Relationship Between Cathepsin B and Thrombin in Rheumatoid Arthritis

TAKUYA MISHIRO, SHUNJI NAKANO, SHIGEYUKI TAKAHARA, MARI MIKI, YOICHI NAKAMURA, SUSUMU YASUOKA, TAKESHI NIKAWA, and NATSUO YASUI

ABSTRACT. ***Objective.*** To investigate the pathophysiological significance of cathepsin B and thrombin in synovial fluid (SF) from patients with rheumatoid arthritis (RA).

Methods. Thrombin and cathepsin B activities of samples from patients with RA and osteoarthritis (OA) were measured using fluorogenic synthetic substrates. The concentration of interleukin 8 (IL-8) in SF was measured by ELISA. The effect of thrombin on the proliferation of synovial fibroblast-like cells (SFC) was examined by measuring ^{3}H-thymidine incorporation. The effect of thrombin on the release of IL-8 and cathepsin B from SFC was investigated. The expression of IL-8 mRNA in SFC after stimulation with thrombin was evaluated using real-time quantitative RT-PCR. The effect of recombinant IL-8 on the activation of cathepsin B was examined using the knee joints of rabbits.

Results. In SF supernatants, cathepsin B and thrombin-like activity was significantly higher in RA than in OA, and there was a significant correlation between them. Cathepsin B activity was also significantly higher in SF cells and synovial tissue extracts from RA patients than in those from OA patients. There was a significant correlation between cathepsin B activity and the concentration of IL-8 in RA SF. Thrombin enhanced the proliferation of SFC in a dose-dependent manner. Thrombin significantly enhanced the release of IL-8 from SFC as well as the expression of IL-8 mRNA in SFC. IL-8 induced activation of cathepsin B in the knee joints of rabbits. However, thrombin did not directly increase cathepsin B activity in SFC.

Conclusion. In RA, thrombin was found to be related to the enhanced growth of SFC and the release of IL-8 from these cells; thus thrombin is probably related to worsening of inflammation through the recruitment of leukocytes (neutrophils), which release cathepsin B into the SF. Thrombin can induce activation of cathepsin B in SFC via increased expression of IL-8. (J Rheumatol 2004;31:1265–73)

Key Indexing Terms:
RHEUMATOID ARTHRITIS CATHEPSIN B THROMBIN INTERLEUKIN 8
NEUTROPHIL SYNOVIAL FIBROBLAST-LIKE CELL

Rheumatoid arthritis (RA) is a complicated inflammatory disease characterized by chronic joint inflammation that leads to the destruction of articular tissues. Studies suggest that various proteases, activated or increased, that are present in RA joints take part in the degradation of the articular cartilage extracellular matrix[1-13]. Under physiological conditions, the synovial fluid (SF) supplies the articular cartilage with nutrients. However, in patients with RA the SF contains various proteases that exacerbate arthritis and destroy the cartilage matrix[1-13].

We previously reported that thrombin and cathepsin B-like protease activity was higher in SF of RA patients than in patients with osteoarthritis (OA)[1,2]. Both proteases appear to be related to the progression of synovitis and the destruction of the extracellular matrix. Thrombin is a multifunctional protease, and activated thrombin stimulates the proliferation of fibroblasts[14-18], numerous cellular responses, synthesis of the extracellular matrix[16], cytokine release from fibroblasts[14,19-21], and degradation of the extracellular matrix[6]. Moreover, while thrombin receptor-positive cells have been shown to be present in rheumatoid synovial tissues, very few are found in osteoarthritic and normal synovial tissues[22].

Cathepsin B is the best-characterized member of the lysosomal cysteine protease family. Studies have shown that cathepsin B is present in increased quantities in SF[7,23] and synovial lining tissue[8] of patients with RA. Increased concentrations of cathepsin B have also been detected in articular tissues during the course of experimental arthritis in animals[9,10]. Cathepsin B is known to degrade not only collagen[11,24] but also proteoglycans[12,13].

From the Department of Orthopedics, School of Medicine, The University of Tokushima; Clinical Research Center, Kochi National Hospital, Kochi; Hakuai Kinen Hospital, Tokushima; and Department of Nutrition, School of Medicine, The University of Tokushima, Tokushima, Japan.

Supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology.

T. Mishiro, MD; S. Nakano, MD, PhD; S. Takahara, MD, Department of Orthopedics, School of Medicine, University of Tokushima; M. Miki, MD, PhD; Y. Nakamura, MD, PhD, Kochi National Hospital; S. Yasuoka, MD, PhD, Hakuai Kinen Hospital; T. Nikawa, MD, PhD, Department of Nutrition, School of Medicine, University of Tokushima; N. Yasui, MD, PhD, Department of Orthopedics, School of Medicine, University of Tokushima.

Address reprint requests to Dr. S. Nakano, Department of Orthopedics, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan.
E-mail: nakano@clin.med.tokushima-u.ac.jp

Submitted February 5, 2003; revision accepted December 2, 2003.

Few studies have examined the relationship between these 2 proteases, which may be related to the inflammatory process and degradation of the extracellular matrix in RA. We investigated these protease activities and the cellular origin of the cathepsin B-like protease present in SF and synovial tissue of patients with RA. We also estimated the effect of thrombin on the production/release of IL-8 and cathepsin B from leukocytes as well as synovial fibroblast-like cells (SFC) prepared from patients with RA. Our results suggest that thrombin can induce activation of cathepsin B in SFC via increased expression of interleukin 8 (IL-8).

MATERIALS AND METHODS

Patient characteristics. All patients with RA had knee joint arthritis and met the American Rheumatism Association 1987 revised criteria[25]. Patients with OA met the clinical and radiological diagnostic criteria[26]. All patients with OA had effusion in their knee joints, and the SF had a clear appearance and high viscosity. The age, sex, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) concentration in serum, and rheumatoid factor status of the patients from whom SF was obtained are shown in Table 1.

SF and synovial tissue specimens. SF was collected from the knee joints of RA and OA patients by puncture at the University of Tokushima. A portion of the SF was used to count the number of cells in the sample. The rest of the SF was then centrifuged at 1000 g for 10 min at 4°C within 1 h after collection. The supernatants were immediately frozen at –20°C until use. Cell pellets obtained from SF were washed twice with 5 ml phosphate buffered saline (PBS), then cells were resuspended in a volume of PBS equal to that of the original sample, homogenized, sonicated, and centrifuged at 3000 g for 5 min at 4°C. The resultant supernatants were designated SF cell extracts. Synovial tissues obtained from RA patients at joint surgery were washed with PBS, blotted on filter paper, homogenized using a Polytron homogenizer in 5 ml PBS per g wet weight of tissue in an ice-bath for 1 min, and centrifuged. The resultant supernatants were designated synovial tissue extracts. The SF, SF cell extract, and synovial tissue extracts were diluted 10-fold, 10-fold, and 100-fold, respectively, with saline and used as the enzyme source to measure thrombin and cathepsin B-like activities. For measurement of cathepsin C and H activity, the SF samples were not diluted. Materials obtained from OA patients were subjected to the same procedures, and used as controls for RA samples.

