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Inhibition of Furin-Like Enzymes Blocks Interleukin-1α/Oncostatin M–Stimulated Cartilage Degradation

J. M. Milner, A. D. Rowan, S.-F. Elliott, and T. E. Cawston

Objective. **To investigate the role of furin-like enzymes in the proteolytic cascades leading to cartilage breakdown and to examine which collagenase(s) contribute to collagen degradation.**

Methods. **Bovine nasal cartilage was stimulated to resorb with the addition of interleukin-1α (IL-1α)/oncostatin M (OSM) in the presence or absence of a furin inhibitor, Dec-RVKR-CH_2Cl, or selective matrix metalloproteinase 1 (MMP-1) inhibitors. Collagen and proteoglycan levels were determined by assay of hydroxyproline and sulfated glycosaminoglycan, respectively. Collagenase and gelatinase activity were measured using ^{3}H-acetylated collagen and gelatin zymography, respectively.**

Results. **The addition of Dec-RVKR-CH_2Cl to stimulated cartilage reduced the release of collagen fragments and the levels of active collagenase and MMP-2, suggesting that furin-like enzymes are involved in the cascades leading to activation of procollagenases. At MMP inhibitor concentrations that selectively inhibit MMP-1, no inhibition of collagen release was observed, but increasing the concentration to the 50% inhibition concentration for MMP-13 resulted in a 50% blockage of collagen release. The addition of Dec-RVKR-CH_2Cl to resorbing cartilage also partially blocked proteoglycan release, thus demonstrating a role for furin-activated enzymes in the pathways leading to proteoglycan degradation.**

Conclusion. **Furin-like enzymes are involved in cascades leading to activation of procollagenases and degradation of collagen. MMP-13, which can be activated by furin-processed membrane-type 1 MMP-1, appears to be a major collagenase involved in collagen degradation induced by IL-1α/OSM. Furin-like enzymes also appear to play a role in the pathways leading to proteoglycan degradation. These findings are of importance when considering proteinase inhibition as a target for therapeutic intervention in arthritic diseases.**

Irreversible destruction of the cartilage matrix is a major feature of osteoarthritis and rheumatoid arthritis (RA). Cartilage tissue consists of a single cell type, the chondrocyte (1), which is embedded within an extracellular matrix (ECM) of predominantly proteoglycans and collagens. Aggrecan, a large aggregating proteoglycan, and type II collagen are the major constituents of articular cartilage. In experimental cartilage models, chondrocytes respond to proinflammatory cytokines such as interleukin-1 (IL-1), resulting in the breakdown of the ECM. Degradation of proteoglycan is rapid and reversible (2); however, the breakdown of collagen is slow and is thought to be essentially irreversible (3).

The principal proteinases responsible for aggrecan degradation in cartilage are the aggrecanases (4). These enzymes cleave aggrecan at the Glu^{373}–Ala^{374} bond. Aggrecan fragments cleaved at this bond have been identified in cultures undergoing cartilage matrix degradation (5,6) and in arthritic synovial fluids (7,8). Two cartilage-derived aggrecanases have so far been identified that belong to a disintegrin and metalloproteinase with thrombospondin motifs (ADAM-TS) subfamily of proteinases; these are ADAM-TS4 (aggrecanase 1) (9) and ADAM-TS5 (aggrecanase 2) (10).

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are implicated in the pathologic destruction of cartilage collagen,

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J. M. Milner, PhD, A. D. Rowan, PhD, S.-F. Elliott, PhD, T. E. Cawston, PhD: The Medical School, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, UK.

Address correspondence and reprint requests to A. D. Rowan, PhD, Department of Rheumatology, Floor 4 Cookson Building, The Medical School, University of Newcastle-upon-Tyne, Framlington Place, Newcastle-upon-Tyne NE2 4HH, UK. E-mail: A.D.Rowan@ncl.ac.uk.

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leading to a loss of joint function (11,12). This family of proteinases is subdivided into groups: collagenases, stromelysins, gelatinases, and membrane-type MMPs (MT-MMPs). The collagenases (MMPs 1, 8, and 13) are considered to be key enzymes involved in cartilage collagen turnover and specifically cleave fibrillar collagen. In addition, MT1-MMP (MMP-14) has also been shown to cleave type II collagen (13).

MMP activity is regulated at 3 distinct levels: synthesis, proenzyme activation, and inhibition. All MMPs are synthesized as zymogens that must undergo cleavage to produce the active enzyme. Once activated, MMPs are subject to further control by a group of inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (14). The production of MMPs and inhibitors is mediated by a variety of cytokines and growth factors. Proinflammatory cytokines, such as tumor necrosis factor α (TNFα), IL-1α, and oncostatin M (OSM), have been shown to stimulate chondrocytes and synovial cells to produce MMPs, and increased levels of these cytokines are present in the arthritic joint (15,16).

Activation of proMMPs is an important control point in cartilage collagen breakdown (17). The precise mechanisms of activation of procollagenases in cartilage are not fully understood. The serine proteinase plasmin is a known activator of several proMMPs, including proMMP-1 (collagenase 1), proMMP-13 (collagenase 3), and proMMP-3 (stromelysin 1) (18–21). MMP-3 itself can activate other proMMPs, including proMMP-1, proMMP-8 (collagenase 2), proMMP-9 (gelatinase B), and proMMP-13 (22–25).

