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Fibrinolytic Enzymes in Experimental Gout Induced by Urate Crystal (MSU)

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Abstract

Acute arthritis was induced by injected synthesized monosodium urate (MSU) crystals into dog's knee joints. Maximum fibrinolytic activity in the synovia occurred 6 hours later. The fibrin plate method, substrate specificity, inhibitor effects and zymography indicated mediation by tissue-type and urokinase-type plasminogen activators (t-PA and u-PA). Lysosomal β-glucuronidase and catepsin G revealed peaks 12 hours after the injection. The synovium then showed strong inflammation histologically.

Increased fibrinolytic activity by plasminogen activator may be involved in this acute inflammation.

Introduction

Concerning the relation between acute gouty attracks and monosodium urate (MSU) crystals, numerous studies have been carried out since the early work of McCarty (1, 2) and Seegmiller (3). When MSU crystals were injected intra-articularly and subcutaneously into humans or dogs, it was confirmed that acute inflammation which resemble gouty arthritis occured (4—9).

However, there are several unexplained aspects of acute gout including the detailed mechanisms of MSU crystal formation within the body and the occurrence of articular inflammation due to the crystal component. Kosugi et al. (10) found that an increase of fibrinolytic activity was related to deterioration of inflammation. Although some papers (11—17) have examined the fibrinolytic system in joints, there are few reports on the fibrinolytic system in arthritis induced by MSU crystal models.

The present study investigated some of the characteristics of the activated fibrinolytic enzyme in the synovia and the histological changes in the synovial membrane after MSU crystal injection.

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Materials and Methods

Reagents and chemicals: Synthetic p-nitroanilide substrates consisting of pyro-Glu-Pro-Val-pNA (S-2484) for leucocytic elastase, H-D-Phe-Pip-Arg-pNA · 2HCl (S-2238) for thrombin, H-D-Val-Leu-Arg-pNA · 2HCl (S-2266) for glandular kallikrein, H-D-Val-Leu-Lys-pNA · 2HCl (S-2251) for plasmin, H-D-Ile-Pro-Arg-pNA · 2HCl (S-2288) for tissue-type plasminogen activator (t-PA), pyro-Glu-Gly-Arg-pNA · HCl (S-2444) for urokinase-type plasminogen activator (u-PA), H-D-Pro-Phe-Arg-pNA · 2HCl (S-2302) for plasma kallikrein, and MeO-Suc-Arg-Pro-Tyr-pNA · HCl (S-2586) for cathepsin G were purchased from Kabivitrum AB (Stockholm, Sweden). The protease inhibitors, diisopropyl fluorophosphate (DFP), soybean trypsin inhibitor (SBTI), tosyllysine chloromethyl ketone (TLCK) and tosylamide phenylethylchloromethyl ketone (TPCK) were obtained from Sigma Chemical Company (St. Louis, USA), elastatinal from the peptide Institute, Inc. (Osaka, Japan), aprotinin from Bayer (Leverkusen, West Germany), and epsilion-amino-n-caproic acid (EACA) and trans-4-amino-methylcyclohexane carboxylic acid (t-AMCHA) from Daiichi Seiyaku Co., Ltd. (Tokyo, Japan). Uric acid and agar were from Wako Pure Chemical Industries Ltd., urokinase and thrombin (bovine) from Mochida Pharmaceutical Co. Ltd., and bovine fibrinogen (75% or 80% clottable) from Miles Inc. or from Sigma Chemical company, respectively. All other chemicals used were of analytical grade.

Assay methods.

Fibrinolytic activity: For determination of the fibrinolytic activity, both the plasminogen-rich fibrin plate method of Astrup and Müllertz (18), and additionally when necessary, the plasminogen-poor fibrin plate method were employed. Plasminogen-poor fibrinogen solution was prepared by passing plasminogen-rich fibrinogen (Miles) solution through a lysine-Sepharose column. The solution was then used to make plasminogen-poor fibrin plates. Each sample volume was 0.03 ml and incubation was carried out for 18 hours at 37°C. The fibrinolytic activity was determined from the lytic area (mm^2) on the fibrin film.

