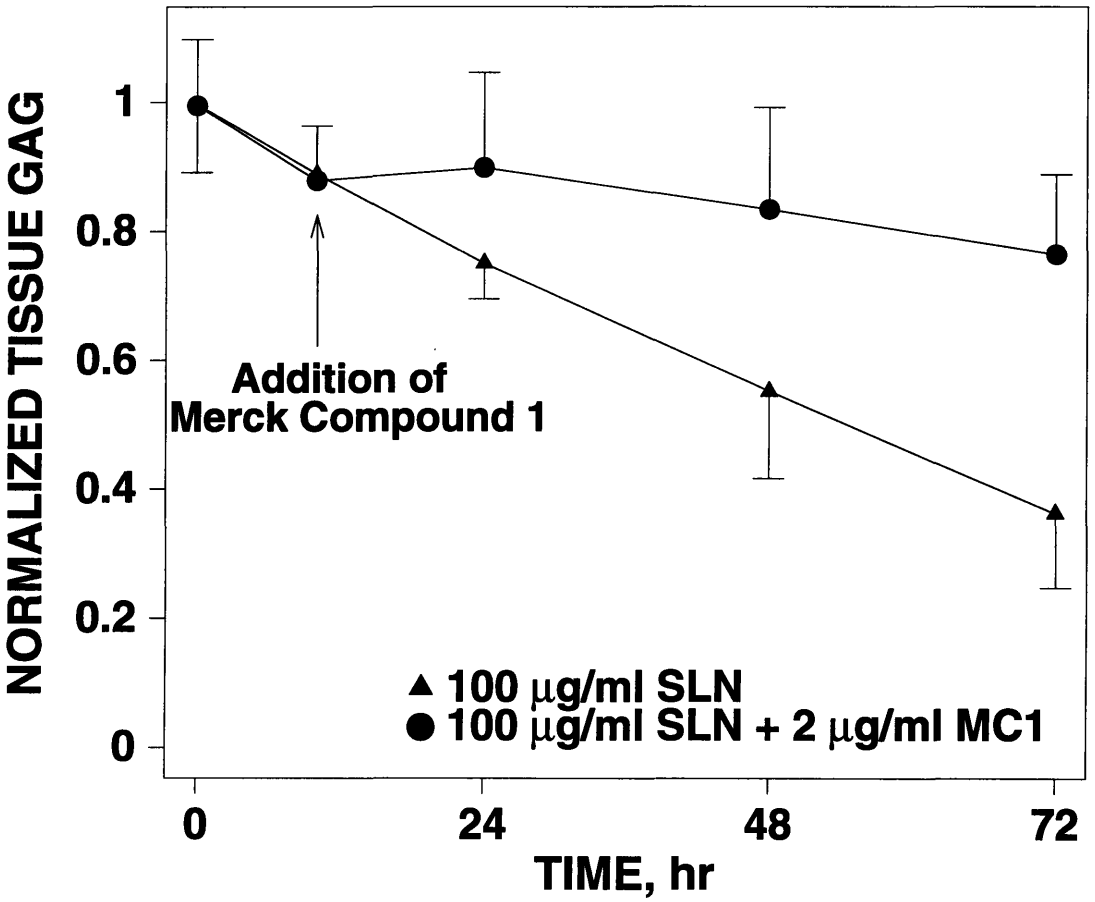

Figure D.1: Normalized GAG loss from cartilage disks incubated in DMEM with 100 μg/ml recombinant human stromelysin in the presence and absence of 2 μg/ml MC1, added 12 hours after the start of culture. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

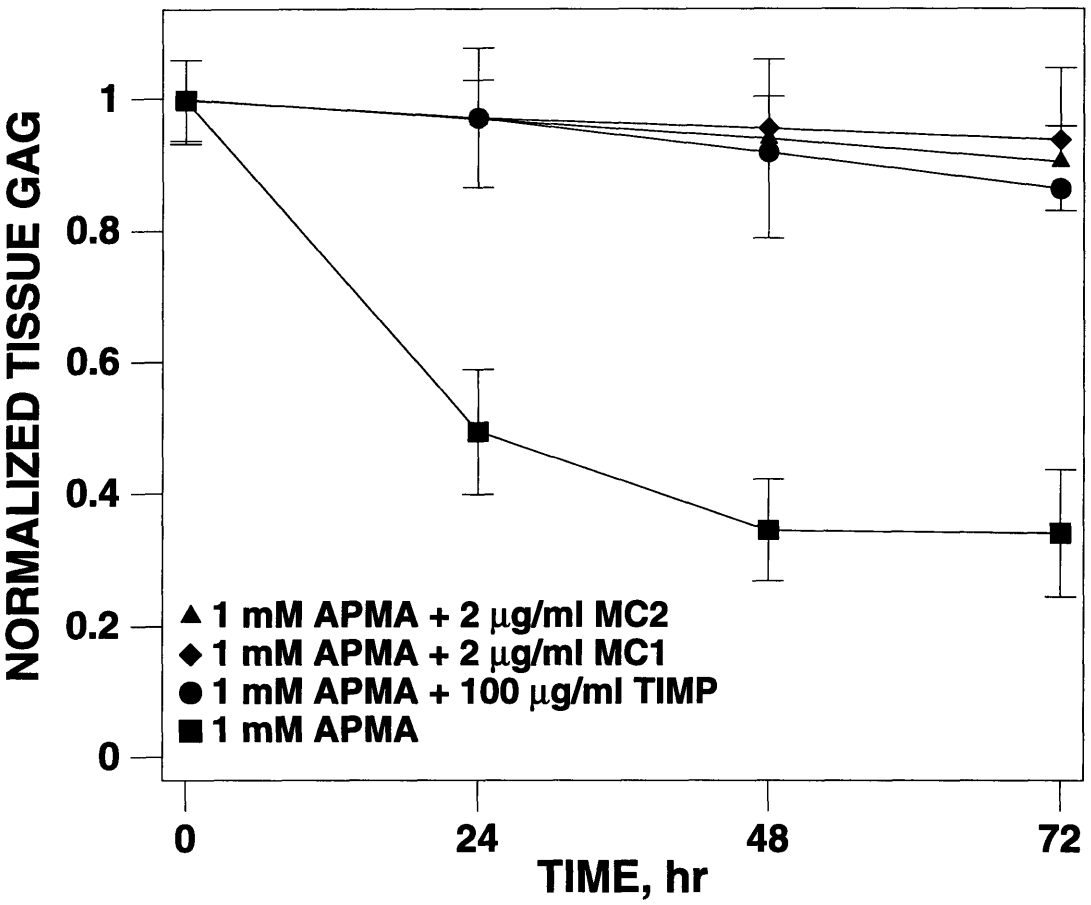

Figure D.2: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA plus 2 μg/ml MC1, or DMEM with 1 mM APMA plus 2 μg/ml MC2. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

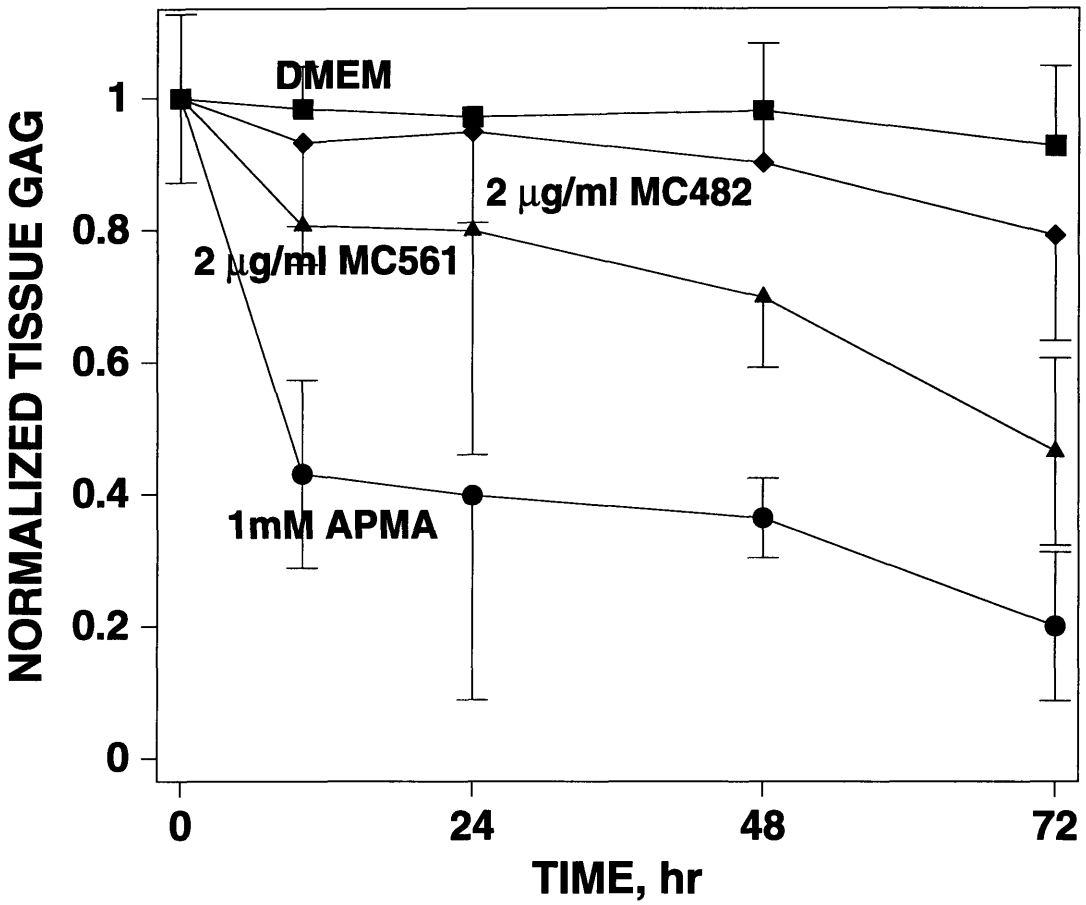

Figure D.3: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA plus 2 μg/ml MC561, or DMEM with 1 mM APMA plus 2 μg/ml MC482. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

Tissue samples cultured in the presence of 1 μM RA lost >60% of tissue GAG content after 8 days in culture, while samples treated with 1 μM RA plus 100 μM MC354 exhibited <30% loss of GAG, compared to ~20% for controls (Fig D.10). Cartilage disks treated with 100 ng/ml IL-1β demonstrated an increase in swelling ratio after 8 days in culture, which was inhibited by the presence of 100 μM MC1. Tissue samples treated with 1 μM RA with or without 100 μM MC1 did not exhibit changes in swelling behavior over 8 days in culture (Fig D.11).