Recently, it has been shown that several members of the MT-MMP family (MTs 1–3, 5, and 6 MMPs) can activate proMMP-2 (gelatinase A) (26–30). MT1-MMP, the best characterized of the MT-MMPs, appears to form a complex with TIMP-2 on the cell surface, to which proMMP-2 binds. An adjacent free and active MT1-MMP molecule initiates processing of proMMP-2 and then autoproteolysis proceeds. MT1-MMP has also been shown to activate proMMP-13, and this is enhanced in the presence of MMP-2 (20). Thus, complex activation cascades are established because active MMPs are able to process other proMMPs.

At the C-terminal end of the propeptide, all MT-MMPs and MMP-11 (stromelysin 3) contain a conserved RX(K/R)R motif which is a recognition motif for the pro-protein convertase furin. MT1-MMP, MT3-MMP, and MMP-11 can all be activated by furin (31–33). In addition, the aggrecanases ADAM-TS4 and ADAM-TS5 contain this furin cleavage site (9,10), and the pro-domain of ADAM-TS4 can be removed by furin (34). Furin has been detected in normal cartilage, and elevated levels are found in osteoarthritic cartilage (35). Thus, furin-activated enzymes may play an important role in the mechanisms leading to cartilage degradation.

In this study, we used a bovine nasal cartilage explant culture system as a model of cartilage degradation. The addition of a combination of IL-1α and OSM to bovine nasal cartilage in explant culture induced an increase in the synthesis of procollagenases and a reproducible release of collagen and proteoglycan fragments. In these assays, the majority of proteoglycan fragments were released by day 3 of culture, and >90% of the total collagen was released by day 14 (36). We investigated the role of furin-activated enzymes in the breakdown of the cartilage matrix by the addition of the furin inhibitor Dec-RVKR-CH_2Cl to resorbing cartilage. This inhibitor is a peptidyl chloromethylketone that irreversibly binds to the catalytic site of furin (37). We also investigated which collagenase contributes predominantly to cartilage collagen degradation by the use of selective MMP-1 inhibitors.

MATERIALS AND METHODS

Recombinant human IL-1α and OSM were donated by Glaxo SmithKline (Stevenage, UK) and Professor J. Heath (Birmingham, UK), respectively. IL-1α (1 μg/ml) in Dulbecco's modified Eagle's medium (DMEM) (with 0.1% [weight/volume] bovine serum albumin [BSA]) was stored at −20°C. OSM (10 μg/ml) in phosphate buffered saline (PBS) (with 0.1% [w/v] BSA) was stored at −80°C. Immediately prior to use, IL-1α and OSM were diluted into culture medium. Dec-RVKR-CH_2Cl was purchased from Bachem (Merseyside, UK). Immediately before use, Dec-RVKR-CH_2Cl was dissolved in methanol and then diluted into culture medium (final concentration of methanol in the cartilage assay was 0.07%). The α_1-proteinase inhibitor (α_1PI) was purchased from Calbiochem (Nottingham, UK) and was dissolved in culture medium. Synthetic MMP inhibitors BRL-54418 and BRL-56613A were generously donated by Glaxo SmithKline. BRL-54418 was dissolved in DMSO so that the highest concentration of DMSO in the cartilage cultures was 0.01%. BRL-56613A was dissolved in culture medium. All reagents added to cartilage cultures were filter-sterilized through a 0.2-μm filter immediately before use. Human plasminogen and high molecular weight human urokinase plasminogen activator (uPA) were from Calbiochem, and Suc-AFK-MCA and Boc-RVRR-MCA were purchased from Bachem. Furin was purchased from Cambridge Bioscience (Cambridge, UK). All other chemicals and biochemicals were commercially available analytical grade reagents available from Sigma (Poole, UK), BDH Chemicals (Poole, UK), Gibco (Paisley, UK), or Fisher Scientific (Loughborough, UK).

Plasmin assay. Plasminogen (2.5 n*M*) and uPA (0.44 n*M*) were incubated at 37°C in 2,940 μl of Tris assay buffer (50 m*M* Tris HCl, pH 7.5, 0.1% Triton X-100) for 15 minutes, with

stirring. Thirty microliters of the substrate Suc-APK-MCA (1 m*M*) (dissolved in DMSO) was added and fluorescence was read every 20 seconds (excitation and emission wavelengths of λ_{ex360} and λ_{em460}). When a continuous rate was achieved (10 minutes), 30 μl of inhibitor or control samples was added.

Furin assay. Furin activity was measured using a quenched fluorescent substrate, Boc-RVRR-MCA, based on the method described by Molloy et al (38) and Hatsuzawa et al (39). Furin (2 units/ml) with or without Boc-RVRR-MCA (0.5 m*M*) and Dec-RVKR-CH_2Cl (0–10 μM) was incubated in assay buffer (100 m*M* HEPES, pH 7.5, 0.5% Triton X-100, 1 m*M* $CaCl_2$, 1 m*M* β-mercaptoethanol) for 1 hour at 30°C. The reaction was stopped by the addition of 2.5 ml of 10 m*M* EDTA and the fluorescence was read (λ_{ex360} and λ_{em460}). The percentage of inhibition of furin activity in the presence of Dec-RVKR-CH_2Cl was then calculated.

