

## Matriptase Is a Novel Initiator of Cartilage Matrix Degradation in Osteoarthritis

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**Objective.** Increasing evidence implicates serine proteinases in pathologic tissue turnover. The aim of this study was to assess the role of the transmembrane serine proteinase matriptase in cartilage destruction in osteoarthritis (OA).

**Methods.** Serine proteinase gene expression in femoral head cartilage obtained from either patients with hip OA or patients with fracture to the neck of the femur (NOF) was assessed using a low-density array. The effect of matriptase on collagen breakdown was determined in cartilage degradation models, while the effect on matrix metalloproteinase (MMP) expression was analyzed by real-time polymerase chain reaction. ProMMP processing was determined using sodium

dodecyl sulfate–polyacrylamide gel electrophoresis/N-terminal sequencing, while its ability to activate proteinase-activated receptor 2 (PAR-2) was determined using a synovial perfusion assay in mice.

**Results.** Matriptase gene expression was significantly elevated in OA cartilage compared with NOF cartilage, and matriptase was immunolocalized to OA chondrocytes. We showed that matriptase activated proMMP-1 and processed proMMP-3 to its fully active form. Exogenous matriptase significantly enhanced cytokine-stimulated cartilage collagenolysis, while matriptase alone caused significant collagenolysis from OA cartilage, which was metalloproteinase-dependent. Matriptase also induced MMP-1, MMP-3, and MMP-13 gene expression. Synovial perfusion data confirmed that matriptase activates PAR-2, and we demonstrated that matriptase-dependent enhancement of collagenolysis from OA cartilage is blocked by PAR-2 inhibition.

**Conclusion.** Elevated matriptase expression in OA and the ability of matriptase to activate selective proMMPs as well as induce collagenase expression make this serine proteinase a key initiator and inducer of cartilage destruction in OA. We propose that the indirect effects of matriptase are mediated by PAR-2, and a more detailed understanding of these mechanisms may highlight important new therapeutic targets for OA treatment.

Metalloproteinases, especially matrix metalloproteinases (MMPs), are considered to be the most important class of proteinase in terms of cartilage degradation, because collectively they can degrade all components of this complex extracellular matrix (ECM) (1). Indeed, type II collagen is a major structural component of this ECM, and collagenolysis is an essentially irreversible step (2), making such proteolysis a major therapeutic target (3). Collagens are remarkably resistant to prote-

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Dr. Milner and Mr. Patel contributed equally to this work.

Drs. Milner, Cawston, and Rowan have submitted a patent application for matriptase as a therapeutic target for osteoarthritis.

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olysis, and relatively few enzymes are able to effect their hydrolysis. MMP-1, MMP-8, and MMP-13 are "classic collagenases," with MMP-1 and MMP-13 most strongly linked to cartilage degradation in rheumatoid arthritis (RA) and osteoarthritis (OA) (3).

Articular cartilage provides a friction-free articulating joint surface and is an unusual tissue, because it is only sparsely populated by a single cell type, the chondrocyte. These cells are responsible for ECM development and maintenance and are subject to a variety of stimuli in both normal and pathophysiologic settings. During disease states, abnormal stimuli, including abnormal loading, as well as proinflammatory stimuli such as interleukin-1 (IL-1) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (4,5) prevail, which leads to uncontrolled ECM turnover. These factors are known to be present in both RA and OA, and we have demonstrated that the IL-6 family cytokines IL-6 and oncostatin M (OSM) markedly exacerbate the catabolic potential of these mediators. Indeed, we have demonstrated synergistic, MMP-dependent cartilage catabolism *in vitro* and *in vivo* (6–9), and we and other investigators have used IL-1 plus OSM as a potent stimulus for inducing cartilage destruction.

In the last few years, the full repertoire of human proteinases, termed the degradome, has been defined (10). Studies revealed many new serine proteinases, such that there are almost as many as there are metalloproteinases. Evidence is now emerging that serine proteinases have many diverse roles in both normal and pathologic scenarios, and this also applies to destructive joint diseases (for review, see ref. 11). We have previously reported that serine proteinase inhibition with inhibitors of urokinase plasminogen activator (uPA) (12) and furin-like enzymes (13) can block cytokine-stimulated collagenolysis. Our findings have consistently supported the notion that serine proteinase pathways activate latent, inactive proMMPs (12–14); such evidence suggests that proteolytic networks exist in resorbing cartilage. These findings therefore make serine proteinases a potential therapeutic target, although the identity of specific enzymes remains elusive.

In OA, collagenolysis initially occurs around the chondrocytes (15), implicating proteinases associated with the cell surface. Using a serine proteinase-active site-specific probe, we previously identified fibroblast activation protein  $\alpha$  (FAP $\alpha$ ), an integral membrane serine proteinase. Of note, FAP $\alpha$  expression is significantly higher in OA cartilage compared with phenotypically normal articular cartilage (16,17), although the role of this transmembrane dipeptidylpeptidase has not

yet been deduced. We have now extended our initial study (16) and performed a screen of OA and normal cartilage for serine proteinase gene expression in order to identify potentially important candidates in the proposed serine proteinase pathways. This screening highlighted matriptase 1 (hereafter referred to as matriptase), one of 20 type II transmembrane serine proteases (TTSPs) (for review, see ref. 18). The biologic functions of the matriptase subfamily, which includes matriptases 1, 2, and 3 as well as polyserase 1, are poorly defined, although matriptase is known to be important in a variety of processes, including embryonic development and tumor invasion (18). This enzyme is not expressed in normal cartilage (19), while its activation and activity are regulated by the endogenous transmembrane Kunitz-type serine proteinase inhibitors hepatocyte growth factor activator inhibitor type 1 (HAI-1) and HAI-2 (20).

We propose that matriptase is an important mediator of catabolic events in diseased cartilage. Specifically, we demonstrate that the expression of matriptase and HAI-1 is increased in OA cartilage, and that the addition of matriptase to cartilage enhances collagenolysis and induces the expression of both MMP-1 and MMP-3. We show that matriptase is an activator of proMMP-1 and proMMP-3 as well as proteinase-activated receptor 2 (PAR-2). Thus, matriptase has direct and indirect roles that drive cartilage breakdown in OA.