D.4 Summary and Discussion

The inhibition of SLN- and APMA-induced GAG loss by MC1 (Figs D.1 and D.2) demonstrated that the *N*-carboxyalkyl peptides are cablable of inhibiting MMPs in an organ culture environment. It is likely, however, that inhibition took place in the culture media for SLN degradation (Fig D.1), given that previous studies have shown this degradation proceeds inward from the tissue surface [8]. This is not the case, however, for APMA-induced degradation, which likely occurs throughout the tissue. It is noteworthy then that MC1, MC2, and TIMP all were able to completely inhibit GAG loss in this system. The levels of TIMP and MC1 are identical to those used in previous experiments [9] which also resulted in complete inhibition. Given that values of k_i for MC1 and MC2 are similar, it is not surprising MC2 was able to inhibit GAG loss is this system as well.

Given that APMA activates a wide range of MMPs, the use of inhibitors specific to only one enzyme can give information about the relative impact of an enzyme on a given process. Used in similar doses, MC482 was more effective at inhibiting APMA-induced GAG loss than MC561 (Fig D.3). Given that MC482 is more potent against stromelysin, MC561 is more potent against collagenase, this suggests that in this system, inhibition of stromelysin is more critical for maintaining tissue proteoglycans. Curiously, MC482 was also more effective at inhibiting tissue swelling than MC561, seemingly implying that inhibition of stromelysin is critical to the maintenence of the collagen network also. Given that stromelysin is known to degrade type IX collagen but only slightly degrade type II

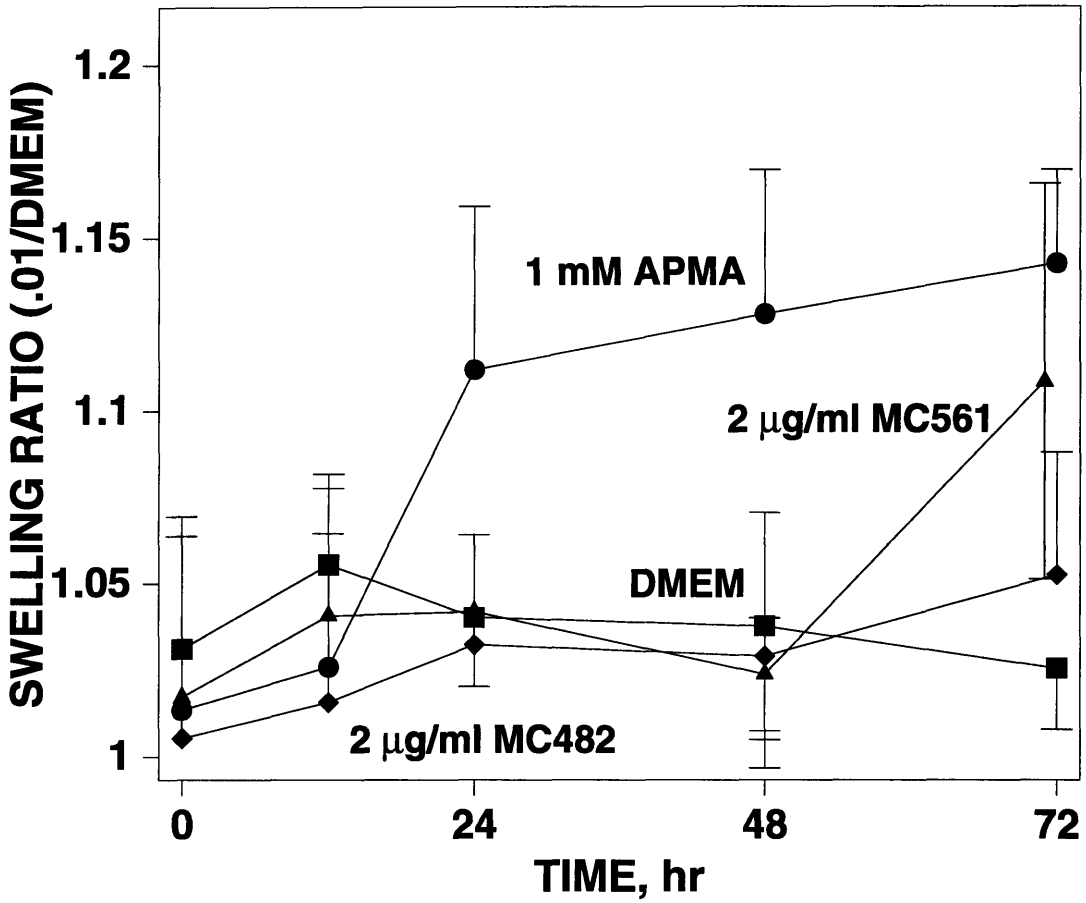

Figure D.4: Swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA plus 2 μg/ml MC561, or DMEM with 1 mM APMA plus 2 μg/ml MC482. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

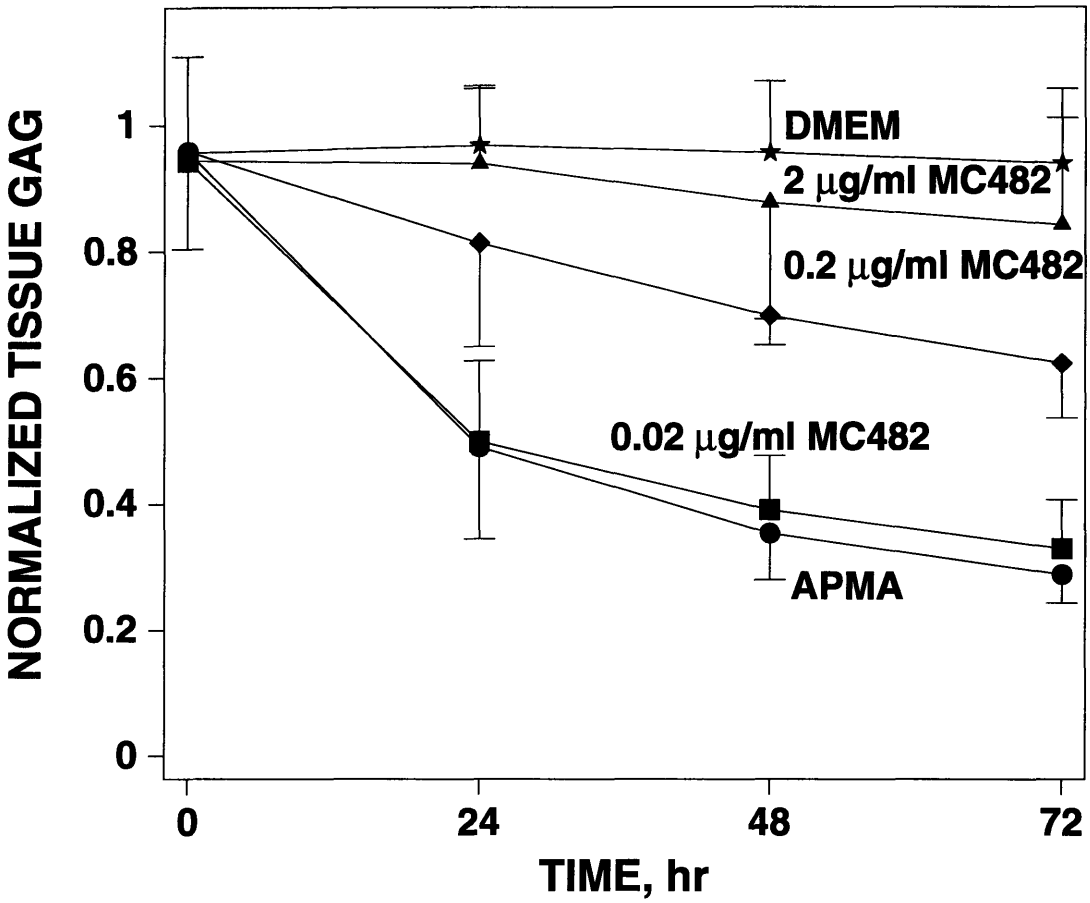

Figure D.5: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, or DMEM with 1 mM APMA plus graded levels of MC482 ranging from 0.02 μg/ml to 2 μg/ml. Data are mean ± SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

collagen, while collagenase in known to cleave type II but not type IX collagen this may indicate that type IX collagen plays an important role in maintaining collagen network integrity. While this is potentially a very interesting hypothesis, the spread of the swelling data make it difficult to make definite conclusions.

The dose response curves for MC482 (Fig D.5) and MC561 (Fig D.6) are consistent with previous data for these inhibitors gathered at high concentration. Doses of 2 and 0.02 μg/ml MC482 yield 85% and 50% inhibition of GAG loss respectively, while the 2 μg/ml MC561 yields <50% inhibition. MC482 is more than an order of magnitude more effective at inhibiting GAG loss in this system, presumably due to the more significant role of stromelysin in degrading matrix proteoglycans.