Cartilage degradation assay. Bovine nasal cartilage was cultured as previously described (35). Briefly, discs (~1 mm^3) were punched from bovine nasal septum cartilage. Three discs per well in a 24-well plate were incubated in control medium overnight at 37°C in 5% CO_2/humidified air. Control culture medium was DMEM containing 25 m*M* HEPES supplemented with glutamine (2 m*M*), streptomycin (100 μg/ml), penicillin (100 units/ml), gentamicin (2.5 μg/ml), and nystatin (40 units/ml). Fresh control medium with or without test reagents (4 wells for each condition) was then added (day 0). When assaying for proteoglycan release, the cartilage assay was stopped on day 3, supernatants were harvested, and cartilage was digested with papain. When assaying for collagen release, cartilage was incubated until day 7, and supernatants were harvested and replaced with fresh medium containing the same test reagents as on day 0 (unless otherwise stated). On day 14, the supernatants were harvested, and the remaining cartilage was digested with papain.

Culture conditions. Viability of cartilage explants was assessed by screening for the production of lactate dehydrogenase (LDH) using the CytoTox 96 assay (Promega, Southampton, UK). No increase in LDH was found with any of the cytokine or inhibitor combinations. This is consistent with the findings of Maquoi et al (40), who demonstrated that the addition of Dec-RVKR-CH_2Cl (100 μM) to cell cultures did not affect cellular metabolism and/or proliferation. Serum was excluded from cartilage explants since it can increase cartilage metabolism in the absence of exogenous cytokine(s) (41). The aim of this study was to create a model of cartilage breakdown; therefore, the presence of serum was avoided because it contains chondroprotective agents such as insulin-like growth factor 1 (42,43). The absence of serum was shown not to affect the viability of the tissue, and previous studies have shown that cartilage in serum-free culture for 8 or 9 days can respond to serum and other growth factors (44). In the present study, cytokines were added after 1 day of serum-free culture.

Hydroxyproline assay. Hydroxyproline release was assayed (as a measure of collagen degradation) using a microtiter modification of the method as described previously (45,46).

Collagenase assay. Collagenase activity was determined by the ^{3}H-acetylated collagen diffuse fibril assay (47) using a 96-well plate modification (48). One unit of collagenase activity degraded 1 μg of collagen per minute at 37°C.

Gelatin zymography. Samples were electrophoresed on 7.5% sodium dodecyl sulfate–polyacrylamide gel copolymerized with 0.1% gelatin. Gels were washed twice for 1 hour in 20 m*M* Tris HCl, pH 7.8, and 2.5% Triton X-100 and then for 1 hour in 20 m*M* Tris HCl, pH 7.8, 1% Triton X-100, 10 m*M* $CaCl_2$, and 5 μM $ZnCl_2$ at 4°C. Gels were then incubated for 16 hours at 37°C in 20 m*M* Tris HCl, pH 7.8, 1% Triton X-100, 10 m*M* $CaCl_2$, and 5 μM $ZnCl_2$. In some instances, an identical gel was run and 1,10-phenanthroline (2 m*M*) was added to the incubation buffers to inhibit metalloproteinases in order to confirm metalloproteinase-dependent activity. All gels were stained with Coomassie blue G250.

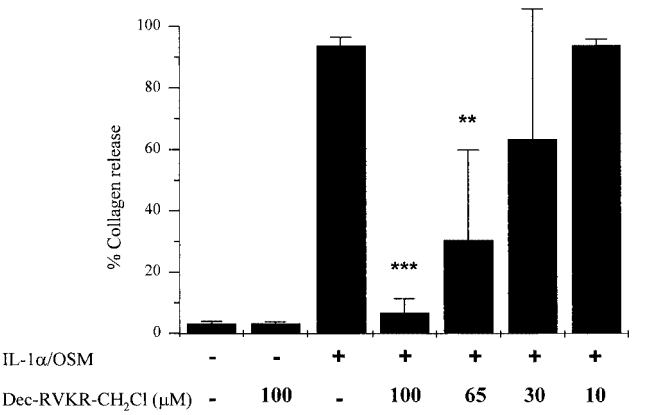

Figure 1. Collagen release inhibited by addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Bovine nasal cartilage discs were cultured in medium with or without interleukin-1α/oncostatin M (IL-1α/OSM) and with or without Dec-RVKR-CH_2Cl. As a measure of collagen, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release by days 7 and 14, expressed as a percentage of the total collagen. Values are the mean and SD. ** = $P < 0.01$; *** = $P < 0.001$ versus IL-1α/OSM.

Proteoglycan assay. Sulfated glycosaminoglycan was assayed (as a measure of proteoglycan) using a dimethylmethylene blue binding assay, based on the method of Farndale et al (49).

Statistical analysis. Statistical analysis was performed using Student's 2-tailed *t*-test, assuming equal variance. Values are expressed as the mean and SD.