Reagents. Boc-Val-Pro-Arg-methylcoumarylamide (MCA), Z-Phe-Arg-MCA, and Arg-MCA, synthetic fluorogenic MCA substrates for thrombin and cathepsins B and H, respectively, were purchased from the Peptide Institute (Osaka, Japan). Gly-Phe-ß-naphtylamide (NA), a synthetic fluorogenic substrate for cathepsin C (dipeptidyl peptidase I), human thrombin, hyaluronidase, and human recombinant IL-8 (rIL-8) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G sodium, streptomycin, and amphotericin B were purchased from Gibco BRL (Gaithersburg, MD, USA). Trypsin (0.25% solution) was obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Assay of protease activity. Thrombin-like activity was measured by spectrofluorophotometry using the synthetic fluorogenic peptide Boc-Val-Pro-Arg-MCA as the substrate, by the method of Yasuoka, *et al* with minor modifications[27,28]. The assay mixture (1.0 ml), which contained 0.05 M Tris-HCl (pH 8.6), the substrate at 50 µM, and 100 µl enzyme solution, was incubated at 37°C for 60 min, and the reaction was stopped by adding 1 ml of 30% acetic acid. The fluorescence intensity of aminomethylcoumarin released into the resultant supernatants was measured by spectrophotometry using a fluorescence spectrophotometer (Hitachi F-3010, Tsukuba, Japan) with excitation at 370 nm and emission at 460 nm. Cathepsin B, C, and H-like activities were measured using Z-Phe-Arg-MCA, Gly-Phe-ß-NA, and Arg-MCA, respectively, as the substrates, according to the method of Barrett and Kirschke[29] or Nikawa, *et al*[30] with some minor modifications. The assay buffer (0.1 M acetate buffer, pH 5.5, for cathepsin B and 0.1 M sodium phosphate buffer, pH 6.0, for cathepsins C and H) contained the appropriate substrate (50 µM Z-Phe-Arg-MCA, 200 µM Gly-Phe-ß-NA, and 50 µM Arg-MCA, respectively), 100 µl of enzyme solution, 4 mM EDTA, and 8 mM dithiothreitol. The mixtures were incubated 1 h at 37°C and the reaction was stopped by adding 1 ml of 0.1 M sodium monochloroacetate, followed by centrifugation at 3000 g for 5 min. Fluorescence intensity was then measured. The amount of AMC released was calculated from a standard curve. One unit of enzymatic activity was defined as that releasing 1 µM of AMC per min. Cathepsin B-like activity in the extracts of SF cells and synovial tissues was expressed as activity per mg protein.

Assay of IL-8 in SF. The concentration of IL-8 in SF was tested using a specific IL-8 ELISA kit (Endogen, Rockford, IL, USA) according to the manufacturer's instructions. Before the assay each supernatant of SF was treated with hyaluronidase at a concentration of 10 U/ml for 1 h at 37°C and centrifuged at 15,000 rpm. The standard of recombinant human IL-8 was used at concentrations of 25–1000 pg/ml; samples were diluted 20-fold. The color reaction was performed with premixed tetramethylbenzydine substrate solution and absorbance was read at 450 nm and 540 nm on an ELISA reader (ImmunoMini NJ-2300, Nalge Nunc International, Tokyo, Japan).

Cell cultures. Synovial fibroblast-like cells were obtained from fresh synovial tissues of RA patients at joint surgery. The synovial tissues were washed 3 times in PBS to remove blood components, dissected free of fat, cut into 2 mm pieces, and then plated in 6-well culture plates. Synovial tissues were cultured in DMEM containing 10% FBS, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The media were replaced twice a week with fresh media until the primary SFC had grown to 90–100% confluence. Within about 2 weeks, cultured SFC were treated with 0.25% trypsin at 37°C and then washed and cultured. Cells from the first to third passages were used for subsequent experiments.

Effect of thrombin on proliferation of SFC. The effect of thrombin on the proliferation of SFC was estimated by the incorporation of [^{3}H]-thymidine. In the [^{3}H]-thymidine incorporation assay, SFC were plated at a cell density of 5000 cells/well into a 96-well flat-bottom culture plate and cultured in 200 µl of DMEM/10% FBS at 37°C in a CO_2 incubator for 2–3 days to about 50–60% confluence. Then the cells were starved for 24 h in DMEM/0.5% FBS to arrest the cells at the G_0 phase. After that they were stimulated with 200 µl DMEM/0.5% FBS containing various concentrations of human thrombin (0.1–10 unit/ml). [^{3}H]-thymidine (0.67 µCi/ml/well) was added to the cultures one day later. After the plates were

Table 1. Patients' characteristics.

Disease	Sex M/F	Age, yrs	ESR, mm/h	CRP, mg/dl	RF +/–
RA, n = 39	9/30	61.6 (26–81)	71.9 (22–140)	4.2 (0.04–15.74)	33/6
OA, n = 33	7/26	71 (50–91)			

Age, ESR, and CRP are expressed as mean (range). ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, RF: rheumatoid factor.

cultured for 3 days, 50 µl of 1 N NaOH was added to each well. The lysed cells were transferred to a specific glass fiber filter using a harvester (Filtermate 196/micromate196; Packard, Meriden, CT, USA). Then incorporation of [^{3}H]-thymidine by the cells was measured using a Matrix 9600 direct counter, and expressed as cpm. The effect of thrombin was tested in 6 wells and 6 wells without thrombin as the control.

Effect of thrombin on release of IL-8 from SFC. SFC were plated at a cell density of 10,000 cells/well into 96-well flat-bottom culture plates and cultured in 200 µl DMEM/10% FBS at 37°C in a CO_2 incubator for 5–7 days to about 100% confluence. After the cells were starved for 24 h in DMEM/0.5% FBS, 200 µl DMEM/0.5% FBS containing various concentrations of human thrombin (0.1–10 unit/ml) were added to each well. After 2 days, the concentration of IL-8 in the culture medium was measured using a specific ELISA kit (Endogen).

Effect of thrombin on expression of IL-8 mRNA in SFC. The expression of IL-8 mRNA was estimated by real-time quantitative polymerase chain reaction as follows. SFC were seeded into 6 cm culture dishes at a cell density of about 1.0×10^5 cells, cultured in DMEM/10% FBS to 100% confluence, and starved for 24 h in DMEM/0.5% FBS. Then they were stimulated with 200 µl DMEM/0.5% FBS containing various concentrations of human thrombin (0.1–10 unit/ml). After 3–24 h, the culture medium was removed by aspiration, and the cells were lysed in 1 ml of Isogen (Nippon Gene, Tokyo, Japan), a mixture of guanidium isocyanate and phenol. From the lysed cells, total RNA was extracted with chloroform and precipitated with isopropanol. cDNA from total RNA was synthesized with TaqMan reverse transcription reagent (Applied Biosystems, Tokyo, Japan) using random hexamers according to the protocol provided by the manufacturer. Predeveloped TaqMan assay reagents (Applied Biosystems) were used for the specific primer and probe of IL-8 (labeled with FAM) and GAPDH (labeled with VIC). cDNA standard curves were generated using serial dilutions of cDNA obtained from the RNA samples. Then the cDNA sample, no-template control, and cDNA standard were amplified using TaqMan universal PCR master mix and the ABI Prism 7000 sequence detection system (Applied Biosystems). Thermal cycler conditions included initial holding at 50°C for 2 min, then at 95°C for 10 min. A 2-step Taqman PCR program consisting of 95°C for 15 s and 60°C for 60 s for 40 cycles followed this.