Given this data, it is logical to use k_i against stromelysin as criteria for predicting the ability of an inhibitor to prevent GAG loss. Comparing several of the inhibitors tested here (Fig D.7) demonstrates that this thought model is not sufficient to explain the observed phenomena. While MC482 was more effective at all concentration than MC561, it was not as effective as MC1, an inhibitor which was shown to be less effective against stromelysin than MC482 in solution studies [22]. In addition, TIMP, which is known to be several orders of magnitude more potent against MMPs in solution than any of the synthetic inhibitors used here was in fact less effective at preventing GAG loss than MC1. While the difference in transport characteristics between TIMP and the small peptides may account for differences in efficacy in intact tissue, it serves to emphasize the fact that there may not be a one to one correspondence between efficacy of a given inhibitor in solution and in tissue.

While inhibitor size is a factor which clearly contributes to transport characteristics, other factors, such as non-specific binding of inhibitors to endogenous proteins can dramatically alter inhibitor transport and ultimately inhibitor efficacy. In experiments assessing inhibition of APMA-induced GAG loss by MC1, the addition of 0.1% BSA to the culture media significantly decreased the level of inhibition. The level of inhibition of 2 μg/ml MC1 in the presence of 0.1% BSA was similar to that of 0.02 μg/ml MC1 with no BSA (see chapter 4). Given that MC1 does exhibit non-specific protein binding (Dr. Michael Lark,

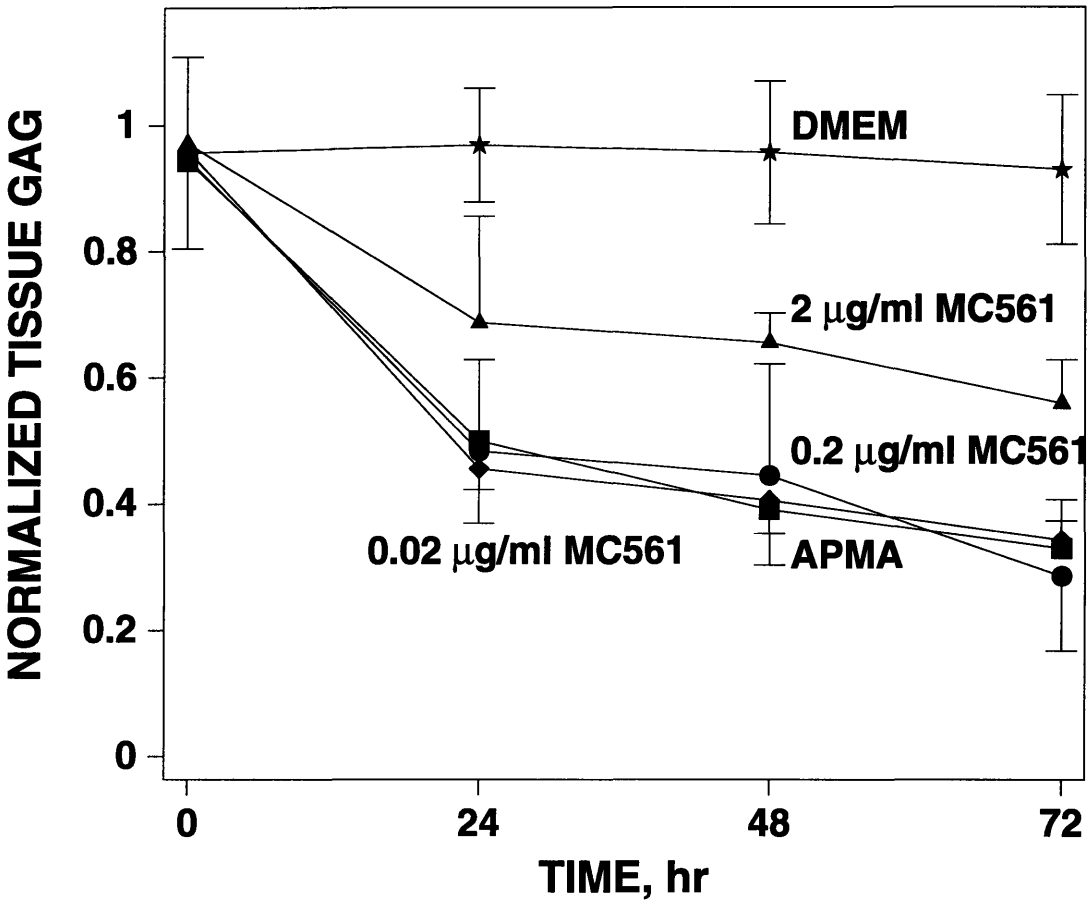

Figure D.6: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, or DMEM with 1 mM APMA plus graded levels of MC561 ranging from 0.02 μg/ml to 2 μg/ml. Data are mean ± SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

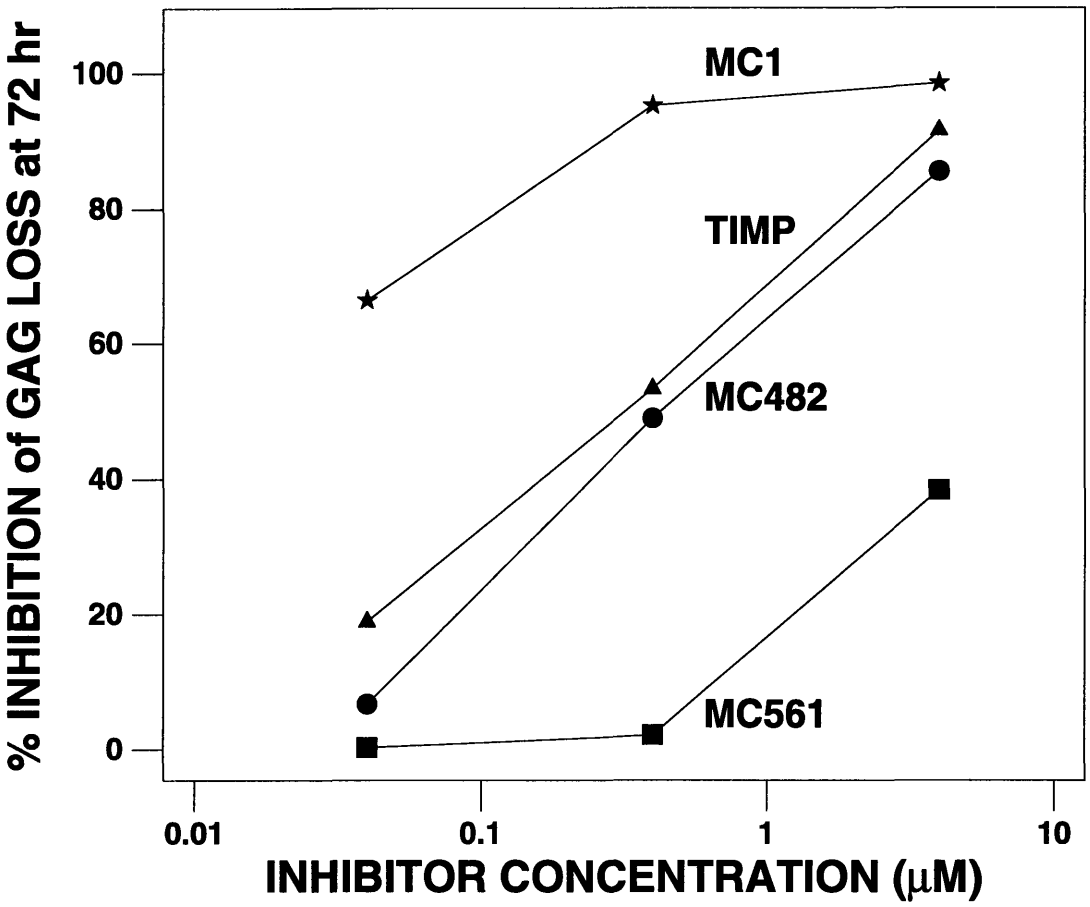

Figure D.7: Percentage inhibition of GAG loss due to APMA treatment plotted against inhibitor concentration, for inhibitors MC1, MC482, MC561, and TIMP.

