Effects of matrix metalloproteinase-13 on extracellular matrix molecule synthesis and small GTPase activation in articular chondrocytes

Biological Sciences Honors Program in the College of Agriculture and Life Sciences

Jennifer M. Battista, Class of 2007
Lisa A. Fortier, Advisor

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Abstract

The degerative joint disease, osteoarthritis (OA), is a leading cause of disability in both humans and animals. OA involves the degradation of articular cartilage, which functions to dissipate tensile forces and cushion the joint[1]. Cartilage degeneration in OA is the result of multiple events including enzymatic degradation of matrix components and malfunction of the intracellular signaling pathways in chondrocytes [2]. Traditionally, the catabolic cytokine interlukin-1, also called catabolin, has been used to induce an OA phenotype for in vitro experiments[3,4]. Recent studies, however, indicate that matrix metalloproteinase-13 (MMP-13), also termed collagenase-3, is chiefly responsible for cartilage deterioration[2,4,5]. Though MMP-13 has been shown to play an integral role in OA, most studies to date have focused on proteoglycan cleavage mechanisms and the characterization of MMP-13 cell-surface binding and internalization [2,3,6-9]. Thus it is the broad objective of this Honors Research Project to determine the effects of equine MMP-13 on the matrix molecule expression of chondrocytes and on the activation states of the small G-proteins of the Rho family (Cdc42, Rac, RhoA), and compare the results to those achieved after treatment with IL-1. To accomplish these aims, equine recombinant MMP-13 (eqrMMP-13) was cloned, inserted into a protein expression vector, and expressed as the active enzyme. To evaluate the expression of extracellular matrix molecules, chondrocytes were plated in monolayer, treated with eqrMMP-13, and total RNA was extracted and evaluated using quantitative RT-PCR. Assessment of the effect of MMP-13 GTPase activation was accomplished by treating chondrocytes in monolayer, retaining active GTP-bound GTPases from cell lysates using a GST fusion protein containing the binding domain of a down-stream target, in pull-down assays, and analyzing the retained proteins by polyacrylamide gel electrophoresis (PAGE) and western analysis, in addition to confocal microscopy. This study showed that MMP-13 induced similar changes in matrix molecule expression as IL-1 and significantly upregulated gene expression of the catabolic factor MMP-3, while down regulating gene expression of the anabolic factor collagen II B (Col2A1). Preliminary data from the pull-down assays indicate that IL-1 and MMP-13 decrease the activation status of Rac, and increase the activation status of RhoA. Confocal microscopy images support these findings. Together the results of this study suggest utilizing MMP-13 to induce the OA phenotype is an acceptable model of cartilage degradation in vitro. This may better mimic the native articular environment, and lead to more clinically translatable results than studies using IL-1 chondrocyte cultures.

In oduction

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microenvironment and determining the types and concentration of molecules present such as growth factors and cytokines [1,12,15].

Cartilage degeneration in OA is the result of events including enzymatic degradation of matrix components and malfunction of the intracellular signaling pathways in chondrocytes [2]. The breakdown of collagen fibrils causes matrix instability while the degradation of aggrecan results in the loss of fixed charges important in maintaining the cushioning function of cartilage[2] Normally, mechanical stress and enzymatic activities in joints cause molecular damage that is compensated by the natural turnover of matrix components [1]. However if catabolic activities begin to outpace anabolic activities, the matrix degenerates and the cartilage becomes damaged [1] The initial stages of cartilage damage begin in the superficial and upper middle zones and then progress to deeper cartilage layers as OA develops in the joint. In severe disease, cartilage may be absent in some areas of the joint and lesions may extend to the bone [7]. In addition to matrix perturbations, the integrity of chondrocytes is also compromised in OA. In vitro studies of articular cartilage degradation induced by the catabolic cytokine, interleukin (IL-1), indicate signal transduction pathways mediated by the Rho subfamily of small G-proteins, or GTPases that includes Cdc42, Rac, and RhoA, are altered[16]. Thus the Rho GTPases may also be affected in naturally occurring OA. These GTPases are important to cartilage biology since they control the organization of the actin cytoskeleton and morphology is tightly linked to control of cell phenotype [17]. Cartilage is an avascular and anerval tissue, which limits its regenerative capabilities [1]. Thus, understanding the regulation of the extracellular matrix and actin cytoskeleton of chondrocytes is vital to elucidating the mechanisms involved in the development of OA.

Interleukin-1

The pro-inflammatory cytokine IL-1, also referred to as catabolin, has been used since 1980 to study the molecular mechanisms that cause cartilage degradation in OA [1,4]. IL-1 is

predominately produced by monocytes[15] in two isoforms (IL-1α and IL-1β), both of which have contributed to the current understanding of cartilage degradation [4]. An increase in the concentration of IL-1 in synovial fluids, which bathe the joint, has been observed in rheumatoid arthritis (an autoimmune disease), and to a lesser extent in OA [1]. IL-1 has been shown to induce the OA phenotype in vitro by causing a down-regulation in the expression of the anabolic matrix components aggrecan and type II collagen, and an up-regulation of catabolic factors such as matrix metalloproteinases (MMPs) and the "A disintegrin and metalloproteinases with thrombospondin type 1 motif" (ADAMTSs) [1,2]. In addition, IL-1 activates other cytokines such as IL-6 and leukaemia inducing factor (LIF) that induce synergistic effects [1]. While IL-1 certainly causes cartilage degradation and the OA phenotype in chondrocytes, resent studies do not indicate a significant up-regulation of IL-1 in naturally occurring OA[2,4,5].

