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Matrix Metalloproteinase-3 In Articular Cartilage Is Upregulated By Joint Immobilization And Suppressed By Passive Joint Motion

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Abstract

Both underloading and overloading of joints can lead to articular cartilage degradation, a process mediated in part by matrix metalloproteinases (MMPs). Here we examine the effects of reduced loading of rat hindlimbs on articular cartilage expression of MMP-3, which not only digests matrix components but also activates other proteolytic enzymes. We show that hindlimb immobilization resulted in elevated MMP-3 mRNA expression at 6 hours that was sustained throughout the 21 day immobilization period. MMP-3 upregulation was higher in the medial condyle than the lateral, and was greatest in the superficial cartilage zone, followed by middle and deep zones. These areas also showed decreases in safranin O staining, consistent with reduced cartilage proteoglycan content, as early as 7 days after immobilization. One hour of daily moderate mechanical loading, applied as passive joint motion, reduced the MMP-3 and ADAMTS-5 increases that resulted from immobilization, and also prevented changes in safranin O staining. Intra-articular injections of an MMP-3 inhibitor, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH), dampened the catabolic effects of a 7 day immobilization period, indicating a likely requirement for MMP-3 in the regulation of proteoglycan levels through ADAMTS-5. These results suggest that biomechanical forces have the potential to combat cartilage destruction and can be critical in developing effective therapeutic strategies.

Keywords

MMP-3; Immobilization; Passive motion; Cartilage

1. Introduction

Joints have evolved to adequately handle a range of mechanical loading consistent with their physiologic function; however, both reduced loading and overloading of joints may have deleterious effects, particularly on their cartilaginous components (Saxon, et al. 1999,

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Vanwanseele, et al. 2002). Both acute and chronic high-intensity loads cause cartilage degeneration, which may eventually lead to osteoarthritis (Buckwalter and Martin 2006, Saxon, et al. 1999). Likewise, reduced loading, occurring commonly as a result of joint immobilization following spinal cord injuries or secondary to treatments for acute musculoskeletal injury (Jones and Amendola 2007, McCarthy and Oakley 2002), or as a result of joint diseases such as arthritis (Fontaine, et al. 2004), also creates catabolic responses within articular cartilage. Previous studies have demonstrated that *in vivo* immobilization causes cartilage thinning (Haapala, et al. 1999, Jurvelin, et al. 1986), tissue softening (Haapala, et al. 2000, Jurvelin, et al. 1986), and reduced proteoglycan content (Haapala, et al. 1999, Haapala, et al. 1996), leading to cartilage matrix fibrillation, ulceration, and erosion (Evans, et al. 1960, Hagiwara, et al. 2009). In patients with spinal cord injury, articular cartilage atrophies at a rate greater than that reported in age-associated osteoarthritis (Vanwanseele, et al. 2003). Despite numerous studies on articular cartilage changes following immobilization, the early stages of the degradative process in particular remain poorly understood. Elucidating these early events therefore remains an unfulfilled goal essential for the development of treatment strategies to prevent or limit cartilage degradation in joint diseases.

The articular cartilage consists of chondrocytes embedded in an abundant extracellular matrix (ECM) composed primarily of type II collagen and the proteoglycan aggrecan. Classic features of cartilage destruction include the degradation of both collagen fibrils and proteoglycans (Aigner and McKenna 2002). Catabolism of the ECM involves a variety of degradative enzymes, many of which are members of the matrix metalloproteinase family (MMP) (Cawston and Wilson 2006). MMPs vary in substrate specificity, primary structure and cellular localization, but many are first synthesized as proenzymes, that themselves require proteolytic cleavage for activation (Chakraborti, et al. 2003). Certain MMPs have been shown to activate others, and so may play key initiating roles in both normal ECM remodeling and pathological degradation.

