Cathepsins B and L in synovial fluids from patients with rheumatoid arthritis and the effect of cathepsin B on the activation of pro-urokinase

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Abstract : To clarify the pathophysiological role of cathepsins in rheumatoid arthritis (RA), we investigated whether cathepsin B or cathepsin L was increased in synovial fluid (SF) of RA joints, and whether the cathepsin isolated from SF of RA patients activated pro-urokinase or not. Thus, we estimated the content of cathepsins in SF of RA patients by measuring their activities by fluorospectrometry, using Z-Phe-Arg-MCA as the substrate. Cathepsin activity was approxymately 4-fold higher in the SF of RA patients than in those of patients with osteoarthritis. Cathepsin B and cathepsin L were separated by cation-exchange column chromatography. As a result, a large peak corresponding to cathepsin B and a very small peak correponding to cathepsin L were detected.

Biochemical sequential fractionation of the cathepsin purified from the SF showed that the large peak was mainly composed of cathepsin B. This purified enzyme induced conversion of pro-urokinase to urokinase, and the Km for pro-urokinase was approximately 8.27μM.

These findings indicated that an imbalance between cathepsin B and its inhibitors occurred due to increased concentrations of active cathepsin B in RA articular lesions, and that cathepsin B might be related to the degradation of cartilage in RA by activating the fibrinolytic cascade. J. Med. Invest. 47 : 61-75, 2000

Key words **:** *synovial fluid, cysteine protease, cathepsin B, rheumatoid arthritis, pro-urokinase*

INTRODUCTION

Collagens and proteoglycans are the main extracellular matrix components of articular cartilage and bone. Cathepsin B and cathepsin L are two of the best-characterized members of the lysosomal cysteine protease family. Both are known to degrade not only collagens (1-6) but also proteoglycans (7-9). Previous studies have reported that cathepsin B is present in increased quantities in synovial fluids (10-13) and synovial lining tissues (14-16) of patients with rheumatoid arthritis (RA). Increased levels of cathepsin B have also been detected in joint tissues during the course of experimental arthritis in experimental animals (16-18). While elevated levels of cathepsin L have been detected in the synovial fluids of patients with arthritis (10) and in the synovial lining of rabbits with antigen-induced arthritis (17).

These studies indicated that cathepsin B and L were implicated in the pathological degradation of the articular cartilage matrix in chronic inflammatory joint diseases such as RA, playing a major role in intracellular protein turnover.

On the other hand, the extracellular metalloprotease family, especially collagenase and stromelysin have been suggested to be involved in the destruc-

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tion of articular tissues by directly degrading the articular extracellular collagens and proteoglycans in chronic inflammatory arthritis (19-21). These metalloproteases are activated by plasmin (22), and plasmin is activated by urokinase (23). Recently, cathepsin B was shown to activate soluble and tumor cell receptor-bound forms of pro-urokinase to urokinase (24, 25). In addition, cathepsin B was shown to directly activate the latent procollagenase (19).

These findings strongly suggest that cathepsins are involved in the destruction of articular tissues at least by the two following mechanisms : direct degradation of the articular extracellular matrix, and indirect degradation of the matrix via activation of the fibrinolytic cascade.

The pathophysiological significance of cathepsin B in RA probably differs from that of cathepsin L, because the biochemical properties of cathepsin B differ markedly from those of cathepsin L (26-28). To compare the pathophysiological significances of cathepsin B with that of cathepsin L in RA, we considered that it was necessary to clarify which of them was mainly increased in the joint lesions of RA patients. However, there have been very few studies in which both cathepsin B and cathepsin L were determined in synovial fluids of the same RA patients (10). Moreover, there have been no studies that examined whether cathepsins isolated from the affected joints of RA patients activated pro-urokinase or not.

In the present study, we attempted to biochemically separate cathepsin B and cathepsin L found in synovial fluids from RA patients using several fractionation procedures, to clarify which of them was predominantly increased in the affected joints. As a result, synovial fluids were found to contain more cathepsin B than cathepsin L. Therefore, we purified cathepsin B and examined whether the purified enzyme activated pro-urokinase or not.

MATERIALS AND METHODS

Patients characteristics

All patients with RA were suffering from knee joint arthritis and fulfilled the American Rheumatism Association 1987 revised criteria (29, 30). Patients with OA, had primary or post-traumatic OA at the knee joints, and fulfilled the clinical and radiological diagnostic criteria (31). All patients with OA had effusion in their knee joints and the synovial fluids showed clear appearance and high viscosity. The clinical background of the study population is shown in Table 1.

Synovial fluid

Synovial fluids were collected from the knee joints by needle puncture and drainage into a plastic syringe and, unless otherwise stated, centrifugated for 10 min at 1,500 rpm at 4°C within one hour after collection. The resultant supernatants were immediately frozen at -20°C until use. Samples distinctly containing blood were discarded. The total numbers of cells in the synovial fluids from 19 RA patients and 10 OA patients were counted using Burker-Turk hemocytometer before centrifugation. The cells were centrifuged on glass slides in a cytocentrifuge (Cytospin 3, Shandon, England), and stained with May-Grunwald-Giemsa solution, and then analyzed.

Reagents

Sephadex G-75, CM Sephadex C-50 and Concanavalin A-Sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The synthetic fluo-

Table 1. Patients 'characteristics

Disease	Sex Male/Female	Age (years)			
RA (n=40)	5/35	56.4 (24-79)	CRP (mg/dl)	ESR (mm/h)	RF (IU/ml)
			3.23 (0.26-14.1)	67 (6-146)	161 (2-1220)
OA (n=27)	10/17	71 (44-81)	OA grade		
			Ⅰ	Ⅱ	Ⅲ	Ⅳ
			11	9	5	2

Age, CRP, ESR and RF are expressed as the mean (range).
CRP=C-reactive protein ; ESR=erythrocytes sedimentation rate ; RF=rheumatoid factor.

rogenic substrates, Z-Arg-Arg-methylcoumarylamide (MCA) and Z-Phe-Arg-MCA, which had MCA at their COOH-termini, other MCA-substrates, CA-074 (a specific cathepsin B inhibitor) (32, 33) and E-64 (an inhibitor of all cysteine proteases), were purchased from the Peptide Institute (Osaka, Japan). Purified human liver cathepsin B was obtained from Sigma (St. Louis, MO) and human liver cathepsin L was from Calbiochem (La Jolla, CA). Single-chain urinary-type plasminogen activator (scu-PA or pro-urokinase) was obtained from American Diagnostica Inc (Greenwich, CT).