RESULTS

Inhibition of the release of collagen fragments by the addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Dec-RVKR-CH_2Cl is a potent inhibitor of furin and other members of the pro-protein convertase (PC) family, including PACE-4, PC2, PC3, PC6, and PC7 (50). The addition of Dec-RVKR-CH_2Cl to bovine nasal cartilage explant cultures that had been stimulated to resorb with IL-1α and OSM produced a dose-dependent reduction in the release of collagen fragments (Figure 1). This inhibition was statistically significant at concentrations $\geq$65 μM. The higher concentration

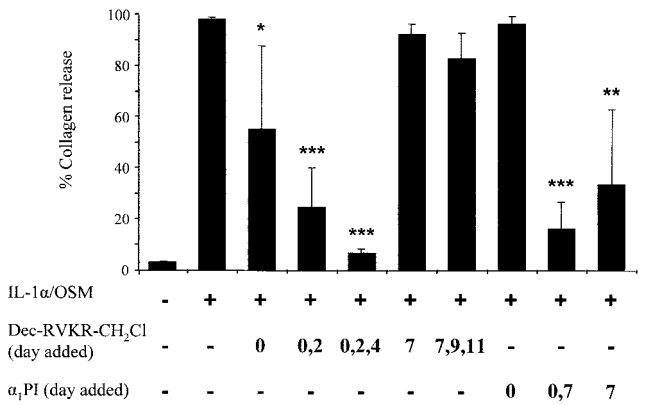

Figure 2. Collagen release inhibited by addition of Dec-RVKR-CH_2Cl at the beginning of culture to stimulated bovine nasal cartilage. Bovine nasal cartilage discs were cultured in medium with or without IL-1α/OSM (0.8/8 ng/ml). Dec-RVKR-CH_2Cl (100 μM) was added on day 0, days 0 and 2, days 0, 2, and 4, day 7, or on days 7, 9, and 11. Then, α_1-proteinase inhibitor (α_1PI; 100 μg/ml) was added on day 0, days 0 and 7, or day 7. Medium was removed on day 7 and replenished with medium, with or without IL-1α/OSM, with or without Dec-RVKR-CH_2Cl, and with or without α_1PI. On day 14, medium was removed, and the remaining cartilage was digested with papain. As a measure of collagen, the levels of hydroxyproline in day 7 and day 14 media and in cartilage digests were assayed. Shown is the cumulative collagen release by days 7 and 14, expressed as a percentage of the total collagen. Values are the mean and SD. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus IL-1α/OSM. See Figure 1 for other definitions.

of Dec-RVKR-CH_2Cl required to block collagen release in the cartilage assay, compared with that actually required to inhibit furin and other PCs (50), is likely to be due to this compound having a short half-life and having to penetrate the highly charged cartilage matrix.

Using fluorometric assays with Boc-RVRR-MCA and Suc-APK-MCA as substrates for furin and plasmin, respectively, we determined the 50% inhibition concentration values (IC_{50}) for furin to be ~0.03 μM and for plasmin to be ~0.1 μM (data not shown). Dec-RVKR-CH_2Cl has a short half-life ($T_{1/2}$ = 3 or 4 hours in DMEM at 37°C; data not shown). Hence, for a starting concentration of 100 μM, the effective concentration after 48 hours in culture will be below the IC_{50} values for both furin and plasmin. Since the inhibitor was added to the cartilage cultures on days 0 and 7 only, the target of this inhibitor is most likely to be present during days 0–2 and/or 7–9 of culture. To further determine when the inhibitor must be present to block collagen release, Dec-RVKR-CH_2Cl was added to IL-1α/OSM–stimulated cartilage on different days of culture (Figure 2). In comparison, α_1PI, a general inhibitor of trypsin-like serine proteinases, but not furin (data not shown) (51), was also added on different days of culture.

The addition of Dec-RVKR-CH_2Cl only on day 0 partially blocked collagen release after 14 days of culture, while addition on days 0 and 2 further reduced collagen release. Complete inhibition of collagen release was observed following the addition of Dec-RVKR-CH_2Cl on days 0, 2, and 4. However, when Dec-RVKR-CH_2Cl was added on day 7 of culture or even on days 7, 9, and 11, collagen release was not significantly reduced. This demonstrates that the target of Dec-RVKR-CH_2Cl is present at the beginning of the culture period. In contrast, the addition of α_1PI on day 0 of culture to cartilage stimulated to resorb did not block collagen release. However, as previously shown (17), addition of α_1PI to stimulated cartilage only on day 7 can still block collagen release. These data demonstrate that these inhibitors target different enzymes at separate time points. Dec-RVKR-CH_2Cl, an inhibitor of furin-like proteinases, targets an enzyme(s) present at the beginning of the culture period. In contrast, the general serine proteinase inhibitor α_1PI targets enzymes that are upregulated after day 7 of culture.

Inhibition of the release of collagenase activity by the addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Media from the same experiment as shown in Figure 2 were assayed for collagenase activity. A reduction in active collagenase was seen in media from cartilage treated with cytokines and Dec-RVKR-CH_2Cl compared with cytokines alone (Figure 3). This suggests that activation of collagenases is inhibited and implicates furin-activated enzymes in the cascades leading to the activation of procollagenases. The addition of Dec-RVKR-CH_2Cl on days 7, 9, and 11 resulted in a significant reduction in the levels of active collagenase but no reduction in collagen release. This result suggests that these reduced levels were, however, not below the threshold required to prevent collagen degradation.