Amidolytic activity: The amidolytic activity was determined essentially by the end-point method of Friberger (19).

The reaction mixture contained 0.05 ml of sample solution (synovial fluid after 6 hours), 0.01 ml of 5mM synthetic substrate and 0.85 ml of 0.1 mM phosphate buffered saline, pH 7.4. After incubation at 37°C for an adequate time, the reaction was stopped with 0.1 ml of 50% acetic acid. The amidolytic activity was defined as the absorbance at 405 nm of the p-nitroaniline released per min.

Inhibitory activity by protease inhibitors: A volume of 0.03 ml of sample was placed on the surface of a fibrinogen-rich (or -poor) fibrin plate containing protease inhibitors at

known concentration and incubated at 37℃ for 18 hours. The fibrinolytic activity was then determined as described above.

Exceptionally, two inhibitors (DFP and elastatinal) was mixed directly with the sample before application to the fibrin plates.

Gel filtration: The synovia after 6 hours were dissolved in 0.01 M phosphate buffer, pH 7.4, and centrifuged at 5,000 rpm for 20 min. 0.5 ml of the supernatant was applied to a column (1.0 x 41 cm) of Sephacryl S-200 (Phamacia Chemicals) equilibrated with the same buffer containing 0.15 M NaCl, pH 7.4. The elution was performed at room temparature and the elute was collected in fractions of 0.5 ml at a flow rate of 0.21 ml/min.

Zymographic method: Zymographic analysis was performed using fibrin agar plates according to the method of Granelli-Piperno and Reich (20). Each fibrin agar plate was prepared with 8.5 ml of 1.6% fibrinogen (Sigma) pretreated on a lysine-Sepharose column or non-pretreated, 8.5 ml of 1.2% agar and 0.5 ml of 20 U/ml thrombin. Human urine and a commercial urokinase preparation were emproyed as references.

Lysosomal enzymes: Cathepsin G was assayed with S-2586 as the substrate, as indicated above. β-Glucuronnidase was assayed with phenolphthalein-β-glucuronidide as the substrate, based essentially on the method of Person and Kar (21). The reaction mixture contained 0.2 M acetate buffer (pH 4.5), 0.03 M substrate and the sample in a total volume of 1 ml. Following incubation at 56℃ for 1 hour, the reaction was stopped with 5 ml of 0.1 M 2-amino 2-methyl 1-propanol (AMP) buffer, pH 11. The absorbance at 550 nm was measured and referred to phenolphtalein standards treated in the same manner.

Sampling and histology: Sixteen mongrel dogs weighing 7 to 16 kg, from the Experimental Animal Center Miyazaki Medical College and maintained at a controlled ambient temperature of 23℃ with a relative humidity of 50%, were used. Anesthesia was induced by intravenous injection of pentobarbital sodium (50 mg/kg). Additional small does were given as needed. We employed uric acid, from which MSU crystals (needle-shaped and amorphous) were prepared according to the modified method of Seegmiller et al. (3). Crystals (30 mg) suspended in 0.9 ml of PSS (pyrogen-free, physiological saline solution) were placed in 2-ml ampoules, and sterilized in an autoclave. Ten mg of the crystalals in 0.3 ml injected aseptically into the knee joint capsule of each of three legs. Simultaneously, control solution (0.3 ml) was injected into the other knee joint. As the control, supernatant from PSS saturated with MSU crystals was used.

Aspiration of synovia was performed from the canine knee joints at 0, 1, 3, 6, 12, 24 and 36 hours after the injection. All samples of synovia were immediately centrifuged at 3,000 rpm for 10 min. The supernatants were frozen at -80℃ until use.

The synovium was examined histologically from knee joints of 4 dogs at 6 and 12 hours after crystal injection and at 6 hours after injection of control solution. The knee joints were

immediately opened, and specimens obtained for light microscopy were fixed in buffered formalin. The animals with opened knee joints were sacrificed by administering an overdose of intravenous sodium pentobarbital after the sampling. Sections of synovial tissue were stained with hematoxylin and eosin.