Assay of protease activity

Cathepsin activity was measured with Z-Phe-Arg-MCA (a substrate for both cathepsin B and L) (34) by the method of Barrett and Kirschke (34) with some minor modifications. The synovial fluid samples were diluted 10-fold with saline. The assay mixture contained 10 mM sodium acetate buffer (pH 5.5), 4 mM EDTA, 100 μl of the enzyme solution, 50 μM Z-Phe-Arg-MCA and 8 mM dithiothreitol in a total volume of 1.0 ml. Dithiothreitol was added at the time the enzyme assay was carried out. The mixtures were incubated for one hour at 37°C, and the reaction was stopped by adding 1 ml of 0.1M sodium monochloroacetate, then they were centrifuged at 2,000 rpm for 5 min. The fluorescence intensity of aminomethylcoumarin (AMC) released into the resultant supernatants was measured by spectrophotometry using a fluorescence spectrophotometer (Hitachi F-3010, Tsukuba, Japan) at 460 nm with excitation at 370 nm. The amount of AMC released was calculated from a standard curve. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of AMC per min.

Partial purification of cysteine proteases from synovial fluid

Cathepsin B and cathepsin L in the pooled synovial fluids from RA patients were isolated using a modified method for the purification of these enzymes from rat liver, described by Barrett and Kirschke (34).

Unless otherwise stated, the following procedures were performed at 4°C.

1) About 40 ml of pooled 10,000×g supernatant of synovial fluid was diluted 10-fold with 10mM potassium phosphate buffer (pH 5.8) -5 mM 2-mercaptoethanol (buffer A). Ammonium sulfate was then added to the solution to 30% saturation, and the precipitate was removed by centrifugation at 10,000×g for 10 min. Next, ammonium sulfate was added to the supernatant to 65% saturation and the precipitate obtained by centrifugation was dissolved in 400 ml of the same buffer. The mixture was transferred into five 100 ml separating funnels, and a 0.4 volume of cold butanol was added to each separating funnel. The mixtures were shaken for 20 min by hand and allowed to stand at 4°C for 2 hours. The lower aqueous phases were combined, transferred into a beaker, and subjected to acetone fractionation. The 40-65% acetone precipitate was collected by centrifugation at 5,000×g for 5 min, dissolved in buffer A to a total volume of 10 ml and dialyzed against 1 L of the original buffer overnight. This solution was centrifuged at 10,000×g for 10 min. The resultant supernatant was applied to a column (3.3×100 cm) of Sephadex G-75 equilibrated with buffer A containing 150 mM KCl, and gel filtration was carried out at a flow rate of 20 ml/h. Four milliliter fractions were collected. The fractions were monitored at 280 nm for protein and assayed for cathepsins B and L-like activities. One peak of Z-Phe-Arg-MCA-hydrolyzing activity was eluted. The active fractions were concentrated by ultrafiltration through an Amicon membrane (YM-10) and dialysed against 1.5 L of buffer A.

2) Cation exchange column chromatography: This sample was subjected to cation exchange column chromatography using a CM Sephadex C-50 equilibrated with buffer A according to the method described by Barrett and Kirschke (34). They described that in this column chromatography, liver cathepsin B was eluted with 200 mM KCl, whereas liver cathepsin L was eluted with a gradient from 200 to 600 mM KCl. After the active fraction obtained by gel filtration using Sephadex G-75 was applied on a column (1.2×13 cm) of CM Sephadex C-50 and unbound material was eluted, the Z-Phe-Arg-MCA-hydrolyzing activity absorbed on the column was eluted with a gradient from 0 to 200 mM KCl at a flow rate of 0.5 ml/min, and then eluted with 600 mM KCl. The eluate was collected in 5 ml fractions. A large peak of unbound protein was eluted and most of the Z-Phe-Arg-MCA-hydrolyzing activity absorbed on the column was eluted with a gradient from 0 to 200 mM KCl, while a very small amount of the activity was eluted with 600 mM KCl. The former cathepsin peak was designated as fraction I, and the latter cathepsin peak as fraction II. Fraction I was concentrated by ultrafiltration and dissolved in 5 ml of 20 mM sodium phosphate

buffer (pH 6.0)/0.2 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$ (buffer B).

As controls, purified human liver cathepsin B and cathepsin L were each dissolved in 2 ml of buffer A, dialyzed against 1 L of this buffer and subjected to the same cation-exchange column chromatography as described above.

3) Affinity chromatography using Concanavalin A-Sepharose : Fraction I, obtained by CM Sephadex C-50 chromatography, was subjected to affinity chromatography using a Concanavalin A-Sepharose column (1.2×8.4 cm) equilibrated with buffer B, according to Barrett and Kirschke (34). Four milliliter fractions each were collected at a flow rate of 0.25 ml/min. In this affinity chromatography, liver cathepsin B does not bind to the coulmn, and liver cathepsin L is eluted with buffer B containing 100 mM methyl α-D-mannoside. The cathepsin of fraction I passed through this column.

These findings showed that fraction I contained mainly cathepsin B, and fraction II mainly cathepsin L, indicating that the synovial fluids from RA patients contained more cathepsin B than cathepsin L. Therefore, cathepsin B-like activity was purified from synovial fluids by ammonium sulfate fractionation, butanol treatment, acetone fractionation, gel filtration through Sephadex G-75 and cation exchange column chromatography using CM-Sephadex C-50, as described above, and biochemical characterization of the purified enzyme was carried out.

Biochemical characterization of the purified enzyme

1) Determination of the Michaelis constants (Km) for each Z-Phe-Arg-MCA and Z-Arg-Arg-MCA

The Km values of the purified enzyme for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA (a substrate for cathepsin B) were examined using liver cathepsin B and liver cathepsin L as controls. The MCA-substrate-hydrolyzing activities of each enzyme when the MCA-substrates were added to the assay mixture at concentrations of 0.25, 0.50, 1.0, 2.0 and 4.0×10^{-4}M, were measured by the fluorometric method. The Km value was calculated from the Lineweaver-Burk plot.

2) Effects of cathepsin inhibitors

Effects of E-64, an inhibitor of both cathepsins B and L, and CA-074, a specific cathepsin B inhibitor, on the purified enzyme activity were examined using liver cathepsin B and cathepsin L as controls.

Before the substrates were added to the cathepsin assay mixture, the mixtures containing each enzyme and its inhibitor were allowed to stand for 10 min at room temperature. Thereafter, the substrates were added, and enzyme activity was measured as described above.

3) Activation of single-chain urinary type plasminogen activator (pro-urokinase) by the purified enzyme.

To examine the time course of activation of pro-urokinase by the purified enzyme, pro-urokinase (final concentration 0.46 μM) was preincubated with each purified enzyme (200 microunits) in 88 mM KH_2PO_4 -12 mM Na_2HPO_4/1.33 mM EDTA/2.7 mM cysteine (pH 5.9) for 10-90 min at 37°C in a total volume of 20 μl, and then the activity of urokinase was measured with 50 μM of Glt-Gly-Arg-MCA (a urokinase substrate) as the substrate by the fluorometric method described above.

To examine the effects of the amount of the purified enzyme on activation of pro-urokinase, this was preincubated with various amounts of the purified enzyme in the same reaction mixture for 60 min, and then the activity of urokinase was measured by the same method.

When we examined the Km value of the purified enzyme for pro-urokinase, this was preincubated at concentrations of 0.25, 0.50, 1.0, 2.0 and 4.0 μM with the purified enzyme (200 microunits) for 60 min. Then the activity of urokinase was measured. The Km value was calculated from the Lineweaver-Burk plot.