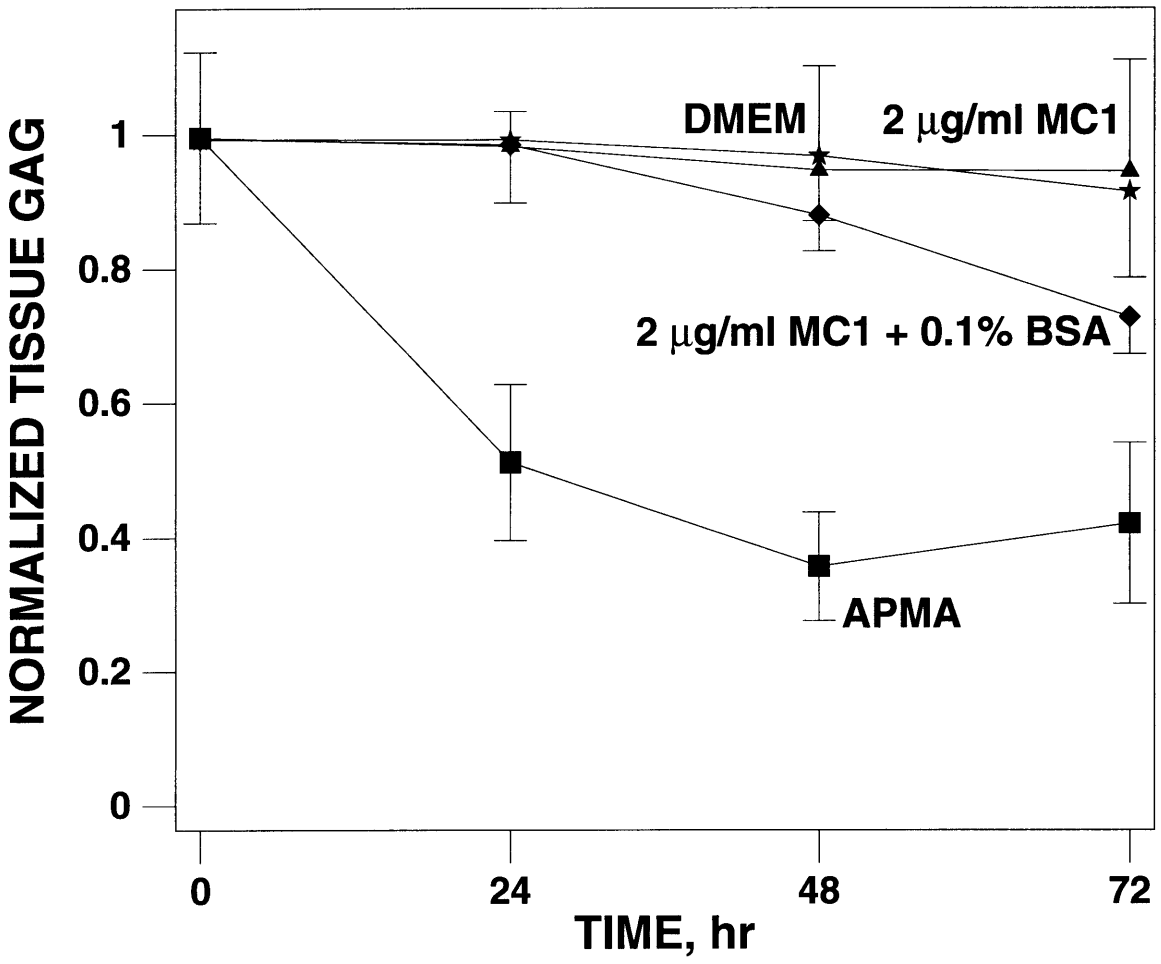

Figure D.8: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 mM APMA, DMEM with 1 mM APMA plus 2 μg/ml MC1, or DMEM with 1 mM APMA plus 2 μg/ml and 0.1% BSA. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

personal communication), it seems likely that this binding decreases the amount of peptide which is free to inhibit MMPs. This is potentially significant to inhibition of MMPs *in vivo*, where synovial fluid contains high concentrations of protein such as albumin.

The inhibition of PG loss by MC354 consistent with previous studies using MMP inhibitor to prevent degradation induced by treatment with IL-1β or RA [1,87,113]. While the inhibition observed here is not complete (Fig D.8 and D.9), it is substantial and suggests that MMPs play a role in the mechanisms of degradation in these systems. Given previous studies, it is reasonable to hypothesize that complete inhibition of GAG loss could be achieved by optimizing the level of MC354 used in the system. The difference in swelling response between IL-1β and RA treatments is consistent with previous results in these systems (Chapter V). The fact that the swelling response in these systems can be completely inhibited suggests that collagen damage induced by IL-1β treatment can be prevented more easily than damage to tissue PGs. This is similar to previous studies in which an MMP inhibitor was able to inhibit loss of tissue hydroxyproline but not GAG [1]. Andrews *at al* have hypothesized that since collagen damage generally occurs later than PG damage, it is possible that the PG matrix must be significantly degraded before collagen-degrading enzymes have access to their substrates. Consequently, the proteases which degrade collagen are more easily inhibited since they require longer times to take action.

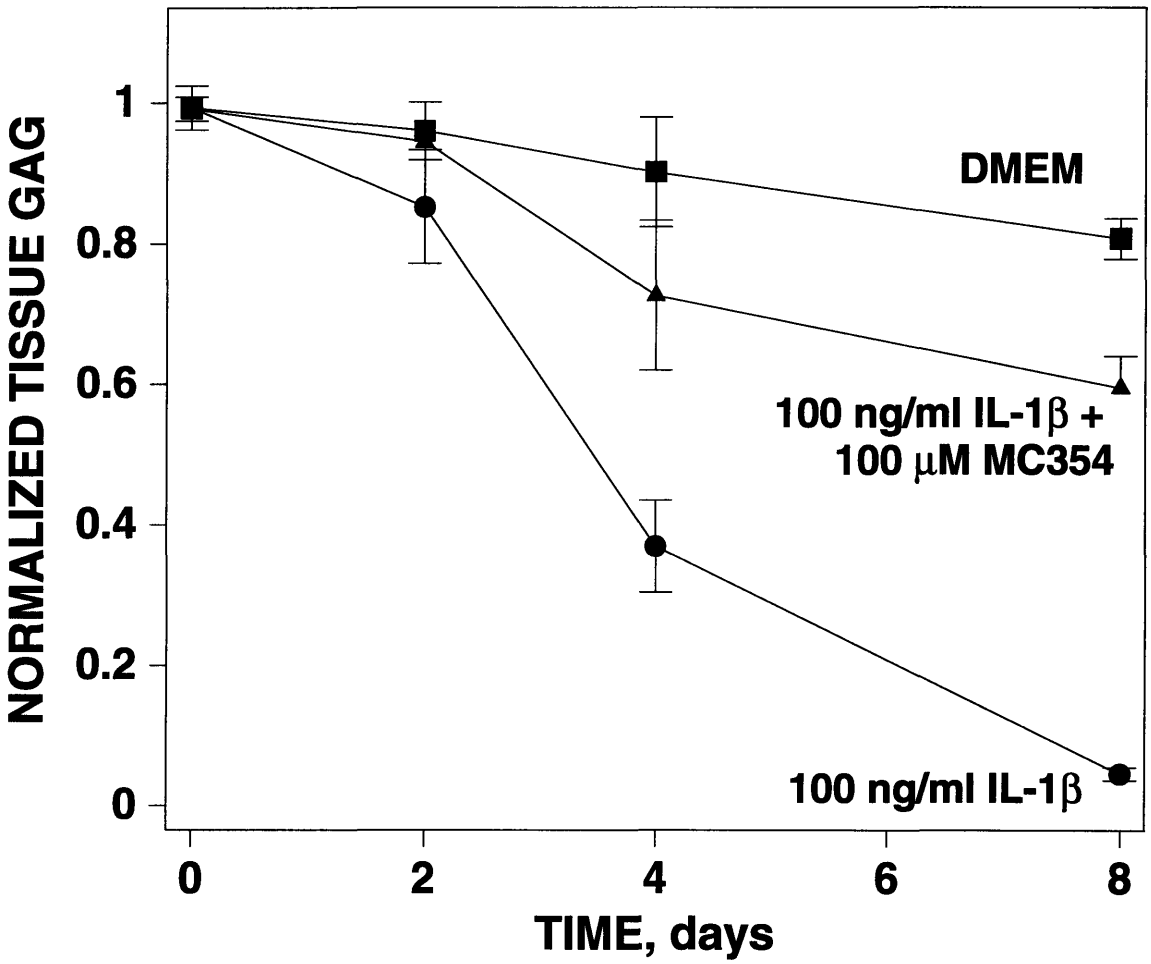

Figure D.9: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 100 ng/ml IL-1β, or DMEM with 100 ng/ml IL-1β plus 100 μM MC354. Data are mean $\pm$ SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

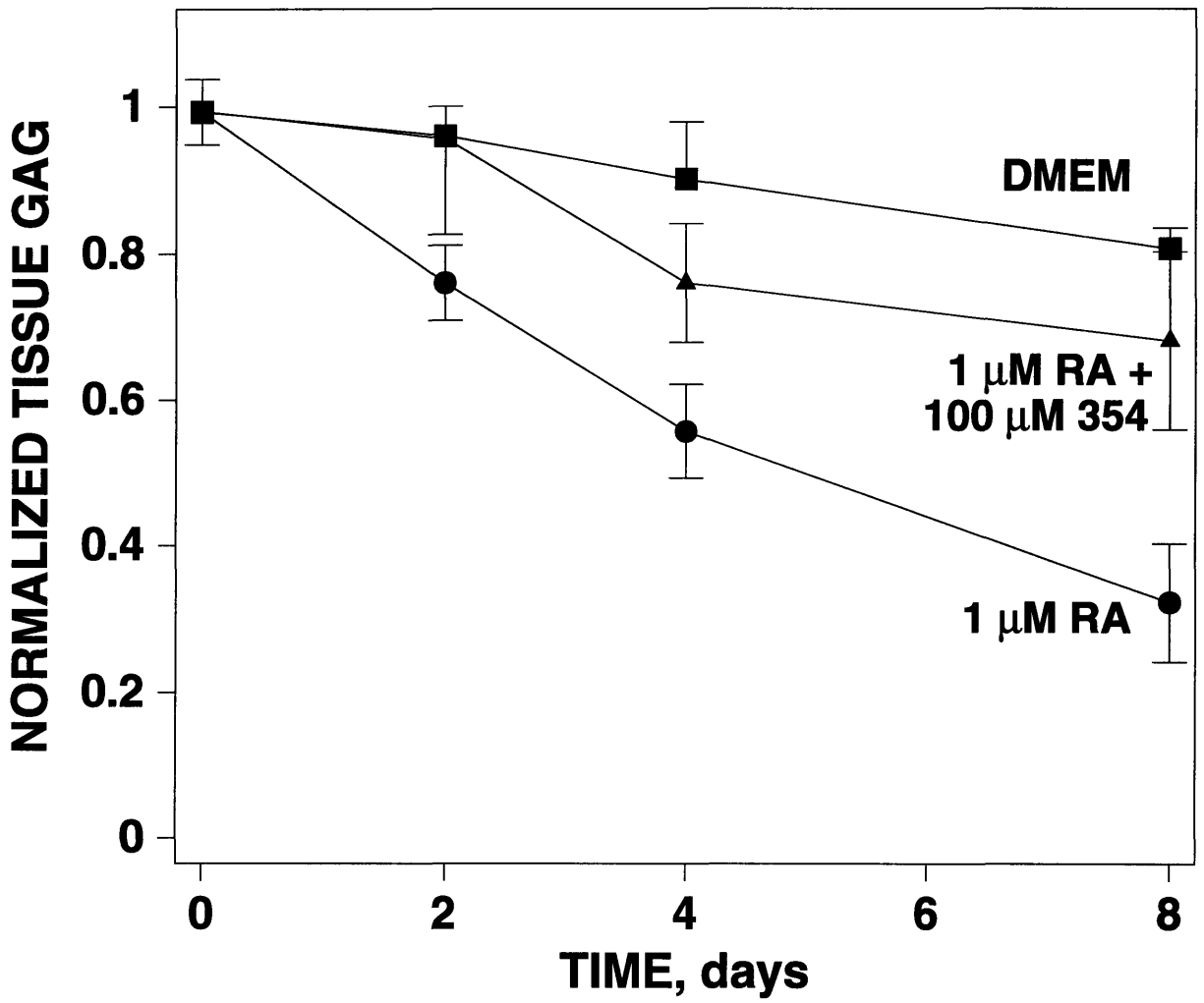

Figure D.10: Normalized GAG loss from cartilage disks incubated in DMEM alone, DMEM with 1 μM RA, or DMEM with 1 μM RA plus 100 μM MC354. Data are mean ± SD (n=4), reported as GAG remaining in the tissue normalized to total GAG (tissue + media GAG) at each time point.