Effect of thrombin on cathepsin B activity in SFC. SFC were plated at a cell density of about 50,000 cells into 6-well flat-bottom culture plates. SFC were cultured to 100% confluence and starved for 24 h in DMEM/0.5% FBS to arrest cells at the G_0 phase. Then they were stimulated with 1.2 ml of DMEM/0.5% FBS containing various concentrations of human thrombin (0.1–10 unit/ml). After 2 days, cultured supernatants were collected, centrifuged at 3000 g for 5 min at 4°C, and stored frozen. SFC on the plate were washed 3 times with ice-cold PBS, collected with a cell scraper, and stored in PBS at –80°C until analysis. The activity of cathepsin B in the cultured supernatants was also measured as described above. Stored cells were disrupted on ice by sonication for 20 s before measurement.

Intraarticular injection of rIL-8. Male Japanese white rabbits (weight 2.0–2.4 kg) were anesthetized with ketamine (4 mg/kg) and xylazine (1 mg/kg), then 10 µg of rIL-8 in 500 µl of endotoxin-free saline was injected through the infrapatellar ligament into the knee joints. An equal amount of BSA in 500 µl saline was also injected into the contralateral knee joint as control. Nine hours after injection, the joints were washed with 1 ml saline, and joint fluid was collected and centrifuged at 3000 g for 3 min at 4°C. The cell-free SF were assayed for cathepsin B-like activity. Cell pellets were resuspended in saline and differential cell analysis was done by Wright-Giemsa staining.

Cell counts and measurement of total protein. The total numbers of cells in SF from 40 RA patients and 12 OA patients were counted using a Burker-Türk hemocytometer before centrifugation. The cells were centrifuged on glass slides in a cytocentrifuge (Cytospin 3, Shandon, England), stained with May-Grünwald-Giemsa solution, and then analyzed.

Total protein was measured by the method of Lowry, *et al*[31] using BSA as the standard.

Statistical analysis. The significance of differences between the 2 groups was assessed by nonparametric Wilcoxon-Mann-Whitney test. The significance of correlations between 2 parameters was assessed by Pearson's correlation coefficient. A p value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Cathepsin B and thrombin-like activity. Consistent with our previous study, the thrombin and cathepsin B-like activity in the SF supernatants was significantly higher (about 6.3 and 3.5-fold, respectively) in the RA group compared to the OA group (Figure 1A, 1B), indicating that these proteases may play an important role in arthritis of RA. To elucidate this, we measured other proteases such as cathepsins C and H, which have been reported to be activated in arthritis[32,33]. Cathepsin C and cathepsin H activities were significantly higher in the RA group than the OA group, but not to the same extent as observed regarding thrombin and cathepsin B activities. Based on these results, we focused on thrombin-mediated activation of cathepsin B in RA SF, and found a significant correlation between thrombin and cathepsin B activity in SF of RA patients ($n = 39$, $r = 0.619$, $p < 0.0001$; Figure 2).

In the SF cell extracts, cathepsin B activity expressed as milliunit/mg protein was also significantly higher (about 5.6-fold) in the RA group (6.33 ± 7.26 milliunit/mg protein, $n = 14$) than in the OA group (1.13 ± 1.40 milliunit/mg protein, $n = 10$; $p = 0.0017$; Figure 3A). In the synovial tissue extracts, cathepsin B activity was also significantly higher (about 2.7-fold) in the RA group (77.8 ± 59.4 milliunit/mg protein, $n = 21$) than in the OA group (29.2 ± 17.5 milliunit/mg protein, $n = 10$; $p = 0.018$; Figure 3B).

Leukocytes, cathepsin B activity, and concentration of IL-8 in SF. As leukocytes are thought to be one of the cellular sources that produce cathepsin B, we investigated the relationship between leukocytes and cathepsin B-like activity. The mean count of leukocytes in SF was about 9.96 ± 6.00 $\times 10^3/mm^3$ in RA patients ($n = 40$) and about 0.25 ± 0.36 $\times 10^3/mm^3$ in OA patients ($n = 12$; $p < 0.0001$). In the RA group, there was a significant correlation between cathepsin B-like activity in SF cell extracts and the leukocyte count in SF ($n = 19$, $r = 0.489$, $p = 0.033$; Figure 4). However, there was no significant correlation between cathepsin B activity in the SF supernatant and the leukocyte count in SF (data not shown).

As IL-8 is one of the cytokines that recruit leukocytes (neutrophils), we also investigated the relationship between the concentration of IL-8 and cathepsin B-like activity in SF. In the RA group, the concentration of IL-8 in SF ranged from 70.7 pg/ml to 13,300 pg/ml. There was a significant correlation between the concentration of IL-8 and cathepsin B-like activity ($n = 36$, $r = 0.688$, $p < 0.001$; Figure 5).