Assay of protein

Protein concentration was measured by the method of Lowry *et al.* (35) with bovine serum albumin as the standard.

Statistical analysis

The significance of differences between groups was assessed by the non-parametric Wilcoxon-Mann-Whitney test. A p value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

I. Activity of cathepsins B and L in synovial fluids from patients with RA and OA, and their relation to other laboratory findings.

The activity of cathepsins B and L in synovial fluid from 40 RA patients was compared with that in synovial fluid from 27 patients with OA.

The activity measured using Z-Arg-Arg-MCA as

the substrate was lower than that measured with Z-Phe-Arg-MCA as the substrate in all the patients examined. Therefore, in subsequent experiments, unless otherwise stated, Z-Phe-Arg-MCA was used as the substrate.

As shown in figure 1, the mean value (mU/ml) of cathepsins B and L activity was approxymately 4-fold higher in the synovial fluids of the RA group (2.83±1.63, mean±SD) than in the OA group (0.77 ±0.52) (P<0.05). Although the RA patients (mean age 56.5 year) was younger than the OA patients (mean age 71.0 year), there was no significant correlation between the patient age and the cathepsins activity in synovial fluids in either group (data not shown).

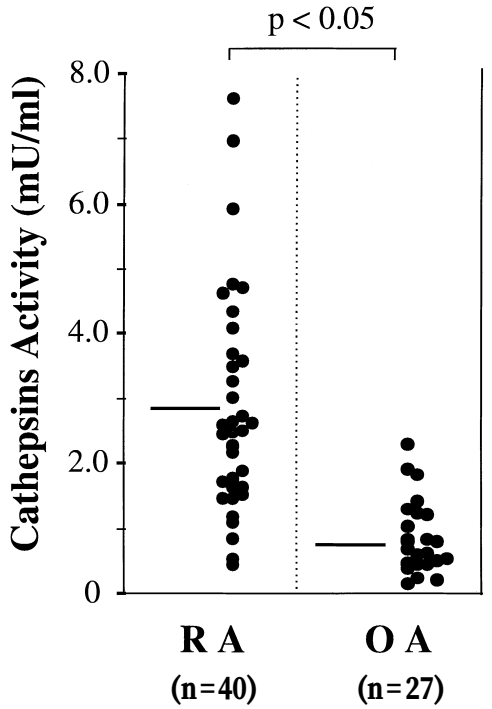

Fig.1. Activities cathepsins B and L in synovial fluids from patients with RA and OA.
Cathepsins activity was measured by spectrofluorophotometry using 50 μM Z-Phe-Arg-methycoumarylamide (MCA) as the substrate.
Horizontal lines show mean values in each group of patients.

Table 2 shows the finding from the analysis of the cells in the synovial fluid from RA patients (n=19) and OA patients (n=10). The total cell count was approxymately 20-fold higher in the RA patients than in the OA patients. Neutrophils constituted approxymately 74% of the cells in the synovial fluid from RA patients while they accounted for approxymately 5% of the cells in those from OA patients. In the synovial fluids from RA patients, the cathepsin activity (mU/ml) was not significantly correlated with neutrophils count (cells/ml) (r=0.23, data not shown).

II. Partial purification of cysteine protease from synovial fluids, and its biochemical characterization

Cathepsins B and L were partially purified by ammonium sulfate fractionation, butanol treatment and acetone fractionation from 40ml of the 10,000×g supernatants of the synovial fluids, as described in Materials and Methods, and subjected to gel filtration on Sephadex G-75.

Fig.2 shows the elution pattern of cathepsins B and L activity after gel filtration using Sephadex G-75. In this chromatography, the void volume was located at tube 50. One peak of cathepsin activity was eluted from tube 74 to tube 100 after a large peak of protein was eluted. The fractions from tube 74 to tube 100 were combined as the active fraction.

The active fraction was applied onto a column of CM Sephadex C-50. Fig.3 shows the elution pattern of cathepsins B and L activity after gel filtration using a CM Sephadex C-50 column. As shown in this figure, a large peak of unbound protein passed through the column, and a prominent peak of cathepsin activity was eluted by a linear 0 to 200 mM

Table 2. Analysis of the cells in synoial fluids from RA and OA patients

Group	Total cell count ($\times 10^6$/ml)	Neutrophil	
		Percentage of total cells	cell count ($\times 10^6$/ml)
RA (n=19)	8.82±5.28 * p<0.01	73.9±19.3 * p<0.01	6.47±4.36 * p<0.01
OA (n=10)	0.44±0.38	4.7± 1.0	0.16±0.13

The total number of cells in the synovial fluids was counted using the Burker-Turk hemocytometer before centrifugation. The cells were centrifuged on glass slides in a cytocentrifuge (Cytospin 3, Shandon), and stained with May-Grunwald-Giemsa solution. Values are means±S.D.
*Significance by Wilcoxon-Mann-Whitney test for difference between RA group versus OA group.