In cartilage, MMP-3 not only digests many cartilage ECM components, but can also activate the pro-forms of several MMPs and also contribute to the activation of aggrecanase II (ADAMTS-5) (Cawston and Wilson 2006, Echtermeyer, et al. 2009). MMP-3 appears to be one of the few genes upregulated very early in the degeneration process (Aigner, et al. 2001), and multiple studies have shown that MMP-3 levels are elevated in various joint pathologies including osteoarthritis and acute injury (Lohmander, et al. 1993, Lohmander, et al. 1993, Walakovits, et al. 1992). Furthermore, aged MMP3^{-/-} mice show a 67% reduction in cartilage damage occurring through spontaneous osteoarthritis (Blom, et al. 2007), suggesting an important role for MMP-3 in pathological cartilage matrix degradation. Therefore, reducing pathologic MMP-3 activity could translate into substantial clinical benefit, but there is still no effective MMP inhibitor for treating arthritis.

Mechanical stimulation of human articular chondrocytes *in vitro* was shown to decrease MMP-3 mRNA expression (Millward-Sadler, et al. 2000); however, the effects of mechanical loading on MMP-3 expression in joint articular cartilage have not been explicitly investigated *in vivo*. In this study, we tested the hypothesis that joint immobilization in rats would lead to an upregulation of MMP-3, and that experimental joint mobilization would mitigate this response, indicating a chondroprotective effect.

2. Results

2.1. Immobilization induces an early and sustained increase in expression and activity of MMP-3

To determine the effect of reduced loading on MMP-3 expression *in vivo*, we immobilized the right hind limb of rats, which fixed the knee in full flexion, and found a significant increase of

MMP-3 mRNA expression 6 hours after immobilization. Elevated levels of MMP-3 were sustained until at least 21 days after immobilization, with a 10-fold increase over unimmobilized controls (Fig. 1A). As gene expression does not always correlate with protein expression, MMP activity was also measured. Similar expression patterns were observed in enzyme activity, as activity increased approximately 4.7 times the control level at 21 days (Fig. 1B).

2.2. Spatial and temporal changes in MMP-3 expression and safranin-O staining in articular cartilage following immobilization

Morphological changes due to immobilization have previously been reported to affect the lateral and medial compartments differently (Vanwanseele, et al. 2002). Therefore, we analyzed MMP-3 expression from cartilage extracts dissected from the lateral and medial condyles. In control joints, no differences in MMP-3 gene expression or enzyme activity were found between the lateral and medial femoral condyles (Figs. 2B and 2C). Following immobilization, MMP-3 mRNA and activity increased significantly above controls in both condyles; however, levels in the medial condyle were approximately 60% higher than in the lateral condyle after 21 days (Fig. 2B). Similar differences between the two condyles were also seen in MMP-3 activity; levels were 62% higher in medial than lateral condyle (Fig. 2C).

In osteoarthritis, MMP-3 expression has been shown to be depth-dependent (Tetlow, et al. 2001), but whether a similar pattern is exhibited in reduced loading conditions is unknown. Further analysis of MMP-3 mRNA levels in chondrocytes isolated by LCM from different zones within each condyle revealed depth-dependent changes in gene expression 21 days after immobilization. The highest level of expression was detected in the superficial zones, followed by the middle and deep zones of the medial condyle (Fig. 3B). In the lateral condyles, no significant zonal differences were observed. Depth-dependent expression pattern of MMP-3 protein was also observed with time after immobilization, as assessed by immunohistochemical staining (Fig. 4A).

The spatial pattern of changes in MMP-3 expression and activity in articular cartilage corresponded with changes in safranin O staining. Cartilage from immobilized limbs showed irregular patches of reduced staining, compared to stronger, more uniform staining in naïve controls (Fig. 4B). Moreover, the reduction in staining was more evident in the medial than lateral condyles. The earliest differences in safranin O staining appeared after 7 days of immobilization, and were clearly evident by 21 days (Fig. 4B). As safranin O staining in cartilage reflects tissue proteoglycan content (Rosenberg 1971), this pattern is consistent with early stages of matrix degeneration.