Effect of thrombin on cultured SFC. As shown in Figure 6,

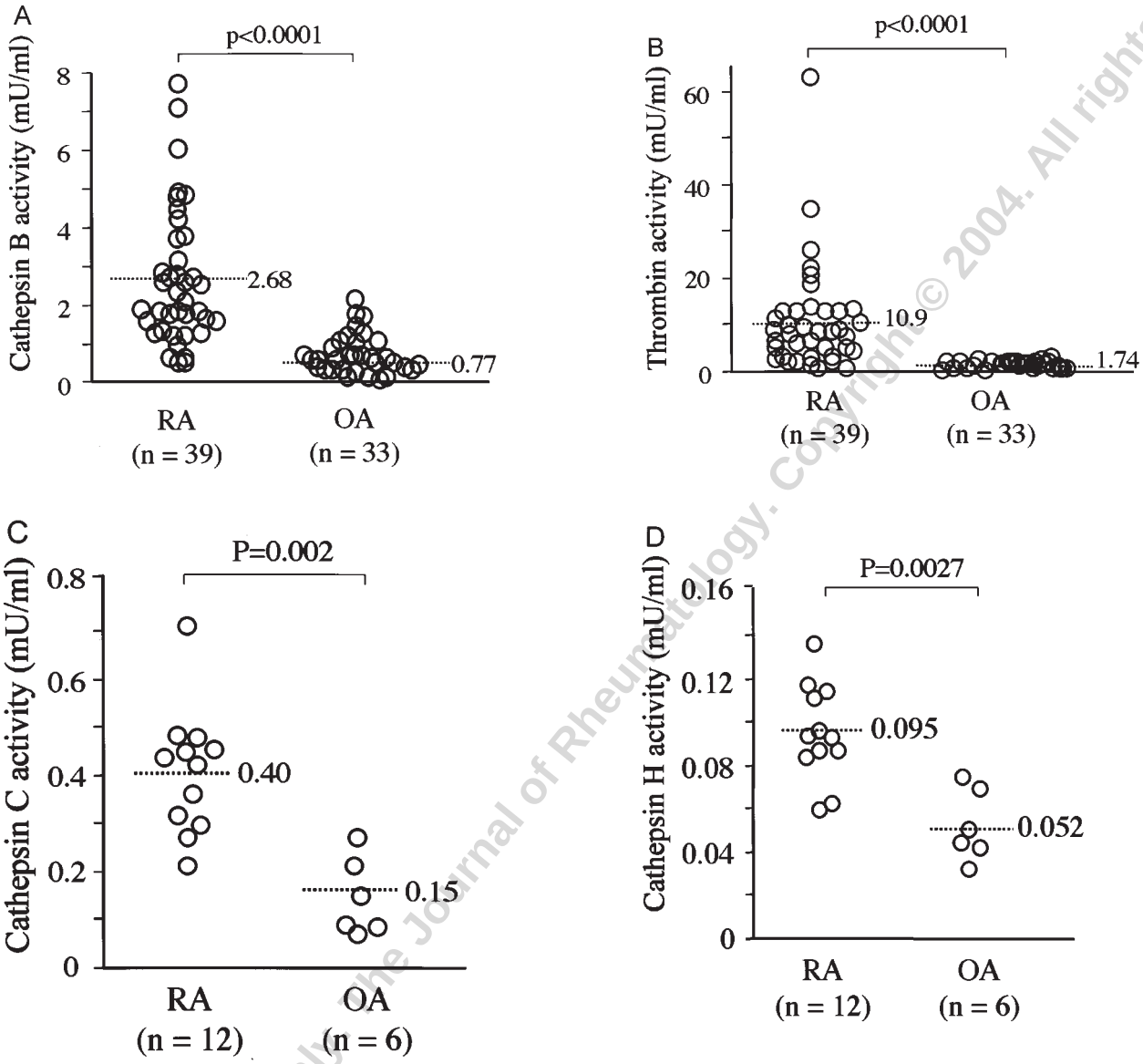

Figure 1. Cathepsin B, C, H, and thrombin-like activity in SF supernatant of individual patients with RA and OA. Protease activities in SF supernatant were measured by spectrofluorophotometry, using fluorogenic synthetic peptides, which had methylcoumarylamide (MCA) at their COOH- termini, as substrates. Horizontal lines show mean values in each group. A. Cathepsin B-like activity was significantly higher (about 3.5-fold) in RA than in OA ($p < 0.0001$). B. Thrombin-like activity in SF supernatant was significantly higher (about 6.3-fold) in RA than in OA ($p < 0.0001$). C. Cathepsin C-like activity was significantly higher (about 2.7-fold) in RA than in OA. D. Cathepsin H-like activity was significantly higher (about 1.8-fold) in RA than in OA. As for the difference in the levels of protease activity between the RA and OA groups, that of cathepsin B was the largest among those examined.

human thrombin enhanced RA SFC proliferation in a dose-dependent manner at concentrations of 0.1 to 10 unit/ml.

As shown in Figure 7, thrombin markedly increased the release of IL-8 from RA SFC in a dose-dependent fashion. To characterize the effects of thrombin on SFC, the expression of IL-8 in SFC was measured by real-time quantitative PCR. The time-dependent change in IL-8 mRNA level after treatment with thrombin was quantified by densitometric analysis. Peak IL-8 mRNA level was observed 8 h after treatment with thrombin (Figure 8). The dose-dependent change in IL-8 mRNA level after treatment with thrombin was quantified using the same method. Thrombin increased the IL-8 mRNA level in a dose-dependent manner in the range of 0.1 to 10 unit/ml (Figure 9).

Effect of thrombin on activation of cathepsin B in cultured SFC extract. Cathepsin B activity was not detectable in the culture medium. A high level of cathepsin B activity was detected in the extracts of RA SFC. The level of activity did not change with the passage of cells. The mean value of cathepsin B activity in the SFC extracts was 125.4 ± 46.5

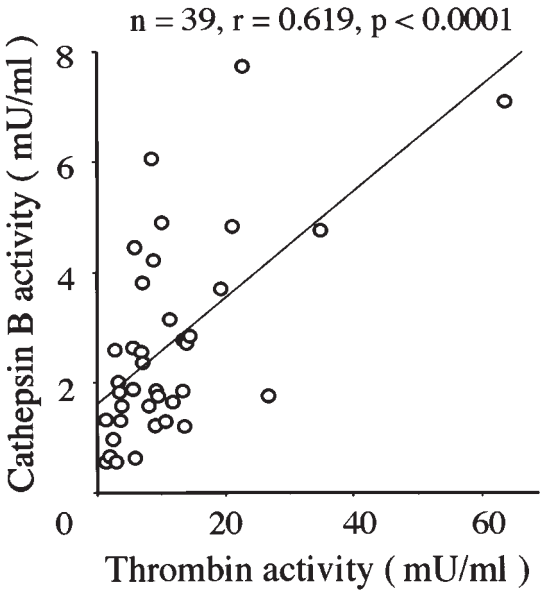

Figure 2. Relationship between cathepsin B and thrombin-like activity in SF supernatant from patients with RA. Cathepsin B and thrombin-like activity was measured by spectrofluorophotometry using 50 µM Z-Phe-Arg-MCA and Boc-Val-Pro-Arg-MCA as substrates, respectively. Spearman rank correlation testing revealed a significant correlation between thrombin and cathepsin B-like activity in RA SF supernatant (n = 39, r = 0.619, p < 0.0001).

milliunit/mg protein without thrombin stimulation (n = 5). Treatment with thrombin did not increase the activity of any cysteine protease, such as cathepsins B, C, or H, in SFC (Figure 10 and data not shown), suggesting that thrombin had no direct effects on the activity of lysosomal cysteine proteases in SFC.

Effect of rIL-8 on release of cathepsin B into rabbit knee joints. We attempted to confirm that IL-8 can enhance the activation of cathepsin B-like activity by means of leukocyte (neutrophils) migration using the knee joints of rabbits.

Nine hours after injection of rIL-8, the cathepsin B activity in SF was 2.93 ± 1.08 milliunit/ml, which was significantly higher than in the contralateral control joints (1.07 ± 0.58 milliunit/ml; $p < 0.0295$). We confirmed by examining Giemsa-stained smears that most cells recruited into the rabbit knee joints injected with rIL-8 were neutrophils.