## MATERIALS AND METHODS

**Materials.** All chemicals of the highest purity available were obtained from Sigma unless stated otherwise. All cytokines and proteins used were recombinant human. IL-1 $\alpha$  was a generous gift from Dr. Keith Ray (GlaxoSmithKline). OSM was produced in-house (21). ProMMP-3 $\Delta$ C was a generous gift from Dr. Rob Visse (Kennedy Institute, London, UK), while human matriptase (catalytic domain, residues 596–855) was prepared as previously described (22), as were proMMP-1 and proMMP-13 (23). A matriptase antibody (catalog no. IM1014), GM6001, and its negative control and the FS-6 substrate were obtained from Calbiochem. A PAR-2 antibody (SAM11, azide-free) was purchased from Santa Cruz Biotechnology. ENMD-1068 (24) was obtained from Enzo Life Sciences. The cathepsin K inhibitor L-873724 was a kind gift from Dr. Cameron Black (Merck Frosst).

**Animals.** Experiments were performed on adult wild-type (PAR-2<sup>+/+</sup>) C57BL/6J mice (body weight 25–30 gm), which were housed in standard cages with food and water available *ad libitum*, with maintenance of a thermoneutral environment. PAR-2-deficient (PAR-2<sup>-/-</sup>) mice were genetically modified as described previously (25) and were maintained under the same conditions used for the wild-type mice.

All procedures were performed in accordance with current UK Home Office regulations. OA in C57BL/6J mice was induced following surgical destabilization of the medial meniscus by sectioning of the medial meniscotibial ligament; this results in medial and posterior rotation of the medial meniscus, leading to a mild form of OA (26). Sham operations were performed in a subset of mice. The mice remained housed under the same conditions for 4 weeks, after which the knee joints were harvested for histologic examination.

**Cartilage degradation assays.** Bovine nasal septum cartilage (obtained from a local abattoir) was dissected into  $\sim 2 \times 2 \times 2$ -mm discs, plated into 24-well tissue culture plates (3 discs/well;  $n = 4$ ) in serum-free medium, and incubated for 14 days in the presence of IL-1 with or without OSM (with or without matriptase), changing the medium after 7 days, as previously described (27). Cartilage remaining on day 14 was digested with papain (27), and all samples were stored at  $-20^{\circ}\text{C}$  until assayed. The viability of cartilage explants was assessed by screening for adenylate kinase (AK) production using the ToxiLight BioAssay Kit (Lonza). No increase in AK levels with any of the treatments, including inhibitors, was observed (data not shown).

Macroscopically normal articular cartilage was obtained from patients with hip OA or from patients with fracture of the femur neck who had no history of OA and were undergoing total joint replacement surgery in hospitals in either Newcastle or Norwich. The cartilage specimens were prepared and treated as described above for bovine cartilage. Cartilage samples were pretreated for 72 hours with PAR-2 inhibitors to allow efficient tissue penetration prior to stimulation. All subjects gave informed consent, and the study was performed with ethics committee approval.

**Collagen and collagenolytic activity assays.** Hydroxyproline measurements (28) were used as an estimate of cartilage collagen, and the cumulative release was calculated and expressed as a percentage of the total for each well (29). Collagenolytic activity present in the culture media from cartilage explants was determined using a diffuse fibril assay with  $^3\text{H}$ -acetylated collagen, as previously described, where 1 unit of collagenase activity degrades 1  $\mu\text{g}$  of collagen per minute at  $37^{\circ}\text{C}$  (30).

**RNA extraction from cartilage.** Total RNA from hip cartilage samples was prepared as previously described (31); cartilage specimens obtained from patients with fracture of the femur neck were phenotypically normal and lesion-free. For experiments in which human OA cartilage was cultured prior to RNA isolation, RNA was prepared as previously reported (16).

**Real-time polymerase chain reaction (PCR) of relative messenger RNA (mRNA) levels.** For TaqMan and SYBR Green PCRs, mRNA levels for each gene were obtained from standard curves and corrected using 18S ribosomal RNA levels. Cycling conditions (7900HT system; Applied Biosystems) for the SYBR Green PCR (using TaKaRa SYBR Ex Taq premix; Lonza) were  $95^{\circ}\text{C}$  for 10 seconds, then 40 cycles at  $95^{\circ}\text{C}$  for 5 seconds, then  $60^{\circ}\text{C}$  for 30 seconds, followed by a standard dissociation curve analysis. Cycling conditions for the TaqMan PCR (JumpStart Taq ReadyMix; Sigma) were 2 minutes at  $50^{\circ}\text{C}$ , 10 minutes at  $95^{\circ}\text{C}$ , then 40 cycles of 15 seconds at  $95^{\circ}\text{C}$  and 1 minute at  $60^{\circ}\text{C}$ . The human primer/

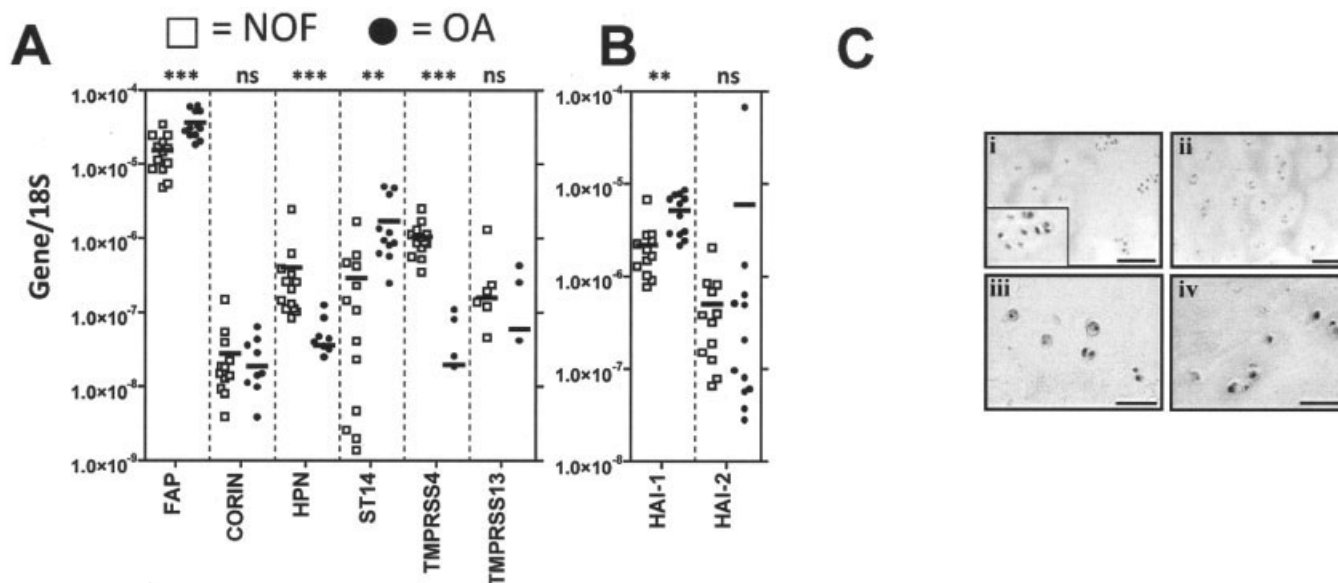
probe sequences used for the TaqMan PCR have been previously described (32).