Results

Progressive changes in fibrinolytic activities. When 10 mg of MSU crystals (needle-shaped) was injected into dog's knee joints and the fluctuations in the synovial fibrinolytic activities were observed over the time course with plasminogen-rich fibrin plates, no changes in fibrinolytic activities were noted at 0 and 1 hour after the administration. However, the fibrinolytic activities began to increase considerably from 3 hours, reached a peak at 6 hours, began to decrease gradually after 12-24 hours, and returned almost to the original level after 36 hours (Fig.1). On the other hand, no fibrinolytic activity was usually observed in the synovia after injection the control substance, although there were a few exceptions.

When amorphous MSU crystals are injected, the degree of inflammation is generally known to be weak in comparison with that following needle-shaped crystal injection. In the present experiments also, although an increase in fibrinolytic activities was demonstrated in the synovia at 6 hours after the injection of 10 mg of amorphous crystals, the levels were lower than those after the injection of needle-shaped crystals. Furthermore, when the changes in fibrinolytic activities were studied with plasminogen- poor fibrin plates, almost parallel fluctuations to those on the plasminogen- rich fibrin plates were observed (Fig.2). However, the level of fibrinolytic activities on the plasminogen-poor fibrin plates amounted to about 1/5 of those on the plasminogen-rich fibrin plates.

Biochemical characteristics. Since the fibrinolytic activity of the synovia revealed a peak at 6 hours after MSU crystal injection, biochemical characterization of the fibrinolytic enzyme in the synovia was carried out at 6 hours.

Among the amidolytic activities for the various synthetic substrates, that for the t-PA substrate S-2288 was the highest, followed by that for the thrombin substrate S-2238, while that for the elastase substrate S-2484 was very low. The amidolytic activity for S-2288 was significantly dominant as compared with that for the u-PA substrate S-2444 (Table 1).

When 1 mM EACA (or 1 mM t-AMCHA) was added to the plasminogen-rich plates, the fibrinolytic activities in the synovia after 6 hours decreased to about 50%. Addition of 1 mM DFP reduced them to 10%. Aprotinin and SBTI demonstrated very strong inhibitory effects. In particular, SBTI repressed the activities to 0% at a concentration of 1 mg/ml. These substances are all plasmin inhibitors. Almost no difference in inhibitory effects was observed between plasminogen-rich and -poor fibrin plates. On the other hand , TLCK, elastatinal

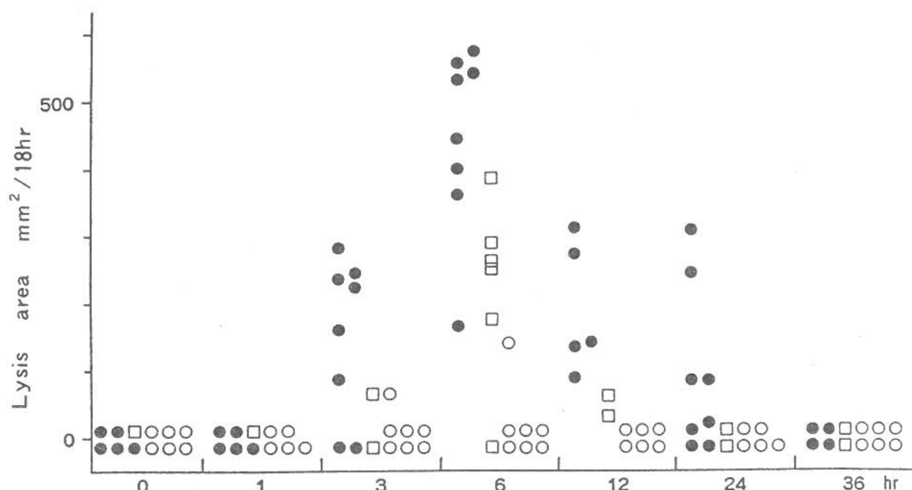

Fig. 1. Changes in fibrinolytic activities of the synovial fluid after MSU crystal injection.
●, MSU crystals (needle-shaped); □, MSU crystals (amorphous);
○, control (supernatant of PSS saturated with MSU crystals).