Matrix metalloproteinase-13

Recent studies of catabolic mediators in naturally occurring OA suggest that collagenase-3 also named matrix metalloproteinase-13 (MMP-13), not IL-1, is principally responsible for matrix degradation[4]. Many microarray studies involving normal and OA human articular cartilage and primary chondrocytes have indicated that MMP-13 genes were up-regulated while those of IL-1 were not. In addition, immunohistochemistry experiments showed that chondrocytes in the superficial areas of OA cartilage expressed MMP-13 to a greater extent and more uniformly than IL-1[4]. Also, the overexpression of MMP-13 alone induces OA in mice[5]. Thus, utilizing exogenous MMP-13 to produce in vitro cartilage degradation may be a valuable tool in developing therapeutics.

Metalloproteinases (MMPs) constitute a protein superfamily of endopeptidases made up of closely related families of collagenases, gelatinases, and stromelysins that contain a zinc-binding motif in their catalytic sites and require calcium for full activation [6,18]. Collagenases, including MMP-13, which was discovered in the early 1990s in human breast carcinomas[19], have

the ability of cleaving all three α chains of triple helical collagen fibers. The preferred substrate of MMP-13 is type II collagen, which is cleaved into characteristic 3/4 and 1/4 fragments[7]. MMP-13 is the most potent collagenase against type II collagen and it is also observed to cleave type II collagen faster than any other member. In addition to directly degrading the matrix, MMP-13 has also been shown to activate signal transduction pathways such as ERK ½, which causes dedifferentiate of chondrocytes and activates pro-inflammatory cytokines [20]. MMP-13 has a restricted distribution in the body relative to other MMPs [14]and is principally found during cartilage development, extracellular matrix remodeling, and is highly expressed in pathological contexts including OA [21]

MMP-13 is synthesized as an inactive pro-enzyme that is activated by cleavage of a peptide at the N-terminus. MMP-13 can be activated by autoproteolytic mechanisms, several other MMPs (including MMP-2, and MMP-3), aminophenylmercuric acetate (APMA), and trypsin [6]. MMP-13 can also be activated with joint damage and the resulting products of cartilage degradation such as collagen fragments[4] and fibronectin fragments via MAP kinase cascades and PKC [22]. Once activated, MMP-13 can be regulated by tissue inhibitors of metalloproteinases (TIMPs) [20]or receptor-mediated endocytosis and degradation [13, 20]. It has been shown that OA chondrocytes have a reduced ability to bind and degrade MMP-13, further contributing to the overexpression of the protein in the joint [20].

Despite these studies that document that MMP-13 plays an integral role in producing the OA phenotype, most studies of MMP-13 focus on proteoglycan cleavage mechanisms and the characterization of MMP-13 cell-surface binding and internalization [2, 3, 7-9]. The focus of this honor's thesis was to investigate the potential of MMP-13 as an acceptable, and potentially more clinically relevant, method for in vitro studies of cartilage degradation.

Rho GTPases

Rho GTPases are a subgroup of the Ras superfamily, which in mammals comprise at least ten distinct proteins [23]. Small GTP-binding proteins cycle between a GTP bound active state, and a GDP bound inactive state. Activity of GTPases is regulated by the ratio of GTP versus GTD-bound forms within the cell [23]. Hydrolysis of GTP to GDP is catalyzed by GTPase activating factors (GAPs) while the exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs) [23,24].

Three members of Rho GTPases in particular, RhoA, Rac, and Cdc42, have been shown to be important regulators of actin cytoskeleton [23-25]. Filamentous actin can be organized into various discrete structures induced by different Rho GTPases [23]. Rac has been observed to activate the formation of lamellipodia that appear as thin protrusive sheets [23,25]. Cdc42 has been shown to induce the formation of filopodia, finger-like protrusions containing a tight bundle of long actin filaments [23,25]. RhoA causes the formation of actin stress fibers, which are actin bundles that transverse the cell and are linked to the extracellular matrix via focal adhesions [23,25].

IL-1 has been observed to up-regulate the activation RhoA, resulting in the increased formation of stress fibers while down-regulating the activation of Rac and Cdc42 in articular chondrocytes[16]. Since IL-1 has been shown to induce an OA phenotype in chondrocytes, this may suggest that in naturally occurring OA there is an increase in the formation of actin stress fibers. Morphology is tightly linked to phenotype of chondrocytes[17], thus it is of interest how MMP-13 affects the activation status of the Rho GTPases and the organization of the cytoskeleton in articular chondrocytes.

Objective

The objective of this honor's thesis project was to investigate the potential of MMP-13 as an acceptable method for in vitro studies of cartilage degradation. The Fortier Lab has shown that human recombinant MMP-13 (hrMMP-13) induced the OA phenotype in chondrocytes from

equine articular cartilage[4]. However, hrMMP-13 was synthesized in an inactive pro-enzyme form and trypsin was used to cleave the protein into its active form. Trypsin cleaved at nonspecific sites and created a mixture of inactive, active, and degraded MMP-13. In our study, equine MMP-13 was cloned, inserted into a protein expression vector to express the active protein. It was the broad objective of this honor's thesis to determine the effects of eqrMMP-13 on matrix molecule expression of chondrocytes, and on the activation states of the small G-proteins of the Rho family (Cdc42, Rac, RhoA), and to compare the results to those achieved after treatment with IL-

The specific aims of this study used complimentary approaches to identify the level of expression of extracellular matrix molecules and the activation status of Cdc42, Rac, and RhoA in equine articular chondrocytes treated with eqrMMP-13. To evaluate the expression of extracellular matrix molecules, chondrocytes were plated in monolayer and treated with eqrMMP-13, and total RNA was extracted and evaluated using quantitative RT-PCR. The effect of MMP-13 on activation status of the small GTPases was evaluated by treating chondrocytes in monolayer, retaining active GTP-bound GTPases from cell lysates using a GST fusion protein containing the binding domain of a down-stream target, and analyzing the retained proteins by polyacrylamide gel electrophoresis (PAGE) and western analysis. The hypothesis was that MMP-13 would produce similar effects as IL-1α on matrix molecule expression and activity of small GTPases, and thus be an acceptable model to study the molecular mechanisms involved with OA and better mimic the native articular environment.

