J. Biochem. 122, 49-54 (1997)

# Collagen Degradation Induced by the Combination of IL-1α and Plasminogen in Rabbit Articular Cartilage Explant Culture

**Shigeki Saito,[1] Masao Katoh, Mari Masumoto, Shun-ichiro Matsumoto, and Yasuhiko Masuho**

*Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305*

Received for publication, January 14, 1997

**To investigate the effect of plasminogen on cartilage catabolism, we assessed collagen degradation in rabbit articular cartilage explants treated with or without plasminogen and interleukin-1α (IL-1α). The combination of IL-1α and plasminogen induced rapid collagen degradation, amounting to more than 60% of the total collagen by day 7, while neither IL-1α nor plasminogen alone had any effect. To examine the mechanism of collagen degradation induced by IL-1α and plasminogen, the matrix metalloproteinases (MMPs) in the culture supernatants were examined by ELISA, Western blotting and gelatin zymography. We found that the treatment with IL-1α induced MMP-1, MMP-3, and MMP-9. In addition, plasminogen converted the pro form of MMPs into the active form. Both a tissue inhibitor of metalloproteinases-1 (TIMP-1) and a synthetic hydroxamate MMP inhibitor prevented this collagen release. These results suggest that plasminogen causes collagen degradation *via* activation of MMPs induced by IL-1α.**

**Key words: cartilage, collagen, interleukin-1α, matrix metalloproteinase, plasminogen.**

Cartilage matrix is composed primarily of type II collagen fibrils and highly charged proteoglycan. Degradation of cartilage matrix, which is a major feature of osteoarthritis (OA) and rheumatoid arthritis (RA), leads to the functional loss of joints. Proteoglycan retains water in the cartilage matrix, and its loss, a phenomenon observed in the early stage of OA (*1, 2*), results in reduced stiffness of the cartilage (*3*). In addition, type II collagen, which comprises 95% of the total collagen in articular cartilage (*29*), plays a role in maintaining the integrity of the cartilage matrix and allows proteoglycan to be held in the matrix (*4*). In view of these facts, investigation into the importance of cartilage degradation should include the monitoring of both collagen and proteoglycan catabolism. Proteoglycan degradation in chondrocytes and cartilage explants has been well studied to date. The inflammatory cytokine IL-1, which is suggested to be involved in the pathogenesis of arthritis (*28*), induces rapid release of proteoglycan from chondrocytes within 24 h (*5*). On the other hand, collagen catabolism has not been well characterized. Bovine cartilage explants cultured in the presence of IL-1 alone required 15 to 25 days for the release of collagen fragments corresponding to 60% of their initial total amount of collagen (*6, 7*). Thus, collagen degradation in explants has, to date, been demonstrated in only an extremely slow and irreproducible way.

The involvement of matrix metalloproteinases (MMPs) in cartilage degradation has been deduced from their ability to degrade all the components of the extracellular matrix (*8*). In addition, MMPs appear to be involved in connective tissue diseases because of their presence in the cartilage (*9*), synovium (*10*), and synovial fluid (*11, 12*) of OA and RA patients. MMPs are synthesized and released in response to stimulants, including IL-1 and TNFα, but as inactive proenzyme forms which require extracellular activation (*8*). Their activation is thus a regulatory point for their proteolytic activity. The slow collagen degradation observed in the earlier studies might have been due to the absence or inadequacy of MMP activation. Although the mechanisms by which MMPs are activated *in vivo* remain unclear, plasmin is a candidate for a physiological activator of MMPs (*8*) and its precursor, plasminogen, is abundant in the plasma and synovial fluid of patients with arthritis (*13*).

In this study, therefore, we examined the effect of plasminogen on collagen degradation in the presence of IL-1α in a rabbit cartilage explant culture system.

## MATERIALS AND METHODS

*Chemicals and Reagents*—Human recombinant IL-1α and human Lys-type plasminogen were purchased from R and D Systems (Minneapolis, MN, USA) and TechnoClone GmbH (Vienna, Austria), respectively. Peroxidase-conjugated rabbit antibodies against mouse IgG were from Zymed Laboratories (San Francisco, CA, USA), and Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY, USA). CI-139 ($N^{\alpha}$-[[3-(*N*-hydroxycarbamoyl)-4-methylthio-2-propoxymethyl] butanoyl]-*N,O*-dimethyl tyrosinamide), a hydroxamate derivative, which is a potent inhibitor of many MMPs, was synthesized at

[1] To whom correspondence should be addressed. Fax: +81-298-52-5412, Tel: +81-298-54-1612

Abbreviations: MMP, matrix metalloproteinase; IL-1α, interleukin-1α; TNFα, tumor necrosis factor α; TIMP, tissue inhibitor of metalloproteinases; FBS, fetal bovine serum; OH-Pro, hydroxyproline; OA, osteoarthritis; RA, rheumatoid arthritis; APMA, 4-aminophenyl mercuric acetate; cDNA, complementary deoxyribonucleic acid.