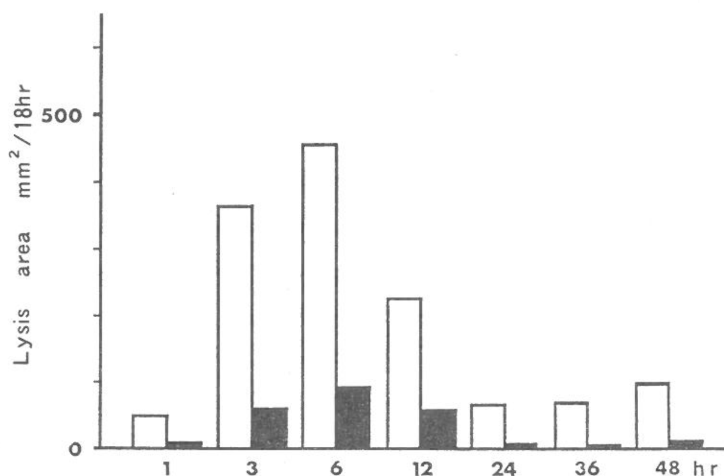

Fig. 2. Changes in mean fibrinolytic activities of MSU crystal-injected synovia on plasminogen-rich fibrin plates (□) and plasminoren -poor fibrin plates (■).

and TPCK exerted no inhibitory effects on the fibrinolytic activities with either plasminogen-rich or plasminogen-poor plates (Table 2).

Six different synthetic substrates were added to each of the fractions which were collected by gel filtration of the synovia after 6 hours, and their amidolysis was assayed. Each of the amidolytic activities was separated into two peaks (peak I, fr. 25-27; peak II, fr. 33-35) (Fig. 3). On the other hand, no activity was observed when the synovia at 0 and 48 hours after injection of MSU crystals were applied to the same colomn (not shown). The

Table 1. Substrate specificity of the fibrinolytic enzyme after 6 hours, employing various synthetic substrates

Substrate	CD_{405}/min	Relative %
S-2288 (t-PA)	0.0173	100
S-2238 (thrombin)	0.0135	78
S-2266 (t-kallikrein)	0.0101	58
S-2251 (plasmin)	0.0098	57
S-2302 (p-kallikrein)	0.0098	57
S-2444 (u-PA)	0.0037	21
S-2484 (elastase)	0.0007	4

Table 2. Inhibitory effects of various protease inhibitors on the fibrinolytic activity in the synovia after 6 hours

Inhibitor	Concentration	% Fibrinolysis	
		Plasminogen-rich plates	Plasminogen-poor plates
None		100	100
EACA	1(mM)	56	51
	10	50	7
t-AMCHA	1(mM)	43	0
		28	0
SBTI	1(mg/ml)	0	0
	10	0	0
DFF	0.01(mM)	74	98
	0.1	52	79
	1	10	0
Aprotinin	10(KIU)	59	69
	100	0	0
TLCK	0.1(mM)	117	100
	1	100	168
Elastatinal	0.01(mg/ml)	75	100
	0.01	114	100
TPCK	0.01(mM)	126	113
	1	84	77

Each of the protease inhibitors was mixed in the fibrin plate at each concentration , except in the case of two inhibitors (DFP and elastational; see Materials and Methods) .