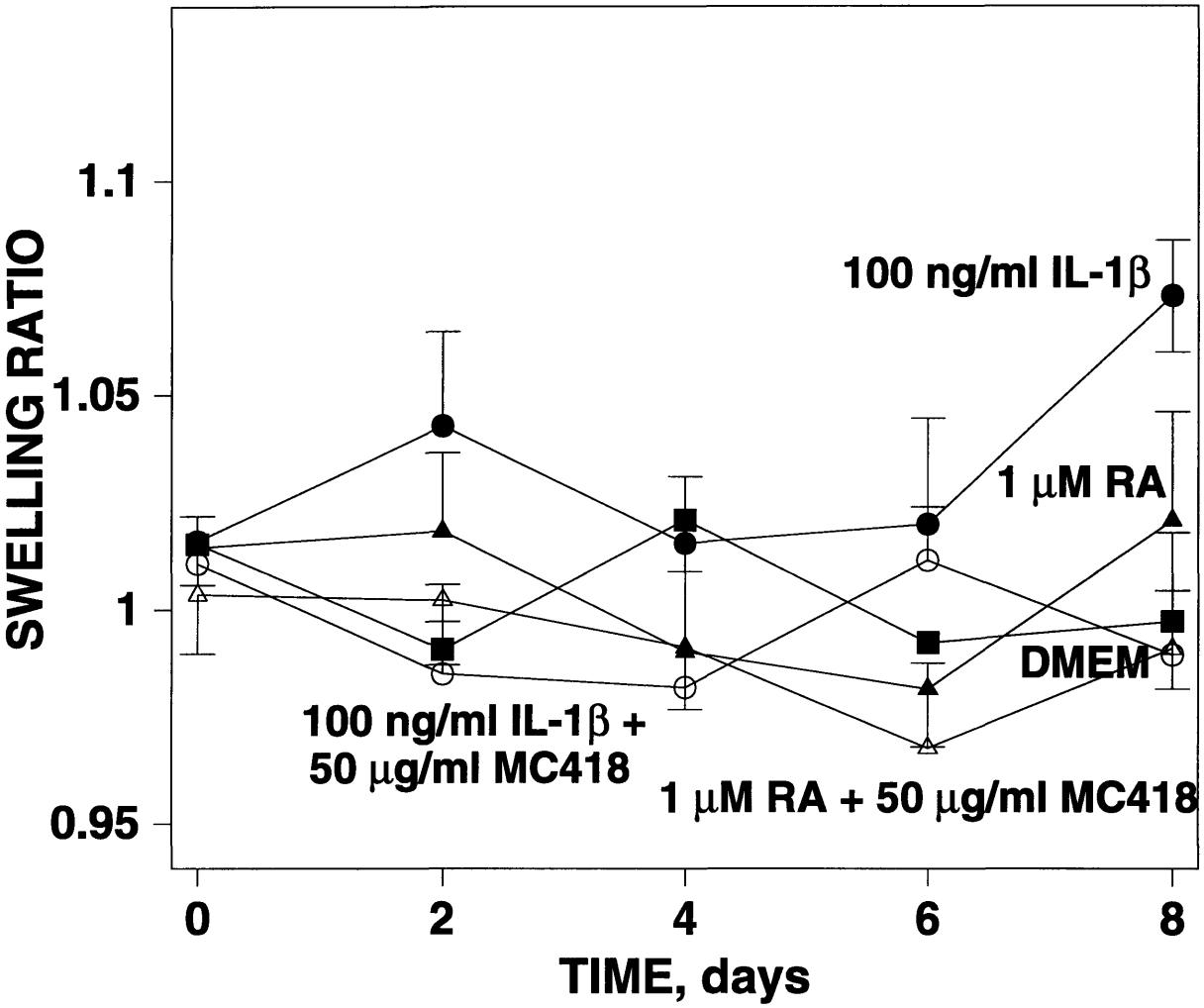

Figure D.11: Swelling ratio (wet weight in 0.01 M NaCl normalized to wet weight in DMEM) plotted against time in culture for samples incubated in DMEM alone, DMEM with 100 ng/ml IL-1β, DMEM with 100 ng/ml IL-1β plus 100 μM MC1, DMEM with 1 μM RA, or DMEM with 1 μM RA plus 100 μM MC1. Data are mean $\pm$ SD (n=4).

Appendix E

Indentation Studies

E.1 Introduction

Articular cartilage provides a load-bearing, low-friction surface that transmits and distributes stress in synovial joints. One of the earliest events in osteoarthritic degeneration of cartilage is a molecular-level alteration of the tissue's extracellular matrix involving loss of highly charged proteoglycan (PG) molecules and fibrillation and swelling of the collagen network. The decrease in tissue fixed charge density that accompanies loss of matrix PGs results in a decrease in tissue stiffness [79] and a marked decrease in cartilage's electrokinetic transduction properties. Detection of early degenerative changes, when therapeutic intervention might be most beneficial, is not presently available. We hypothesized that detection of subtle changes in cartilage electrokinetic behavior might provide a sensitive measure of early stages of OA-degeneration. It is advantageous to develop methods for non-invasive surface electrokinetic spectroscopy that can image focal degradation of cartilage during arthroscopy.

E.2 Methods

A 1 mm diameter plane-ended indentor was fabricated from plexiglas and implanted with a 50 μm silver/silver chloride electrode at the center of the indentation surface. The indentor was mounted in the Dynastat mechanical spectrometer and used to characterize cartilage on intact guinea pig stifle joints and 9 mm diameter by 1 mm thick cartilage disks explanted from the femoropatellar groove of young calves.

Guinea pig joints or bovine cartilage disks were placed in insulating chambers and bathed in phosphate buffered saline (PBS) at room temperature. The indentor was positioned at the center of the bovine cartilage disks or at several points along the guinea

pig femoropatellar groove. Sequential increments of static strain were imposed on the tissue up to 15% total compressive strain and dynamic strains of $<1\%$ amplitude were superimposed on the static strains at frequencies ranging from 0.001 to 1 Hz. The static and dynamic load signals from the load cell and streaming potential measured between the indentor electrode and a reference electrode in the PBS bath were simultaneously recorded. Measured load and streaming potential were normalized to imposed strains to give dynamic stiffness and normalized streaming potential data.

E.3 Results

The magnitude of the dynamic stiffness and streaming potential increased with frequency for both guinea pig and bovine cartilage. Values of dynamic stiffness ranged from $\sim$4 MPa at 0.002 Hz to 11 MPa at 1 Hz for both guinea pig (Fig E.1A) and bovine cartilage (Fig E.1C). Streaming potential ranged from $\sim 5\,\mu$V/% at 0.008 Hz to $85\,\mu$V/% at 1 Hz (Figs E.2A and E.2C). The phase angles of the dynamic stiffness had peaks at 0.03 Hz and 0.003 Hz for guineau pig (Fig E.1B) and bovine cartilage (Fig E.1D), respectively. Similarly the phase angles of the streaming potential had peaks at 0.03 Hz and 0.003 Hz for guineau pig (Fig E.2B) and bovine cartilage (Fig E.2D), respectively.

E.4 Summary and Discussion

Both types of cartilage exhibit a frequency response typical of poroelastic behavior. This is characterized by high and low frequency plateaus in magnitude, where the stiffness is relatively constant with respect to frequency and the phase angle is low; and a range of frequency in which there is transition from low to high frequency behavior where, the phase angle is high due to friction between and moving fluid and the stationary matrix.