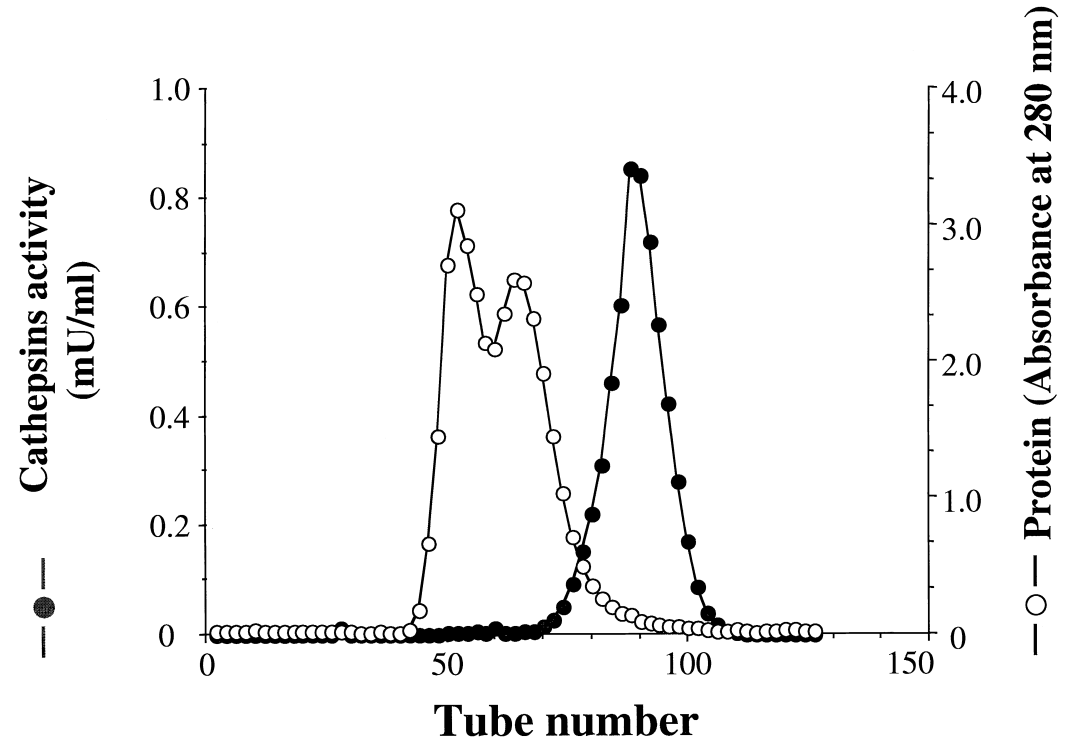

Fig. 2. Gel filtration of cysteine protease present in the synovial fluids of RA patients through Sephadex G-75
The cysteine protease fraction obtained by successive procedures including ammonium sulfate fractionation, butanol treatment and acetone fractionation from 40 ml of pooled synovial fluids of RA patients, was applied onto a column (3.3×100 cm) of Sephadex G-75 equilibrated with 0.01 M potassium phosphate buffer (pH 5.8)/0.15 M KCl/5 mM 2-mercaptoethanol. Elution was carried out at a flow rate of 20 ml/h and fractions of 5 ml each were collected.
Cathepsin activity was measured using 50 μM Z-Phe-Arg-MCA as the substrate.

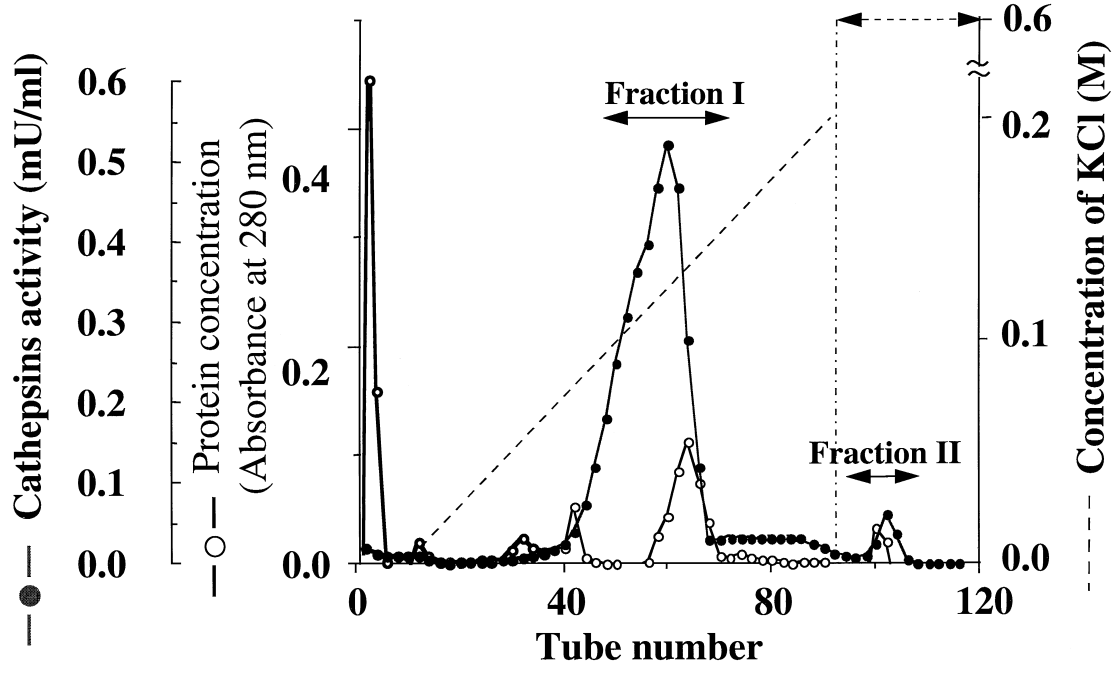

Fig. 3. CM Sephadex C-50 column chromatography of cysteine protease present in the synovial fluids of RA patients
The active fraction obtained after gel filtration through a Sephadex G-75 column, was applied onto a column (1.2×13 cm) of CM Sephadex C-50 equilibrated with 0.01 M potassium phosphate buffer (pH 5.8)/5 mM 2-mercaptoethanol. After the column was washed with 50 ml of the equilibration buffer, elution was carried out with a linear 0 to 200 mM KCl gradient, and thereafter with 600 mM KCl. Five milliliter fractions each were collected.

KCl gradient. Thereafter, a small peak of cathepsin activity was eluted with 600 mM KCl.

Each human liver cathepsin B and L was then subjected to CM Sephadex C-50 column chromatography as described for the cathepsins in synovial fluid. Figs.4 and 5 show the elution patterns of human liver cathepsins B and L subjected to column chromatography, respectively. Cathepsin B was eluted with a linear 0 to 200 mM KCl gradient (Fig.4), while cathepsin L was not eluted with the linear 0 to 200 mM KCl gradient but with 600 mM KCl (Fig. 5).

Judging from the description of Barrett and Kirschke, who isolated cathepsins B and L from rat liver (34), and from the results illustrated in Figs. 4 and 5, it was considered that the first large activity peak (fraction I) corresponded to cathepsin B, and the second small activity peak (fraction II) corresponded to cathepsin L (Fig. 3). This column chromatography was found to be very useful for the isolation and purification of individual cysteine proteases present in specimens containing both cathepsin B and cathepsin L.

Fraction I was subjected to affinity chromatography using Concanavalin A-Sepharose. As shown in Fig. 6, most of the cathepsin activity in fraction I was not absorbed to the Concanavalin A-Sepharose column. This result supports the assumption that fraction I contained mainly cathepsin B.

Cathepsin B-like enzyme was purified from 40 ml of the 10,000×g supernatants of pooled synovial fluids of RA patients by the above-described methods. Table 3 shows a summary of the purification procedure.

When the purified enzyme was subjected to SDS-PAGE under denaturing and reducing conditions by the method of Laemmli (36), several bands with molecular weights from 92 to 29 kDa were detected (data not shown).

III Biochemical characterization of the purified enzyme

1) Effects of cathepsin inhibitors

Table 4 shows the effects of CA-074 (a specific inhibitor of cathepsin B) and E-64 (an inhibitor for all cysteine proteases) on Z-Phe-Arg-MCA-hydrolyzation by the purified enzyme and human liver cathepsins B and L. The activity of liver cathepsin B was almost completely inhibited by both CA-074 and E-64 at both concentrations of 10^{-7} and 10^{-6} M. The activity of liver cathepsin L was almost completely inhibited by E-64 at both concentrations of 10^{-7} and 10^{-6} M, but only slightly (8.8%) inhibited by CA-074 at the same concentrations. The activity of the purified enzyme was almost completely inhibited by both