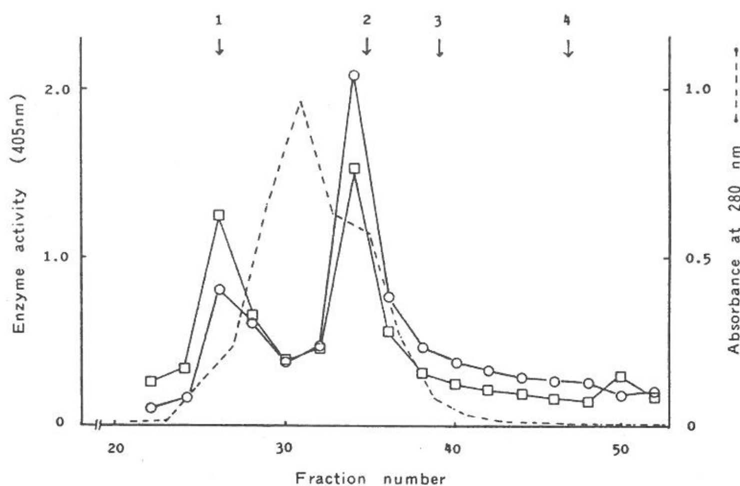

Fig. 3. Gel filtration patterns ynovia of after 6 hours employing various synthetic substrates. The enzyme activities of each fraction were assayed with 6 different synthetic substrates. However, date for 4 of the synthetic substrates (S-2302, S-2266, S-2251 and S-2586) , i.e. except S-2288 (○) and S-2238 (□) are not shown in the figure since they displayed only low activities. 1. bovine serum albumin; 2. ovalbumin; 3. α-chymotrypsinogen; 4. cytochrome c. These were used as protein markers.

fibrinolytic activities of peak II were higher than those of peak I, and an enzyme digesting S-2288, a t-PA substrate, was present chiefly in peak II. The estimated optimum pH of the enzymatic activities in the peak I and peak II fractions was 7.0 (substrate, S-2586) and 7.5 (substrate, S-2288), respectively.

The fibrinolytic enzymes in the peak I and peak II fractions obtained by gel filitration were subjected to zymography respectively. The peak II fraction displayed two lytic bands of about MW. 50,000 (similar to urokinase from normal human urine) and about MW. 80,000-90,000. The fibrinolytic bands of the peak I fraction appeared chiefly at a position of about MW. 50,000 and also at about MW. 80,000-90,000 or more (Fig. 4).

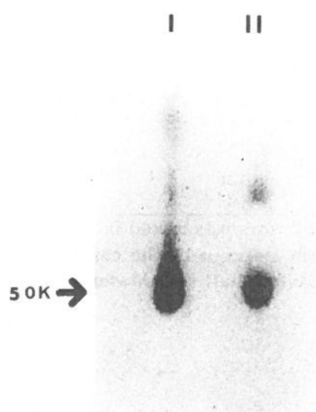

Fig. 4. Patterns of lytic bands observed by zymography of the peak (I, II) fractions. The main band in the two peak (I, II) fractions appeared at about the position of M 50,000 similarly to urokinase from normal human urine.

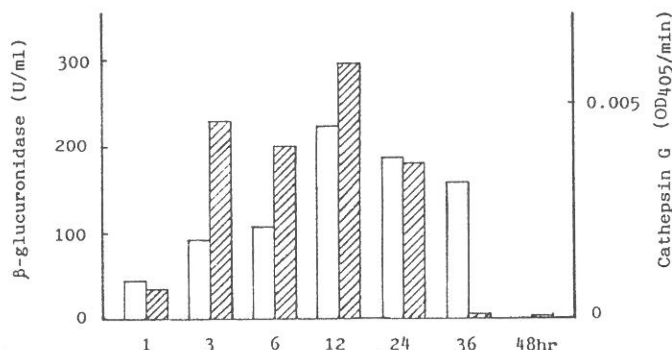

Fig. 5. Time course of changes in mean levels of lysosomal enzymes (□, β-glucuronidase; ▨, cathepsin G) in the synovia after MSU crystal injection. Each value represents the average of samples from 3 dogs.

Lysosomal enzymes and histological findings. The activity changes of two lysosomal enzymes (β-glucuronidase and cathepsin G) were observed. Although they began to increase from 3 and 6 hours, the peaks of both these activities were found in the synovia at 12 hours after the crystal injection (Fig. 5). Their appearance was thus later than the peak of fibrinolytic activity in the synovia which occurred after 6 hours.

When control solution was injected into the knee joint capsule, synovial cells were distributed continuously in one or two layers on the synovial surface. Sticking to the blood vessel walls and minimal emigration of neutrophils into extravascular connective tissue of capsule were observed (Fig. 6A). In the case of the synovium at 6 hours after crystal

injection, plasma exudation with fibrin deposition and marked neutrophil infiltration were noted throughout the joint cavity.