Inhibition of furin blocks MMP-2 activation in bovine nasal cartilage stimulated to resorb. All MT-MMPs contain the furin recognition motif, and furin has been shown to activate MT1-MMP and MT3-MMP (31,32). MT1–3-MMP, MT5-MMP, and MT6-MMP can all activate proMMP-2 (26–30). Therefore, we investigated whether the addition of Dec-RVKR-CH_2Cl to IL-1α/OSM–stimulated bovine nasal cartilage results in the blockade of proMMP-2 activation. Gelatinase activity in day 7 media (from the same experiment shown in Figure 2) was analyzed by gelatin zymography (Figure 4). Western blotting confirmed the identity of MMP-2 (data not shown). In day 7 control cartilage medium,

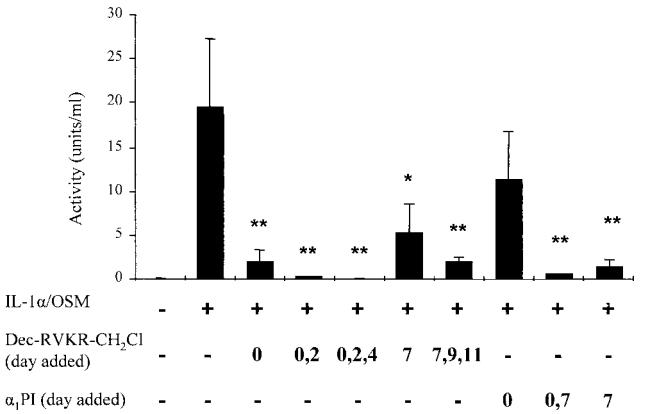

Figure 3. Levels of active collagenase reduced by addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Bovine nasal cartilage discs were cultured in medium with or without IL-1α/OSM (0.8/8 ng/ml). Dec-RVKR-CH_2Cl (100 μM) was added on day 0, days 0 and 2, days 0, 2, and 4, day 7, or on days 7, 9, and 11. Then, α_1-proteinase inhibitor (α_1PI) (100 μg/ml) was added on day 0, days 0 and 7, or day 7. Medium was removed on day 7 and replenished with medium, with or without IL-1α/OSM, with or without Dec-RVKR-CH_2Cl, and with or without α_1PI. On day 14, medium was removed, and the remaining cartilage was digested with papain. Active collagenase was analyzed in day 14 medium by ^{3}H-acetylated collagen diffuse fibril assay. Values are the mean and SD. $* = P < 0.05$; $** = P < 0.01$ versus IL-1α/OSM. See Figure 1 for other definitions.

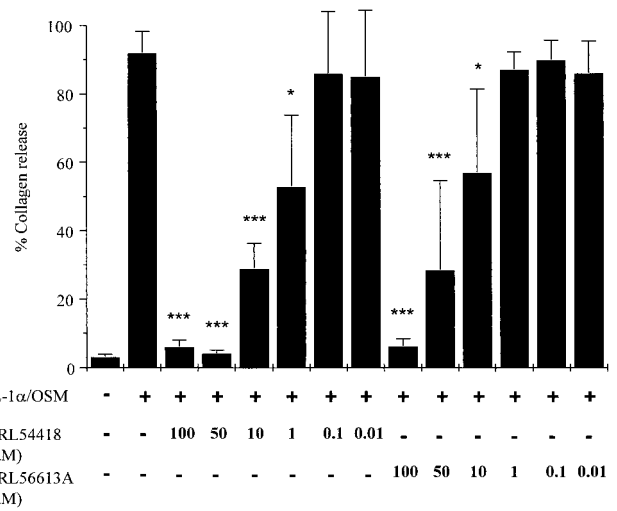

Figure 5. Addition of selective matrix metalloproteinase 1 inhibitors to resorbing bovine nasal cartilage. Bovine nasal cartilage discs were cultured in medium with or without IL-1α/OSM (0.2/2 ng/ml) with or without BRL54418 or BRL56613A (0.01–100 μM). Medium was removed on day 7 and replenished with identical reagents. On day 14, medium was removed, and the remaining cartilage was digested with papain. As a measure of collagen, the levels of hydroxyproline in day 7 and day 14 media and in cartilage digests were assayed. Shown is the cumulative collagen release by days 7 and 14, expressed as a percentage of the total collagen. Values are the mean and SD. $* = P < 0.05$; $*** = P < 0.001$ versus IL-1α/OSM. See Figure 1 for definitions.

only latent MMP-2 was present, while IL-1α/OSM–treated cartilage contained both latent and active MMP-2. Inclusion of Dec-RVKR-CH_2Cl with IL-1α/OSM resulted in a reduction of the levels of active MMP-2. This suggests that Dec-RVKR-CH_2Cl inhibits furin, which prevents furin-dependent activation of proMT-MMPs, which, in turn, blocks proMMP-2 activation.