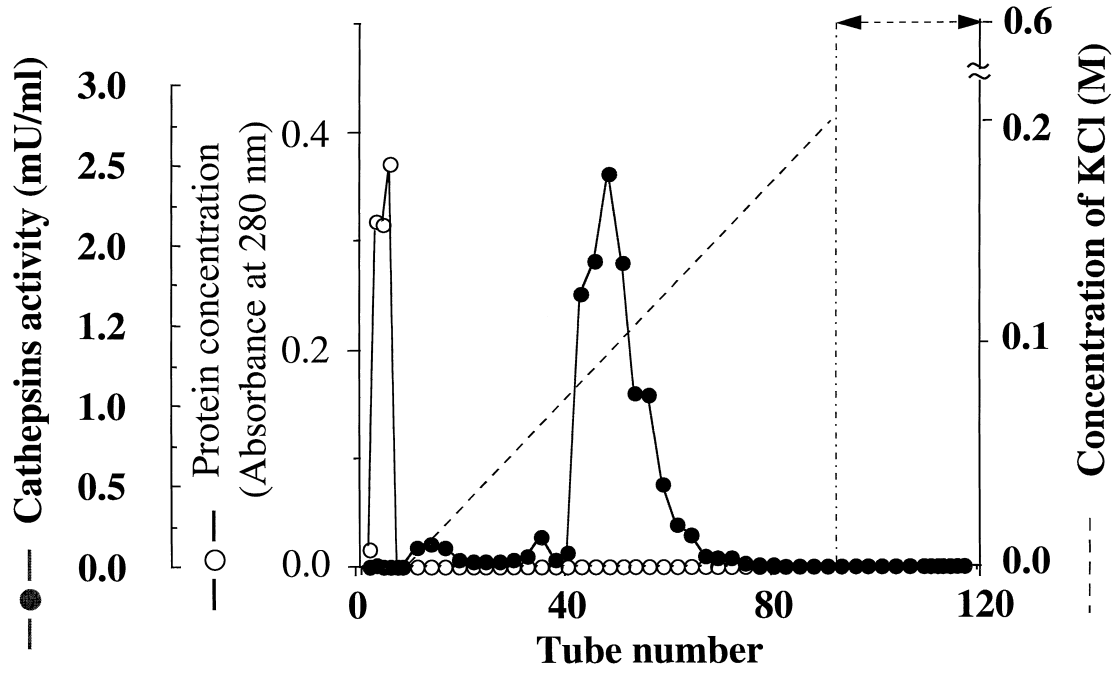

Fig. 4. Elution pattern of human liver cathepsin B subjected to CM Sephadex C-50 column chromatography
Purified human cathepsin B was subjected to CM Sephadex C-50 column chromatography described in Fig. 3. After the column was washed with 50 ml of the equilibration buffer, elution was carried out with a linear 0 to 200 mM KCl gradient, and thereafter with 600 mM KCl.

CA-074 and E-64 at both concentrations.

2) The Michaelis constants (Km) for synthetic substrates

Table 5 shows the Km for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA hydrolyzed from the purified enzyme and human liver cathepsins B and L. In the case of the purified enzyme, the Km for Z-Phe-Arg-MCA (0.118) was lower than that for Z-Arg-Arg-MCA

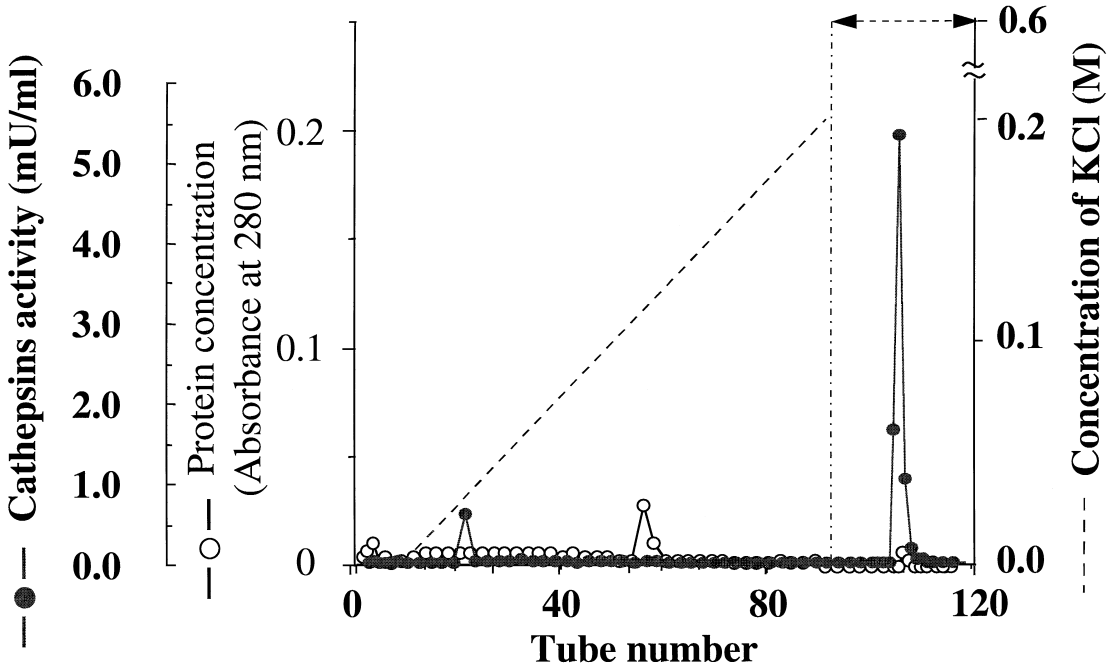

Fig. 5. Elution pattern of human liver cathepsin L subjected to CM Sephadex C-50 column chromatography
Purified human cathepsin L was subjected to CM Sephadex C-50 column chromatography in the same way as described in Fig. 4.