Methods

Cloning and expression of eqrMMP-13 in *Escherichia coli*

Equine chondrocytes were treated with hrIL-1 141ng/μL hrIL-1α (R&D, Minneapolis, MN) for 48 hours to increase expression of MMP-13[1, 18, 21, 26, 27]Total RNA

was isolated using a monophasic solution of phenol and guanidine isothicyanate according to the manufacturer's instructions for RNA extraction from cells in monolayer (Invitrogen, Carlsbad, CA). cDNA of full length MMP-13 was constructed using gene specific ThemoScript™ RT-PCR System (Invitrogen), a primer for equine MMP-13 5` GGT TCC AGC CAC GCA TAG C 3`(Integrated DNA Technologies, INC., Coralville, IA), and the Perkin Elmer Gene Amplification PCR system 9600 set at 55° for 30 minutes. cDNA of active MMP-13 was amplified with PCR by creating a *Eco*RI site immediately upstream of the first codon after the propeptide cleavage site with the forward primer 5' GGA ATT CTA CAA TGT TTT CCC TCG AAC TCT CA 3' and reverse primer 5` TTC AGA GAG CGG CCG CTT CCT CTG AT 3`(Integrated DNA Technologies, INC.,), using Platinum *Taq* (Invitrogen) and the Perkin Elmer Gene Amplification PCR system 9600 set at 95° for 2 minutes, (95° 30 seconds, 55° 30 seconds, 72° 1 min)x 30 cycles. The PCR product was purified using the QIAquik PCR Purification Kit (Qiagen, Valencia, CA). Then the insert was ligated into the TOPO TA vector using the TOPO TA Cloning Kit (Invitrogen) and transformed into TOP 10 cells according to manufactures instructions. Next, the TOPO TA plasmid was purified with the Qiagen miniprep kit (Qiagen). Finally, the insert was restricted digested with *Eco*RI and *Not* 1 (Invitrogen) and ligated into the pGEX 4T-1 plasmid (Amersham Biosciences, Piscataway, NJ) and transformed into the JM109 strain of E. *coli* (Stratagene, La Jolla CA) with heat shock at 42° for 45 seconds.

E. *coli* pGEX plasmids express foreign DNA sequences as glutathione *S*-transferase (GST) fusion proteins, thus GST:eqrMMP-13 was expressed and purified by batch purification. The protein was resolved on a 12% PAGE (180V, 90minutes),

transferred to polyvinylidene difluoride membranes (200mA, 60 minutes), and probed using rabbit anit-MMP-13 1° antibody (Chemicon International, Temecula, CA) and donkey anti-rabbit 2° antibody (Amersham Biosciences) . The Fluorokine® E Human Active MMP-13 Fluorescent Assay (R&D, Minneapolis, MN) was then used to test the activity of the purified protein according to manufacturer's instructions, except samples were diluted by a factor of 25 with the Caibrator Diluent RD5-25 (contained in the fluorescent assay kit)

Chondrocyte culture

Cartilage was harvested from 4 horses, aged 6-1 months euthanized for reasons unrelated to this study. Chondrocytes were isolated by digestion with collagenase (Worthington Biochemicals, Lakewood, NJ) as described [28]. For the extracellular matrix molecule study, cells were plated on a 12 well plate at 75% confluence, in Ham's F-12 medium containing 10% Fetal Bovine Serum (FBS), 30mg/ml L-glutamine, 3mg/ml α-ketoglutaric acid, 5mg/ml ascorbic acid, and 0.025% HEPES. Thirty-six and 84 hours after plating, samples were treated in duplicate with hrIL-1α (10ng/mL), eqrMMP-13 (50ng/mL or 100ng/mL) in fresh Ham's F-12 media containing 2% FBS. Chondrocytes were harvested five days after plating by rinsing with 1mL 1x PBS and lysed with 1mL of a monophasic solution of phenol and guanidine isothicyanate (Invitrogen) and stored at -80°C until utilized for RNA isolation.

For the small GTPase activation study, cells were plated on 100mm plates at 95% confluencey in Ham's F-12 medium containing 10% FBS. Twenty-four hours after plating the media were exchanged for fresh Ham's F-12 medium containing 10% FBS

Seventy-two hours after plating the chondrocytes were treated with GST:eqrMMP-13 (50 ng/mL) for 2 or 30 minutes.

