

# Activation of progelatinase B in synovial fluids of patients with rheumatoid arthritis, with reference to stromelysin-1 and tissue inhibitor of matrix metalloproteinase-1

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## Abstract

### Objective

*To clarify whether stromelysin-1 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) are involved in the modulation of activation of progelatinase B in the synovial fluid of patients with rheumatoid arthritis (RA).*

### Methods

*Gelatinases in the synovial fluid of patients with RA were analyzed by gelatin zymography. Concentrations of stromelysin-1 and TIMP-1 were measured using a specific sandwich enzyme linked immunosorbent assay.*

### Results

*Forty-three rheumatoid synovial fluids containing progelatinase B were examined to clarify whether the enzyme was activated by incubation. Incubation at 37°C caused the conversion of progelatinase B to the active form in 22 of the 43 synovial fluids. The levels of both stromelysin-1 and TIMP-1 were determined for each group and the concentration ratio of stromelysin-1/TIMP-1 in the synovial fluids of each group was highly correlated to the activation of progelatinase B.*

### Conclusion

*The balance between the concentrations of stromelysin-1 and of TIMP-1 in the synovial fluid appears to determine whether the progelatinase B molecule causes conversion into the active form.*

### Key words

Progelatinase B, stromelysin-1, tissue inhibitor of metalloproteinase-1, synovial fluid, rheumatoid arthritis.

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## Introduction

Rheumatoid arthritis (RA) is a disease in which proliferating synovial pannus degrades the cartilage extracellular matrix, causing joint tissue damage. A number of proteolytic enzymes are secreted into the synovial fluids of RA patients and they participate in the degradation of components of the matrix. Matrix metalloproteinases (MMPs) are thought to play a crucial role in the cartilage destruction of joints in patients with RA. More than fifteen members of the MMP family have been reported (1). The gelatinases represent a subgroup of the MMP family consisting of two distinct enzymes, the gelatinases A (MMP-2) and B (MMP-9), which have similar activity. Gelatinase B is expressed by inflammatory cells and the enzyme has been found at high levels in the synovial fluid of patients with RA (2-5). The activity of gelatinase B is thought to be regulated by the levels of activation of progelatinase B and inhibited by TIMP-1. Although progelatinase B is known to be activated by many proteases, stromelysin-1 (MMP-3) is thought to be one of the physiological activators of the enzyme (6). Stromelysin-1 activates progelatinase B in a stepwise manner. The initial cleavage at the Glu<sup>40</sup>-Met<sup>41</sup> bond of the pro-peptide sequence of progelatinase B by stromelysin-1 is required for the second cleavage at the Arg<sup>87</sup>-Phe<sup>88</sup> bond. TIMP-1 is not only a potent inhibitor for gelatinase B, but the progelatinase B and TIMP-1 complex is highly resistant to activation by stromelysin-1 (7).

In this study, to investigate the regulatory mechanism of progelatinase B activation in the synovial fluid of patients with RA, we examined the relationship between progelatinase B activation and the concentrations of stromelysin-1 and TIMP-1. Our findings demonstrate that the concentration ratio of stromelysin-1/TIMP-1 plays an important role in the positive regulation of progelatinase B activation in synovial fluids.

## Materials and methods

### Human synovial fluid specimens

Patients who satisfied at least 4 of the 7 criteria for the classification of RA as revised by the American Rheumatism

Association (9) were entered in this study. No patients were being treated with steroids. Arthrocentesis was performed for therapeutic injury. Synovial fluid aspirated from knee joints was centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were then subdivided and immediately stored at -70°C until use. All fluids were used only once, because the progelatinase B present in synovial fluids may sometimes be converted to the active form by repeated freezing and thawing.

### Chemicals and proteins

Human recombinant TIMP-1 was purchased from Cosmo Bio. (Tokyo). Rat N-[propionate-2,3-<sup>3</sup>H]-labeled type I collagen (0.0052 GBq/mg protein, 2.2 MBq/ml) and Aquasol-2 were obtained from Du Pont-New England Nuclear (Wilmington, DE). Stromelysin-1 was purified from the synovial fluid of RA patients as described previously (2). All other reagents used were of the highest grade available from commercial sources.