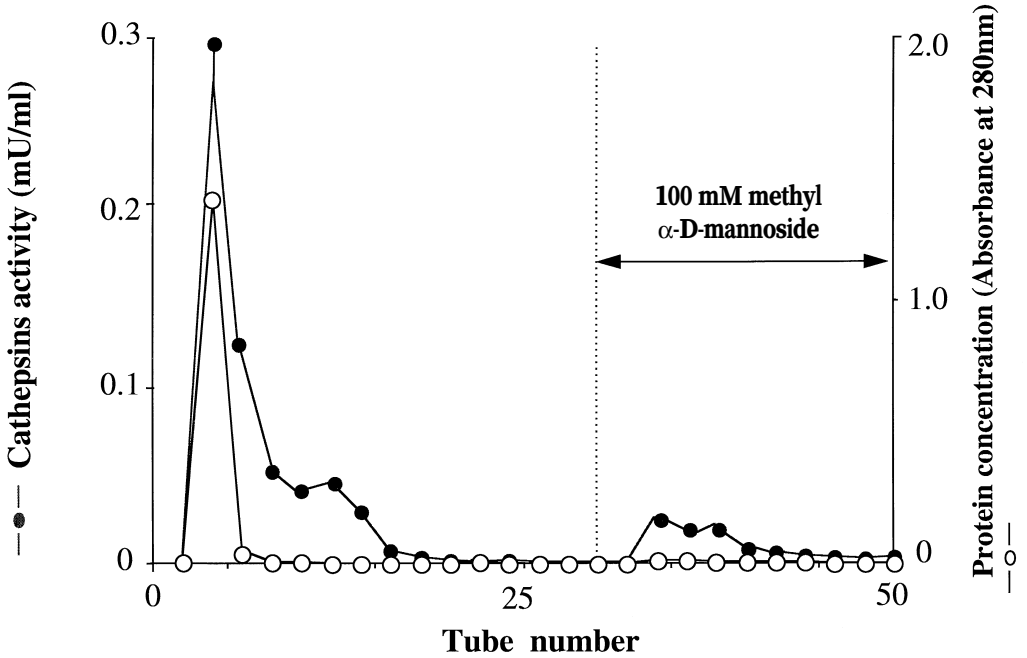

Fig. 6. Affinity chromatography of fraction I obtained after CM Sephadex C-50 column chromatography on Concanavalin A-Sepharose
The active fraction (fraction I) obtained by CM Sephadex C-50 column chromatography was dissolved in 0.02 M sodium phosphate buffer (pH 6.0)/0.2 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$, and applied to a column (1.2×8.4 cm) of Concanavalin A-Sepharose. After elution of unbound protein with equilibration buffer, the bound protein was eluted with starting buffer containing 100 mM methyl α-D-mannoside. Five milliliter fractions each were collected at a flow rate of 0.25 ml/min.

Table 3. Summary of purification of cathepsin B-like protease from synovial fluid of RA patients

Step	Total protein (mg)	Total activity (milliunits)	Specific activity (mU/mg)	Yield (%)	Purification factor
Original	2618.9	161.1	0.062	100	1.00
Ammonium sulfate fractionation (30~65%)	550.2	125.8	0.229	78.0	3.71
Butanol treatment	311.1	117.4	0.377	72.8	6.14
Acetone fractionation (40~65%)	55.1	57.1	1.036	35.4	16.85
Sephadex G-75	2.3	24.2	10.52	15.0	171.0
CM Sephadex C-50	0.43	8.4	19.53	5.2	317.6

Cathepsin B activity was measured against 50 μM Z-Phe-Arg-MCA at pH 5.8.

Table 4. Effects of the cathepsin inhibitors, CA-074 and E-64 on the hydrolyzing activity of the purified enzyme, human liver cathepsins B and L

Inhibitor	Concentration (M)	Percent inhibition (%)		
		Purified enzyme	Human liver Cathepsin B	Human liver Cathepsin L
1) CA-074	1×10^{-7}	99.4	99.8	8.85
	1×10^{-6}	99.8	99.9	14.8
2) E-64	1×10^{-7}	99.9	100	96.8
	1×10^{-6}	100	100	99.6

The purified enzyme (200μU) was leaved to stand in the assay mixture for the cathepsin activity with the specific inhibitors for 10 min at room temperature, and then reaction mixture was incubated with 50μM Z-Phe-Arg-MCA as substrate for 60 min at 37°C.

(0.345), and both values were similar to those determined for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA when human liver cathepsin B was used.

In the case of human liver cathepsin L, the Km for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were markedly lower, compared with each of the purified enzyme and liver cathepsin B.

3) In vitro activation of pro-urokinase by the purified enzyme

The activity of the purified enzyme (200 microunits) on various MCA-substrates, which were synthesized for the assay of serine proteases and collagenase in previous investigators, were examined (Table 6). Of the substrates tested, Z-Phe-Arg-MCA (a substrate for cathepsins B and L) was most preferentially hydrolyzed, and Z-Arg-Arg-MCA (a substrate for cathepsin B), Boc-Phe-Ser-Arg-MCA (a substrate for trypsin), Boc-Gln-Ala-Arg-MCA (a substrate for trypsin), Boc-Val-Leu-Lys-MCA (a substrate for plasmin) were hydrolyzed to different extents, the relative activity of the purified enzyme for these

Table 5. Michaelis constants (Km) for synthetic substrates hydrolyzed by the purified enzyme and human liver cathepsins B and L

Substrate	Purified Enzyme	Human liver Cathepsin B	Human liver Cathepsin L
Z-Arg-Arg-MCA (μM)	0.345	0.38	0.091
Z-Phe-Arg-MCA (μM)	0.118	0.157	0.037

Activity of the purified enzyme (200 μU) and human liver cathepsins were measured using Z-Arg–ArgMCA or Z-Phe-Arg-MCA at concentrations of 0.25, 0.50, 1.0, 2.0 and 4.0×10^{-4}M as the substrate.

substrates being 66.0, 24.5, 17.7 and 14.8%, respectively. Glt-Gly-Arg-MCA (a substrate for urokinase) and Boc-Val-Pro-Arg-MCA (a substrate for thrombin) were hardly hydrolyzed by the purified enzyme. As shown in Table 6, the relative activities of human liver cathepsin B on these MCA-substrates were almost the same as those of the purified enzyme.

Moreover, in the same assay system, the hydrolyzation of Boc-Phe-Ser-Arg-MCA, Boc-Gln-Ala-Arg-MCA and Boc-Val-Leu-Lys-MCA by the purified enzyme was completely inhibited by 10^{-5} M CA-074, like the activities that hydrolyzed Z-Arg-Arg-MCA and Z-Phe-Arg-MCA of the purified enzyme.

As shown in Fig. 7, when pro-urokinase was incubated with the purified enzyme for 10-90 min, the urokinase activity appeared and increased with incubation time, indicating that the purified enzyme

Table 6. Activities of the purified enzyme on synthetic model substrate

Substrate (50 μM)	Enzyme*	Relative activity** (%)			
		Purified enzyme		Human liver Cathepsin B	Human liver Cathepsin L
		without CA-074	with CA-074 (10^{-5}M)		
Z-Phe-Arg-MCA	Cathepsin L	100	1.1	100	100
Z-Arg-Arg-MCA	Cathepsin B	66.0	0.2	69.0	9.3
Boc-Phe-Ser-Arg-MCA	Trypsin	24.5	0.7	27.3	3.4
Boc-Gln-Ala-Arg-MCA	Trypsin	17.7	0.9	19.8	2.9
Boc-Val-Leu-Lys-MCA	Plasmin	14.8	0.3	17.0	16.1
Boc-Val-Pro-Arg-MCA	Thrombin	1.1	0.4	1.5	0.3
Glt-Gly-Arg-MCA	Urokinase	0.3	0.4	0.2	0
Boc-Ile-Gln-Gly-Arg-MCA	Factor Xa	0.4	0.4	0.3	0.1
Suc-Ala-Pro-Ala-MCA	Elastase	0.1	0	0.3	0
Suc-Ala-Ala-Pro-Phe-MCA	Chymotrypsin	0	0	0.2	0
Suc-Gly-Pro-Phe-MCA	Collagenase	0	0	0.2	0.1

* Each MCA-substrate was synthesized by previous investigators for the assay of the enzyme specified.
* * Relative activity is expressed as a percentage of that obtained with Z-Phe-Arg-MCA.