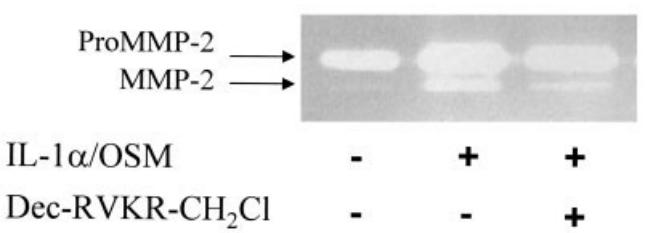

Figure 4. Levels of active matrix metalloproteinase 2 (MMP-2) reduced by addition of Dec-RVKR-CH_2Cl to IL-1α/OSM–treated bovine nasal cartilage. Day 7 media from bovine nasal cartilage discs cultured in medium with or without IL-1α/OSM (0.8/8 ng/ml) and with or without Dec-RVKR-CH_2Cl (100 μM; added on day 0 only) were analyzed for gelatinolytic activity by gelatin zymography. Gelatinolytic activity appears as clear zones on a gray background. ProMMP-2 = pro–matrix metalloproteinase 2 (see Figure 1 for other definitions).

Investigating the role of MMP-1 in cartilage collagen degradation. In this study, we showed that collagenolytic activity is reduced in the presence of a furin inhibitor, as is MMP-2. ProMMP-13, but not proMMP-1 or proMMP-8, can be activated by furin-processed MT1-MMP (20). These results suggest that MMP-13 may be responsible for collagen turnover in the bovine nasal cartilage model. To investigate which collagenases contribute predominantly to cartilage collagen degradation, we added selective MMP-1 inhibitors to resorbing cartilage. BRL54418 (IC_{50} for MMP-1 = 0.134 μM and for MMP-13 = 3.1 μM) and BRL56613A (IC_{50} for MMP-1 = 0.0564 μM and for MMP-13 = 3.94 μM) have a 23- and 70-fold selectivity for MMP-1 over MMP-13, respectively. Bovine nasal cartilage was cultured in medium, with or without IL-1α (0.2 ng/ml)/OSM (2 ng/ml), with or without a range of concentrations of these selective inhibitors (0.01–100 μM) (Figure 5). At concentrations that selectively inhibit only MMP-1, the levels of collagen release were unaffected. Increasing the inhibitor concentration produced a dose-dependent reduction in collagen release. Approximately 50% inhibition of collagen release occurred at inhibitor

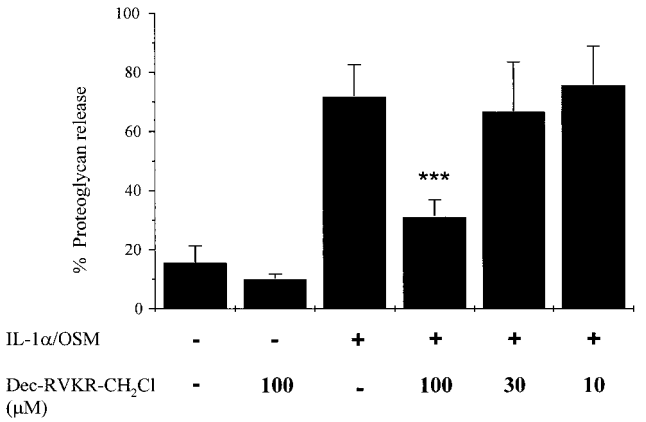

Figure 6. Release of proteoglycan fragments inhibited by addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Bovine nasal cartilage discs were cultured in medium with or without IL-1α/OSM (1/10 ng/ml) with or without Dec-RVKR-CH_2Cl (100, 30, 10 μM) for 3 days. On day 3, medium was removed, and the remaining cartilage was digested with papain. As a measure of proteoglycan, sulfated glycosaminoglycan was assayed in culture medium and cartilage digests. Shown is the release of proteoglycan fragments, expressed as a percentage of the total proteoglycan. Values are the mean and SD. *** = $P < 0.001$ versus IL-1α/OSM. See Figure 1 for definitions.