### Gelatin zymography

For the detection of gelatinase activity, synovial fluid (equivalent to 0.4 µl) was run, without reduction, in a 0.1% SDS, 7.5% polyacrylamide gel impregnated with 0.3 mg/ml gelatin. Gel electrophoresis was performed at 4°C. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 15 min and then incubated for 16 hrs at 37°C in a buffer containing 50 mM Tris-HCl, pH 7.6, 5 mM CaCl<sub>2</sub>, 1% Triton X-100 and 0.02% NaN<sub>3</sub>. Following this incubation, the gel was stained for proteins with 0.1% Coomassie brilliant blue in 40% (v/v) isopropanol and destained in 7% (v/v) acetic acid. A minigel apparatus from ATTO (Tokyo) was used for the gel.

### <sup>3</sup>H-gelatin degradation assay

The substrate, rat N-[propionate-2,3-<sup>3</sup>H]-labeled type I collagen, was first denatured for 30 min at 60°C. All reaction mixtures contained 1.1 Bq substrate, 0.2M NaCl, 5 mM CaCl<sub>2</sub>, 30 mM Tris-HCl, pH 7.6, and 30 µl of synovial fluid samples in a total reaction volume of 100 µl. The assay mixtures were incubated for 2 hrs at 37°C and reactions were stop-

ped by the addition of 50  $\mu$ l of 30% TCA. After standing in an ice bath for 10 min, the tubes were centrifuged at 10,000  $\times$  g for 10 min and the radioactivity in 100  $\mu$ l of each supernatant was measured in Aquasol-2 solution with an Aloka liquid scintillation counter (model LSC-1000).

#### Assay of stromelysin-1 and TIMP-1

The concentrations of stromelysin-1 and TIMP-1 in the synovial fluid were determined by specific sandwich EIA using a human MMP-3 ELISA kit (Fuji Chemical Industries, LTD, Toyama) and an hTIMP-1 determination kit (Daiichi Pure Chemicals, Tokyo), respectively.

#### Results

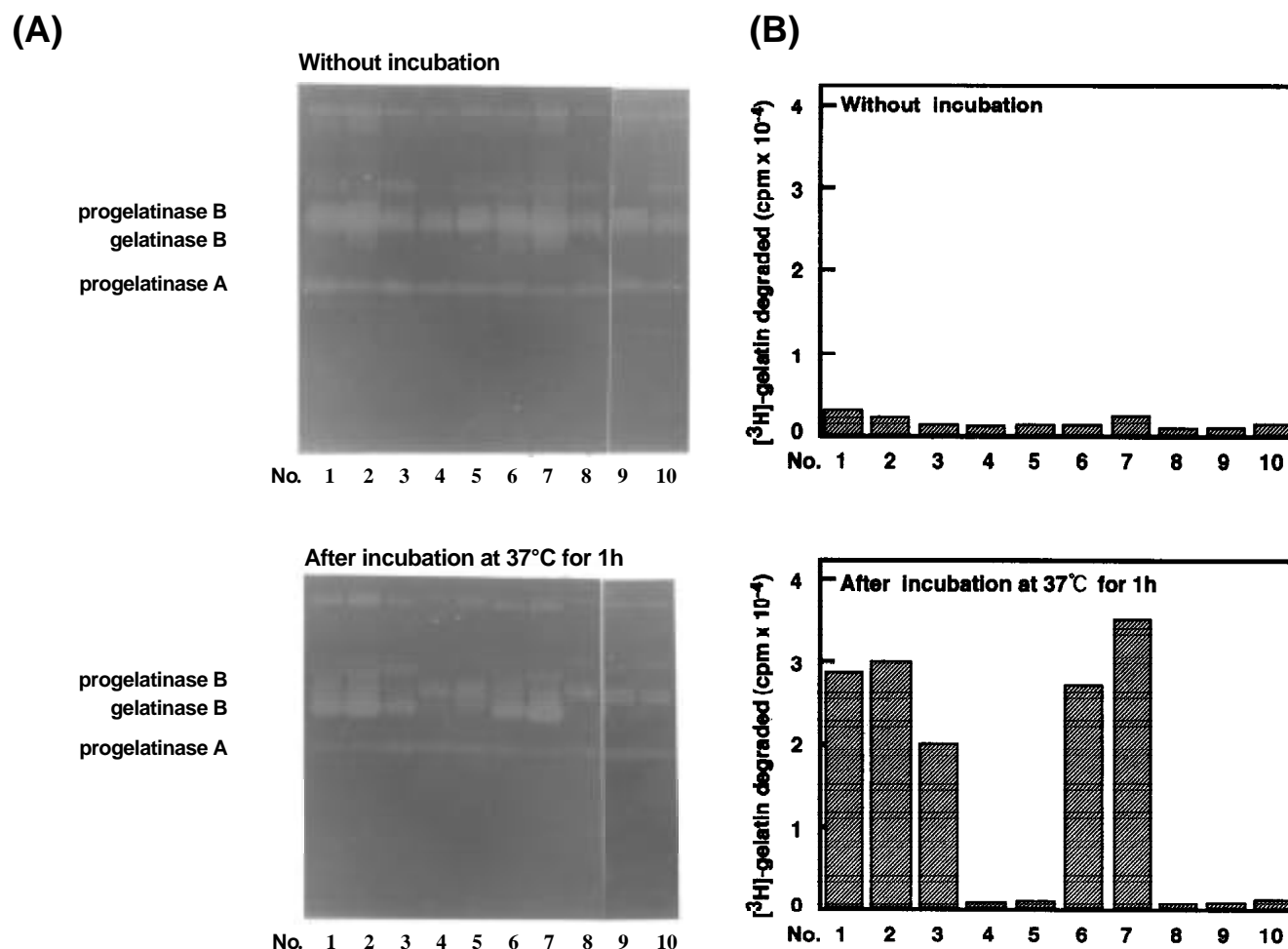
In our previous study, we analyzed gelatinases in synovial fluid samples from