**ProMMP activation assays.** Human recombinant and titered matriptase (catalytic domain) was incubated with equal volumes of either proMMP-1 (1:5 molar ratio) or proMMP-3 $\Delta\text{C}$  (1:15 molar ratio) at  $37^{\circ}\text{C}$  in a 50- $\mu\text{l}$  final volume. Working dilutions (25  $\mu\text{l}$ ) of each enzyme were prepared using 25 mM sodium cacodylate, 10 mM  $\text{CaCl}_2$ , 0.05% (weight/volume) Brij-35, and 0.02% (w/v) sodium azide, pH 8.0. Enzyme stocks were stored in the following: for matriptase, 50 mM Tris HCl, pH 9.0, 10% (v/v) glycerol, 1 mM  $\beta$ -mercaptoethanol, 0.4M NaCl; for proMMP-1, 20 mM Tris HCl, pH 7.2, 5 mM  $\text{CaCl}_2$ , 0.05% (w/v) sodium azide, 0.01% (w/v) Brij-35, 0.5M NaCl; for proMMP-3, 50 mM Tris HCl, pH 7.5, 0.15M NaCl, 10 mM  $\text{CaCl}_2$ , 0.02% (w/v) sodium azide, 0.05% (w/v) Brij-35, 10% (v/v) glycerol. Equal aliquots (8  $\mu\text{l}$ ) were removed at time points up to 24 hours and snap-frozen until being resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. For visualization, proteins were silver stained. For sequence determination, incubations were performed with or without GM6001 (50  $\mu\text{M}$  final concentration), proteins were transferred to polyvinylidene fluoride membrane, and bands of interest were excised after being stained briefly with 0.1% (w/v) Coomassie Blue R-250 and subjected to amino-terminal sequencing and/or mass spectrometry.

**Substrate assays.** Enzymatic assays of MMP activity were performed in 0.1M Tris HCl, pH 7.5, 0.1M NaCl, 10 mM  $\text{CaCl}_2$ , 0.05% (w/v) Brij-35, 0.1% polyethylene glycol 6000. Enzyme activity was monitored by measurement of the increase in fluorescence (excitation 324 nm, emission 400 nm) from 5  $\mu\text{M}$  Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> (FS-6) at  $37^{\circ}\text{C}$  in an LS-50B fluorometer with microplate accessory reader (PerkinElmer). Matriptase activity was confirmed using 25  $\mu\text{M}$  Boc-Gln-Ala-Arg-AMC (excitation 360 nm, emission 460 nm) in 0.1M Tris HCl, pH 9.0, 500  $\mu\text{g}/\text{ml}$  bovine serum albumin, 0.01% (w/v) Brij-35.

**Immunohistochemical analysis.** For human cartilage, tissue was frozen in ice-cold isopentane. Serial cryostat sections (10  $\mu\text{m}$ ) on 3-aminopropyltriethoxysilane-coated (2%) slides were prepared and immunostained as previously described (23). Normal rabbit serum (1.5%) was used for blocking (10 minutes), and sections were incubated with anti-matriptase antibody (1:1,000 dilution) for 90 minutes at room temperature. Washed sections were then incubated with biotinylated secondary antibody (rabbit anti-sheep IgG, diluted 50-fold in phosphate buffered saline [PBS] according to the Vectastain kit instructions) in 1.5% rabbit serum in PBS for 30 minutes, followed by incubation with avidin-biotin complex for 30 minutes using Vectastain kit PK-6105 (Vector) according to the manufacturer's instructions. Decalcified murine knee joints were embedded in paraffin wax, and, subsequently, sections (6  $\mu\text{m}$ ) were deparaffinized, rehydrated, and stained with SAM11 antibody at 1  $\mu\text{g}/\text{ml}$ , using ARK (Animal Research Kit) Peroxidase (Dako) according to the manufacturer's instructions. Signal development was achieved with diaminobenzidine tetrahydrochloride (Dako), following the manufacturer's protocol. Images were captured using a 3-CCD color video camera (JVC).

**PAR-2 activation assay.** Synovial perfusion was measured from the exposed medial aspect of the knee joint capsules of parallel groups of wild-type and PAR-2<sup>-/-</sup> mice,



**Figure 1.** A and B, Elevated matriptase expression in osteoarthritis (OA) cartilage. Gene expression levels of several membrane proteinases (A) or hepatocyte growth factor activator inhibitor type 1 (HAI-1) and HAI-2 (B) (analyzed in a separate set of samples, where  $n = 12$  for both OA and neck of femur fracture [NOF]) were assessed in hip cartilage obtained from patients with OA ( $n = 13$ ) or in normal control cartilage obtained from patients with NOF ( $n = 12$ ). Levels were determined as described in Materials and Methods and normalized to the level of 18S ribosomal RNA. Since all primer/probe combinations amplify with essentially equal efficiencies, gene expression levels are directly comparable within each separate set of samples. All detectable data points are shown; horizontal lines show the means. NS = not significant. \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ , OA versus NOF, by Mann-Whitney 2-tailed U test. C, Detection of matriptase and proteinase-activated receptor 2 (PAR-2) in human OA cartilage. Specimens were embedded in OCT compound, and 10- $\mu\text{m}$  sections were obtained using a cryostat. Matriptase was detected in 3 separate cartilage samples using rabbit antimatriptase polyclonal antibody (panels i–iii), and PAR-2 was detected using SAM11 monoclonal antibody (panel iv). Panels iii and iv show the same sample. Boxed area in panel i shows a higher-magnification view. Bars = 100  $\mu\text{m}$  in panels i and ii and 50  $\mu\text{m}$  in panels iii and iv.