Yamanouchi Pharmaceutical (WO patent No. 9309090-A1).

*Protein Source*—Using the rabbit synovial cell cDNA library (*17*), rabbit MMP-3 cDNA (*18*) was obtained with an RNA LA PCR Kit (Takara, Kyoto). The cDNA was ligated into expression vector pEF-BOS-dhfr, which has an expression unit of dihydrofolate reductase at the AatII site of pEF-BOS (*19*). The expression plasmid was transfected to CHO/dhfr$^-$ cells and a clone stably expressing MMP-3 was obtained by sequentially increasing the methotrexate concentration. MMP-3 was purified from the culture supernatants of the transfectants using the procedure described by Housley *et al.* (*20*).

Rabbit MMP-1 cDNA (*21*) was also obtained from the rabbit synovial cell cDNA library, and MMP-1-expressing CHO transfectants were obtained as described above. Recombinant rabbit MMP-1 was purified from the culture supernatants of MMP-1-expressing CHO transfectants, as described by Suzuki *et al.* (*22*).

Rabbit MMP-2 cDNA (*17*) and MMP-9 cDNA (*23*), which was kindly provided by Dr. H. Kawashima, were ligated into expression vector pEF-BOS-dhfr and expressed in COS cells.

Human TIMP-1 cDNA (*24*) was obtained from the total RNA of HeLa S3 cells, and TIMP-1-expressing CHO transfectants were obtained as described above. Recombinant TIMP-1 was purified from the conditioned medium of TIMP-1-expressing CHO transfectants, as described by Roswit *et al.* (*25*).

*Explant Culture*—Rabbit articular cartilage was obtained from the knee joints of 5-6-week-old male Japanese white rabbits. The cartilage was cut into slices, whose weight was adjusted to approximately 5 mg, and they were placed in the wells of 24-well plates. Quadruplicate cartilage slices (explants) were used for each point. Explants were cultured in 0.5 ml of DMEM containing 50 U/ml penicillin, 50 $\mu$g/ml streptomycin, 2.5 $\mu$g/ml amphotericin B, 25 mM HEPES, and 1% FBS.

*Collagen Degradation Assay*—Cartilage explants were cultured in the presence of 1 ng/ml IL-1$\alpha$ and various concentrations of plasminogen. Degradation of collagen matrix was determined by measuring hydroxyproline (OH-Pro), a marker specific for collagen, as follows. At the end of culture, the residual cartilage explants were removed from the culture wells and completely digested with 4.5 mg/ml of papain for 18 h at 55°C. The culture supernatants and digested cartilage were hydrolyzed in 6 N HCl at 105°C for 20 h. Their OH-Pro contents were then measured using the method of Ellis *et al.* (*7*). The hydrolysates were dried, dissolved in water and incubated with chloramine-T reagent in microtiter plates at room temperature for 4 min, followed by the addition of DAB (dimethylaminobenzaldehyde) reagent. The microtiter plates were heated for 35 min at 60°C, and then the absorbance of individual wells at 560 nm was measured with a 96-well plate reader. The results are expressed as the percentage of the OH-Pro amount in the culture supernatant with respect to the total amount.

*Quantitation of MMP-1 and MMP-3 by ELISA*—The MMP-1 in the culture supernatants was measured using a double-antibody sandwich ELISA system. Chicken IgY generated against rabbit MMP-1 was used as the primary trapping antibody. The secondary antibody was a mouse polyclonal IgG generated against rabbit MMP-1. These antibodies specifically recognized MMP-1. Plates were coated with the chicken anti-MMP-1 IgY and blocked with 1% BSA in 10 mM Tris-Cl, 150 mM NaCl, pH 7.4. The plates were then incubated with samples, followed by the mouse anti-MMP-1 IgG. The plates were incubated with peroxidase-conjugated rabbit antibodies against mouse IgG at a dilution rate of 1:1,000, followed by addition of tetramethylbenzidine.

The MMP-3 in the culture supernatants was also measured using a double-antibody sandwich ELISA system. Mouse monoclonal antibodies MP1819 and MP3049 were generated against rabbit MMP-3. These antibodies specifically recognized MMP-3. ELISA was performed as described above. Briefly, plates were coated with MP1819 and blocked with 1% BSA. The plates were then incubated with samples, followed by biotinylated MP3049. The plates were incubated with peroxidase-conjugated streptavidin at a dilution rate of 1:1,000, followed by addition of tetramethylbenzidine.