Vasodilation and neutrophil infiltration in proliferated, edematous villous synovium were also found (Fig. 6B). After 12 hours, plasma effusion, stronger infilration of neutrophils and fibrin deposition intermingled with neurophil aggregate were seen. Overall, neutrophil emigration in the synovium after 12 hours was stronger than that after 6 hours and apparent acute inflammatory changes were induced in the synovial tissues after 12 hours (Fig. 6C).

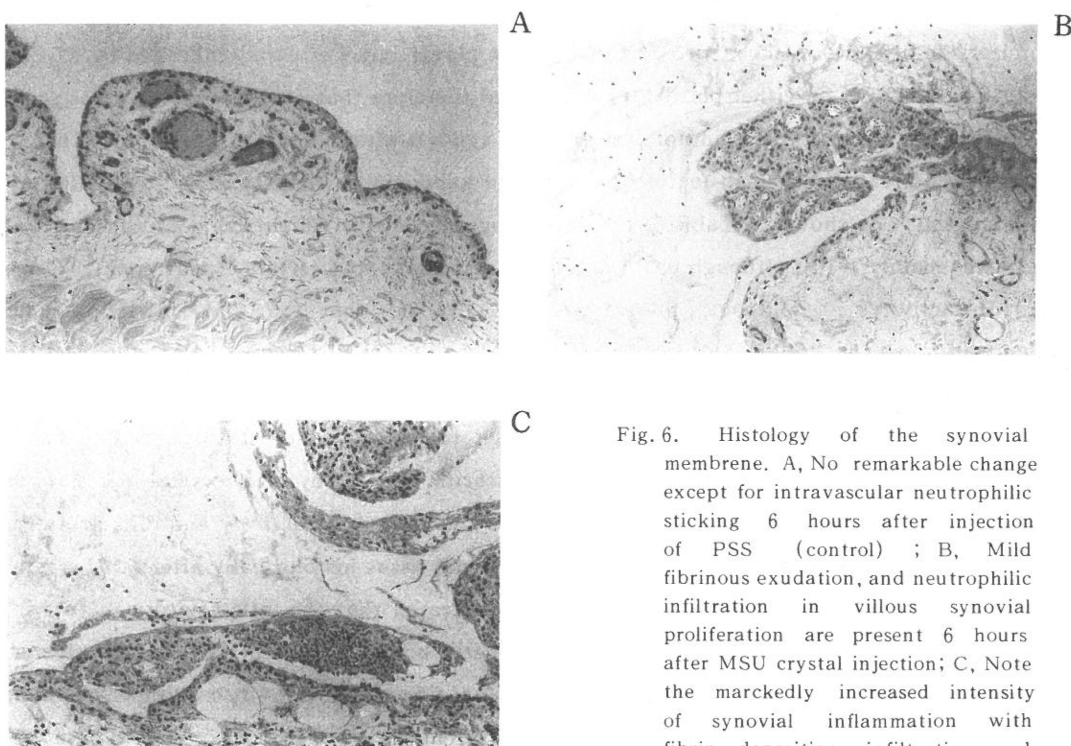

Fig. 6. Histology of the synovial membrene. A, No remarkable change except for intravascular neutrophilic sticking 6 hours after injection of PSS (control); B, Mild fibrinous exudation, and neutrophilic infiltration in villous synovial proliferation are present 6 hours after MSU crystal injection; C, Note the marckedly increased intensity of synovial inflammation with fibrin deposition, infiltration and aggrezation of neutrophils 12 hours after MSU crystal injection. (H and E. ×170)

Discussion

Many investigations have examined the arthritis induced by urate crystal injection (22-24) and it has been confirmed that injection of MSU crystals into the joints of rabbits (25), dogs (26-28) and humans (3, 8, 29) causes an acute inflammatory response.