patients with RA by gelatin zymography, and we classified the patients into three distinct groups according to the appearance of the enzymes (2). Although a similar quantity of inactive gelatinase A was present in all of the synovial fluids, including those from the control group, the appearance of gelatinase B varied between individuals.

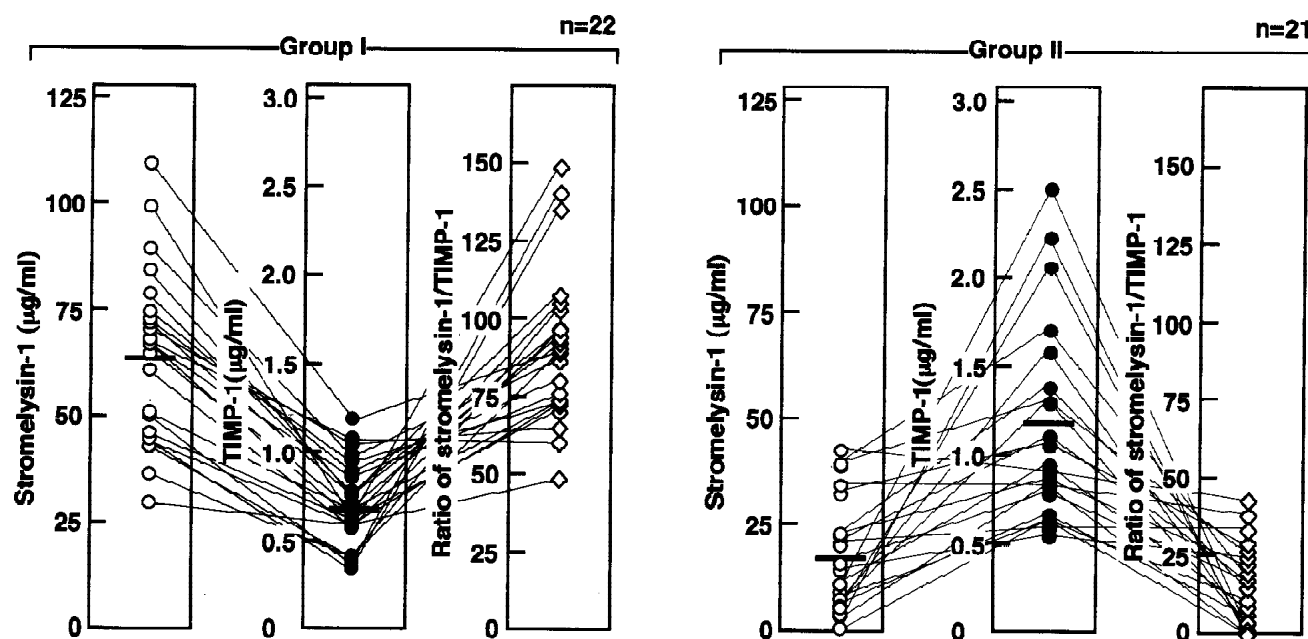
We selected 10 different synovial fluids containing progelatinase B from patients with RA and compared progelatinase B activation after incubation at 37°C using gelatin zymography (Fig. 1A). Activated gelatinase B is discriminated from progelatinase B by its reduced molecular weight. After incubation for 1 hr, five samples (lanes 1, 2, 3, 6, and 7 in Fig. 1A) effectively converted progelatinase B to the active forms, while the other five