*Western Blot Analysis of MMPs Secreted from Explants*—Culture supernatants were collected from cartilage explants cultured for 7 days in the presence or absence of plasminogen and/or IL-1$\alpha$. The culture supernatants were heat-denatured in boiling water and subjected to 10-20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred onto a PVDF membrane and blocked with Blockace (Snow Brand, Sapporo). The membrane was then incubated with 1 $\mu$g/ml of a monoclonal antibody, MP1006. This antibody was obtained from the culture supernatants of hybridoma cells that were established by fusing mouse myeloma P3U1 with spleen cells from mice immunized with keyhole limpet hemocyanin conjugates of the MMP-3 peptide (NYTPDLPRDAVDAAIEKALK), which corresponds to residues 121 through 140 of rabbit proMMP3. This MP1006 antibody reacts with rabbit MMP-1 and MMP-3 in both their latent and active forms. After washing, the membrane was incubated with peroxidase-conjugated rabbit antibodies against mouse IgG at a dilution rate of 1:2,000, followed by staining by the enhanced chemiluminescent detection method (NEN, Boston, MA, USA) according to the manufacturer's instructions. Standard active forms of rabbit MMP-1 and MMP-3 were generated by incubation with 1 mM 4-aminophenyl mercuric acetate (APMA) at 37°C for 1 h.

*Gelatin Zymography*—Culture supernatants were applied without reduction to a 10% polyacrylamide slab gel impregnated with 1 mg/ml gelatin. Gel electrophoresis was performed at 4°C. After electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 for 1 h and then for 18 h at 37°C in 50 mM Tris-Cl, pH 7.5, containing 200 mM NaCl, 10 mM $CaCl_2$, 10 $\mu$M $ZnCl_2$, and 0.02% Brij-35. The gel was then stained with Coomassie Blue.

## RESULTS

*Collagen Degradation Induced by Treatment with IL-1$\alpha$ and Plasminogen in Rabbit Cartilage Explant Culture*—Rabbit cartilage explants were cultured in the presence of IL-1$\alpha$ and/or plasminogen to determine whether plasminogen affects collagen degradation. Explants were cultured for up to 10 days, and the collagen released into the

media was assessed by measuring the release of OH-Pro. As shown in Fig. 1, no collagen release was detected from cartilage treated with IL-1α alone until day 10 and at least until day 21 in another experiment (data not shown). Treatment with plasminogen alone hardly released collagen, *i.e.*, only 5% on day 10. In contrast, collagen degradation was observed as early as day 2 when both 1 ng/ml of IL-1α and 100 μg/ml of plasminogen were added, and more than 60% of the initial collagen content was degraded and released by day 7. The total amount of OH-Pro in the culture supernatant and explant was constant during culture for up to 10 days. In the next experiment, the concentration of plasminogen was varied in the presence of 1 ng/ml IL-1α in culture for 7 days. As shown in Fig. 2, the release of OH-Pro reached a maximum at a plasminogen concentration of 10 μg/ml. These results suggest that the combination of IL-1α and plasminogen is essential for degradation of the collagen in rabbit cartilage.

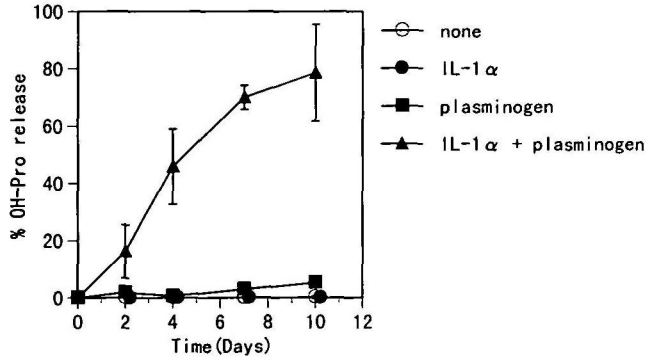


Fig. 1. **Time-course of release of collagen fragments from rabbit cartilage in the presence of IL-1α and/or plasminogen.** Rabbit cartilage explants were stimulated with IL-1α (1 ng/ml) and/or plasminogen (100 μg/ml). At various times up to day 10, the culture supernatants were removed and the percentages of total collagen released into the media were determined by OH-Pro assay. Values are expressed as the mean and standard deviation of quadruplicate assays.