Although some reports (10, 17, 30) have indicated that the fibrinolytic system may be

responsible for the deterioration of inflammation, so far as we know, there are few reports on the fibrinolytic system in the arthritis induced by MSU crystal injection. We therefore investigated this problem in dogs with acute synovitis induced by intra-articular MSU crystal injection.

Since it was confirmed that the fibrinolytic activity showed a maximum value in the synovia after 6 hours, we examined the biochemical characterristics of the fibrinolytic enzyme after 6 hours. As demonstrated in Fig. 2, the fibrinolytic activity on plasminogen-poor fibrin plates appeared to be mediated by a plasmin-like enzyme, whereas most of the remainder of the fibrinolytic activity was found to be mediated by plasminogen activator. The experiments with protease inhibitors clarified that the enzyme after 6 hours was well inhibeted by plasmin inhibitors on both plasminogen-rich and -free fibrin plates, but was barely inhibited by TLCK, elastatinal or TPCK on either plate. It is assumed therefore that non-plasmic enzymes such as elastase and chymotrypsin did not appear in the synovia after 6 hours. It is known that t-PA, u-PA, activated Hageman factor (XIIa) and kallikiein participate in the activation of plasminogen. As shown in Table 1, the plasminogen activator which was confirmed in this study was mainly t-PA, although u-PA activity was present to a certain extent (ca. 20% of the t-PA activity). Moreover, there are reports (31, 32) that u-PA also participates in inflammation, and molecullar forms of both t-PA like and u-PA like enzymes were observed by zymography.

Accordingly, both t- PA and u- PA, although t- PA exhibited the dominant activity compared to u-PA, appear to be involved in the arthritis induced by MSU crystal injection. In order to clarify the relation between the intensity of fibrinolytic activity and inflammatory findings of the synovial tissue, we examined the synovial tissue histologically after MSU crystal injection. The results of these observations demonstrated that the inflammatory picture in the synovium was stronger after 12 hours than after 6 hours. In other words, the clearer inflammatory picture did not correspond to the peak of fibrinolytic activity but appeared later than this peak (Fig. 6B, C). This could imply a latent period of 6 hours or more before the inflammatory response becomes more evident following the peak in the fibrinolytic activity. It is thought that plasma proteins may migrate into the synovial space during this intervals, activating a series of enzymes required for large accumulations of leukocytes, so that the inflammatory reactions progress. Both of the lysosomal enzymes investigated (β-glucuronidase and cathepsin G) also displayed maximal activity in the synovia after 12 hours, and the progressive changes in these enzymatic activities were nearly parallel to the increase in inflammation in the synovial tissue (Fig. 6B, C). These lysosomal enzymes are therefore considered to have been derived from the lysosomes of neutrophils and other related cells (33). Nevertheless, the question still remains as to what the joint pain results from, especially since the violent pain commonly accompanies gouty arthritis. There are

considerable restrictions and difficulties in evaluating the pain per se induced by MSU crystal injection in animal experiments. However, Okuda et al. (34) have examined the time course of fluctuations in pain induced by MSU crystal injection in cats and have demonstrated that the joint pain reached a peak at about 6 hours after the crystal injection.

Thus, the peak of pain caused by intra-articular MSU crystal injection in the cat corresponded closely with the rise in fibrinolytic activity induced by crystal injection into the dog's knee joint. These findings suggest that there might be some correlation between joint pain and a rise in fibrinolytic activity. Since plasmin is a kinin-forming enzyme, it is possible that an increase of plasminogen activator could be related to the intensity of pain. However, the detailed mechanisms remain to be determined, and further experiments on the significance of the rise in fibrinolytic activity in acute inflammation are needed. It is also possible that plasmin activated from plasminogen might promote a series of acute inflammatory reactions through the activation of complements (C3a, C5a) which results in vasodilation, an increased vascular permeability, and neutrophil emigration through the vessel wall.

It is speculated that a fibrinolytic factor (plasminogen activator-plasmin) could play an important role in the manifestation of the acute inflammatory response. Such a proposal for the development of acute gouty arthritis remains attractive and will serve as a hypothesis to stimulate further experiments on the mechanisms of inflammation in gout and other arthritides.

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