(lanes 4, 5, 8, 9, and 10 in Fig. 1A) resisted activation. Activation of progelatinase B by incubation was also assessed by measuring the hydrolysis of heat denatured  $^3$ H-labeled type I collagen as the substrate (Fig. 1B). Increased levels of activity were only observed in synovial fluid samples converted into the active form. Five samples which were unconverted after incubation for 1 hr were still unchanged after further incubation for 24 hrs at 37°C (data not shown).

Although progelatinase B is known to be activated by many proteases, stromelysin-1 is thought to be a physiological activator of the enzyme (6). Furthermore, progelatinase B is known to form a complex with TIMP-1, and the progelatinase B-TIMP-1 complex is highly resistant to activation by stromelysin-1 (7). There-



**Fig. 1.** Activation of progelatinase B in the synovial fluid of patients with RA. Ten selected synovial fluids containing progelatinase B were incubated at 37°C for 1 hr. (A) After incubation, aliquots from each incubation were removed and electrophoresed in SDS-PAGE gels containing 0.3 mg/ml gelatin. After electrophoresis, the gels were incubated for 16 hrs at 37°C and stained for proteins with 0.1% Coomassie brilliant blue. Migration positions of standard progelatinases A and B are indicated on the left. (B) The same samples were analyzed for gelatinolysis activity using a heat-treated N-[propionate-2,3- $^3$ H]-labeled type I collagen as substrate.



**Fig. 2.** Comparison of stromelysin-1 and TIMP-1 levels, and their concentration ratio between two groups of synovial fluid sample patients with RA. The concentrations of stromelysin-1 and TIMP-1 were measured using specific sandwich enzyme linked immunosorbent assay. Horizontal bars indicate mean of concentrations of stromelysin-1 and TIMP-1 of each group.

fore, to clarify whether stromelysin-1 and TIMP-1 are involved in the modulation of activation of progelatinase B in the synovial fluid of patients with RA, we analyzed their stromelysin-1 and TIMP-1 levels. For the experiments, 43 randomly chosen synovial fluids containing progelatinase B were investigated. Gelatin zymograms indicated that in 22 samples progelatinase B was converted to the active form after incubation at 37°C and in 21 other samples it remained unconverted. We classified the converted and unconverted samples as groups I and II, respectively. Levels of stromelysin-1 and TIMP-1 in the synovial fluids of groups I and II, as determined by the specific ELISAs, are shown in Figure 2.

The concentration of stromelysin-1 in the synovial fluids of group I was significantly higher than in the synovial fluids from group II. It ranged from 27.9 to 111.6 µg/ml (mean  $\pm$  SD,  $63.99 \pm 21.06$  µg/ml) for group I and ranged from 1.35 to 39.6 µg/ml (mean  $\pm$  SD,  $17.60 \pm 1.386$  µg/ml) for group II. Some synovial fluids in group I contained low concentrations of stromelysin-1. However, levels of TIMP-1 in the synovial fluid of patients classified in group I tended to be lower than those observed in group II. It ranged from 0.308 to 1.204 µg/ml (mean