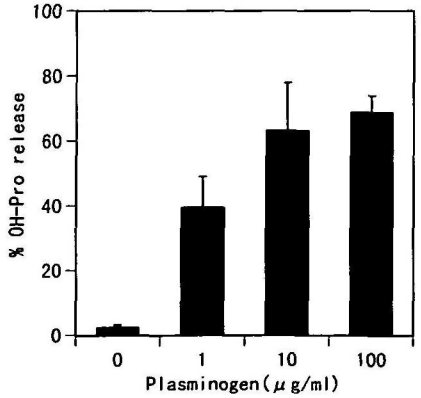


Fig. 2. **Effect of increasing plasminogen concentration on release of collagen fragments from rabbit cartilage in the presence of IL-1α.** Rabbit cartilage explants were cultured in medium containing various concentrations of plasminogen in the presence of IL-1α (1 ng/ml). On day 7, the culture supernatants were removed, and the percentages of the total collagen released were determined by OH-Pro assay. Values are expressed as the mean and standard deviation of quadruplicate assays.

*Induction and Activation of MMP-1 and MMP-3 by IL-1α and Plasminogen*—To study the mechanism of collagen degradation induced by the combination of IL-1α and plasminogen, MMP-1 and MMP-3 in the culture supernatants were analyzed using ELISA. The treatment with IL-1α induced the production of MMP-1 and MMP-3, whereas no MMP-1 or MMP-3 protein was detected in the control medium (Fig. 3, A and B). Then, we examined whether MMP-1 and MMP-3 were activated by the addition of plasminogen or not by Western blotting using the monoclonal antibody MP1006, which reacts with both the latent and active forms of rabbit MMP-1 and MMP-3 (Fig. 3C). Both standard rabbit proMMP-1 (lane 7) and proMMP-3 (lane 9) moved the same distance on SDS-PAGE because they have almost the same molecular weight (55 and 57 kDa, respectively) (*8*). Induction of a band corresponding to proMMP-1 and proMMP-3 was observed in the culture supernatant of an explant stimulated with IL-1α alone (lane 2). An unknown band just above proMMP-1 and proMMP-3 (lanes 1, 2) did not seem to be important for collagen degradation, because the same band was also observed in the medium containing 1% FBS that had not been exposed to explants (data not shown). The addition of plasminogen to IL-1α reduced the molecular

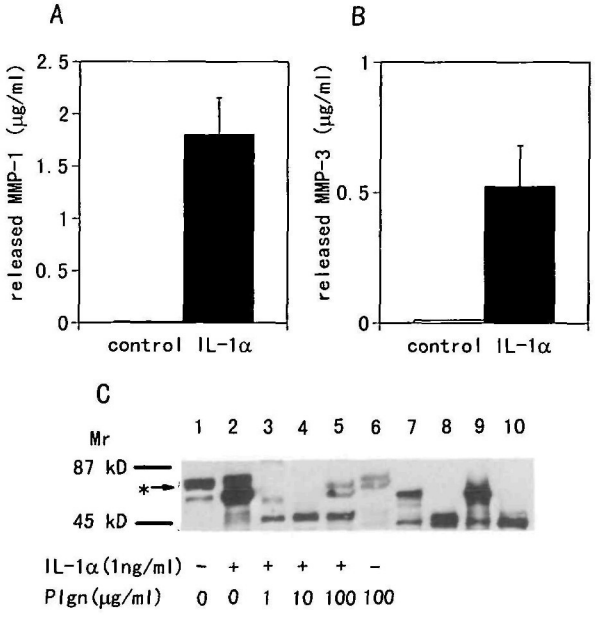


Fig. 3. **Induction and activation of MMP-1 and MMP-3 by IL-1α and plasminogen.** Rabbit cartilage explants were cultured in the presence of IL-1α (1 ng/ml) and/or plasminogen at the indicated concentrations. The culture supernatants, after incubation for 7 days in control medium or medium containing IL-1α (1 ng/ml), were analyzed by (A) ELISA for MMP-1 and (B) ELISA for MMP-3. (C) Western blotting with monoclonal antibody MP1006, which reacts with MMP-1 and MMP-3. The band marked with an asterisk in lanes 1 and 2 was derived from FBS included in the medium. Lane 1, control culture; lane 2, IL-1α (1 ng/ml); lane 3, IL-1α (1 ng/ml) + plasminogen (1 μg/ml); lane 4, IL-1α (1 ng/ml) + plasminogen (10 μg/ml); lane 5, IL-1α (1 ng/ml) + plasminogen (100 μg/ml); lane 6, plasminogen (100 μg/ml); lane 7, proMMP-1 standard; lane 8, APMA-activated MMP-1 standard; lane 9, proMMP-3 standard; and lane 10, APMA-activated MMP-3 standard. In the culture supernatants with 100 μg/ml plasminogen, unknown doublet bands were detected near 60 kDa (lanes 5 and 6).