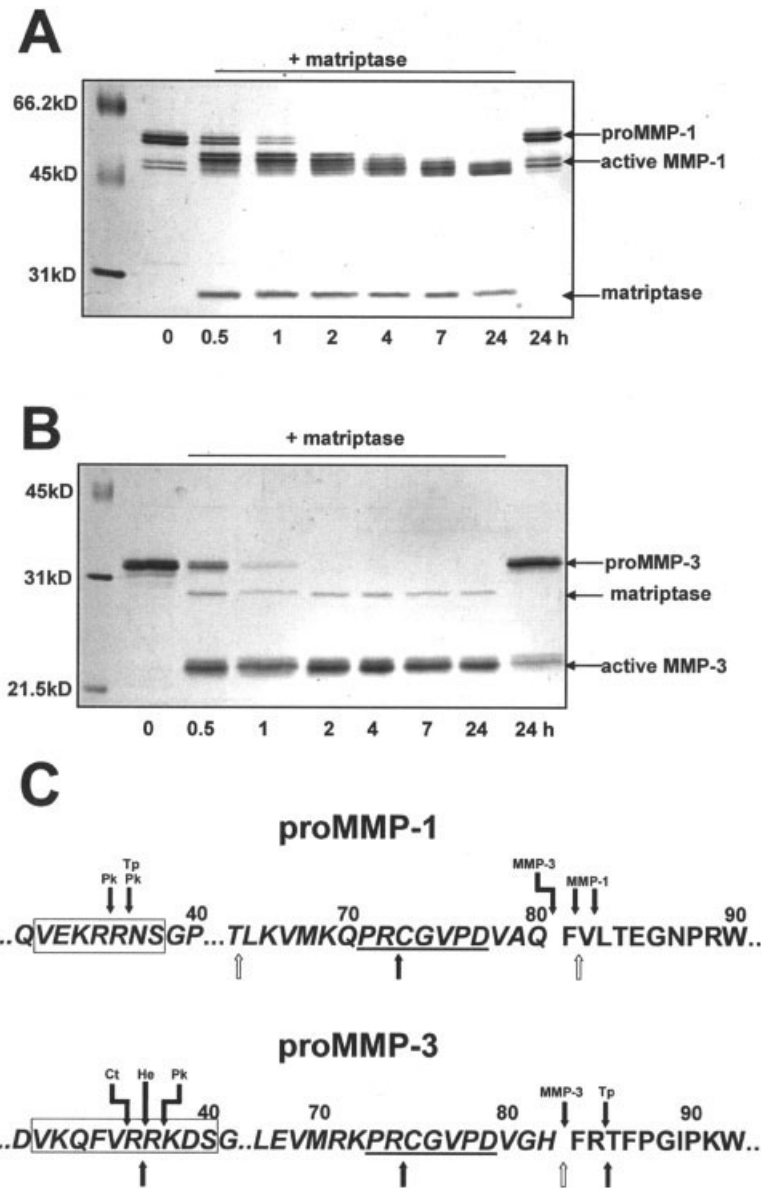
using high-resolution laser Doppler imaging (Moor Instruments) as previously described (33). A series of scans were obtained immediately following topical application of saline vehicle, and once a stable baseline was achieved, a further series of scans was obtained immediately after topical administration of matriptase. Images were later analyzed by dedicated software to obtain median flux values over the knee joint region. Vascular perfusion responses (in arbitrary perfusion units) are presented as the percentage change from baseline. The carotid artery was cannulated to allow blood pressure monitoring.

**Statistical analysis.** Significant differences between patient groups were determined using a Mann-Whitney 2-tailed U test. Standard TaqMan experiments were performed at least in triplicate for a minimum of 3 separate samples, with data analyzed using Student's 2-tailed *t*-test. Cartilage experiments were performed in quadruplicate for 3 different cartilage specimens, and significance was assessed using analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test, using commercial software (SPSS, v15.0). Blood flow measurement data were similarly assessed by ANOVA. For clarity, only selected comparisons are presented in some figures.

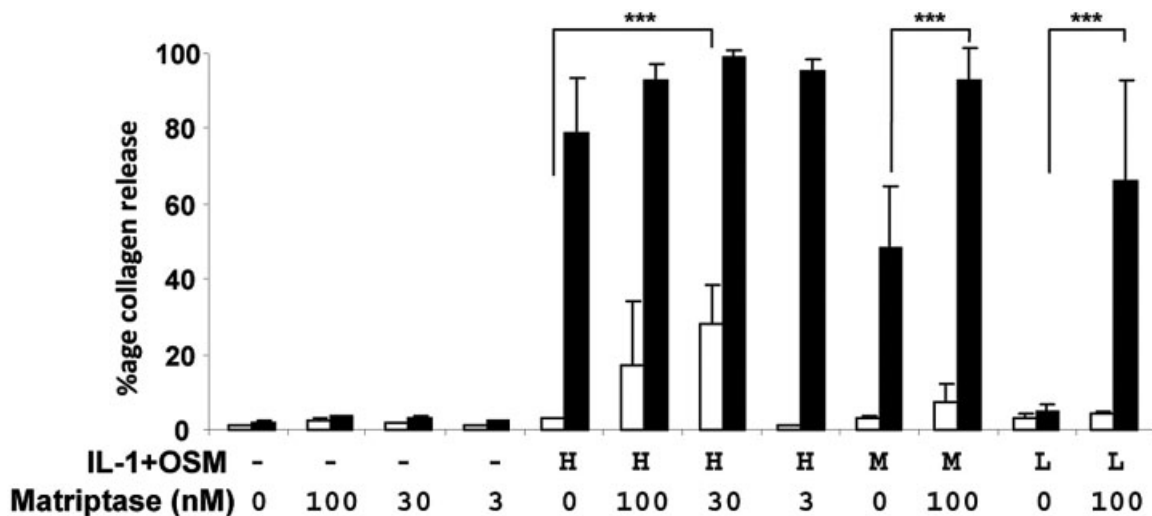
## RESULTS

**Elevated matriptase gene expression in OA tissue.** Analyses of total RNA from OA joint tissue and normal joint tissue obtained from patients with femur neck fracture revealed a statistically significant increase in matriptase (ST14) gene expression in OA cartilage ( $P = 0.0039$ ) (Figure 1A). In a separate experiment, HAI-1 gene expression, but not HAI-2 gene expression, was also significantly elevated ( $P = 0.0039$ ) in OA cartilage (Figure 1B). The increase in matriptase gene expression was reflected in detectable matriptase protein in several OA cartilage specimens (Figure 1C, panels i–iii); PAR-2 was also detected in OA cartilage (Figure 1C, panel iv).

Of the TTSP genes assessed, none other than matriptase was up-regulated in OA cartilage, although both HPN (hepsin;  $P = 0.00006$ ) and TMPRSS4 (MTSP2;  $P = 0.00002$ ) were markedly suppressed in OA cartilage compared with normal cartilage (Figure 1A).