RNA isolation and gene expression by relative quantification RT-PCR

Total RNA was isolated using a monophasic solution of phenol and guanidine isothicyanate according to the manufacturer's instructions for RNA extraction from cells in monolayer (Invitrogen) according to the manufacturer's instructions for RNA extraction from cells in monolayer. Total RNA of duplicate samples were then reversed transcribed and amplified using a one-step relative quantification RT-PCR technique and sequence detection system (Applied Biosystems, Foster City, CA) with primers and dual-labeled fluorescent probes designed using designated software (Applied Biosystems) from equine specific sequences published in Genbank or sequenced in the Fortier laboratory. The RNA was assessed for expression of aggrecan, collagen type IIB (Col2A1), MMP-3, MMP-13, and 18s. Values for the threshold cycle (C_T) for RT-PCR duplicates were averaged. All samples were normalized relative to the 18s values by subtracting 18s C_T values from sample C_T values, tissue culture duplicates were averaged, and evaluated using the $2^{-\Delta\Delta CT}$ method as described [29].

Rac and Cdc42 activation studies

Following treatment and lysis, whole cell lysates were centrifuged at 16,100 × *g*, 4°C, for minute. Protein concentration in each sample were quantified using a Bradford assay. 1.0 mg (range .0mg-1.5mg) of each sample was loaded onto 40 μg of glutathione S-transferase- p21-activated kinase binding domain (GST-PBD) fusion proteins and

rocked at 4°C for 30 minutes. GST-PBD fusion proteins were purified by standard techniques and immobilized on glutathione-agarose beads. The glutathione beads were rinsed with the pGEX lysis buffer four times and 20 μL 5X western blot loading dye was added to the remaining pellet. Retained proteins were resolved by a 15% PAGE (180V, 90minutes) and transferred to polyvinylidene difluoride membranes (200mA, 60 minutes). Nonspecific binding was blocked with 5% milk in TBS/Tween, and the membranes were probed with 1:500 dilution of mouse anti-Cdc42 (BD Transduction Labs, San Diego, CA) in TBS/Tween for a minimum of 15 hours. The membranes were then probed with a 1:1000 dilution of sheep anti-mouse antibody (Amersham Biosciences) with 1% milk in TBS/Tween for a minimum of 30 minutes followed by 3 ten-minute washes with TBS/Tween. Western blots resolved with chemiluminescence signals were assessed using a ChemiDoc station with Quant1 Software (BioRad, Hercules, CA). Finally, membranes were stripped and re-probed with mouse anti-Rac antibody (BD Transduction Labs).

RhoA activation study

The methods used to evaluate the activation of RhoA after treatment with GST:eqrMMP-13 were similar to those of the Rac and Cdc42 activation studies. Following treatment and lysis, the whole cell lysates were centrifuged at 5000× *g*, 4°C, for 1 minute. The protein concentration in each sample was quantified using a Bradford assay. 3.5 mg of each sample was loaded onto 40 μg of glutathione S-transferase-rhotecktin-binding-domain (GST-RBD) fusion protein and rocked at 4°C for 30 minutes. GST-RBD fusion proteins were purified by standard techniques and immobilized on

glutathione-agarose beads. The glutathione beads were rinsed with lysis buffer four times. Retained proteins were analyzed as described for Rac and Cdc42 studies with PAGE and western analysis using mouse anti-RhoA primary antibody (Cytoskeleton Inc., Denver, CO).

Confocal microscopy

Chondrocytes were grown on coverslips in monolayer at 50% confluence, treated with IL-1α (10 ng/ml) or GST:eqrMMP-13 (50 ng/ml) for 0 or 2 minutes, and fixed in 4% paraformaldehyde. Chondrocytes were subsequently incubated with 0.1% triton and then 7% BSA to block non-specific antibody binding. The actin cytoskeleton was stained with Alexa Fluor 488 phalloidin (Invitrogen), and the nucleus was identified with To-PRO-3 nucleic acid stain (Invitrogen). Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen), the cells were visualized using confocal microscopy.

Statistical analysis

Results from replicate samples were averaged and expressed as a mean plus/minus one standard deviation of the four independent experiments that were performed at separate times using tissues from different animals. Data from the extracellular matrix molecule study were analyzed by a one-way analysis of variance (ANOVA) to compare values between treatments (+/-IL-1α or MMP-13), all of which were included in the model. When an ANOVA had a significant F-test, Tukey's post hoc procedure was performed with $p<0.05$ considered significant. Data from the small

GTPase activation studies will be evaluated after four independent experiments have been completed.

Results

GST:erqMMP-13

A 74 kD protein was purified from the E. *coli* transformed with the pGEX-4T-1:erMMP-13 plasmid (**Figure 1**). This protein was bound by an anti-MMP-13 antibody and was the expected size of the active enzyme (47kD)[7] plus the GST tag (23kD). In addition the Fluorokine® E Human Active MMP-13 Fluorescent Assay showed that the two batches of purified GST:erMMP-13 used in these studies contained 334 and 400 ng/ml of active MMP-13 of the total 216 μg/ml protein, respectively.

Extracellular matrix molecule expression

As hypothesized, the effects of treatment with MMP-13 generally paralleled the changes in matrix molecule expression produced by IL-1α (**Figures 2-5**). Both the 50 and 100 ng/ml doses of MMP-13 significantly increased the mRNA expression of the catabolic mediator MMP-3 (**Figure 2**). Neither 10 ng/ml IL-1α nor ≥ 50 ng/ml MMP-13 changed the level of endogenous MMP-13 expression at a significance level of $p<0.05$ (**Figure 3**).

The expression of the anabolic factor Col2A1 was significantly decreased by both ≥ 50 ng/ml MMP-13 and 10 ng/ml IL-1α (**Figure 4**). There was also no significant difference in aggrecan expression with treatment of either MMP-13 or IL-1α (**Figure 5**).