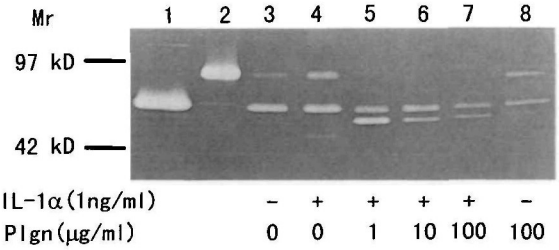


Fig. 4. **Induction and activation of gelatinase by IL-1$\alpha$ and plasminogen.** Rabbit cartilage explants were cultured in the presence of IL-1$\alpha$ (1 ng/ml) and/or plasminogen at the indicated concentrations. The culture supernatants were subjected to gelatin zymography. Lane 1, proMMP-2 standard; lane 2, proMMP-9 standard; lane 3, control culture; lane 4, IL-1$\alpha$ (1 ng/ml); lane 5, IL-1$\alpha$ (1 ng/ml)+plasminogen (1 $\mu$g/ml); lane 6, IL-1$\alpha$ (1 ng/ml)+plasminogen (10 $\mu$g/ml); lane 7, IL-1$\alpha$ (1 ng/ml)+plasminogen (100 $\mu$g/ml); and lane 8, plasminogen (100 $\mu$g/ml).

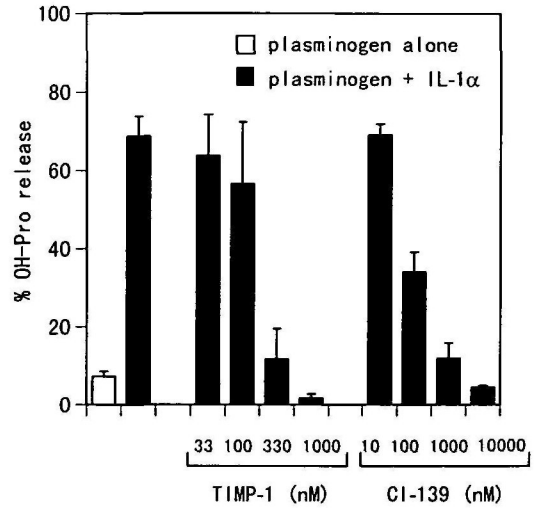


Fig. 5. **Effect of MMP inhibitors on the collagen degradation in the presence of IL-1$\alpha$ and plasminogen in rabbit cartilage explants.** Rabbit cartilage explants stimulated with IL-1$\alpha$ (1 ng/ml) and plasminogen (100 $\mu$g/ml) were cultured in the presence of TIMP-1 or the hydroxamate MMP-inhibitor, CI-139, for 7 days. Percentages of total collagen released were determined by OH-Pro assay and expressed as the mean and standard deviation of quadruplicate assays.

weight to about 45 kDa (lanes 3, 4, and 5), which corresponds to the active truncated forms of MMP-1 (lane 8) and MMP-3 (lane 10), while MMP-1 and MMP-3 were not detected in the culture supernatants of explants cultured in the absence of IL-1$\alpha$ (lane 6). This suggested that MMP-1 and MMP-3 induced by IL-1$\alpha$ were activated by the addition of plasminogen, although MP1006 did not distinguish MMP-1 and MMP-3. In the explants cultured with IL-1$\alpha$ and 1 $\mu$g/ml of plasminogen, both the pro and active forms of MMP were detected (lane 3), while only the active form was detected in the explants treated with 10 $\mu$g/ml of plasminogen (lane 4). This increase in the active forms between 1 and 10 $\mu$g/ml of plasminogen correlated with the increase in collagen degradation, as shown in Fig. 2. Although the active form in lane 5 appeared to be less than the pro form in lane 2, this was because this antibody had the character of reacting more weakly with the active form of MMP than with the pro form of MMP. Thus, plasminogen was essential for the activation of MMP-1 and MMP-3, which were produced by stimulation with IL-1$\alpha$.

*Induction and Activation of Gelatinase by IL-1$\alpha$ and Plasminogen*—Gelatin zymography of the culture supernatants from control explants (Fig. 4, lane 3) and explants stimulated with IL-1$\alpha$ (lane 4) demonstrated that treatment with IL-1$\alpha$ increased the gelatinase activity, which corresponded in electrophoretic mobility to the proMMP-9 standard (lane 2). Although activity corresponding to proMMP-2 standard (lane 1) was detected in the culture supernatants from control explants (lane 3), the level of proMMP-2 activity was not increased by IL-1$\alpha$ treatment (lane 4).