2.3. Passive motion loading suppresses immobilization-induced MMP-3 upregulation and histological changes in articular cartilage

Accumulating studies suggest mechanical loading at physiological levels is beneficial and might limit cartilage degradation in arthritic conditions (Ferretti, et al. 2005, Salter 2004). Therefore, we tested whether intervals of passive joint motion during hind limb immobilization would reduce cartilage degradation and inhibit MMP-3 upregulation. In this experiment, immobilized rat hind limbs were subjected to 1 hour of passive motion in the middle of 6 or 24 hour immobilization periods, or to daily 1 hour periods of passive motion during 7 days of immobilization. MMP-3 mRNA levels in the immobilized cartilage were upregulated; however, these increases were prevented in limbs subjected to passive motion (Fig. 5A). Passive motion loading also completely blocked the increase in total articular cartilage MMP-3 activity (Fig. 5B). Histological evaluation of cartilage using safranin O-fast green staining revealed that the reduced staining intensity resulting from immobilization, particularly in the superficial and middle zones, was markedly diminished in animals that had undergone passive

motion loading (Fig. 5C). Since ADAMTS-5 contributes to the depletion of proteoglycans and is activated by MMP-3 (Echtermeyer, et al. 2009), we investigated the effect of loading on ADAMTS-5 in immobilized cartilage. ADAMTS-5 was increased due to 7 days of immobilization and its expression was dampened upon passive motion loading (Fig. 5C).

To determine the relationship between MMP-3, ADAMTS-5, and proteoglycan content, we injected an MMP-3 inhibitor, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH), in 50% PBS/50% ethanol into the joint of immobilized hind limbs to suppress MMP-3. Inhibiting MMP-3 prevented increases in ADAMTS-5 as well as proteoglycan depletion, (Fig. 5C), while injections of PBS/ethanol alone did not (data not shown). This demonstrates that the downregulation of MMP-3 is required for the load-induced attenuation of ADAMTS-5 and the prevention of cartilage degradation.

3. Discussion

The concepts that physiologic loading is necessary for proper joint maintenance, that joint immobilization leads to cartilage degradation, and that joint motion can protect cartilage from degradation due to immobilization or other causes, are generally accepted (Ferretti, et al. 2005, Hagiwara, et al. 2009, Salter 1989). The results of this study are consistent with those concepts, and further support a role for MMP-3 as a mediator of cartilage breakdown resulting from immobilization. MMP-3 gene expression and enzyme activity in articular cartilage were upregulated within 6 hour following immobilization, remained elevated during a 21 day immobilization period, and exhibited a spatial distribution that was mirrored by increases in ADAMTS-5 and histological changes in cartilage suggestive of proteoglycan loss. Inhibition of MMP-3 by NNGH treatment prevented these immobilization-induced catabolic changes, demonstrating the requirement of MMP-3 in regulating proteoglycan homeostasis through ADAMTS-5.

Notably, MMP-3 mRNA levels increase within 6 hours following the onset of immobilization, suggesting that immobilization may trigger a catabolic gene expression response at an early phase under reduced loading conditions. Since MMP-3 regulates proteoglycan homeostasis, the fact that increased MMP-3 expression both precedes and spatially corresponds to the apparent proteoglycan loss in cartilage further supports that MMP-3 contributes to the initial molecular events underlying pathologic cartilage degradation. Previously, the earliest changes reported after immobilization were an increase of MMP-1 within 24 hours in antigen-induced-arthritis rabbits (Ferretti, et al. 2005), and a loss of luster on the cartilage surface after 7 days of immobilization in rats (Hagiwara, et al. 2009).