GTPase activity

MMP-13 (50 ng/ml) was sufficient to induce significant differences in matrix molecule expression, thus this dose was chosen for the GTPase activity studies. Preliminary data indicates that MMP-13 reduces the activity level of Rac (**Figures 6B**). The ratio of GTP-bound active Rac, to total Rac in the culture decreased by an average of 44% and 40% after 2 and 30 minutes of treatment, respectively (n=2). The activity of Cdc42 has been observed to be slightly increased by treatment with MMP-13 at this stage in the study (n=2) **(Figure 6B)**. As expected, MMP-13 increased the activity of RhoA after 2 and 30 minutes of treatment **(Figure 6A and B).**

Confocal microscopy

Confocal microscopy images (**Figure 7**) show that treatment with both IL-1α (10 ng/ml) and MMP-13 (50 ng/ml) induce normal cultured chondrocytes to become fibroblastic with increased numbers of actin stress fibers. This increase in stress fiber formation indicates an increase in the activation status of RhoA.

Discussion

For the first time, active recombinant equine MMP-13 was cloned, inserted into a vector, expressed as a GST fusion protein, and confirmed to be active. Thus, additional factors used to cleave the proenzyme in past studies such as APMA [6] and trypsin [4] of MMP-13, were not needed in these experiments.

The results of this study indicate that MMP-13 induced an OA phenotype in chondrocytes, and is thus an acceptable model for in vitro studies of cartilage degradation. The changes in extracellular matrix molecule expression caused by MMP-13 paralleled that of IL-1α. Both cytokines generally increased the expression of catabolic matrix factors while decreasing or unaffecting the expression of anabolic factors.

MMP-13 (≥ 50ng/ml) significantly increased mRNA expression of MMP-3 as observed in other studies of equine cultured chondrocytes[4,27]. No significant difference in MMP-3 expression was observed between chondrocytes treated with IL-1α and chondrocytes untreated or treated with MMP-13. It was surprising that IL-1α did not significantly increase MMP-3 expression because the cytokine has been shown to increase MMP-3 expression in equine cultured chondrocytes [27] and chondrocyte-synoviocyte co-culture systems[4]. The lack of such observation in this study may be due to the variation of response between different animals.

Expression of endogenous MMP-13 was not significantly increased in cultures treated with either IL-1α or exogenous MMP-13. It was unexpected that IL-1α did not increase expression of MMP-13 as such phenomenon has been well documented studies of cultured equine[27]and human chondrocytes [7, 18]. The reason this trend wasn't proven significant by statistical analysis may be due to the variability in the increase of endogenous MMP-13 observed between animals.

It has been shown that chondrocytes react differently to stimuli depending on the age of the horse[27] and their physiological state [14]. Though chondrocytes used in this study were from horses of similar ages, animal specific differences may have caused

differences in the physiological state of the chondrocytes. Treatment with exogenous MMP-13 also did not produce a significant increase in endogenous MMP-13 expression, which is consistent with observations in other studies [4]. Though MMP-13 in chondrocytes is upregulated in naturally occurring OA [7, 18], it was expected that treatment with exogenous MMP-13 would induce an auto-inhibitory affect. The lack of this observation could be the result variability between animals and the low levels of endogenous MMP-13 present in normal chondrocytes [18].

As expected with the OA phenotype, anabolic matrix factors were decreased or unaffected by IL-1 α and MMP-13. Both IL-1α and MMP-13 significantly decreased expression of Col2A1 Our observed decrease in Col2A1 expression induced by MMP-13 is consistent with the standard cartilage degradation model [15, 27].

No significant change in the expression of aggrecan, the other anabolic matrix component observed in this study, was observed with either IL-1α or MMP-13. A lack of change in aggrecan expression when a decrease in Col2A1 expression was observed following treatment with IL-1α has also been observed in other studies using cultured equine chondrocytes [27]. Thus, it is not surprising that MMP-13 also failed to induce a significant change in aggrecan expression. An absence of an observed change in aggrecan expression may also be the result of the temporal effect and differences in metabolism between cartilage and isolated chondrocytes as discussed with Col2A1 expression. The data presented in the study of extracellular matrix molecule expression following treatment with IL-1α and MMP-13 was based only on mRNA expression. While mRNA and protein levels are generally well correlated, one must not forget that

the proteins the mRNA codes for may be further regulated at the translational and posttranslational levels as well as other protein inhibitors [2]

Preliminary data also indicates that MMP-13 produces similar responses to IL-1α in the activation of small GTPases as indicated by the ratio of GTP/GDP-bound forms. Since IL-1α is the typical agent used to induce the OA phenotype in chondrocytes, and IL-1α has been shown to decrease the activation of Rac and Cdc42 while increasing the activation status of RhoA [16], it was expected that MMP-13 would cause similar changes in the activation of the Rho GTPases. Preliminary results show a decrease in Rac activation, parallel to what was observed with treatment of IL-1α [16]. At the current point in the study, a slight increase in the activation status of Cdc42 has been observed after treatment with MMP-13. This result was unexpected and additional studies are needed to verify these results. An increase in RhoA activation has also been observed. Confocal microscopy images confirm an increase in the activation status of RhoA and show a definite increase in actin stress fiber formation following treatment with MMP-13. Therefore, similar to IL-1 α, MMP-13 causes a reorganization of the actin cytoskeleton and stimulates the formation of stress fibers.