DISCUSSION

In previous studies analyzing RA synovial fluid samples, we obtained the following findings: (1) in the SF, thrombin-like activity was highest among the various types of serine protease activities tested, and was due to an effect of thrombin[1]; (2) the purified materials isolated from SF of RA patients were mainly composed of cathepsin B[2]; and (3) the cathepsin B purified from SF of RA patients was capable of activating urokinase-type plasminogen activator *in vitro*[2]. However, it is thought that the degradation of cartilage in

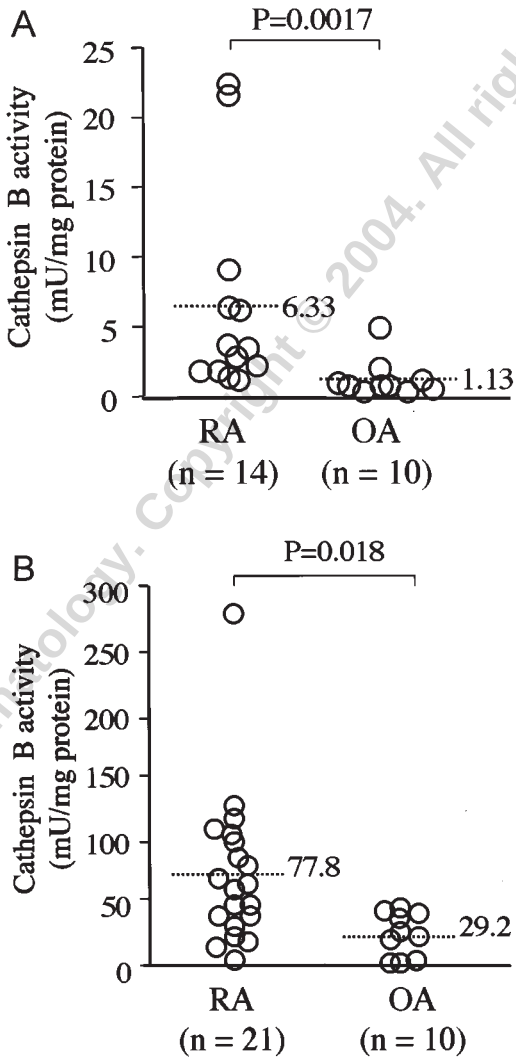

Figure 3. Cathepsin B-like activity in extract of RA and OA samples. Cathepsin B activity was expressed as activity per mg protein. Horizontal lines show mean values in each group. A. Cathepsin B activity in SF cell extracts was significantly higher (about 5.6-fold) in RA than in OA. B. Cathepsin B activity in synovial tissue extracts was also significantly higher (about 2.7-fold) in RA than in OA. Comparisons of mean values by Wilcoxon-Mann-Whitney test.

RA may be due to the action of a number of proteases synthesized and activated in the RA lesion, and of proteases secreted by both synovial tissues and inflammatory cells into the SF. Matrix metalloproteases (MMP) and cysteine proteases are known to be capable of degrading the extracellular matrix[4,34-39]. Recent reports[35-38] suggest that cathepsin K is a critical protease in fibroblast-mediated collagen degradation because cathepsin K is not only expressed by osteoclasts but also by synovial fibroblasts. Moreover, there is a large body of evidence for the role of cathepsins B, L, and S in joint destruction[39]. Except for cathepsin B, we also measured the activities of cathepsins C and H in the SF supernatants. As shown in Figure 1, the

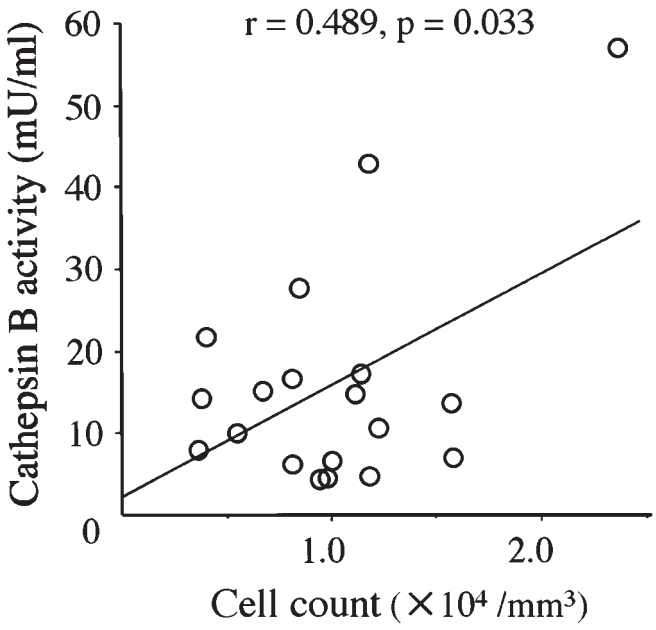

Figure 4. Relationship between cathepsin B-like activity in SF cell extracts and leukocyte count in RA SF. Total number of cells in SF from 19 patients with RA was counted before centrifugation. Spearman rank testing revealed significant correlation between cathepsin B-like activity in SF cell extracts and the leukocyte count in SF (r = 0.489, p = 0.033).

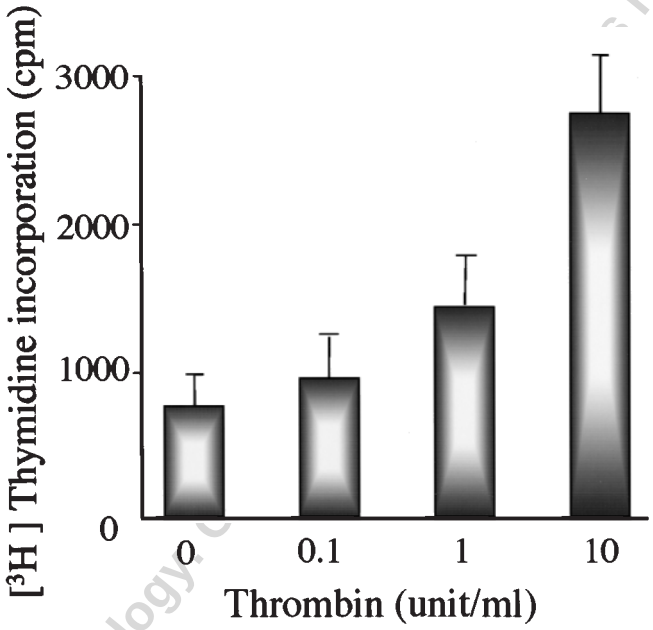

Figure 6. Thrombin enhanced proliferation of SFC from RA patients in a dose-dependent manner at concentrations of 0.1 to 10 unit/ml. Data represent the mean and SD of 3 wells and are representative of 5 independent experiments.