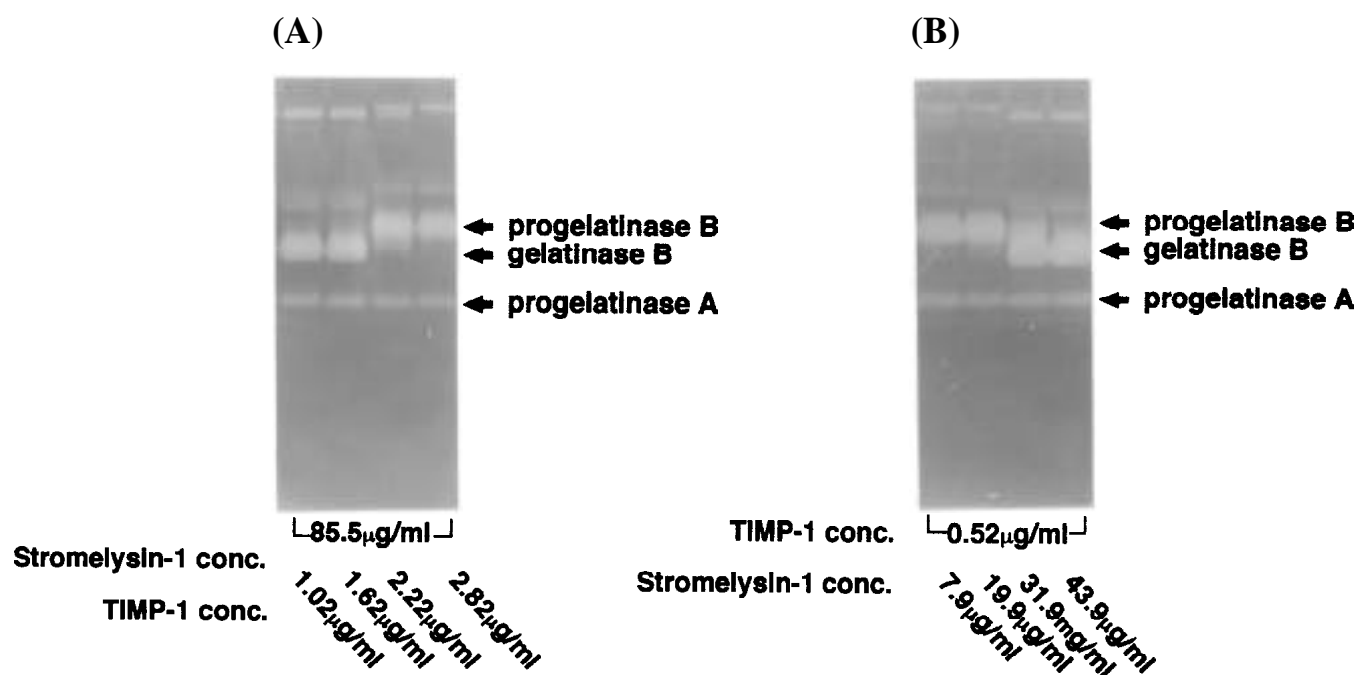
$\pm$  SD,  $0.717 \pm 0.025$  µg/ml) for group I and from 0.560 to 2.518 µg/ml (mean  $\pm$  SD,  $1.229 \pm 0.591$  µg/ml) for group II. However, in some individuals, the level did not always correlate with the activation or inhibition of activation of progelatinase B. Some synovial fluids in group I contained higher TIMP-1 levels than those in group II. The concentration ratio of stromelysin-1/TIMP-1 in the synovial fluids from each patient in group I was greater than for the patients in group II. These findings suggest that both increased stromelysin-1 levels and decreased TIMP-1 levels may lead to the activation of progelatinase B in the synovial fluids of patients with RA.

To evaluate the importance of the ratio of stromelysin-1/TIMP-1 on the activation of progelatinase B in the synovial fluids of patients with RA, synovial fluids from each group were incubated with exogenously added TIMP-1 (Fig 3A) or stromelysin-1 (Fig 3B). In this experiment, recombinant TIMP-1 was obtained commercially and stromelysin-1 was purified from the synovial fluids of patients with RA. Two selected synovial fluids were as follows: in group I, the synovial fluid which converted to active gelatinase B after incubation contained 85.5 µg/ml stromelysin-1 and 1.02 µg/ml TIMP-1 (the ratio of stromelysin-1/

TIMP-1 was 83.8); while in group II, the synovial fluid which did not convert contained 7.9 µg/ml stromelysin-1 and 0.52 µg/ml TIMP-1 (the ratio of stromelysin-1/TIMP-1 was 15.2), respectively. Activation of progelatinase B by incubation did not occur gradually by increasing the concentration of TIMP-1 in the synovial fluid of group I. Progelatinase B resisted activation above 2.22 µg/ml TIMP-1 (the ratio of stromelysin-1/TIMP-1 was 38.5). However, progelatinase B in the synovial fluid of group II converted to the active form when purified stromelysin-1 was added. Progelatinase B converted to the active form when stromelysin-1 was above 31.9 µg/ml (the ratio of stromelysin-1/TIMP-1 was 61.3).