In the explants cultured with 1 $\mu$g/ml of plasminogen and IL-1$\alpha$ (lane 5), no band corresponding to proMMP-9 was detected, but two bands were detected at 72 and 66 kDa. Treatment with APMA or MMP-3 was reported to convert proMMP-9 to the active-form with 67 kDa molecular weight (*26*). Therefore, the band detected at 66 kDa might be the active form of MMP-9. It remains unclear whether MMP-2 was activated. It might be that MMP-2 was not activated in this study; this would be consistent with the finding that MMP-2 was not cleaved by plasmin (*27*). At concentrations ranging from 10–100 $\mu$g/ml of plasminogen (lanes 6 and 7), a decrease in gelatinase activity at 66 kDa was observed, probably due to further degradation of the enzyme. The treatment with 100 $\mu$g/ml plasminogen in the absence of IL-1$\alpha$ (lane 8) did not change the electrophoretic mobilities of gelatinase activities corresponding to proMMP-2 and proMMP-9. These data suggest that the combination of IL-1$\alpha$ and plasminogen was essential for activation of proMMP-9.

*Inhibition of Collagen Degradation by TIMP-1 and a Synthetic MMP Inhibitor*—In order to confirm that the release of collagen fragments by combination of IL-1$\alpha$ and plasminogen is mediated by MMPs, but not by plasmin or other enzymes, rabbit cartilage explants were incubated with MMP-specific inhibitors, TIMP-1 and the hydroxamate derivative, CI-139. The release of collagen fragments from the cartilage was completely inhibited by either TIMP-1 or CI-139, as shown in Fig. 5. The $IC_{50}$ values for TIMP-1 and CI-139 were estimated to be approximately 200 and 100 nM, respectively. These results confirm that the collagen degradation was mediated by MMPs, which were activated by plasminogen.

## DISCUSSION

The present study has demonstrated that the combination of IL-1$\alpha$ and plasminogen induces degradation of collagen in rabbit cartilage, whereas neither IL-1$\alpha$ nor plasminogen alone does (Fig. 1). These findings suggest that the presence of both IL-1$\alpha$ and plasminogen is essential for collagen degradation in explants. Although previous investigators showed that IL-1 alone caused collagen degradation in bovine cartilage explants, release of more than 60% collagen required 15 or 25 days (*6, 7*). In contrast, the combination of IL-1$\alpha$ and plasminogen in our experiments resulted in 60% collagen release as early as day 7. In addition, IL-1$\alpha$ alone did not cause any release of collagen fragments from the rabbit cartilage for at least 21 days. These findings, therefore, indicate that plasminogen is

important for rapid collagen degradation in rabbit cartilage explants stimulated with IL-1α.

Regarding the mechanism by which the combination of IL-1α and plasminogen induced collagen degradation, our data indicate that IL-1α induces expression of MMP-1, MMP-3, and MMP-9 in proenzyme forms and that the addition of plasminogen activates these inactive proenzymes to their active forms. As shown in Fig. 3 and Fig. 4, only the proenzyme forms of MMP-1, MMP-3, and MMP-9 were detected on day 7 in the explant cultures treated with IL-1α alone. In contrast, in the presence of plasminogen, MMP-1, MMP-3, and MMP-9 were detected in their active forms. These results imply that plasmin, which is derived from plasminogen, activates MMPs. In fact, it has been reported that plasmin is able to activate purified MMPs (*14*). These results suggest that the inability of IL-1α alone to cause collagen degradation is due to the lack of MMP activation, and that the activation of MMPs by plasmin results in collagen degradation. Which MMP is responsible for this collagen degradation should be examined in further studies.

The mechanism described above is confirmed by the complete inhibition of collagen degradation by MMP inhibitors, as shown in Fig. 5. This result is consistent with a report which showed that MMP inhibitors prevented collagen degradation induced by IL-1 alone in bovine cartilage explants, although the exposure was continued for as long as 15 or 25 days in that system, as mentioned above (*6, 7*). Thus, these data suggest that the collagen degradation we observed here is totally mediated by MMPs, and exclude the possibility that collagen was degraded directly by plasmin derived from plasminogen.