The streaming potential of cartilage on the intact guinea pig joint and the bovine disk ranged from approximately $5\,\mu$V/% at 0.002 Hz to $90\,\mu$V/% at 1 Hz and exhibited frequency behavior similar to the dynamic stiffness. It is interesting to note that the peak in

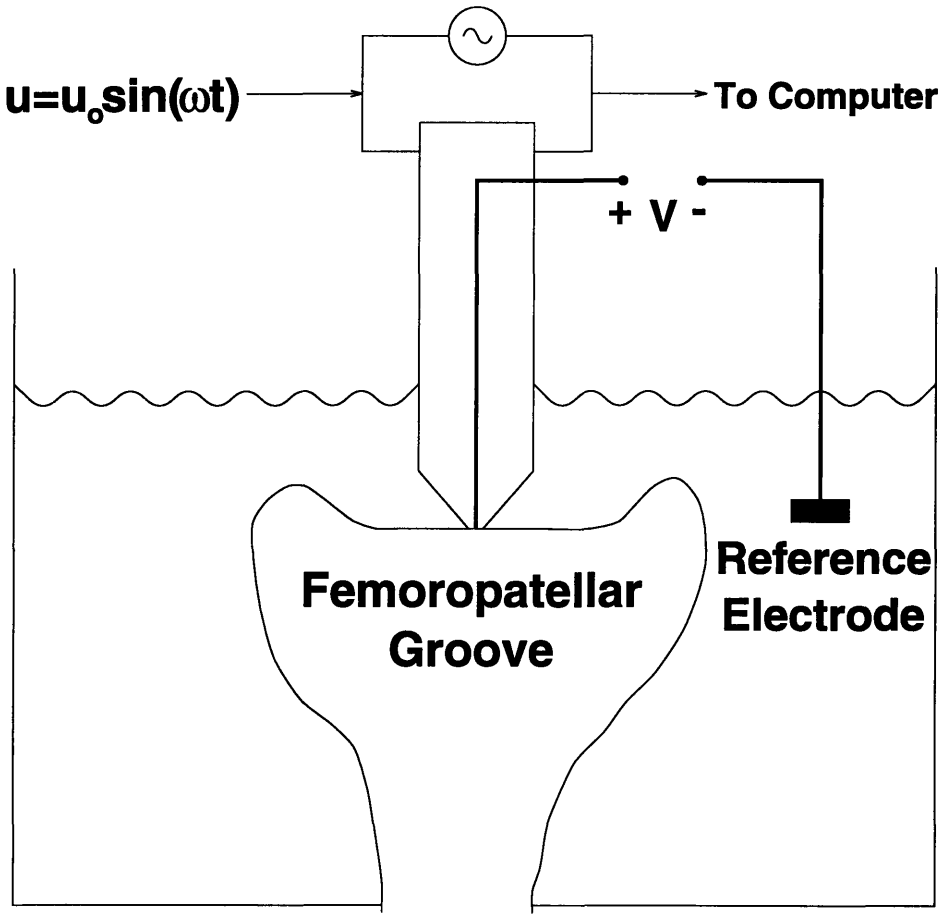

Figure E.1: Schematic illustration of experimental protocol for characterizing electro-mechanical behavior of cartilage on intact joints using a plane-ended indentor.

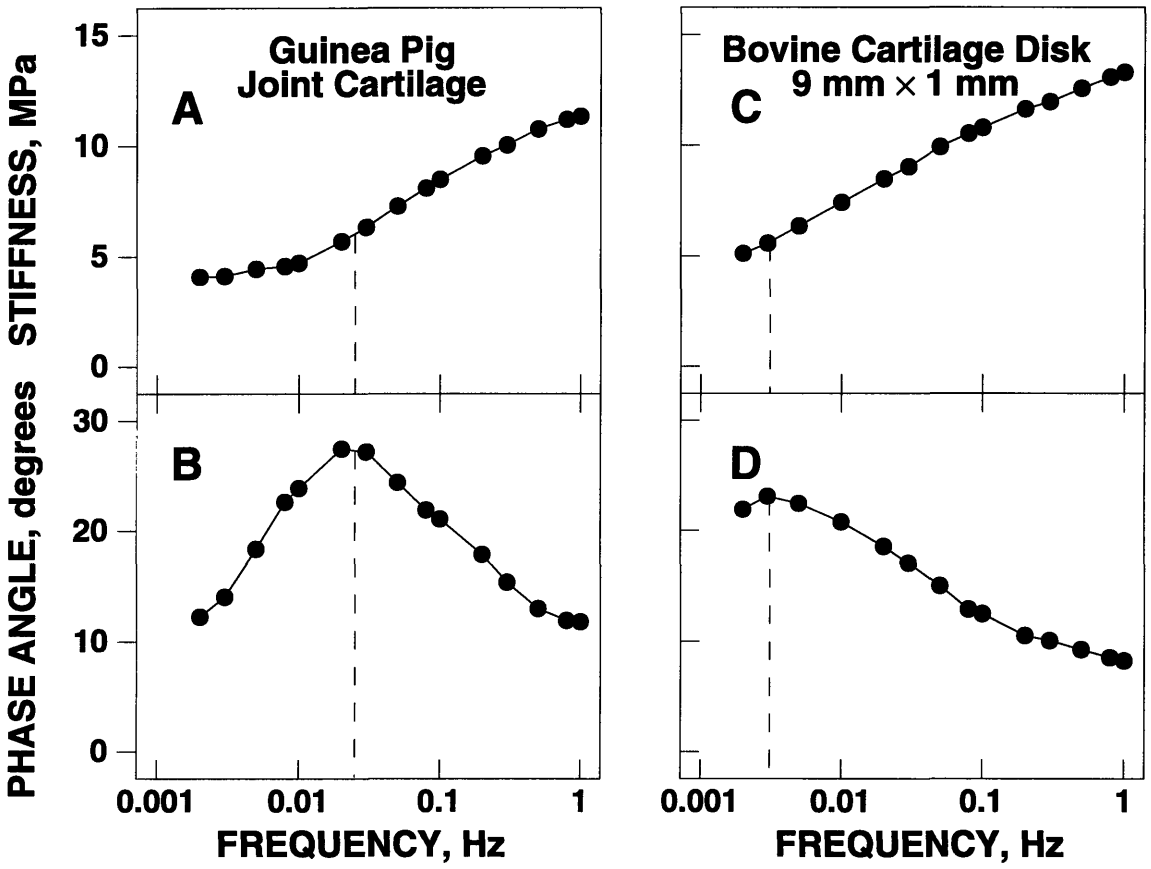

Figure E.2: Frequency response of dynamic stiffness magnitude (**A**) and phase angle (**B**) of guinea pig femoropatellar groove cartilage as measured on bone with plane-ended indentor. Frequency response of dynamic stiffness magnitude (**C**) and phase angle (**D**) of explanted bovine cartilage disk as measured with plane-ended indentor.

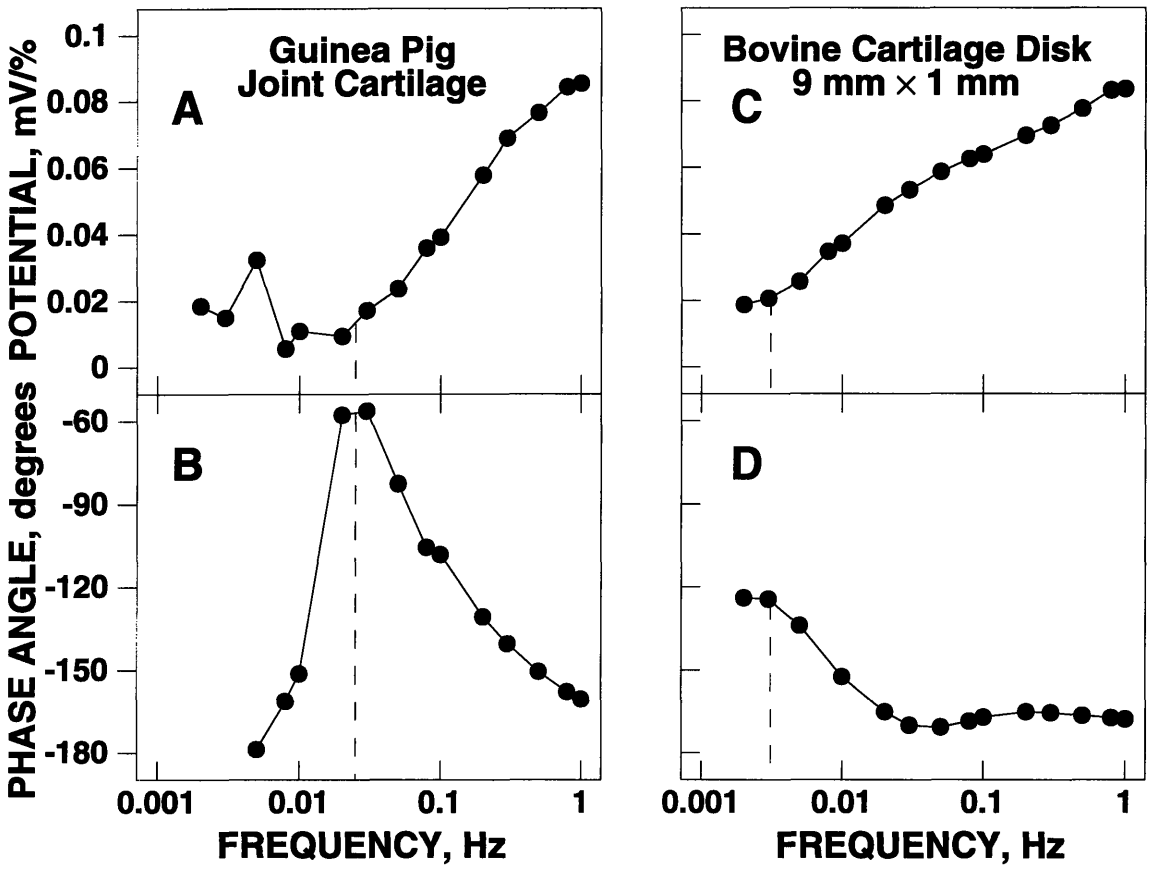

Figure E.3: Frequency response of streaming potential magnitude (**A**) and phase angle (**B**) of guinea pig femoropatellar groove cartilage as measured on bone with plane-ended indentor. Frequency response of streaming potential magnitude (**C**) and phase angle (**D**) of explanted bovine cartilage disk as measured with plane-ended indentor.

the phase angle of both stiffness and streaming potential differed significantly between the guinea pig and bovine cartilage samples. This is consistent with the difference in sample dimensions as well as tissue modulus and permeability. The thickness of the guinea pig cartilage was approximately 100 μm compared to the 1 mm cartilage disk. The increase in path length required for the fluid to leave the bovine samples pushes the peak in phase to lower loading frequencies, where the fluid has time to leave the tissue.

In addition frequency dependence of the fluid flow allows the streaming potential to be used as a detector of the spatial distribution of charge groups and thus as an indicator cartilage degradation. Previous studies using cartilage explants have shown that streaming potential measurements are capable of detecting the initial, focal degradation induced by enzymatic degradation. Studies are underway which are using this indentation device to detect small scale focal degradation of intact joints induced by enzymes and experimental models of osteoarthritis.