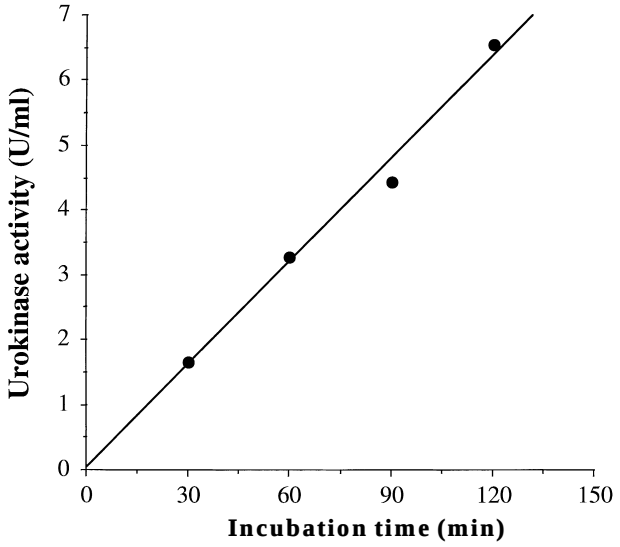

Fig. 7. Activation of pro-urokinase by cathepsin B purified from synovial fluids of RA patients
-Time course-
Recombinant pro-urokinase (final concentration 0.46 μM) was preincubated with the purified enzyme (40 microunits) in 88 mM KH_2PO_4-12 mM Na_2HPO_4/1.33 mM EDTA/2.7 mM cysteine (pH 5.9) for 30, 60, 90 and 120 min at 37°C in a total volume of 20 μl, using a modified method of Kobayashi et al. (24). The activities of the urokinase produced in the mixtures was measured by spectrofluorometry using 50 μM Glt-Gly-Arg-MCA as the substrate, as described in Materials and Methods.

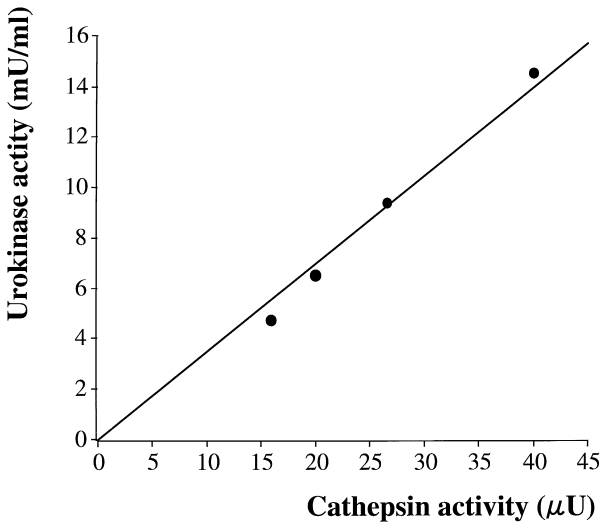

Fig. 8. Activation of pro-urokinase by the cathepsin B purified from synovial fluids of RA patients
-Effect of dose-
Pro-urokinase (0.46 μM) was preincubated with various amounts of the purified enzyme in the same reaction mixtures described for Fig.7, for 60 min, and then the activity of the urokinase produced was measured by the same method.

activates the transformation of pro-urokinase into urokinase, in a time-dependent fashion.

As illustrated in Fig.8, the purified enzyme activated pro-urokinase in a dose-dependent fashion. The Km for pro-urokinase, that reacted with the purified enzyme, was 8.27 μM as calculated from the Lineweaver-Burk plot.

DISCUSSION

Isolation of cathepsins from synovial fluids

The increase of cathepsin B levels in synovial fluids of RA patients has been reported in several studies (10-13), but there has been no report on the activity of cathepsin L in their synovial fluids. There has been only one study in which the content of cathepsin B was compared with that of cathepsin L in synovial fluids from the same RA patients (10).

Both the active form of cathepsin B and that of cathepsin L are composed of protein and an oligosaccharide moiety (27, 28). It is known that minor changes in the biochemical properties of cathepsin B, such as the heterogenous behavior in isoelectric focusing are caused by modifications of the oligosaccharide moieties (37). However, fundamental biochemical properties of the catalytic protein moieties of both active cathepsins are common for enzyme regardless of the tissue in which the enzyme is localized (27, 28). Therefore, this suggests that the biochemical property of each mature cathepsin B and L in the synovial fluids of the RA patients are fundamentally similar to those of liver cathepsins. Based on this concept, in the present study, we attempted to isolate cathepsin B and cathepsin L from synovial fluids, using the same fractionation methods used by Barrett and Kirschke (34) to purify cathepsins B and L from rat liver.

When liver cathepsins B and L are subjected to cation-exchange column chromatography using CM-Sephadex C-50 at pH 5.8, cathepsin B is eluted with a 0 to 200 mM KCl gradient, while cathepsin L is eluted with 600 mM KCl (34). In the present study, it was necessary to separate the cathepsins from lipids before subjecting them to cation exchange column chromatography, because synovial fluids contain large amounts of lipids. A considerable part of the lipids contained in synovial fluids could be removed by butanol treatment and acetone fractionation, without marked loss of cathepsins B and L activity. Therefore, the samples delipided by both butanol and acetone fractionation were subjected to gel filtration using Sephadex G-75. The fluorogenic substrate, Z-Phe-Arg-MCA, which we used as substrate for the assay of cathepsins, is known to be hydrolyzed by both cathepsins B and L. After the gel filtration, only one peak of Z-Phe-Arg-MCA-hydrolyzing activity was detectable.

The results (Fig. 3) from the CM-Sephadex C-50 column chromatography of the active fraction obtained by Sephadex G-75 gel chromatography showed that the Z-Phe-Arg-MCA-hydrolyzing activity, eluted with a 0 to 200 mM KCl gradient, was markedly higher than that eluted with 600 mM KCl. As described in the Results section and from the report of Barrett and Kirschke (34), the first large peak (fraction I) was thought to correspond mainly to cathepsin B, and the second small peak (fraction II) to cathepsin L (Fig. 3). The findings of the control experiments using human liver cathepsin B and cathepsin L (Figs. 4 and 5) supported this idea.

When human liver cathepsin B and cathepsin L were subjected to affinity chromatography using Concanavalin A-Sepharose equilibrated with 20 mM sodium phosphate buffer (pH 6.0)/0.2M NaCl/1mM $CaCl_2$/1 mM $MnCl_2$, the former did not bind to this column, while most of the latter bound to this column and was eluted with a 0 to 100 mM methyl α-D-mannoside gradient (34). In the present study, fraction I from the CM-Sephadex column did not bind to the Concanavalin A-Sepharose column. This results also supported the idea that the cathepsin activity of fraction I was mainly due to cathepsin B.