In summary, this study reports the successful cloning and expression of active equine MMP-13. Treatment with GST:eqrMMP-13 induced the OA phenotype, assessed by altered extracellular matrix molecule expression, in chondrocytes isolated from articular cartilage. Further experiments are currently being conducted to determine the effect MMP-13 has on the activation of small GTPases of the Rho subfamily and the organization of the actin cytoskeleton. De-differentiation of chondrocytes and destruction of the surrounding extracellular matrix leads to irreversible cartilage

damage[20]. Thus using MMP-13 as a model to induce cartilage degradation will better mimic the native articular environment, and this then may lead to more clinically translatable results than studies using IL-1 chondrocyte cultures. Consequently, understanding how MMP-13 affects the actin cytoskeleton via the activation status of the small GTPases of the Rho subfamily may lead to novel OA therapeutics.

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Figure legions

Figure 1: Western blot analysis of purified GST:eqrMMP-13 using rabbit anti-MMP-13 1° antibody and donkey anti-rabbit 2°antibody. WCL represents the whole cell lysate. The protein bound by the anti-MMP-13 antibody is the expected size of active MMP-13 (47kD) with the GST tag (23kD).

Figure 2: MMP-3 gene expression was significantly increased in cultures treated with ≥ 50 ng/ml GST:eqrMMP-13. IL-1α treatment did not result in a significant difference from cultures that were treated with MMP-13. Bars represent the mean of n=4, ± S.D. Letters indicate Tukey's post-hoc classification groups for those ANOVA's with significant F-tests.

Figure 3: Endogenous MMP-13 was not significantly affected by treatment with either IL-1α or exogenous GST:eqrMMP-13. Bars represent the mean of n=4, ± S.D. The ANOVA's lacked significant F-tests at $p<0.05$ between all treatment groups.

Figure 4: Collagen type IIB (Col2A1) gene expression was significantly decreased with treatment of both IL-1α and ≥ 50 ng/ml GST:eqrMMP-13. Bars represent the mean of n=4, ± S.D. Letters indicate Tukey's post-hoc classification groups for those ANOVA's with significant F-tests.

Figure 5: Aggrecan gene expression was not significantly affected by treatment with either IL-1α or GST:erMMP-13. Bars represent the mean of n=4, ±S.D. The ANOVA's lacked significant F-tests at $p<0.05$ between all treatment groups.

Figure 6: **A.** Western blot of active RhoA (RBD) and whole cell lysate (WCL) after treatment with 50 ng/ml GST:eqrMMP13 for 0, 2, and 30 minutes. "C" represents the positive control, which was derived from Cos7 cells transfected with constitutively active RhoA. "M" = MagicMark™ Western Staining (Invitrogen). **B.** Graphic representation of the ratio of active (GTP-bound) to total (GTP-bound and unbound) for Rac (n=2), Cdc42 (n=2), and RhoA (n=1) after treatment with GST:eqrMMP-13 for 0, 2, or 30 minutes. Preliminary data suggest that treatment with MMP-13 decreases the activation of Rac. The activation status of Cdc42 and RhoA after treatment with MMP-13 appears to increase.

Figure 7: Confocal microscopy images of articular chondrocytes after treatment with **A.** No treatment, **B.** IL-1α (10ng/ml), **C.** GST:eqrMMP-13 (50ng/ml). The nucleus was stained with To-PRO-3 nucleic acid stain (red), while the actin cytoskeleton is identified with Alexa Fluor 488 phalloidin (blue). Normally, cultured chondrocytes are polyhedral with a few scattered actin stress fibers as observed in the no treatment control (**A**). Treatment with either IL-1α or MMP-13 (**B, C**) results in more fibroblastic shaped cells and the formation of many cytoplasmic stress fibers, which indicate an increase in RhoA activity. Though no quantitative data were obtained, all images are representative the cells observed on their respective slides.

Figure 1

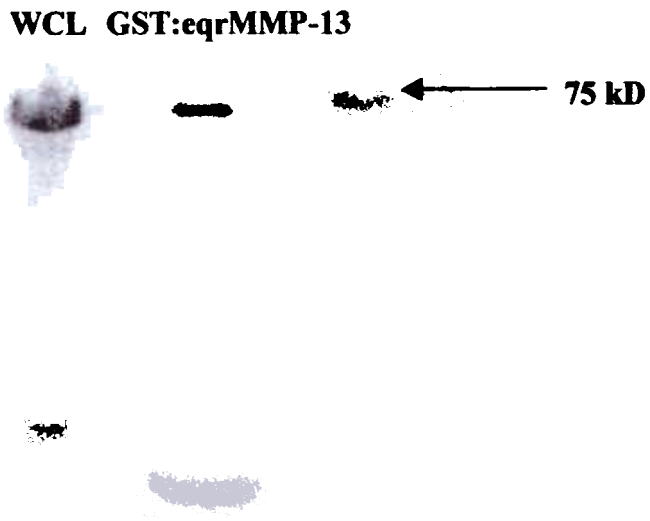
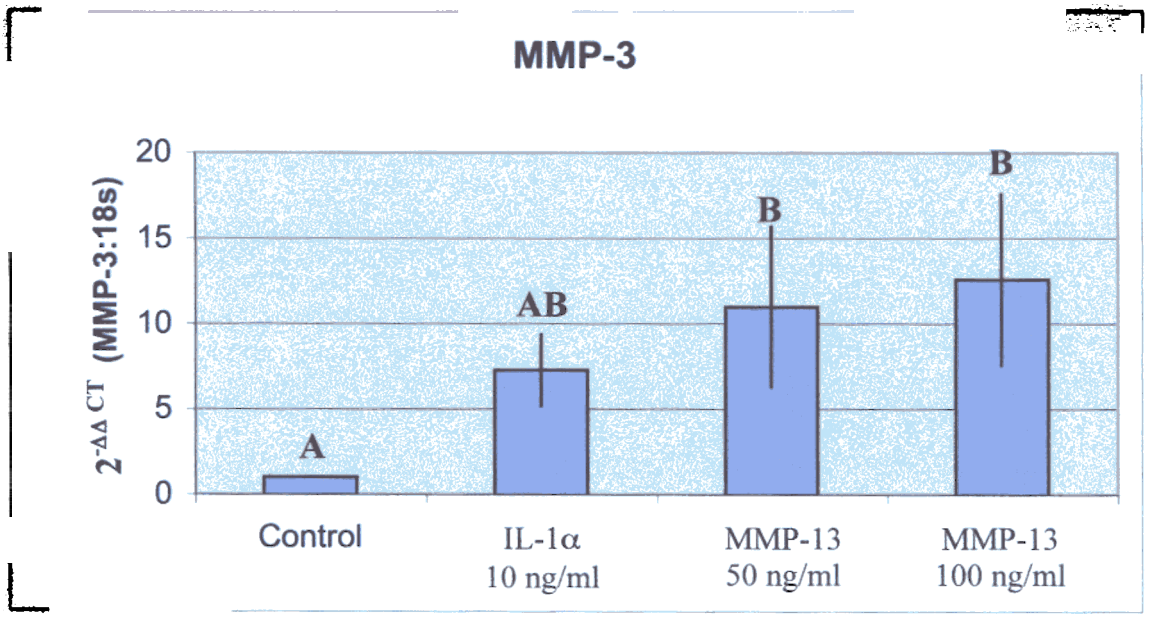