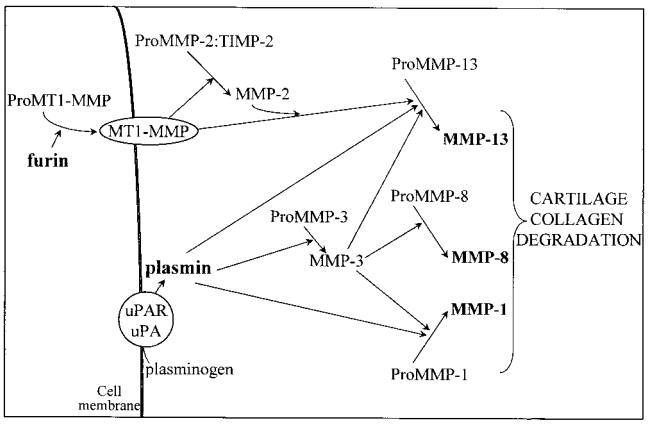

Figure 7. Activation mechanisms of pro–matrix metalloproteinases (proMMPs) leading to cartilage collagen breakdown. Pro–membrane type 1 matrix metalloproteinase (proMT1-MMP) can be activated by furin, which, in turn, can release active MMP-2 from a proMMP-2: tissue inhibitor of metalloproteinase 2 (TIMP-2) complex at the cell surface. MT1-MMP, as well as MMP-2, can also activate proMMP-13. The furin inhibitor Dec-RVKR-CH_2Cl partially blocks collagen release and reduces the levels of active collagenase and active MMP-2. This suggests a role for furin-processed enzymes in the activation cascades of procollagenases that initiate the breakdown of cartilage collagen. Since α_1-proteinase inhibitor also blocks collagen release, a second activation cascade is suggested. Trypsin-like proteinases, such as urokinase plasminogen activator (uPA), bind their cell surface receptor (uPAR) and promote the generation of plasmin from plasminogen. Plasmin can directly activate proMMP-1 and proMMP-13. ProMMP-3 can also be activated by plasmin, and active MMP-3 can process other proMMPs, including the collagenases proMMP-1, proMMP-8, and proMMP-13.

concentrations that corresponded to the IC_{50} values for MMP-13. These data suggest that MMP-1 is not the predominant collagenase involved in cartilage collagen degradation in this bovine nasal cartilage culture system.

Inhibition of the release of proteoglycan fragments by the addition of Dec-RVKR-CH_2Cl to resorbing bovine nasal cartilage. Since the aggrecanases ADAM-TS4 and ADAM-TS5 contain the typical furin recognition motif (9,10) and ADAM-TS4 can be processed by furin (34), we investigated the effect on proteoglycan release of adding the furin inhibitor Dec-RVKR-CH_2Cl to stimulated cartilage. A marked release of proteoglycan was seen from cartilage stimulated for 3 days with IL-1α (1 ng/ml) in combination with OSM (10 ng/ml), as previously reported (15,36). This release was partially blocked following inclusion of Dec-RVKR-CH_2Cl at 100 μM (Figure 6), suggesting that furin is involved in processing enzymes that lead to the breakdown of cartilage proteoglycan.

DISCUSSION

In this study, bovine nasal cartilage stimulated with IL-1α and OSM was used as a model of cartilage resorption. After 14 days in explant culture, this combination of cytokines resulted in an increase in the synthesis of procollagenases and a reproducible release of collagen and proteoglycan fragments (15,36). Moreover, most of the proteoglycan was degraded by day 3 of the culture while the collagen release occurred later, with >90% collagen release by day 14. Although procollagenases were present as early as day 3 of culture, active collagenases and collagen fragments were not detected until after day 7. Addition of pro-MMP activators to IL-1α/OSM–treated bovine nasal cartilage resulted in collagen release by day 7, demonstrating that activation of procollagenases is a rate-limiting step in the degradation of cartilage collagen (17). The mechanisms leading to the activation of procollagenases that occur in vivo are unclear. Several converging pathways are likely to be important (Figure 7). In this study, we investigated the role of furin-like enzymes in the cascade(s) leading to activation of procollagenases and breakdown of the cartilage matrix.

Here we showed that the addition of a furin inhibitor, Dec-RVKR-CH_2Cl, at the beginning of culture to stimulated bovine nasal cartilage resulted in

partial inhibition of collagen release and reduced levels of active collagenase after 14 days of culture. Our data suggest that Dec-RVKR-CH_2Cl targets an enzyme(s) present in cartilage from the beginning of culture. Since procollagenases are not detected until day 3 and active collagenases are not present until after day 7 (17), Dec-RVKR-CH_2Cl appears to target an enzyme(s) involved in the early cascades prior to actual activation of procollagenase itself. Dec-RVKR-CH_2Cl is a potent inhibitor of a furin-like activity, but we cannot rule out another unrecognized serine proteinase present early in the culture period.

In contrast, α_1PI, an inhibitor that does not block furin (data not shown) (51), blocks an enzyme that is up-regulated after day 7 of culture. This may well be a component of the plasminogen activator (PA)/plasmin cascade, since plasmin is inhibited by α_1PI and is a known activator of procollagenases (18–21). These observations suggest that a furin-dependent activation mechanism occurs early in the cartilage assay, while a serine proteinase pathway, possibly the PA/plasmin cascade, is important later in the cartilage assay. These pathways may or may not be linked. Figure 7 summarizes known activation cascades that lead to generation of active collagenases and shows where furin and plasmin may be involved. The activation cascades in vivo are likely to be more complex, possibly involving other enzymes such as cysteine proteinases (52).

Possible furin-activated enzymes involved in cartilage collagen degradation are members of the MT-MMP family since they all contain the furin recognition site (31,32). These MT-MMPs are present in rheumatoid and osteoarthritic tissues (53–55). MT1-MMP can cleave type II collagen (13), which is the major type of collagen in articular cartilage. However, in the bovine nasal cartilage assay, we have shown that the addition of TIMP-1 to resorbing cartilage completely blocks collagen release (17,45). Since TIMP-1 is a poor inhibitor of MT1-MMP (56), these data suggest that MT1-MMP is not directly involved in degrading cartilage collagen. However, MT1-MMP may have a role as an activator of other proMMPs. MT1-MMP can activate proMMP-2 and proMMP-13 (20,26). Indeed, we showed that Dec-RVKR-CH_2Cl reduced proMMP-2 activation in stimulated cartilage. This is consistent with the results of several previous studies, which have shown that the addition of Dec-RVKR-CH_2Cl to cell cultures prevents proMMP-2 activation by blocking furin-dependent activation of proMT-MMPs (39,57–60).