**Figure 2.** Cleavage sites in the propeptide regions of matrix metalloproteinase 1 (MMP-1) and MMP-3 of matriptase. Recombinant human proMMP-1 or proMMP-3ΔC was incubated with recombinant human matriptase (molar ratios 1:5 and 1:15, respectively) for various lengths of time. **A** and **B**, Products generated from proMMP-1 (**A**) and proMMP-3ΔC (**B**) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. **C**, Mass spectrometry and N-terminal sequence analyses were also performed on the 1–4-hour incubations. The data presented include several known cleavage sites within the propeptide regions (shown in italics); residue numbering is for the proform (for review, see ref. 35). Boxed areas show the “bait” region, and the cysteine-switch regions are underlined. Cleavage sites marked with upward-pointing closed arrows are for matriptase (plus GM6001); open arrows mark sites that are matriptase-generated in the absence of GM6001. Pk = plasma kallikrein; Tp = trypsin; Ct = chymotrypsin; He = human neutrophil elastase.



**Figure 3.** Enhanced cytokine-induced cartilage collagenolysis by matriptase. Bovine cartilage explants were incubated with interleukin-1 (IL-1) plus oncostatin M (OSM) with or without matriptase (100 nM unless indicated otherwise) for 14 days, with fresh medium, cytokines, and reagents used on day 7, as appropriate. The concentrations of IL-1 plus OSM used are as follows: high (H; 1 ng/ml plus 10 ng/ml), medium (M; 0.6 ng/ml plus 6 ng/ml), and low (L; 0.25 ng/ml plus 2.5 ng/ml). Collagen release by day 7 (open bars) and cumulative day-14 release (closed bars) were determined by hydroxyproline measurement and expressed as a percentage of the total for each treatment. Bars show the mean and SD results from at least 3 separate experiments ( $n = 4$  samples). \*\*\* =  $P < 0.001$ , IL-1 plus OSM plus matriptase versus IL-1 plus OSM.

These TaqMan Low Density Array data for cartilage were validated by the significantly elevated expression of FAP $\alpha$  expression ( $P = 0.0004$ ), as has been demonstrated previously (16,17). Furthermore, significant elevations of several other serine proteinase genes were detected, including high-temperature requirement A1 peptidase (HtrA1), PRSS23 (SPUVE), CFD and CFI (complement factors D and I), and PCSK6 (proprotein convertase 6), while PCSK1 (proprotein convertase 1) and CFB (complement factor B) were significantly down-regulated in OA cartilage (see Supplementary Figure 1 and Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>).

#### Matriptase-induced activation of proMMP-1.

Because matriptase is a known activator of proMMP-3 (34), we assessed its ability to activate the proforms of the 2 major collagenolytic MMPs relevant to cartilage collagenolysis. SDS-polymerase gel electrophoresis revealed that matriptase failed to process proMMP-13 (results not shown) but did process proMMP-1, albeit at a slower rate than for proMMP-3 (Figures 2A and B). Mass spectrometry and amino-terminal sequence analysis of the processed proMMPs revealed cleavage at Arg74-Cys75 (cysteine-switch region) and at Arg35-Arg36 (bait region) for MMP-3 in the presence of GM6001 (Figure 2C). For proMMP-1, cleavage at

Thr64-Leu65 occurred in the absence of GM6001, reflecting the presence of some active MMP-1 in the preparation. Matriptase generated the [Val<sup>82</sup>]MMP-1 mature form irrespective of the presence of GM6001. Full-length mature MMP-3 ([Phe<sup>83</sup>]MMP-3) was generated in the absence of GM6001, while inclusion of this MMP inhibitor generated [Thr<sup>85</sup>]MMP-3 (Figure 2C).

**Enhancement of IL-1 plus OSM-mediated cartilage collagenolysis by matriptase activity.** Having demonstrated that matriptase is an activator of both proMMP-1 and proMMP-3, we hypothesized that the exogenous addition of active matriptase to stimulated cartilage would enhance collagenolysis. An ex vivo model of cartilage degradation confirmed that on day 7, a time point when little or no collagen release is typically observed (29), significant levels of hydroxyproline (a measure of collagen) were detected in the culture supernatants. Furthermore, even with a low dose of IL-1 plus OSM, matriptase (100 nM) still significantly enhanced the release by day 14 (Figure 3). When collagenolysis was evident at day 7, active collagenase was also detected (results not shown).

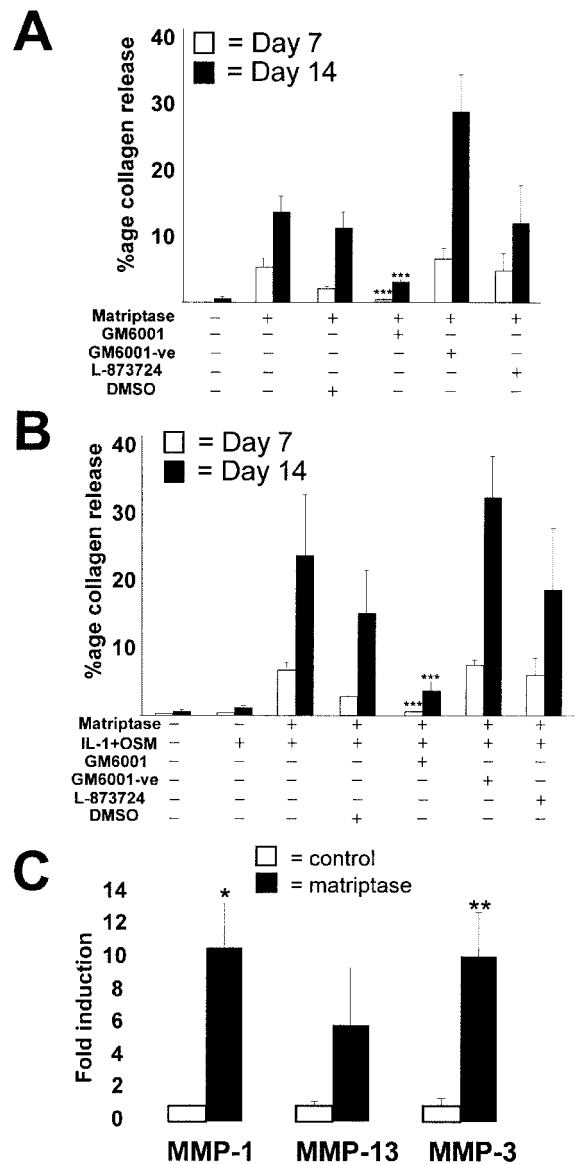
**Matriptase-enhanced OA cartilage collagenolysis and collagenase gene expression in the absence of an inflammatory stimulus.** Although our data confirmed a potential role for matriptase in cytokine-stimulated car-

tilage, previous experiments with matriptase-treated bovine explants failed to demonstrate any collagenolysis (see Figure 3). However, assessment of the effect of exogenous matriptase on human OA cartilage resulted in the striking observation that matriptase alone induced significant collagenolysis that was sensitive to the metalloproteinase inhibitor GM6001 but neither its negative control nor a cathepsin K inhibitor (Figure 4A). Similar results were observed when IL-1 plus OSM was also included, except slightly more collagenolysis was observed (Figure 4B). We also demonstrated significant glycosaminoglycan release from OA cartilage following matriptase treatment compared with control (results not shown).