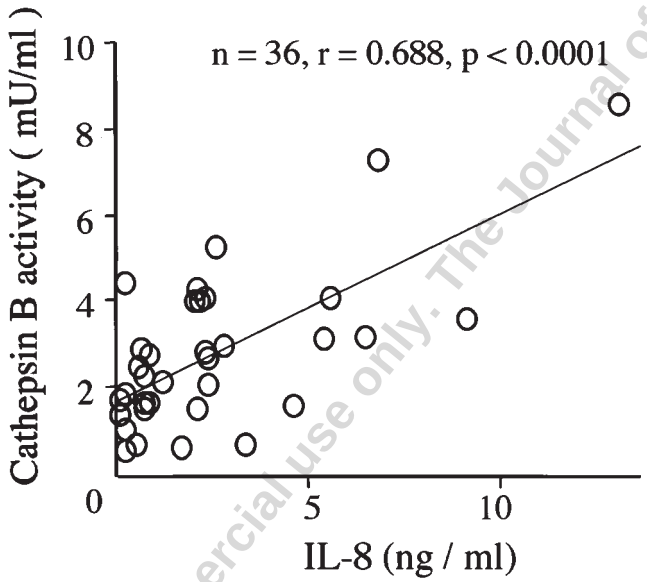

Figure 5. Relationship between cathepsin B-like activity and concentration of IL-8 in RA SF. Concentration of IL-8 in SF samples was measured using a specific IL-8 ELISA. Samples were diluted 20-fold, standards were in the range 25–1000 pg/ml. Spearman rank testing revealed significant correlation between cathepsin B-like activity and concentration of IL-8 in RA SF supernatant (n = 36, r = 0.688, p < 0.001).

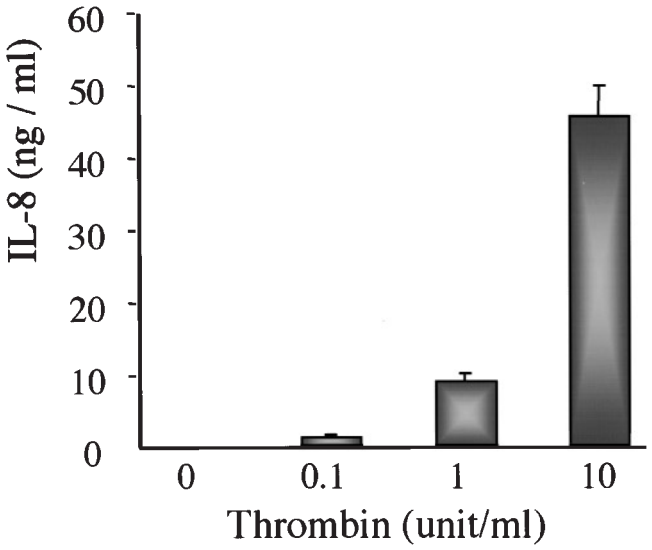

Figure 7. Thrombin markedly increased the release of IL-8 in SFC from RA patients in a dose-dependent fashion. Data represent the mean and SD of 3 wells and are representative of 5 independent experiments.

significant difference in the levels of cathepsin B-like activity observed between the RA group and the OA group was larger than that in the levels of cathepsins C and H in the SF supernatants. We selected cathepsin B-like activity to clarify the relationship between cysteine proteases and thrombin.

The increase in cathepsin B and thrombin concentration in SF of RA patients has been reported in several studies[1,2,7,8,23], but there are no previous reports on the rela-

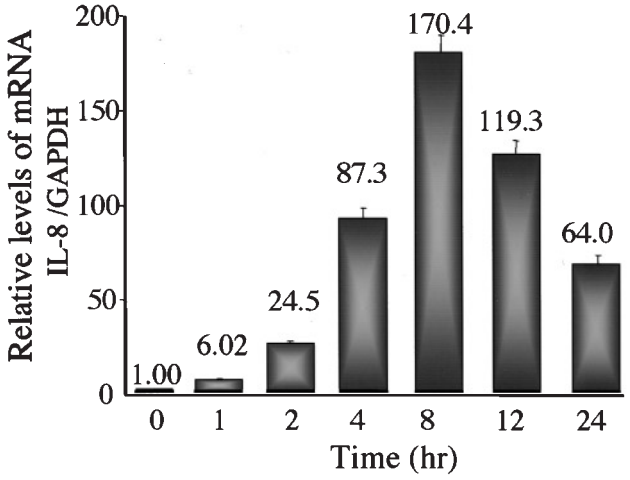

Figure 8. Effect of human thrombin on gene expression of IL-8 in SFC from patients with RA. SFC were treated for 1-24 h with human thrombin (2 unit/ml). Peak IL-8 mRNA level was observed 8 h after treatment with thrombin. Data represent the mean and SD of 3 wells and are representative of 3 independent experiments.

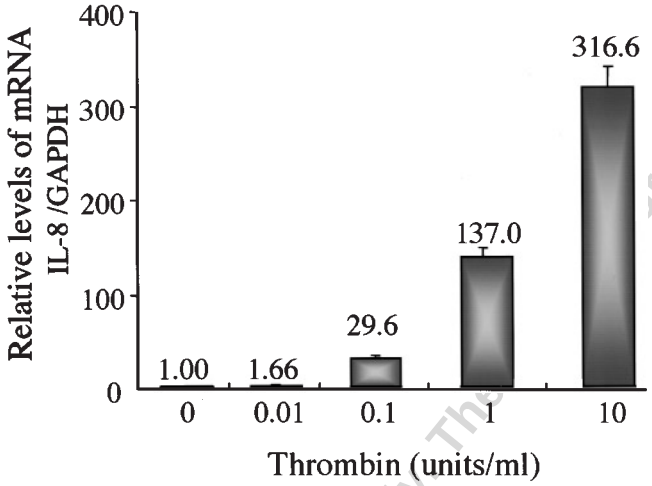

Figure 9. Dose-response effect of human thrombin on gene expression of IL-8 in SFC from RA patients. After incubation of SFC with various concentrations (0–10 unit/ml) of human thrombin for 8 h, PCR analysis was performed and relative amount of IL-8 mRNA was determined. Dose-dependent change in IL-8 mRNA level after treatment with thrombin was quantified by the same method. Thrombin increased IL-8 mRNA level in a dose-dependent manner in the range of 0.1 to 10 units/ml. Data represent the mean and SD of 3 wells and are representative of 3 independent experiments.

tionship between cathepsin B and thrombin in SF and synovial tissue from RA patients. Our study was undertaken to clarify the pathophysiological significance of cathepsin B and thrombin in RA. Figure 2 shows that the activity of cathepsin B released into RA SF correlated with the activity of thrombin activated in the lesion. The following 2 possibilities are considered as the reason for the significant correlation between cathepsin B-like activity and thrombin-like

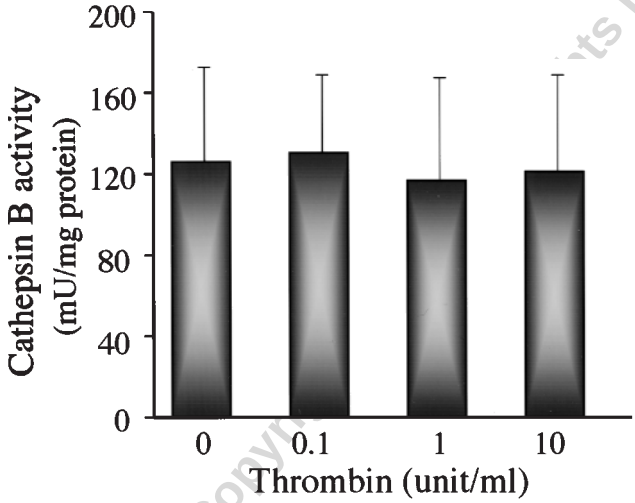

Figure 10. Cathepsin B-like activity in SFC extracts after stimulation by human thrombin (0, 0.1, 1, 10 unit/ml) for 2 days. Thrombin did not increase cathepsin B activity in SFC extracts. Data represent the mean and SD of 5 independent experiments.

activity in RA SF. First, both the activation of thrombin and release of cathepsin B from cells in the RA lesion are independently proportional to the activity of RA; and second, the activated thrombin is intimately related to the release of cathepsin B from cells such as inflammatory cells and synovial cells.