Appendix F

IL-1β In Vivo Studies

F.1 Introduction

The results from Chapter V and previous studies of IL-1β-induced degradation of cartilage explants motivates application of this model to an *in vivo* system. While IL-1β is known to upregulate chondrocyte expression and production of MMPs [63,64], degradation products which result in these culture systems are not those typically associated with MMP cleavage [108]. Given that APMA has been shown to be effective at activating endogenous MMPs, its use in conjunction with IL-1β should result in significant matrix degradation attributable to MMPs. The toxic nature of APMA requires that IL-1β be administered first, upregulating the production of latent MMPs. The susbsequent treatment with APMA should then activate these MMPs which are free to degrade the matrix.

F.2 Methods

6-8 week old NZW female rabbits received intraarticular injections of 100 μg of recombinant human interleukin-1β (IL-1β) in buffer into one hind stifle joint, with the contralateral control joint receiving only buffer. After 16 hours, animals received a second intraarticular injection of 1 mM APMA in both knees to activate endogenous MMPs. One hour after APMA treatment, the animals were sacrificed and joints were lavaged with 1 ml PBS. Upon sacrifice, stifle joints were isolated and stored at -70°C for up to three months. After thawing, stifle joints were dissected and 3 mm cartilage/bone cylinders were cut from femoropatellar groove using a dermal punch.

Immediately prior to mechanical testing, the cartilage was separated from the bone, with care take to ensure that articular surface was placed in the confined compression chamber in anatomic orientation (with articular surface up and bone/cartilage interface

down). Cartilage plugs were tested in uniaxially confined compression using a Dynastat mechanical spectrometer to measure the dynamic stiffness and streaming potential in the 0.005 to 1 Hz range, and equilibrium modulus, hydraulic permeability, and electrokinetic coupling coefficient, using established methods [41]. After mechanical testing, cartilage samples were lyophilized and digested with papain. Testing media and papain-digested plugs were analyzed for GAG by DMB dye binding as a measure of PG loss.

F.3 Results

Intraarticular injection of IL-1β followed by injection of APMA did not induce significant loss of GAG from femoropatellar groove cartilage compared to tissue from contralateral control joints which received injection of only APMA (Fig F.1). With values from 2-3 disks averaged for each joint, the streaming potential at 1 Hz and electrokinetic coupling coefficient of disks decreased significantly ($p<0.05$ and $p<0.05$) for joints treated with 100 ng IL-1β prior to APMA treatment (Fig F.1). Changes in equilibrium modulus, dynamic stiffness at 1 Hz and hydraulic permeability were not significant ($p>0.05$) in these animals (Fig F.1).

F.4 Summary and Discussion

The changes in properties of rabbit knee cartilage exposed to rhIL-1β *in vivo* without intravenous inhibitor resemble changes in calf explants after limited exposure to rhSLN *in vivo* [9]. After 16 hours of IL-1β exposure followed by activation of MMP by injection of APMA, there was little GAG loss and no associated change in mechanical properties, but significant decreases in high frequency streaming potential and electrokinetic coupling coefficient, consistent with PG degradation limited to the tissue *surface* [39]. Due to the size of IL-1β (18 kD), its transport through the cartilage matrix is hindered significantly. Consequently, only cells near the tissue surface were stimulated by IL-1β, resulting in the production of latent MMPs in this region. The subsequent injection of APMA likely

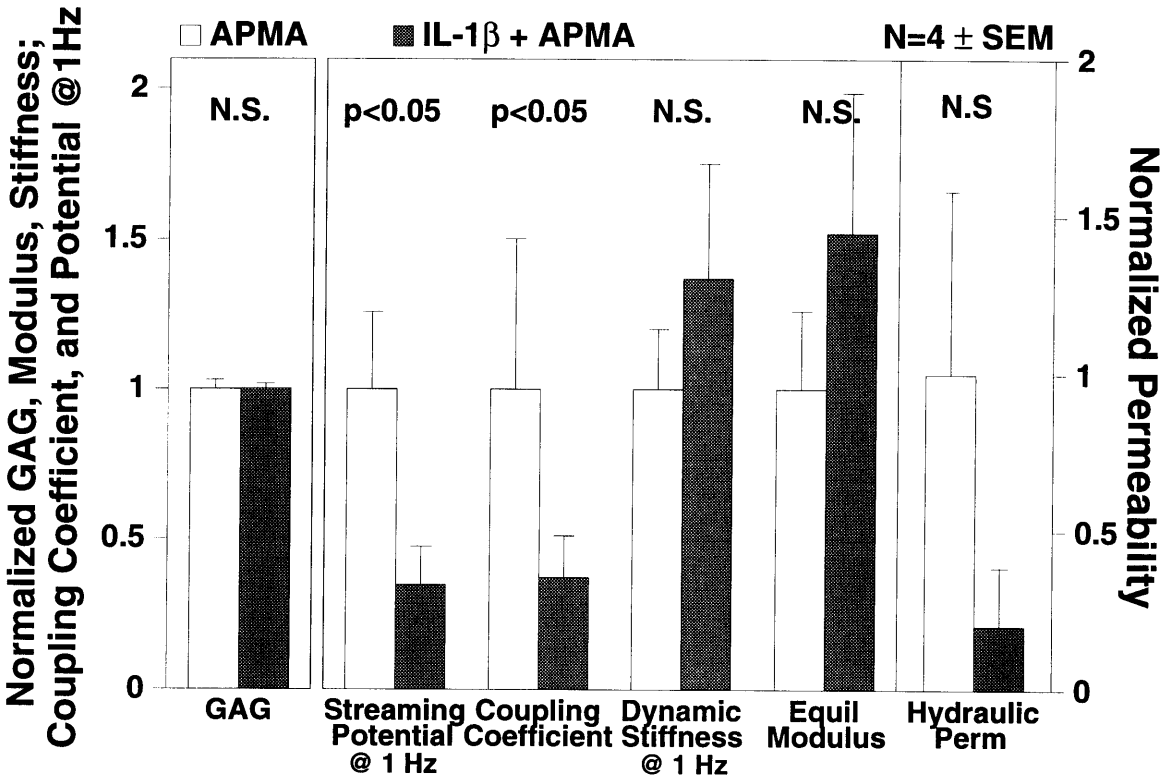

Figure F.1: Normalized streaming potential at 1 Hz, electrokinetic coupling coefficient, dynamic stiffness at 1 Hz, equilibrium modulus, and hydraulic permeability of cartilage disks from lapine stifle joints which received intraarticular injection of 100 μg IL-1β followed by 1 mM APMA and from contralateral control joints which received only 1 mM APMA. Data are mean ± SD (n=4).

resulted in activation of these proenzymes, causing matrix degradation. Previous studies in which SLN treatment caused loss of GAG in regions of the matrix near the articular surface resulted in significant changes in electromechanical behavior, but little change in purely mechanical behavior [9], similar to the results seen here.

Appendix G

IL-1β Media Fragment Analysis

G.1 Introduction

The ability of TIMP and MC354 to partially inhibit proteoglycan loss from IL-1β or RA treated samples, combined with previous reports of inhibition using synthetic MMP inhibitors [16,87,113] necessitates the characterization of proteoglycan fragments in these systems. While stromelysin cleaves at the VDIPEN-FFGVG site in the aggrecan interglobular domain, the predominant site of cleavage observed in these systems as well as in synovial fluid from human OA patients is the NITEGE-ARGSVIL site [108,111]. The activity responsible for the generation of this cleavage has been termed "aggrecanase" activity.

G.2 Methods

Cartilage disks, 3 mm diameter $\times$ 1 mm thick, were harvested from the femoropatellar groove of 1-2 week old calves using a dermal punch and sledge microtome. Groups of 4 plugs were maintained in culture at 37 °C in a 5% CO_2 atmosphere for up to 8 days in 1 ml DMEM containing either 1 mM 100 ng/ml recombinant human IL-1β, or 1 μM retinoic acid (RA) in 0.1% dimethylsulfoxide in the presence or absence of 100 μM MC354, with fresh media changes every two days.

Portions of culture media were treated with chondroitinase ABC to deglycosylate the aggrecan core protein. Degradation products of aggrecan in culture media were detected by western blot analysis. Antibodies raised against aggrecan G1 domain (anti-ATEGQV), and the NITEGE and ARGSVI epitopes in the interglobular domain [36,72] were used to detect the presence of proteoglycan fragments in culture media. Portions of culture media from days 2, 4, 6, and 8 were fractionated on SDS-PAGE and electroblotted onto a PVDF

membrane for western blot detection of aggrecan cleavage fragments.

G.3 Results

Western blot analysis using the anti-ATEGQV G1 antibody revealed the presence of the two band "aggrecanase" cleavage in media from IL-1β treated samples, running at approximately 70 kD (Fig G.1). The release of fragments was slow on days 2 and 4, and much greater on days 6 and 8. The 50 kD band, indicative of stromelysin-generated aggrecan fragments, which was present in bovine meniscus and rat chondrosarcoma A1, was not present in media from IL-1β treated samples.

The use of the anti-NITEGE antibody again demonstrated the slow release of NITEGE fragments on days 2 and 4, with more massive release on days 6 and 8 from samples treated with IL-1β (Fig G.2). The addition of MC354 to the culture media inhibited the release of these aggrecanase-generated cleavage fragments. The Western blot with the anti-ARGSVIL (BC-3 [36]) antibody shows the release of several sized fragments with the N-terminus ARGSVIL due to IL-1β treatment (Fig G.3). The 220 kD product is the intact core protein cleaved at the ARGSVIL site, while the broad bands at 150 and 70 kD are likely species with the N-terminus ARGSVIL and different C-terminii. The release of ARGSVIL fragments of all sizes was slowed by the addition of MC354 to the culture media.