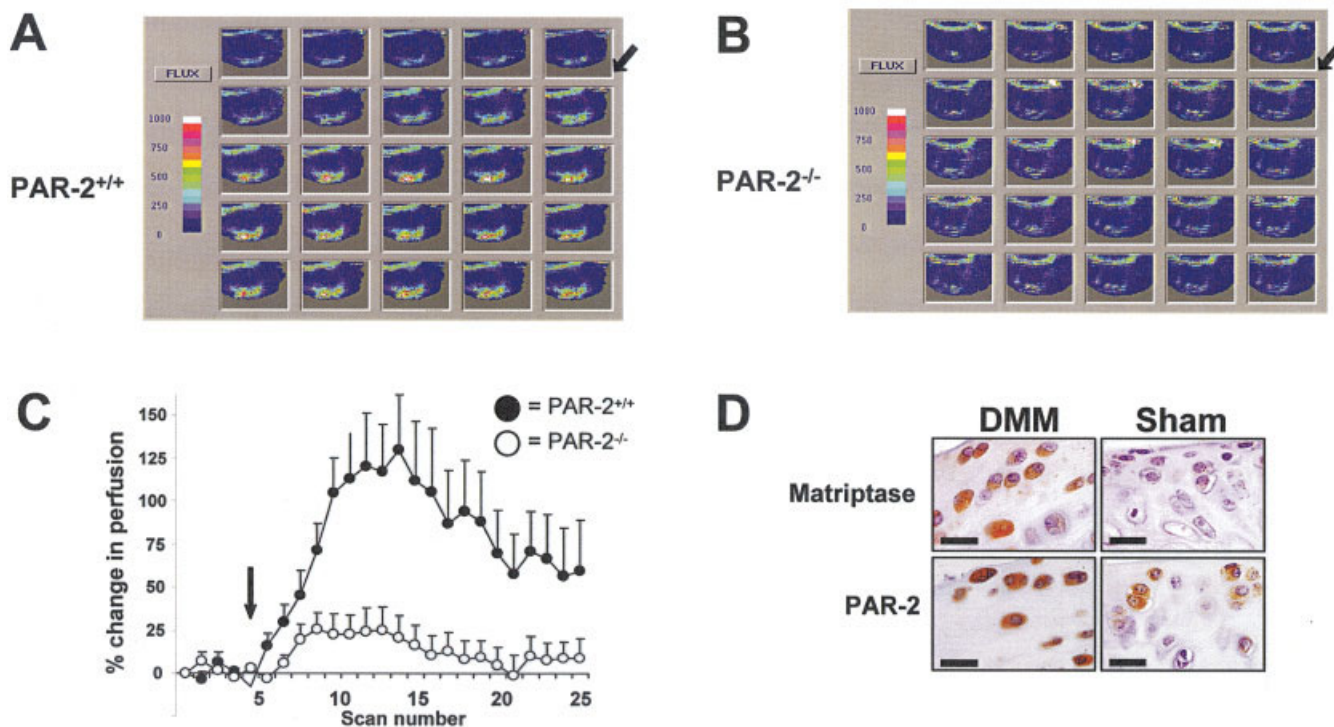
The finding that matriptase alone caused significant collagenolysis, and that this was most likely mediated by MMP, led us to hypothesize that the action of matriptase on OA cartilage led to new collagenase gene expression. Culture of human OA cartilage explants with matriptase for 7 days revealed a significant increase in the expression of both MMP-1 and MMP-3 mRNA, while expression of MMP-13 mRNA was also increased, although the difference was not statistically significant (Figure 4C). All 3 MMP genes were detectable ( $C_t$  value ranges for untreated human cartilage, 37.9–21.2; for treated cartilage, 33.5–20.2). No increases in MMP-14 were seen, while di-isopropyl phosphorofluoridate-treated matriptase failed to induce any MMP expression (results not shown).

**Expression of PAR-2, an in vivo substrate of matriptase, in OA cartilage.** To confirm the ability of recombinant matriptase to act as an in vivo activator of PAR-2, we assessed blood perfusion in murine joints following topical administration of matriptase. This demonstrated a rapid and significantly greater ( $P < 0.0001$  by two-way ANOVA) increase in synovial perfusion in PAR-2<sup>+/+</sup> (wild-type) mice compared with their PAR-2-deficient (PAR-2<sup>-/-</sup>) littermates (Figures 5A–C). Arterial blood pressure was not significantly affected by matriptase administration (mean  $\pm$  SD 1.1  $\pm$  0.3% increase). Immunohistochemical analysis of murine knee joints confirmed the presence of chondrocyte PAR-2 and also revealed matriptase expression in chondrocytes from the OA model (destabilization of the medial meniscus), but not in sham-operated mice (Figure 5D). Indeed, matriptase was detectable only at the medial aspect of the joint, corresponding to where the OA pathology associated with this model occurs.

**Matriptase-enhanced OA cartilage collagenolysis via PAR-2.** Because we had demonstrated an indirect role for matriptase in driving cartilage degradation,



**Figure 4.** Enhanced osteoarthritis (OA) cartilage collagenolysis by matriptase. **A** and **B**, Human OA cartilage explants were incubated with control medium with or without matriptase (100 nM), with or without IL-1 plus OSM (1 ng/ml plus 10 ng/ml), and with or without GM6001 (or its negative control; both 10  $\mu$ M) or L-873724 (10 nM) for 7 days. The collagen release by day 7 and cumulative release by day 14 were determined by hydroxyproline measurement and expressed as a percentage of the total for each treatment. **A**, Matriptase as prime stimulus. **B**, Matriptase plus IL-1 plus OSM as prime stimulus. Bars show the mean and SD results from at least 3 separate experiments (n = 4 samples). **C**, RNA isolated from the day-7 cartilage was subjected to real-time polymerase chain reaction for matrix metalloproteinase (MMP) gene expression. The data were normalized to 18S ribosomal RNA, and the results (representative of 3 separate experiments) are the mean and SEM fold induction compared with basal (n = 4). \* =  $P < 0.05$ ; \*\* =  $P < 0.01$  versus basal, by analysis of variance (ANOVA); \*\*\* =  $P < 0.001$  versus GM6001-negative control, by ANOVA. See Figure 3 for other definitions.