Since the results of the experiments described above indicated that the synovial fluids of RA patients predominantly contained cathepsin B, we purified this enzyme from those fluids using several fractionation methods. To compare the biochemical properties of the purified enzyme with those of human liver cathepsin B, biochemical characterization of the former was carried out with the latter as control, and the following results were obtained : 1) The relative activities of the purified enzyme on several MCA-substrates were almost the same as those of human liver cathepsin B (Table 4), 2) The Km for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA that reacted with the purified enzyme were similar to those obtained with human liver cathepsin B (34), 3) Both the Z-Phe-Arg-MCA hydrolyzing activity and the Z-Arg-Arg-MCA hydrolyzing activity of the purified enzyme were almost completely inhibited by 10^{-7}-10^{-6} M of CA-074, a specific inhibitor of cathepsin B. These findings indicated that the purified enzyme was mainly composed of cathepsin B of the

cathepsins, although it was not pure as shown by the SDS-PAGE pattern of migration.

It is known that there are many molecular forms of cathepsin B in vivo (27). Cathepsin B is synthesized as 339 residue pre-procathepsin B with a molecular weight of 40 to 42 kDa. This pre-procathepsin B is converted into inactive pro-cathepsin B, following removal of the 17 residue signal sequence. Conversion of the pro-cathepsin B into mature cathepsin B (single-chain form) with a molecular weight of 30-33 kDa occurs following cleavage and dissociation of the 62 residue pro-region by the actions of proteases. The final proteolytic event is cleavage between residues 47 and 50 to yield a two-chain form of cathepsin B (25 kDa and 5 kDa) with the excision of a dipeptide.

In the present study, we estimated the contents of cathepsins B and L in synovial fluid by measuring the synthetic substrate-hydrolyzing activities of these enzymes. Previous investigators have shown that the synovial fluids of RA patients contained cathepsin inhibitors such as low-molecular cystatin and high-molecular kininogen, and part of the active cathepsin B contained in the synovial fluids is thought to exist as a complex formed from these cathepsin inhibitors (9, 11). Thus, only when an imbalance between active cathepsin B and its inhibitors occurs in the synovial fluids, cathepsin activity can be measured. The significantly higher concentrations of Z-Phe-Arg-MCA-hydrolyzing activitiy detected in the synovial fluids of RA patients compared to OA patients, indicated both an increase of cathepsin content and an imbalance between active cathepsins and their inhibitors in RA articular lesions. Therefore, in the present study, we probably purified free active cathepsin B and cathepsin B bound to non-inhibitory substances from the synovial fluids of RA patients. The purified enzyme showed several bands in the SDS-PAGE analysis, which is thought to be partly due to that the cathepsin B in the synovial fluid is in complexed forms.

Maciewicz and Etherington (10) reported that there was approximately 10 fold more cathepsin B than cathepsin L in the synovial fluids of patients with RA, OA and other types of arthritis. It is thought that they probably measured total of pro-cathepsin B and mature cathepsin B as well as total pro-cathespin L and mature cathepsin L by ELISA for both enzymes, because the pro-enzyme and mature enzyme of each cathepsin have common antigenic properties. The findings of the present study showed that synovial fluids of RA patients contained more cathepsin B than cathepsin L, and were roughly in accordance with the findings of Maciewicz and Etherington (10) with respect to RA.

Using immunohistochemical methods, previous investigators showed cathepsin B activity in synovial cells such as macrophages and fibroblasts, and in neutrophils and macrophages found in synovial fluids of RA patients (13, 14). The synovial fluids of RA patients contain inflammatory cells including neutrophils and macrophages (38, 39). We confirmed the significant increase of neutrophils in the synovial fluids from RA patients. Recently, Lemaire et al. showed that the cultured synovial fibroblast-like cells from RA patients secrete cathepsins B and L in response to cytokines (40). These findings suggest that the synovial cells and inflammatory cells infiltrating inflammatory lesions may be the source of the cathepsin B found in the synovial fluids of RA patients. However, it is unknown whether joint tissues or inflammatory cells are the main source of the cathepsin B.

In the present study, we observed that cathepsin activity did not significantly correlate with the neutrophils content in the synovial fluids from RA patients. The reason for this finding remains to be elucidated, but there is a possiblity that a considerable part of cathepsin B was derived from the joint tissues, including the synovium.

Activation of pro-urokinase by purified enzyme

Plasmin is activated by urokinase (23), and conversion of pro-urokinase to urokinase is mediated by several proteases including plasmin, kallikrein and mast cell tryptase (41-43). Recently, Kobayashi et al. reported that cathepsin B also activate the soluble and the tumor cell receptor-bound form of pro-urokinase (24, 25).

In the present study, the substrate specificity of the purified enzyme was almost the sames as that of human liver cathepsin B, and the hydrolyzing activities of the purified enzyme when reacted with Boc-Phe-Ser-Arg-MCA (a substrate for trypsin) and Boc-Val-Leu-Lys-MCA (a substrate for plasmin), were completely inhibited by 10^{-5}M CA-074. These results indicated that Boc-Phe-Ser-Arg-MCA-hydrolyzing activity and Boc-Val-Leu-Lys-MCA-hydrolyzing activity of the purified enzyme were mainly due to cathepsin B itself, but not to a trypsin or plasmin-like protease contaminating.

The Glt-Gly-Arg-MCA (a substrate for urokinase) -hydrolyzing activity of the purified enzyme was barely detected in the assay system for cathepsin

activity. When pro-urokinase was incubated with the purified enzyme in vitro, urokinase activity appeared in a time- and dose-dependent fashion. These findings indicated that the cathepsin B purified from the synovial fluids of RA patients may activate pro-urokinase. The Km for pro-urokinase reacted with purified cathepsin B was approximately 8.27 μM. This value was similar to that for the reactivity catalyzed by plasmin (41), but smaller than that (34 μM) for the reaction catalyzed by lung mast cell tryptase (43).

The metalloprotease family including collagenase, is directly related to the destruction of articular tissues (20-22), and plasmin is intimately related to the activation of these metalloproteases (22). Previous investigators have shown increased levels of plasmin and plasminogen activator in the articular cartilages of RA patients by enzymography, ELISA and by detections of messenger RNA levels (44-47). Saxne et al. showed that in the synovial fluids of RA patients the urokinase concentration was elevated while that of tissue activator was low (45). This study indicated that of the plasminogen activators, active urokinase increased more markedly than tissue activator at the articular lesions in RA, strongly suggesting that activation of pro-urokinase to urokinase was a very important step in the destruction of articular tissues in RA. This step was shown to be catalyzed by several kinds of proteases including plasmin (24, 25, 41-43). The results of the present study suggest that cathepsin B may contribute to the destruction of the cartilage extracellular matrix in RA by activating the fibrinolytic cascade via activation of pro-urokinase.

Further studies are necesssary to clarify what kinds of proteases may be most intimately related to the activation of pro-urokinase in RA.

Finally, in the present study, we showed, by measuring the activities of cathepsin B and L, that an imbalance between cathepsin B and its inhibitors occured due to an increased concentration of active cathepsin B in synovial fluids of RA patients. It was also shown that the content of active cathepsin L was significantly lower than that of active cathepsin B, and that cathepsin B purified from the synovial fluids activated pro-rokinase to urokinase.

These findings indicate that cathepsin B is released in large amounts into the articular extra-cellular spaces in active RA, and the cathepsin B may be related to the degradation of cartilage in RA by activating the fibrinolytic cascade.

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