## Discussion

Many studies have focused on the study of proteases in the synovial fluid of patients with inflammatory joint disease such as RA. In many of these studies, comparisons of the content of MMPs were made between RA and non-inflammatory joint disease such as osteoarthritis. The gelatinases consist of two well characterized species, with migration at 72 and 92 kDa, and are termed gelatinase A (MMP-2) and B (MMP-9), respectively. Gelatinase A is constitutively present in all synovial fluids, while



**Fig. 3.** Effects of stromelysin-1 or TIMP-1 on the stimulation or inhibition of activation of progelatinase B in the synovial fluid samples from two groups of patients with RA. A selected synovial fluid sample from group I (A containing 85.5 µg/ml stromelysin-1 and 1.02 µg/ml TIMP-1) and one from group II (B containing 7.9 µg/ml stromelysin-1 and 0.52 µg/ml TIMP-1) were incubated at 37°C in the presence of various concentrations of human recombinant TIMP-1 or purified stromelysin-1, respectively. In the final sample A each synovial fluid contained 1.02 to 2.28 µg/ml stromelysin-1, and in the final sample B each synovial fluid contained 7.9 to 43.9 µg/ml TIMP-1. After incubation for 1 hour, a portion of each mixture was subjected to gelatin zymography as described above.

high levels of gelatinase B have been detected in the synovial fluid of patients with RA. Abnormal levels of gelatinase B may contribute to the destructive processes of the joints in patients with RA. The presence of gelatinase B in synovial fluids reflects the inflammatory condition of the joint, because the appearance of the enzyme positively correlates with the content of polymorphonuclear elastase (2) and local activity score (3). The enzyme is synthesized as proenzyme and is secreted as latent proenzyme. The activation of the proenzyme is the critical step that leads to proteolytic activity. The mechanism of this activation was accomplished *in vitro* by limited proteolysis by some proteases such as cathepsin G (9), trypsin (9,10), chymotrypsin (9), stromelysin-1 (9,10), matrilysin (MMP-7) (10), collagenase-1 (MMP-1) (10), polymorphonuclear elastase (11), tissue kallikrein (12), mast cell chymase (13), gelatinase A (14) and tumor associated trypsin-2 (15). The mechanism of the activation of progelatinase B has not been entirely elucidated but stromelysin-1, together with tumor associated trypsin-2, is thought to be one of the true activators because a

catalytic amount of stromelysin-1 activates inactive gelatinase B (6). Furthermore, if the progelatinase B-TIMP-1 complex is allowed to interact with stromelysin-1, stromelysin-1 dissociates the complex of progelatinase B-TIMP-1 and the resulting progelatinase B, free from TIMP-1, is readily activated by stromelysin-1 (16). The amount of TIMP-1 in the synovial fluid thus influences the activation of progelatinase B by stromelysin-1.

Stromelysin-1, in synovial fluids of patients with RA, may play a major role in the degradation of connective tissue, because the enzyme can directly act on a native type II collagen, a main collagen type in cartilage. Stromelysin-1 acts as a depolymerizer of type II collagen where intermolecular crosslinking occurs (17). The enzyme is also capable of degrading core protein of cartilage aggrecan (18). Furthermore, stromelysin-1 is an activator of procollagenase-1 (19). The loss of structural integrity of the cartilage matrix by the action of stromelysin-1 may increase the susceptibility of type II collagen to collagenase-1. Collagenase-1 cleaves at a single site

with a triple helix resulting in three-quarter and one-quarter fragments (20). The fragments are denatured at body temperature and the resulting gelatin may then be susceptible to active gelatinase B.

Active stromelysin-1 is found in the synovial fluid of patients with RA (21). Stromelysin-1 is activated by many proteinases such as trypsin, chymotrypsin, plasmin, chymase, polymorphonuclear elastase etc., but not MMPs (22). At present, the detailed mechanism of the activation of stromelysin-1 in the synovial fluids of patients with RA is not fully understood. However, it is suggested that after release, the enzyme may activate during a limited time course under the influence of certain proteinases in the synovial fluids. Furthermore, although progelatinase B in synovial fluids containing high stromelysin-1 and low TIMP-1 converted to its active form after short incubation times (within 1 hr at 37°C), many synovial fluids obtained after arthrocentesis did not contain detectable amounts of active gelatinase B. At present, the reason for this is not clear. Some other regulatory mechanism for the activation of progelatinase B may be

present in the synovial fluids of patients with rheumatoid arthritis *in vivo*.

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