Does the effect of plasminogen on collagen degradation which we observed *in vitro* have any significance in diseases such as OA and RA *in vivo*? As shown in Fig. 2, plasminogen in the presence of IL-1α induced collagen degradation even at 1 μg/ml *in vitro*. The plasminogen level was 138 μg/ml in the plasma and 56 μg/ml in the synovial fluid of patients with arthritis (*13*), making it likely that IL-1α and plasminogen are, in fact, involved in the pathogenesis of collagen degradation. The possibility exists that peroxides and other proteases, such as kallikrein, may induce collagen degradation in concert with IL-1α, because, like plasmin, they can activate MMPs *in vitro* by removing the propeptide domain which masks the active site of MMPs, through limited proteolysis or by oxidation of the cysteine switch of the propeptide (*8*). However, tranexamic acid, which prevents plasminogen activator from converting plasminogen to plasmin, ameliorated cartilage destructive lesions in an experimental OA model (*15*), supporting a significant role of plasmin in MMP activation *in vivo*.

IL-1 alone induced 70% proteoglycan degradation within 3 days in bovine cartilage explants (*7*). Thus, unlike collagen degradation, proteoglycan degradation occurs rapidly even without activators of MMPs, such as plasminogen.

With regard to drug discovery, on the basis of its 60% degradation of collagen within one week, our system is useful for the rapid evaluation of potential drugs which inhibit MMP activity as well as those which reduce MMP production from cartilage. In addition, we suggest that inhibitors which block the fibrinolytic cascade may prevent collagen degradation. This idea is supported by the finding that activation of MMPs in vascular smooth muscle cells was inhibited by aprotinin, which can block serine proteases such as plasmin and plasminogen activator (*16*).

In summary, we have presented evidence that the combination of IL-1α and plasminogen causes rapid collagen degradation in cartilage by generating active MMPs.

We thank Dr. Hiroyuki Kawashima for providing rabbit MMP-9 cDNA and Dr. Shigekazu Nagata for providing the pEF-BOS expression vector.

## REFERENCES

1. Carney, S.L., Billingham, M.E.J., Muir, H., and Sandy, J.D. (1984) Demonstration of increased proteoglycan turnover in cartilage explants from dogs with experimental osteoarthritis. *J. Orthop. Res.* **2**, 201-206
2. McDevitt, C.A. and Muir, H. (1976) Biochemical changes in the cartilage of the knee in experimental and natural osteoarthritis in the dog. *J. Bone Joint Surg.* [*Br*] **58**, 94-101
3. Bonassar, L.J., Frank, E.H., Murray, J.C., Paguio, C.G., Moore, V.L., Lark, M.W., Sandy, J.D., Wu, J., Eyre, D.R., and Grodzinsky, A.J. (1995) Changes in cartilage composition and physical properties due to stromelysin degradation. *Arthritis Rheum.* **38**, 173-183
4. Jubb, R.W. and Fell, H.B. (1980) The breakdown of collagen by chondrocytes. *J. Pathol.* **130**, 159-167
5. Yocum, S.A., Lopresti-Morrow, L.L., Gabel, C.A., Milici, A.J., and Mitchell, P.G. (1995) Bafilomycin A1 inhibits IL-1-stimulated proteoglycan degradation by chondrocytes without affecting stromelysin synthesis. *Arch. Biochem. Biophys.* **316**, 827-835
6. Nixon, J.S., Bottomley, K.M.K., Broadhurst, M.J., Brown, P.A., Johnson, W.H., Lawton, G., Marley, J., Sedgwick, A.D., and Wilkinson, S.E. (1991) Potent collagenase inhibitors prevent interleukin-1-induced cartilage degradation in vitro. *Int. J. Tissue React.* **13**, 237-241
7. Ellis, A.J., Curry, V.A., Powell, E.K., and Cawston, T.E. (1994) The prevention of collagen breakdown in bovine nasal cartilage by TIMP, TIMP-2 and a low molecular weight synthetic inhibitor. *Biochem. Biophys. Res. Commun.* **201**, 94-101
8. Docherty, A.J.P., O'Connell, J., Crabbe, T., Angal, S., and Murphy, G. (1992) The matrix metalloproteinases and their natural inhibitors: prospects for treating degenerative tissue diseases. *Trends Biotechnol.* **10**, 200-207
9. Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M.T., Iwata, K., and Nagase, H. (1992) Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. *Lab. Invest.* **66**, 680-690
10. Gravallese, E.M., Darling, J.M., Ladd, A.L., Katz, J.N., and Glimcher, L.H. (1991) In situ hybridization studies of stromelysin and collagenase messenger RNA expression in rheumatoid synovium. *Arthritis Rheum.* **34**, 1076-1084
11. Lohmander, L.S., Hoerrner, L.A., and Lark, M.W. (1993) Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. *Arthritis Rheum.* **36**, 181-189
12. Walakovits, L.A., Moore, V.L., Bhardwaj, N., Gallick, G.S., and Lark, M.W. (1992) Detection of stromelysin and collagenase in synovial fluid from patients with rheumatoid arthritis and posttraumatic knee injury. *Arthritis Rheum.* **35**, 35-42
13. Kummer, J.A., Abbink, J.J., de Boer, J.P., Roem, D., Nieuwenhuys, E.J., Kamp, A.M., Swaak, T.J., and Hack, C.E. (1992) Analysis of intraarticular fibrinolytic pathways in patients with inflammatory and noninflammatory joint diseases. *Arthritis Rheum.* **35**, 884-893
14. He, C., Wilhelm, S.M., Pentland, A.P., Marmer, B.L., Grant, G.A., Eisen, A.Z., and Goldberg, G.I. (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. Natl. Acad. Sci. USA* **86**, 2632-2636
15. Vignon, E., Mathieu, P., Bejui, J., Descotes, J., Hartmann, D.,