Cathepsins, including cathepsin B, are mainly localized in intracellular spaces, and related mainly to hydrolysis of intracellular proteins[29]. However, the pathologies of inflammatory and neoplastic diseases suggest roles for cathepsin B outside the cells[40], although the origin of extracellular active cathepsin B has not been well defined. Thus it is thought that the cathepsin B detected in the SF of RA patients is derived mainly not only from synovial cells but also from inflammatory cells recruited to the RA lesions. We investigated cathepsin B-like activity not only in SF supernatants but also in extracts of SF cells and synovial tissue. Cathepsin B-like activity was higher in samples from RA patients than in those from OA patients. Extracts of synovial tissue and cultured SFC obtained from RA patients showed high cathepsin B-like activity. In the SF of our RA patients, neutrophils accounted for most of the cells that were found, and cathepsin B-like activity in the SF cell extracts correlated significantly with the leukocyte counts. These results support the idea that the cathepsin B present in RA SF derives not only from synovial cells but also from inflammatory cells (neutrophils) recruited to the RA lesion. We also measured cathepsin B-like activity in extracts of articular cartilage, and confirmed that cathepsin B activity was not detectable (data not shown). Previous studies have already indicated that cathepsin B may derive from synovial cells[8-10] and chondrocytes[9,10,41]. To our knowledge, there are few reports about RA that cathepsin B may derive from

leukocytes (neutrophils) in RA. However, Sohar, *et al*[32] recently reported that the activity of cathepsin B was elevated in leukocytes from patients with RA.

On the other hand, thrombin localized in extracellular spaces is related not only to blood coagulation by limited hydrolysis of fibrinogen and extracellular proteins, but also to fibroblast proliferation and regulation of various kinds of cellular functions[16]. Recently, it was clarified that certain serine proteases such as thrombin and trypsin, which have been considered to participate principally in the degradation of extracellular proteins, are also signaling molecules that regulate multiple cellular functions by activating protease-activated receptors (PAR), a family of G-protein-coupled receptors. Three thrombin-activated PAR (PAR1, PAR3, PAR4), which are localized on cell membranes, have been identified, and multiple functions of thrombin have been found to originate from cleavage of the amino-terminal exodomain of PAR[42,43]. In this study we showed that thrombin enhanced the proliferation of SFC in a dose-dependent fashion at concentrations ranging from 0.1 to 10 unit/ml. This result is in accord with previous reports[14,18,20]. As shown in Figure 10, thrombin has no direct role in inducing activation of cathepsin B in cultured SFC.

It is known that thrombin enhances the release of cytokines such as IL-8 from fibroblasts[19,21]. We showed that human thrombin enhanced the release of IL-8 from SFC cultured *in vitro*. We also showed by analyzing IL-8 mRNA by PCR that thrombin enhanced the production of IL-8 in the SFC. IL-8 is a potent chemoattractor and activator of neutrophil-mediated cartilage degradation[44]. Inflammatory cells such as neutrophils may contribute to cartilage destruction through the release of lysosomal enzymes, including cathepsin B, capable of degrading the matrix[3]. We observed the following results: (1) rIL-8 enhanced leukocyte (neutrophils) migration and the activation of cathepsin B-like activity in the knee joints of rabbits; and (2) there was a significant correlation between the concentration of IL-8 and cathepsin B-like activity in RA SF. Since thrombin enhanced IL-8 release from SFC of RA patients, it is plausible that thrombin is related to the enhancement of leukocyte (neutrophils) migration in RA. It is therefore possible that thrombin plays a role in the pathophysiology of arthritis in RA by increasing the release of inflammatory cytokines and cathepsin B from leukocytes (neutrophils). Granulocyte-colony stimulating factor (G-CSF) is a well known chemokine, the same as IL-8. Shin, *et al*[20] have reported that thrombin induces the expression of G-CSF via thrombin receptor in RA synovial fibroblasts. We measured G-CSF using a specific ELISA kit and confirmed that there was no significant correlation between the concentration of G-CSF and cathepsin B-like activity in RA SF (data not shown). It is thought that IL-8 may be more closely related to the activation of cathepsin B than G-CSF.

Our results suggest that active cathepsin B and thrombin play critical roles in RA; thrombin would be activated in articular lesions and cathepsin B would be released from both synovial and inflammatory cells. Both proteases would be related in a complex fashion to the progression of inflammation and joint destruction in RA. Thrombin stimulates synovial fibroblast-like cells to proliferate as well as to increase the release of IL-8; in turn, IL-8 induces the migration of leukocytes (neutrophils) that release cathepsin B into the synovial fluid. This pathway is thought to be important in the degradation of articular cartilage.

ACKNOWLEDGMENT

We thank Drs. Tatsuhiko Henmi (Health Insurance Naruto Hospital), Kazuhito Shinohara (Kochi National Hospital) for valuable advice, and Kimihiko Yukata (The University of Tokushima), Sachiko Kawano, and Shizuka Inoue for excellent technical assistance.