Spatial analysis of changes in MMP-3 gene and protein levels during immobilization revealed that upregulation varied not only among zones within a region of articular cartilage, but also differed between condyles. Using laser capture microdissection to precisely isolate target cells, and immunolocalization, our findings that MMP-3 is elevated mainly in the superficial zone after 21 days of immobilization, reveal a pattern that has been reported in OA cartilage, suggesting that reduced and overloading may share a common progression of cartilage degradation (Okada, et al. 1992, Tetlow, et al. 2001, Tetlow and Woolley 2001). This finding also agrees with the concept that the initial stages of OA are characterized by disorganization and loosening of collagen fibers from the superficial and upper middle zones (Arokoski, et al. 2000). The pattern of increased MMP-3 mRNA expression and activity in the medial compared to the lateral condyle expectedly corresponded to regions of reduced safranin O staining. Interestingly, morphological changes due to immobilization have previously been reported to affect the lateral and medial compartments differently. The mean articular cartilage thickness in patients who experienced complete, traumatic spinal cord injury, decreased significantly in the medial but not lateral tibia 6 months after injury (Vanwanseele, et al. 2002). In animal

models of immobilization, the cartilage thickness of the medial femoral condyle in dogs was more substantially affected compared to the lateral compartment (Haapala, et al. 2000, Haapala, et al. 1999). Moreover, in OA, which is believed to be initiated by abnormal loading (Roos 2005), there are also reports that cartilage degeneration occurs more commonly in the medial femoral condyle than the lateral condyle (Ledingham, et al. 1993). The reason why the medial compartment may be more mechanically sensitive to reduced loading is unknown, but differences in contact forces or pressures (Andriacchi, et al. 2004, Bullough 2004), and differential contact with the menisci between the lateral and medial femoral condyles (Freeman and Pinskerova 2005) may be a major causal factor for condyle-dependent susceptibility to cartilage degradation. Since the load distribution in the joint is primarily transmitted through the medial compartment during weight-bearing activities (Thambyah 2007), in reduced loading conditions, this compartment may see a greater percent decrease in stress, resulting in the greater rate of degradation that we observed.

Finally, our study indicated that a brief (1 hour) period of joint mobilization per day was able to suppress the upregulation of MMP-3 and ADAMTS-5 as well as histological changes suggesting cartilage breakdown. Mechanical stimulation of chondrocytes *in vitro* has been demonstrated to decrease MMP-3 mRNA levels within one hour of loading, a pathway involving integrins, stretch-activated channels, and IL-4 (Millward-Sadler, et al. 2000). Similarly, mechanically responsive genes such as CITED2 were shown to downregulate MMP-1 and MMP-13 expression in response to moderate fluid shear (Yokota, et al. 2003), identifying a possible intracellular signaling pathway for the chondroprotective effects of joint loading. *In vivo*, the anti-catabolic effects of physiologic loading, in the form of passive motion and moderate mechanical loading on articular cartilage have been clearly demonstrated (Salter 1989, Torzilli, et al. 2009). However, the molecular mechanisms through which this mechanical loading modality exerts chondroprotective effects are unknown. The best pathophysiologic estimates suggest that passive motion can inhibit the action and/or release of pro-inflammatory cytokines, such as IL-1 β and TNF- α amongst others, in the joint space (Ferretti, et al. 2006, Ferretti, et al. 2005). In this respect, it is particularly interesting that we observed the duration of loading required to suppress the catabolic effects of immobilization in cartilage to be relatively short. Clinically, passive motion therapies have been reported to have low compliance because they nominally involve 4+ hours of treatment, while the mean duration of therapy patients endure was 126 minutes (Kasten, et al. 2007). Our study suggests that shorter periods of loading might be sufficient for chondroprotective effects, and thus improve compliance.