Patricot, L.M., and Richard, M. (1991) Study of an inhibitor of plasminogen activator (tranexamic acid) in the treatment of experimental osteoarthritis. *J. Rheumatol. Suppl.* **27**, 131-133
16. Lee, E., Vaughan, D.E., Parikh, S.H., Grodzinsky, A.J., Libby, P., Lark, M.W., and Lee, R.T. (1996) Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. *Circ. Res.* **78**, 44-49
17. Matsumoto, S., Katoh, M., Watanabe, T., and Masuho, Y. (1996) Molecular cloning of rabbit matrix metalloproteinase-2 and its broad expression at several tissues. *Biochim. Biophys. Acta* **1307**, 137-139
18. Fini, M.E., Karmilowicz, M.J., Ruby, P.L., Beeman, A.M., Borges, K.A., and Brinckerhoff, C.E. (1987) Cloning of a complementary DNA for rabbit proactivator. A metalloproteinase that activates synovial cell collagenase, shares homology with stromelysin and transin, and is coordinately regulated with collagenase. *Arthritis Rheum.* **30**, 1254-1264
19. Mizushima, S. and Nagata, S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322
20. Housley, T.J., Baumann, A.P., Braun, I.D., Davis, G., Seperack, P.K., and Wilhelm, S.M. (1993) Recombinant Chinese hamster ovary cell matrix metalloprotease-3 (MMP-3, stromelysin-1). Role of calcium in promatrix metalloprotease-3 (pro-MMP-3, prostromelysin-1) activation and thermostability of the low mass catalytic domain of MMP-3. *J. Biol. Chem.* **268**, 4481-4487
21. Fini, M.E., Plucinska, I.M., Mayer, A.S., Gross, R.H., and Brinckerhoff, C.E. (1987) A gene for rabbit synovial cell collagenase: member of a family of metalloproteinases that degrade the connective tissue matrix. *Biochemistry* **26**, 6156-6165
22. Suzuki, K., Enghild, J.J., Morodomi, T., Salvesen, G., and Nagase, H. (1990) Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry* **29**, 10261-10270
23. Tezuka, K., Nemoto, K., Tezuka, Y., Sato, T., Ikeda, Y., Kobori, M., Kawashima, H., Eguchi, H., Hakeda, Y., and Kumegawa, M. (1994) Identification of matrix metalloproteinase 9 in rabbit osteoclasts. *J. Biol. Chem.* **269**, 15006-15009
24. Docherty, A.J., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J., Murphy, G., and Reynolds, J.J. (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* **318**, 66-69
25. Roswit, W.T., McCourt, D.W., Partridge, N.C., and Jeffrey, J.J. (1992) Purification and sequence analysis of two rat tissue inhibitors of metalloproteinases. *Arch. Biochem. Biophys.* **292**, 402-410
26. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. (1992) Matrix metalloproteinase 9 (92-kDa gelatinase/Type IV collagenase) from HT 1080 human fibrosarcoma cells. *J. Biol. Chem.* **267**, 21712-21719
27. Matrisian, L.M. (1992) The matrix-degrading metalloproteinases. *BioEssays* **14**, 455-463
28. McDonnell, J., Hoerrener, L.A., Lark, M.W., Harper, C., Dey, T., Lobner, J., Eiermann, G., Kazazis, D., Singer, I.I., and Moore, V.L. (1992) Recombinant human interleukin-1$\beta$-induced increase in levels of proteoglycans, stromelysin, and leukocytes in rabbit synovial fluid. *Arthritis Rheum.* **35**, 799-805
29. Eyre, D.R., Wu, J.J., and Woods, P. (1992) Cartilage-specific collagens: structural studies in *Articular Cartilage and Osteoarthritis* (Kuettner, K.E., Schleyerbach, R., Peyron, J.G., and Hascall, V.C., eds.) pp. 119-131, Raven Press, New York