Figure 2



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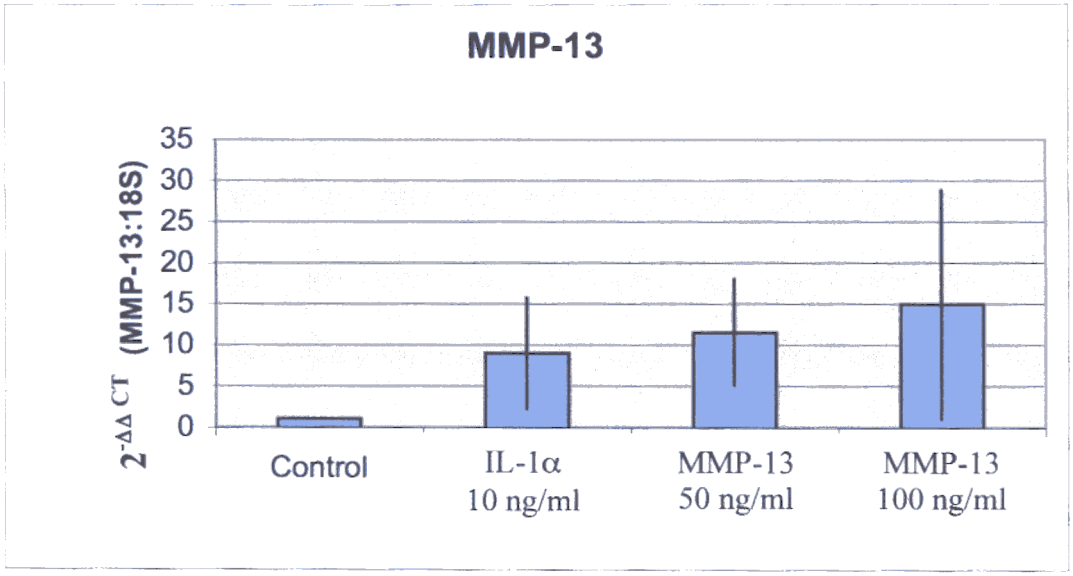

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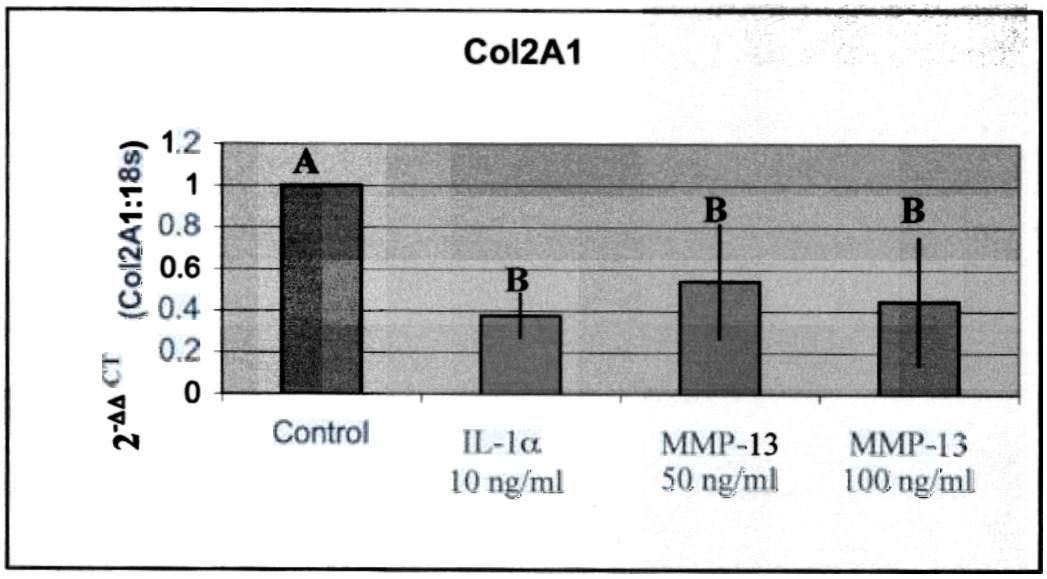