REFERENCES

1. Nakano S, Ikata T, Kinoshita I, Kanematsu J, Yasuoka S. Characteristics of the protease activity in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. Clin Exp Rheumatol 1999;17:161-70.
2. Ikeda Y, Ikata T, Mishiro T, Nakano S, Ikebe M, Yasuoka S. Cathepsin B and L in synovial fluids from patients with rheumatoid arthritis and the effect of cathepsin B on the activation of pro-urokinase. J Med Invest 2000;47:61-75.
3. Barrett AJ. The possible role of neutrophil proteinases in damage to articular cartilage. Agents Actions 1978;8:11-7.
4. Kikuchi H, Shimada W, Nonaka T, Ueshima S, Tanaka S. Significance of serine proteinase and matrix metalloproteinase systems in the destruction of human articular cartilage. Clin Exp Pharmacol Physiol 1996;23:885-9.
5. Werb Z, Mainardi CL, Vater CA, et al. Endogenous activation of latent collagenase by rheumatoid synovial cells. New Eng J Med 1977;296:1017-23.
6. Furmaniak-Kazmierczak E, Cooke TD, Manuel R, et al. Studies of thrombin-induced proteoglycan release in the degradation of human and bovine cartilage. J Clin Invest 1994;94:472-80.
7. Mort JS, Recklies AD, Poole AR. Extracellular presence of the lysosomal proteinase cathepsin B in rheumatoid synovium and its activity at neutral pH. Arthritis Rheum 1984;27:509-15.
8. Trabandt A, Gay RE, Fassbender HG, Gay S. Cathepsin B in synovial cells at the site of joint destruction in rheumatoid arthritis. Arthritis Rheum 1991;34:1444-51.
9. Van Noorden CJF, Smith RE, Rasnick D. Cysteine proteinase activity in arthritic rat knee joints and the effects of a selective systemic inhibitor, Z-Phe-AlaCH2F. J Rheumatol 1988;15:1525-35.
10. Mehraban F, Tindal M, Proffitt M, Moskowitz R. Temporal pattern of cysteine endopeptidase (cathepsin B) expression in cartilage and synovium from rabbit knees with experimental osteoarthritis: gene expression in chondrocytes in response to interleukin-1 and matrix depletion. Ann Rheum Dis 1997;56:108-15.
11. Burleigh MC, Barrett AJ, Lazarus GS. Cathepsin B1: A lysosomal enzyme that degrades native collagen. Biochem J 1974;137:387-98.
12. Morrisson RIG, Barrett AJ, Dingle JT, Prior D. Cathepsins B1 and D. Action on human cartilage proteoglycans. Biochim Biophys Acta 1973;302:411-9.
13. Gabrijelcic D, Annan-Prah A, Rodic B, Rozman B, Cotic V, Turk V. Determination of cathepsin B and H in sera and synovial fluids of patients with different joint diseases. J Clin Chem Clin Biochem 1990;28:149-53.
14. Ohba T, Takase Y, Ohhara M, Kasukawa R. Thrombin in synovial

fluid of patients with rheumatoid arthritis mediates proliferation of synovial fibroblast-like cells by induction of platelet derived growth factor. J Rheumatol 1996;23:1505-11.
15. Pohjanpelto P. Stimulation of DNA synthesis in human fibroblasts by thrombin. J Cell Physiol 1978;91:189-94.
16. Carney DH, Redin W, McCroskey L. Role of high-affinity thrombin receptors in postclotting cellular effects of thrombin. Semin Thromb Hemost 1992;18:91-103.
17. Shin H, Nakajima T, Kitajima I, et al. Thrombin receptor-mediated synovial proliferation in patients with rheumatoid arthritis. Clin Immunol Immunopathol 1995;76:225-33.
18. Maruyama N, Hirano F, Yoshikawa N, Migita K, Eguchi K, Tanaka H. Thrombin stimulates cell proliferation in human fibroblast-like synoviocytes in nuclear factor-κB activation and protein kinase C mediated pathway. J Rheumatol 2000;27:2777-85.
19. Sower LE, Froelich CJ, Carney DH, Fentonii JW, Klimpel GR. Thrombin induces IL-6 production in fibroblasts and epithelial cells. J Immunol 1995;155:896-901.
20. Shin H, Kitajima I, Nakajima T, et al. Thrombin receptor mediated signals induce expressions of interleukin 6 and granulocyte colony stimulating factor via NF-κB activation in synovial fibroblasts. Ann Rheum Dis 1999;58:55-60.
21. Ludwicka-Bradley A, Tourkina E, Suzuki S, et al. Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-1 and protein kinase C-γ activation. Am J Respir Cell Mol Biol 2000;22:235-43.
22. Morris R, Winyard PG, Brass LF, Blake DR, Morris CJ. Thrombin receptor expression in rheumatoid and osteoarthritic synovial tissue. Ann Rheum Dis 1996;55:841-3.
23. Duffy JM, Walker B, Guthrie D, et al. The detection, quantification and partial characterization of cathepsin B-like activity in human pathological synovial fluids. Eur J Clin Chem Clin Biochem 1994;32:429-34.
24. Eeckhout Y, Vaes G. Further studies on the activation of procollagenase, the latent precursor of bone collagenase: Effect of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. Biochem J 1977;166:21-31.
25. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
26. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Arthritis Rheum 1986;29:1039-49.
27. Yasuoka S, Fujisawa K, Ueta Y, et al. Cell profile and elastase activity in diffuse panbronchiolitis investigated by bronchoalveolar and bronchial lavage. Intern Med 1992;31:599-605.
28. Yasuoka S, Ohnishi T, Kawano S, et al. Purification, characterization and localization of a novel trypsin-like protease found in the human airway. Am Respir Cell Mol Biol 1997;16:300-8.
29. Barret AJ, Kirschke H. Cathepsin B, cathepsin H, and cathepsin L. In: Lorand L, editor. Methods in enzymology. New York: Academic Press; 1981:535-61.
30. Nikawa T, Towatari T, Katsunuma N. Purification and characterization of cathepsin J from rat liver. Eur J Biochem 1992;204:381-93.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-70.
32. Sohar N, Hammer H, Sohar I. Lysosomal peptidases and glycosidases in rheumatoid arthritis. Biol Chem 2002;383:865-9.
33. Gabrijelcic D, Annan-Prah A, Rodic B, Rozman B, Cotic V, Turk V. Determination of cathepsins B and H in sera and synovial fluids of patients with different joint diseases. J Clin Chem Clin Biochem 1990;28:149-53.
34. Gabrijelcic D, Gollwitzer R, Popovic T, et al. Proteolytic cleavage of human fibrinogen by cathepsin B. Biol Chem Hoppe Seyler 1988;369 Suppl:287-92.
35. Dodds RA, Connor JR, Drake FH, Gowen M. Expression of cathepsin K messenger RNA in giant cells and their precursors in human osteoarthritic synovial tissues. Arthritis Rheum 1999;42:1588-93.
36. How WS, Li Z, Gordon RE, et al. Cathepsin K is a critical protease in synovial fibroblast-mediated collagen degradation. Am J Pathol 2001;159:2167-77.
37. Hummel KM, Petrow PK, Franz J, et al. Cysteine proteinase cathepsin K mRNA is expressed in synovium of patients with rheumatoid arthritis and is detected at sites of synovial bone destruction. J Rheumatol 1998;25:1887-94.
38. Hou WS, Li W, Keyszer G, et al. Comparison of cathepsins K and S expression within the rheumatoid and osteoarthritic synovium. Arthritis Rheum 2002;46:663-74.
39. Cunnane G, FitzGerald O, Hummel KM, et al. Collagenase, cathepsin B and cathepsin L gene expression in the synovial membrane of patients with early inflammatory arthritis. Rheumatology 1999;38:34-42.
40. Kobayashi H, Sugimura M, Goretzki L, et al. Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-u PA). J Biol Chem 1991;266:5147-52.
41. Zwicky R, Baici A. Cytoskeletal architecture and cathepsin B trafficking in human articular cartilage. Histochem Cell Biol 2000;114:363-72.
42. Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. J Biol Chem 2000;275:25216-21.
43. Ishihara H, Connolly AJ, Zeng D, et al. Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 1997;386:502-6.
44. Elford PR, Cooper PH. Induction of neutrophil-mediated cartilage degradation by IL-8. Arthritis Rheum 1991;34:325-32.