G.4 Summary and Discussion

The partial inhibition by MC354 of GAG loss from IL-1β treated samples suggests that there is either a primary proteinase activity which is being partially inhibited by MC354 or that multiple proteinase activities are taking place, only some of which are being inhibited by MC354. The data from analysis with the anti-ATEGQV antibody shows that there is in fact only one fragment being generated in this system (Fig G.1) and that it is the fragment associated with aggrecanase activity. This is consistent with previous studies of similar

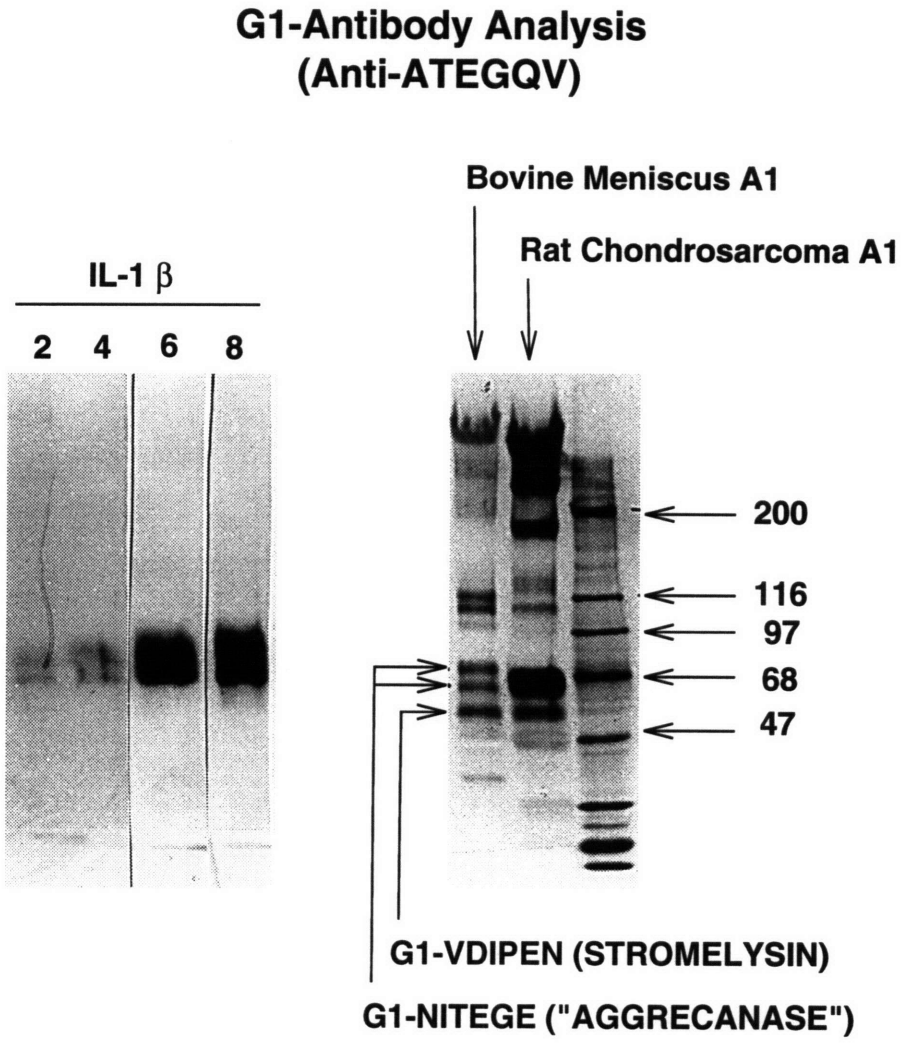

Figure G.1: Western blot analysis of media samples from IL-1β treated samples. Media from days 2, 4, 6, and 8 were analyzed with an antibody to the ATEGQV epitope in the first fold of the G1 domain. Media fractions are compared to extracts from bovine meniscus and rat chondrosarcoma A1.

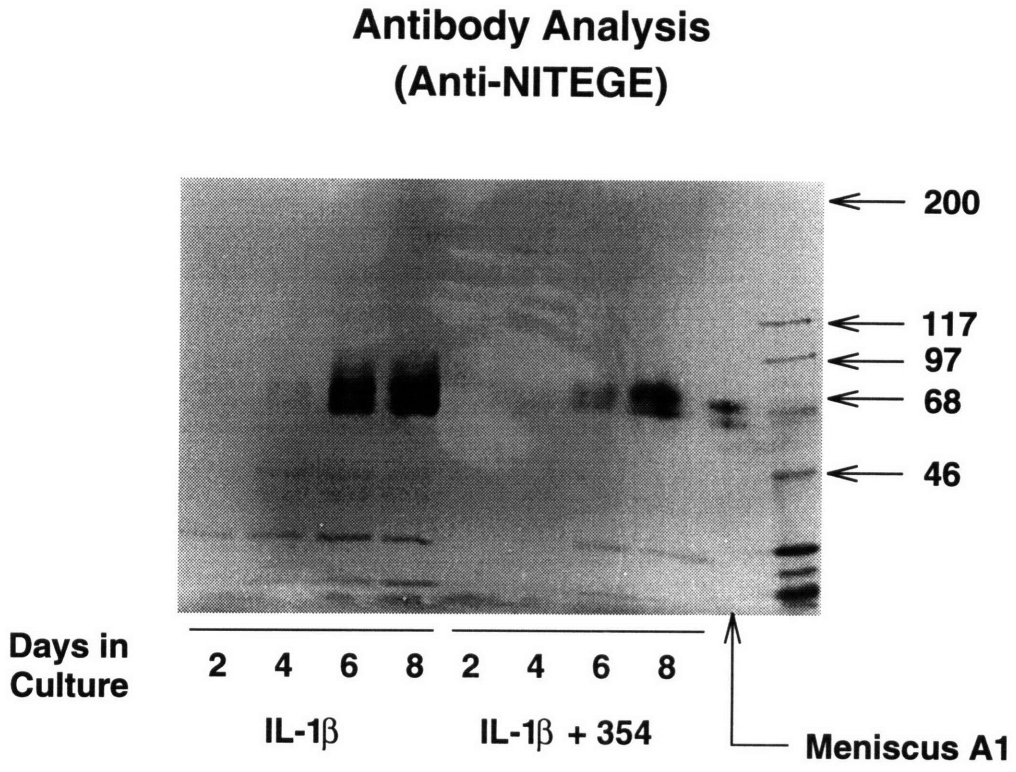

Figure G.2: Western blot analysis of media samples from samples treated with IL-1β or IL-1β + MC354. Media from days 2, 4, 6, and 8 were analyzed with an antibody to the NITEGE epitope in the interglobular domain of aggrecan. Media fractions are compared to extracts from bovine meniscus A1.

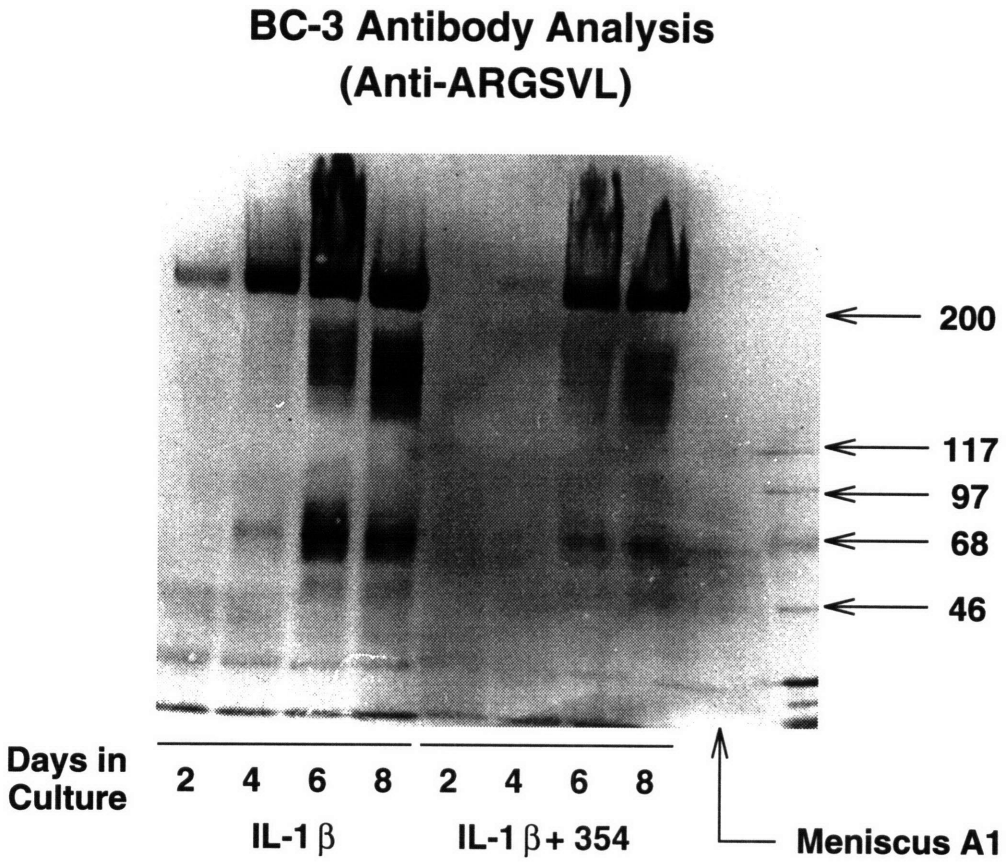

Figure G.3: Western blot analysis of media samples from samples treated with IL-1β or IL-1β + MC354. Media from days 2, 4, 6, and 8 were analyzed with an antibody to the ARGSVIL epitope (BC3) in the interglobular domain of aggrecan. Media fractions are compared to extracts from bovine meniscus A1.

culture systems [111]. Additionally, the generation of the C-terminal NITEGE fragment (Fig G.2) and the N-terminal ARGSVIL fragments (Fig G.3) are clearly inhibited by the addition of MC354 to the culture media. This implies that the mechanism responsible for this "aggrecanase" activity involves MMPs and is consistent with the suggestion of other investigators [36] that the enzyme responsible for this cleavage is an MMP.

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