Figure 5

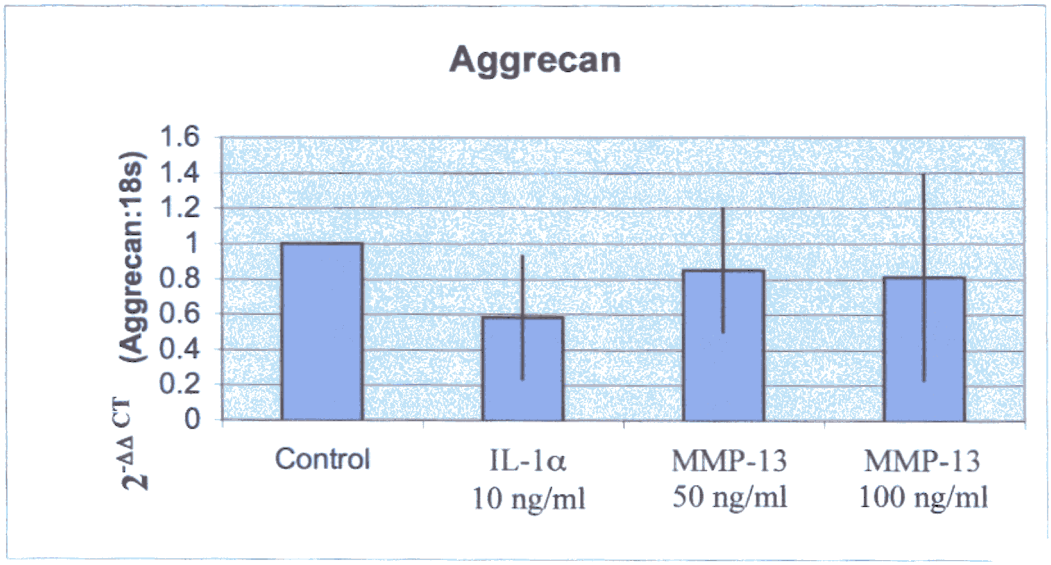
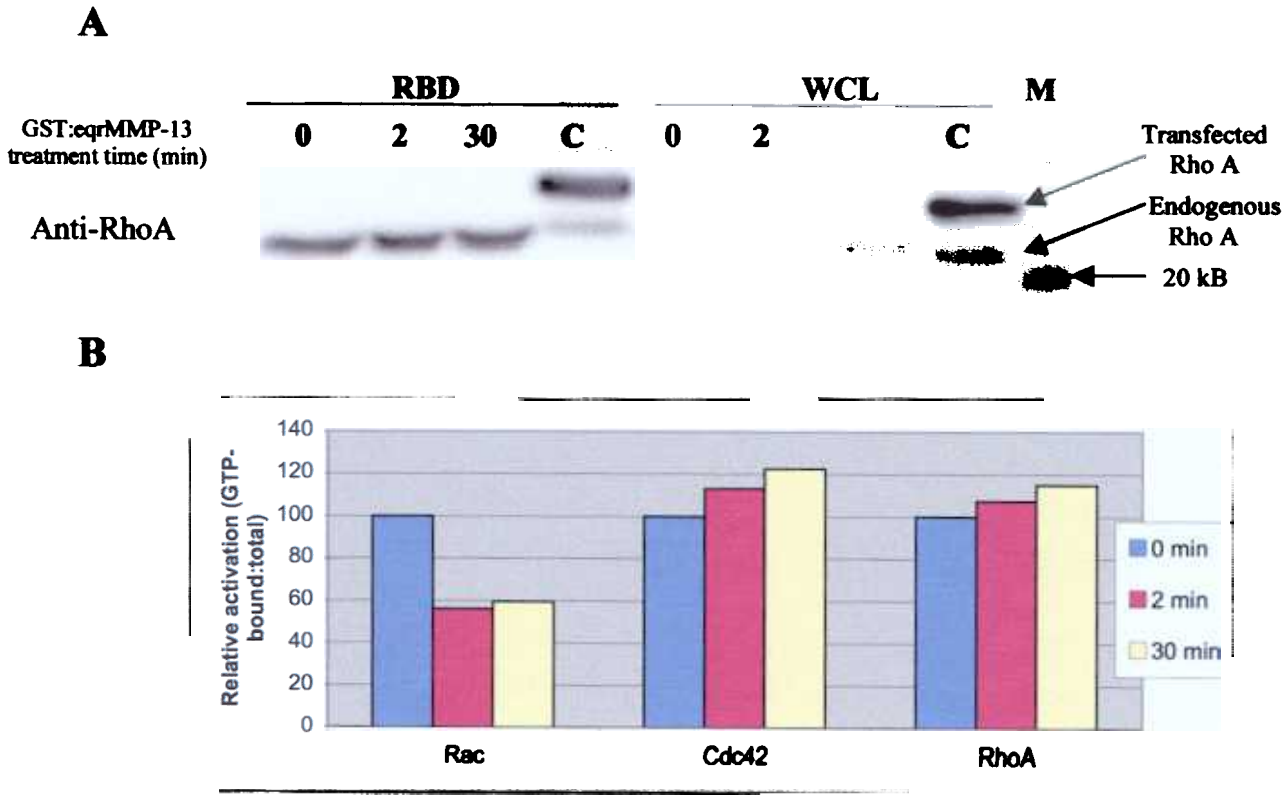


Figure 6



Figure