All 3 known human collagenases, proMMPs 1, 8, and 13, are synthesized by human articular chondrocytes and are up-regulated by proinflammatory cytokines (61–65). Moreover, we recently demonstrated that the combination of IL-1α and OSM was able to synergistically induce messenger RNA (mRNA) expression for all 3 collagenases in chondrocytes, with a concomitant increase in MT1-MMP expression (66). Increased MMP-1 expression has also been detected in rheumatoid synovium and cartilage (67) and in osteoarthritic cartilage (68). MMP-13 protein is present in the pannus–hard tissue junction in RA (69) and in osteoarthritic cartilage (68). However, type II collagen is the principal collagen in cartilage and is cleaved most efficiently by MMP-13 (25,63).

The ability of MT1-MMP to activate the collagenase proMMP-13, but not proMMP-1 or proMMP-8 (20), is of interest. Results obtained in the present study with selective MMP-1 inhibitors suggest that MMP-1 is not the major collagenase involved in IL-1α/OSM–induced cartilage collagen release. Other collagenases involved could be MMP-8 and MMP-13. In addition, gelatinases may be important in the degradation of denatured collagen and the release of fragments into the culture medium. Together with the furin inhibitor data, these results suggest that MMP-13 may be the predominant collagenase involved in cartilage collagen degradation in the bovine system. Clearly, it would be important to use a specific MMP-13 inhibitor to further corroborate this hypothesis. In addition, we cannot rule out the presence of unidentified and uncharacterized furin-activated proteinases that may play a role in cartilage collagen degradation. Inhibitor studies have shown a role for MMP-13 and MMP-8, but not MMP-1, in collagen degradation (70,71). Other studies using bovine nasal cartilage suggest that MMP-3, MMP-8, and MMP-13 do not have a major role (72), while a partial role for MMP-1 in collagen breakdown has been reported (71). The use of inhibitors in cartilage explant cultures has limitations. It is not known to what extent these inhibitors penetrate the cartilage. This may be influenced by the stability and charge of the inhibitors and their interaction with the cartilage matrix.

Furin may play a role in the mechanisms leading to the destruction of cartilage in the arthritic joint because elevated levels of furin are present in osteoarthritic cartilage compared with normal human articular cartilage (35). Furin is localized to zones of cartilage where raised levels of MMP-13 are also detected (73). An activation cascade involving MT1-MMP processing of proMMP-13 is supported by several observations of coordinate expression of these MMPs together with MMP-2. In RA, MMP-13 mRNA is expressed simulta-

neously with MT1-MMP and MMP-2 mRNA in fibroblast-like cells of the synovial membrane and in the pannus–hard tissue junction (74,75). In addition, coordinated activation of proMMP-13 and proMMP-2 in SW1353 chondrosarcoma cells following MT1-MMP up-regulation results in increased collagenolytic activity (76).

The aggrecanases ADAM-TS4 and ADAM-TS5 are considered to be the principal proteinases responsible for cartilage proteoglycan degradation, and these enzymes contain the typical furin recognition motif (9,10). This study shows that the addition of a furin inhibitor to resorbing cartilage can partially block the breakdown of the proteoglycan matrix. This suggests that furin-activated enzymes are involved in proteoglycan breakdown. Gao et al (34) demonstrate that Dec-RVKR-CH_2Cl inhibits the processing of ADAM-TS4, resulting in an inactive enzyme, which is consistent with our results. The observation that Dec-RVKR-CH_2Cl only partially blocked proteoglycan release suggests a role for enzymes that are not furin-activated or that furin inhibition was incomplete. Since Dec-RVKR-CH_2Cl is unstable in culture medium at 37°C, a likely explanation for the partial inhibition of proteoglycan release is the instability of this inhibitor. In addition, the presence of highly charged proteoglycan may hinder the penetration of the inhibitor into the cartilage. Some studies have raised the possibility that aggrecanase activity may be regulated by posttranscriptional mechanisms (77,78). Gao et al (34) demonstrate that the production of active ADAM-TS4 requires the removal of the pro domain by a furin-like activity as well as the removal of a portion of the C-terminal spacer domain by MMP-mediated activity.

In summary, we have shown that furin-like enzymes play a role in the early cascades leading to activation of procollagenases and subsequent cartilage collagen degradation. Up-regulated trypsin-like serine proteinases are also involved in these activation mechanisms. Furin-like enzymes also appear to play a role in the mechanisms leading to degradation of the proteoglycan matrix. The enzymes processed by furin-like enzymes may be members of the ADAM-TS family or MT-MMPs, both of which contain furin recognition motifs. In addition, we present data to suggest that MMP-13, an enzyme that can be activated by furin-processed MT1-MMP, is a predominant collagenase involved in cartilage collagen degradation in the bovine system. An understanding of the mechanisms leading to activation of procollagenases and subsequent degradation of the cartilage matrix is important to identify novel targets for possible future therapeutic intervention.

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