In summary, we demonstrated that MMP-3 expression in rat articular cartilage undergoes a rapid and sustained upregulation during limb immobilization. The pattern of MMP-3 upregulation is non-uniform, being concentrated in the medial condyle in the superficial zone of the articular cartilage. Increased MMP-3 levels consequently contributed to increases in ADAMTS-5 expression and reduced safranin O staining. Finally, a short duration of joint loading suppressed MMP-3 and ADAMTS-5 upregulation and decreased safranin O staining in response to immobilization. Taken together, our findings provide evidence that a lack of biomechanical signals may be critical in the induction of MMP-3-mediated cartilage destruction, whereas physiologic joint loading could be used for developing effective therapeutic interventions to halt cartilage degradation in arthritic diseases. Furthermore, the application of laser capture microdissection and gene expression analysis to the model of immobilization and passive motion loading in rats may be particularly useful to reveal molecular pathways and mechanisms underlying cartilage degeneration.

4. Experimental Procedures

4.1. Animals

Male Sprague-Dawley rats (5-6 months old, weighing 580 ± 35 g) were used in this study. Rats were housed in a 12 hour light/dark environment and were given free access to food and water. Following experiments, all animals were euthanized with CO₂. All procedures were approved by the IACUC of Mount Sinai School of Medicine.

4.2. Immobilization

The right limbs of rats were immobilized as previously described (Coutinho, et al. 2002). Briefly, the rats were anesthetized with isoflurane and were fitted with a cast made of steel mesh and cotton materials which fixed the knee in full flexion. The rats were immobilized for 6 and 24 hours, and 7 and 21 days. A separate group of rats was used as naïve controls. For rats treated with NNGH during immobilization, intra-articular injections of 50μL of 5mM NNGH (Enzo Life Sciences International, Plymouth Meeting, PA) in 50% PBS/50% ethanol were given in the immobilized hind limb with a 28½ gauge needle via the patella tendon on days 1, 3, and 5 of the experiment under anesthesia.

4.3. Passive motion loading

Three groups of rats were subjected to immobilization as described above for 6 or 24 hours, or 7 days. During the middle of each immobilization period (for 6 and 24 hour groups), and daily, 12 hours after the initial application of the casts (for 7 day group), rats were anesthetized, the casts were removed, and the rats were placed on a joint loading system (Gu, et al. 2009) for 1 hour. During that period, these rats were subjected to motion loading at a frequency of two cycles per minute, with a range of motion between 65° and 115°, while a separate group of rats were maintained in the device without motion, at 115° of knee flexion (sham group). After the experimental period, all rats were either sacrificed or re-cast until the next motion loading session.

4.4. Laser capture microdissection

Distal femora were dissected, decalcified with Morse's solution (20% formic acid, 10% sodium citrate) for 2 days at 4°C, fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for 1 hour at 4°C (Shao, et al. 2006), embedded in paraffin, cut into 5-7μm thick sections and mounted on Superfrost/Plus slides. After deparaffinization, slides were air-dried and laser capture microdissection (LCM) was performed with the Arcturus Pixcell Iie (Mountain View, CA). Approximately 500 chondrocytes were microdissected from each of the superficial, middle, and deep zones of the lateral and medial condyles using the following instrument settings: Power – 85mW, Spot size – 7.5μm, Duration – 600μs-2.5ms. Captured cells were collected in microtubes containing lysis buffer (Qiagen, Valencia, CA).

4.5. RNA isolation, cDNA synthesis, real-time PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen) with DNase treatment. RNA was quantitated with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), then reverse transcribed (RT) using oligo(dT) primers. Two nanograms of total RNA were analyzed by real-time PCR with SYBR Green to assess MMP expression, as well as GAPDH and β-actin as housekeepers. PCR primers pairs used were: MMP-3, forward 5'-TCAGCGGATCTTCACAGTTG-3', reverse 5'-ACTTCAGTGCGCCAAGTTTC-3'; GAPDH, forward 5'-GAGGACCAGGTTGTCTCCTG-3', reverse 5'-ATGTAGGCCATGAGGTCCAC-3'; β-actin, forward 5'-TTGCTGACAGGATGCAGAAG-3', reverse 5'-ACATCTGCTGGAAGGTGGAC-3'.

Expression values of GAPDH and β -actin for each treatment condition were averaged and used as a denominator to determine the relative expression level of MMP-3 (Yokota, et al. 2003).