**Figure 5.** Expression of proteinase-activated receptor 2 (PAR-2), an in vivo substrate of matriptase, in an osteoarthritis model. **A** and **B**, Sequential laser Doppler images showing vasodilatation within 1–2 minutes after topical administration of 10  $\mu$ g of recombinant matriptase (arrows) to PAR-2<sup>+/+</sup> mice (**A**) or PAR-2<sup>-/-</sup> mice (**B**). Perfusion was measured in arbitrary flux units and color coded (dark blue = lowest, dark red = highest). **C**, Mean and SEM percentage change in perfusion ( $n = 3$ ). **D**, Consecutive knee joint sections (6  $\mu$ m) from C57BL/6J mice that underwent either destabilization of the medial meniscus (DMM) surgery or sham operations, stained for matriptase or PAR-2. Results are representative of at least 3 separate mice. Bars = 20  $\mu$ m.

we next hypothesized that matriptase mediated this effect by activating PAR-2. We (36) and other investigators (37) have shown that PAR-2 is a target of matriptase, and 2 different PAR-2 inhibitors (SAM11 and ENMD-1068) both significantly prevented collagenolysis (Figure 6).

## DISCUSSION

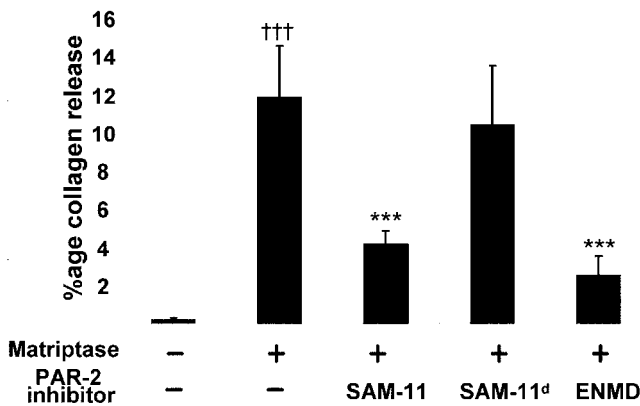
The burgeoning field of degradomics has enabled research into aberrant proteolysis to make important new discoveries. Cartilage disassembly during disease involves multiple proteinase cascades that are likely to be interdependent. We and other investigators have reported that human OA cartilage is highly resistant to proinflammatory stimuli despite increased expression of collagenolytic MMPs; the reasons for this resistance are unclear, although failure to activate latent procollagenases could be an explanation (29). Our previous data strongly implicate serine proteinases with roles in pathologic cartilage turnover, especially procollagenase acti-

vation (12–14), but identifying specific enzymes has been difficult.

Our current screen of  $\sim$ 100 serine proteinases in OA cartilage samples and phenotypically normal cartilage samples (obtained from patients with a femur neck fracture) corroborated previous findings of significantly elevated expression of several serine proteinases in OA cartilage, albeit with differing degrees of significance, as recently outlined (17). The elevated expression of FAP $\alpha$  observed in the present study corroborates previous independent reports (16,17); these data suggest that this post-prolyl peptidase, known to modify chemokines and bioactive peptides (38), may have a protective role in OA. Another up-regulated enzyme to be confirmed was HtrA1 (17), which further implicates this proteinase in catabolic ECM turnover, because treatment of synovial fibroblasts with HtrA1 or HtrA1-generated fibronectin fragments induces MMP-1 and MMP-3 expression (39).

Of the TTSPs screened, most were unaltered and expressed at very low levels. However, the expression of





**Figure 6.** Proteinase-activated receptor 2 (PAR-2)-mediated matriptase-enhanced osteoarthritis (OA) cartilage collagenolysis. Human OA cartilage explants were incubated with control medium with or without either a neutralizing antibody to PAR-2 (SAM11) at 400 ng/ml final concentration or the same antibody that had been previously heat-denatured (SAM11<sup>d</sup>), or ENMD-1068 at 10 mM final concentration for 72 hours. Subsequently, matriptase (100 nM) was added such that the SAM11 antibody and ENMD-1068 remained at final concentrations of 200 ng/ml and 5 mM, respectively, for 7 days. Collagen release was then determined by hydroxyproline measurement and expressed as a percentage of the total for each treatment. Bars show the mean and SD results from 3 separate experiments (n = 4 samples). ††† = *P* < 0.001 versus control; \*\*\* = *P* < 0.001 versus matriptase alone, by analysis of variance.

one TTSP gene was significantly elevated in OA cartilage compared with normal cartilage, namely, matriptase, which is widely expressed in various cancers. A failure to suppress matriptase expression during disease may therefore have catabolic-related implications for cartilage. Several putative substrates of matriptase have been identified, including pro-hepatocyte growth factor and the serine proteinases pro-urokinase plasminogen activator (pro-uPA) and prostasin (40), with a major role as initiators of serine proteinase cascades. We have proposed that such cascades are important in cartilage degradation (11–13); this initiator hypothesis is further supported in that matriptase activation is autocatalytic (41), which is uncommon for a latent serine proteinase. In fact, matriptase activation is a complex process requiring its cognate inhibitor, HAI-1, which enables appropriate trafficking to the cell surface (42). This process therefore directs matriptase activity to the cell surface while providing a means of regulating this activity. In turn, this suggests that increased matriptase expression and subsequent activity would result only with a concomitant increase in HAI-1 expression. We found this situation to exist in OA cartilage, with the

data strongly implying elevated matriptase activity in OA.

Some of the potential consequences of matriptase activity in cartilage are well aligned with our previous data (12–14). MMP-3 is a key activator of procollagenases (43,44), and we confirm that matriptase processes proMMP-3 (34) to a fully active enzyme following intermediate cleavage within the bait region (Arg–Arg bond), consistent with its preference for position P1 (37). This intermediate species generates a fully active MMP-3 with an N-terminal Phe. Interestingly, inclusion of an MMP inhibitor revealed cleavage at the Arg–Cys bond within the cysteine-switch regions for the MMP-1 and MMP-3 proforms; this same bond is cleaved in MMP-7 and MMP-8 by trypsin and in MMP-13 by plasmin (for review, see ref. 35). This presumably results in the loss of MMP latency, because in the absence of MMP activity, matriptase ultimately processed proMMP-1 and proMMP-3 to less active forms similar to those generated by chymase and trypsin, respectively (35). However, MMP-3 is typically coordinately expressed with MMP-1 and MMP-13 (45) such that in vivo, matriptase action on proMMP-3 will generate an MMP-3 enzyme capable of processing collagenolytic MMPs to their most active forms (35). This is evidenced by the enhanced collagenolysis on day 7 when cytokine stimulation alone failed to promote such collagenolysis (29); this therefore provides a mechanism that maximizes the degradative potential of the procollagenase pool and one that preferentially localizes this activity at the cell surface to effect pericellular collagenolysis.