4.6. MMP activity assay

Tissues were dissected, flash-frozen in liquid nitrogen and stored at -80°C . Frozen tissue samples were pulverized (Dismembrator, B Braun Biotech, Germany) and enzyme activity was quantitated in tissue extracts using fluorogenic substrates specific for individual MMP's as described previously (Lee, et al. 2009). To measure total MMP levels in the tissue, assays were carried out in the presence of the activator 4-aminophenylmercuric acetate (APMA); endogenous active MMP levels were determined in assays carried out in the absence of APMA.

4.7. Immunohistochemistry and Safranin O staining

Immunostaining of histological sections prepared as described above was performed using monoclonal antibodies against MMP-3 and ADAMTS-5. Endogenous peroxidase activity was blocked with 3% (vol/vol) H_2O_2 for 5 minutes. Tissues were incubated with primary antibody overnight at 4°C with anti-MMP-3 (1:100 dilution, Abcam, Cambridge, MA) and anti-ADAMTS-5 (1:100 dilution, Abcam) followed by a 30 minute incubation with anti-rabbit secondary antibody (Dako, Carpinteria, CA) and visualization with TrueBlue (KPL, Gaithersburg, MD) for 10 minutes or DAB chromagen for 3 minutes. Negative control sections were prepared using irrelevant isotype-matched antibodies (Dako) in place of the primary antibody. Safranin O-fast green staining was carried out to demonstrate glycosaminoglycans in the articular cartilage.

4.8. Statistical Analysis

Results are expressed as the mean $\pm$ SEM. Statistical analysis was carried out using a one-way ANOVA and Tukey's test for post hoc analysis with significance set at $P < 0.05$.

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Figure 1. MMP-3 mRNA and activity changes in articular cartilage during immobilization. Levels of MMP-3 mRNA by RT-PCR (A) and total MMP-3 activity (B) expressed relative to levels in unimmobilized controls. Whole condyles, rather than tissue sections, were used as tissue sources for MMP-3 activity assays. Data show mean $\pm$ SEM (n=5). * = P < 0.05 versus unimmobilized control.

Figure 2.

MMP-3 expression and activity in medial and lateral condyles. (A) Sites of tissue analysis by laser capture microdissection. Sagittal sections were taken for LCM from the mid-regions of each condyle as indicated by lines. (B) MMP-3 mRNA levels expressed relative to levels in the lateral condyle of non-immobilized controls. (C) MMP-3 total activity expressed relative to activity in lateral condyles of non-immobilized controls. Data in show mean $\pm$ SEM ($n = 5$). * = $P < 0.05$ versus unimmobilized control or indicated comparison.

Figure 3. Zonal analysis of MMP-3 mRNA expression following 21 days of immobilization. (A) Safranin O stained section of cartilage indicating zones selected for analysis by Laser Capture Microdissection. (B) MMP-3 mRNA levels (expressed as copy number/ng RNA). Data show mean and SEM (n = 5). * = P < 0.05.

Figure 4. MMP-3 protein expression in medial and lateral condyles following immobilization. (A). Immunohistochemistry of MMP-3 and (B) Safranin O-fast green staining of medial and lateral condyles. Bar = 100µm.

Figure 5.

Changes in cartilage mRNA, enzyme activity and histology due to immobilization and motion loading. (A) Relative MMP-3 mRNA gene expression after 6 and 24 hours, and 7 days after immobilization, with and without motion loading. (B) MMP-3 activity after 7 days immobilization with and without motion loading. (C) Immunohistochemistry of MMP-3, ADAMTS-5 and Safranin-O staining of control, 7day immobilized, immobilized + passive motion loaded cartilage, and NNGH treated cartilage from medial condyles. Data show mean + SEM (n = 5). * = $P < 0.05$ versus unimmobilized control; + = $P < 0.05$ versus immobilization. Bar = 100 μ m.