Matriptase also activates pro-uPA (37), which generates active plasmin from plasminogen. Although we confirmed the absence of plasminogen expression in OA cartilage (data not shown), this enzyme is expressed in synovial tissue. Both MMP-3 and plasmin activate procollagenases to expedite collagenolysis in resorbing cartilage (12,46), and plasmin has several other roles that contribute to ECM remodeling (11). We have proposed that procollagenase activation represents a key and rate-limiting step in cartilage collagenolysis, and confirmation that matriptase activates proMMP-1 as well as expedites collagenolysis from proinflammatory cytokine-stimulated cartilage (with a concomitant increase in detectable active collagenase) further supports the notion that matriptase could be a major player in such processes. We have also shown reduced collagenolysis from stimulated cartilage following the addition of anti-thrombin III or PA inhibitor 1 (Milner JM, Rowan AD: unpublished observations); these serpins inhibit matriptase (47), albeit not specifically.

Historically, human OA cartilage explants are highly resistant to proteolysis, even following stimulation with highly catabolic cytokine combinations such as IL-1 plus OSM. Indeed, only ~25% of cartilage specimens respond with typically very low collagen release (29). The current study also failed to promote cytokine-induced collagenolysis despite collagenase(s) induction (data not shown). However, the most striking observation was that even in the absence of a proinflammatory stimulus, matriptase induced significant collagenolysis from OA cartilage, which was entirely metalloproteinase-dependent. Inclusion of matriptase with a proinflammatory stimulus (IL-1 plus OSM) promoted slightly more collagenolysis, presumably via the activation of cytokine-induced proMMPs. This new MMP expression occurred as a consequence of matriptase action on OA cartilage that was significant for MMP-1 and MMP-3. Matriptase activity would subsequently activate secreted proMMPs, although we cannot exclude the possibility of concomitant initiation of another activation cascade.

These highly novel observations suggest that matriptase may mediate its effect via a cell surface receptor specifically expressed in OA cartilage, because treatment of healthy bovine or nondiseased human cartilage with matriptase failed to promote collagenolysis (data not shown). Matriptase is a known activator of PAR-2 (36,37); this mechanism involves the proteolytic release of a tethered ligand, which can be mimicked by use of a PAR-2-activating peptide [48]). We confirmed this mechanism *in vivo* using wild-type and PAR-2-deficient mice in synovial perfusion assays, as previously performed for  $\beta$ -tryptase (33). The small amount of vasodilatation in the PAR-2-deficient mice may indicate that matriptase has a minor non-PAR-2-mediated effect. PAR-2 expression is significantly elevated in OA cartilage compared with normal cartilage (49,50), and synthetic activation of PAR-2 in OA cartilage induces both MMP-1 and MMP-13 (49), which is consistent with our observations using matriptase.

We have previously shown that the PAR-2 antagonist, ENMD-1068, is effective at inhibiting PAR-2 when activated proteolytically (trypsin) or via a PAR-2-activating peptide (51). In the current study, PAR-2 inhibition with a neutralizing antibody (SAM11) or ENMD-1068 effectively blocked matriptase-induced collagenolysis, thus confirming PAR-2 as a target for matriptase in OA cartilage. We have previously implicated PAR-2 as having a role in RA, because it is highly expressed in rheumatoid synovium, and the spontaneous synovial expression of both IL-1 $\beta$  and TNF $\alpha$  is markedly reduced following PAR-2 antagonism (24). Further-

more, chondrocyte PAR-2 expression has been shown to be increased following stimulation with IL-1 $\beta$  or TNF $\alpha$ , both of which are proinflammatory cytokines implicated in OA (4,5).

The expression of various cell surface receptors is altered in OA (e.g., some Toll-like receptors [TLRs] [52]), which may well be a consequence of inflammation (IL-1 $\beta$ , TNF $\alpha$ ) and/or aberrant mechanical load. This altered phenotype may then increase susceptibility to further stimuli for ECM degradation. Interestingly, although PAR-2 was detectable in chondrocytes from both sham-operated and OA joints, matriptase was consistently detectable only in the latter. Moreover, matriptase was detected only in locations where PAR-2 was also present. This suggests that PAR-2 may act as a sentinel for proteinase-mediated injury signals, such as matriptase expression due to joint destabilization, leading to PAR-2 activation and signaling, resulting in cartilage degradation. PAR-2 expression is also elevated in OA subchondral bone, and PAR-2 activation increases bone resorption (53). Taken together, these findings clearly support a catabolic role for PAR-2 in both RA and OA.

The ability of matriptase to activate key MMPs makes it an important effector of cartilage degradation. We recently highlighted the importance of serine proteinases in cartilage breakdown (11) and propose matriptase as a key initiator of proteinase cascades, including MMPs, that collectively orchestrate ECM turnover. Furthermore, matriptase activity in OA cartilage leads to the induction of collagenolytic MMPs, a process we suggest occurs via PAR-2 activation.

These properties of matriptase offer a paradigm for OA, in which an initial focal injury or inflammatory event leads to localized, altered chondrocyte receptor expression, which, when combined with matriptase action, leads to PAR-2 activation and subsequent MMP activation (e.g., MMP-1) at the cell surface; this then facilitates pericellular collagenolysis within this focal region of the cartilage. This could have direct implications, because MMP-1 is a known activator of PAR-1, shown to be expressed in cartilage (54), leading to new MMP expression (55). In the context of altered receptor expression in OA cartilage, MMP-mediated proteolysis of the ECM may also generate ligands for TLRs that normally are not abundantly expressed (e.g., TLR-2) (52). All or a combination of these mechanisms could thus perpetuate cartilage degradation in the absence of classic inflammatory cues, especially in OA. Periodic recurrences of such events over time may also explain the slower progression of OA compared with RA, a

disease that is characterized by more persistent inflammatory stimuli and synovial involvement.

In summary, we have shown that the TTSP matriptase is expressed in OA cartilage, and that this enzyme is both an inducer and activator of procollagenases. Therefore, matriptase is a highly attractive new target for preventing pathologic cartilage breakdown.

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### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Rowan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Milner, Patel, Cawston, Ferrell, Lockhart, Rowan.

**Acquisition of data.** Milner, Patel, Davidson, Swingler, Desilets, Young, Kelso, Donell, Clark, Ferrell, Plevin, Lockhart, Leduc, Rowan.  
**Analysis and interpretation of data.** Milner, Patel, Young, Ferrell, Lockhart, Rowan.

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