Bull. Mukogawa Women's Univ. Nat. Sci., **41**, 27－34(1993)
武庫川女子大紀要（自然科学）

SPLEEN FIBRINOLYTIC PROTEINASE IN THE MICE INDUCED BY CARRAGEENAN INFLAMMATION

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The variation of fibrinolytic activity in the spleen was investigated using a carrageenan air-pouch method in mice. After the injection of carrageenan into the air-pouch, numbers of leukocyte on the 6th day and concentration of fibrinogen on the 3rd day were increased, and activated partial thromboplastin time was prolonged at the 6 to 12 hr, but prothrombin time was not changed. Diluted blood clot lysis was decayed on the 1st day and enhanced on the 10th day. Fibrin(ogen) degradation products were increased on the first day. The spleen fibrinolytic activity was significantly enhanced with increasing of spleen weight on the 10th day after carrageenan injection. The comparison of fibrinolytic and amidolytic activities of the spleen extract with those of leukocyte extract were also made on the 10th day after carrageenan injection. Synthetic substrates, suc-L-Ile-L-Tyr-L-Phe-pNA for chymotrypsin-like proteinase was hydrolyzed 3.9 times at the spleen and 3.7 times at leukocyte compared with each control, and suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA for elastase-like proteinase was hydrolyzed 4.9 times at the spleen and 2.9 times at the leukocyte. The fibrinolytic activity of the spleen was increased 4.8 times in comparison with the control, and that of leukocyte was 6.2 times. The fibrinolytic activity was completely inhibited by diisopropyl fluorophosphate, L-phenylmethyl sulfonyl fluoride, p-nitrophenyl p-guadinobenzoate p-toluene sulfonyl L-phenylalanine chloromethyl ketone or soybean trypsin inhibitor, and was not inhibited by tranexamic acid, aprotinin, cysteine or EDTA. The spleen fibrinolytic activities were induced by the carrageenan inflammation, were derived from chymotrypsin-like proteinases but not from plasmin.

Introduction

A fibrinolytic proteinase extracted from the human spleen was identified as elastase in the human leukocyte immunologically(1,2). The fibrinolytic activity other than plasmin was detected in the spleen of conventional rats but not of the germfree ones(3). However, even in the germfree rats the fibrinolytic activity appeared after the surgical operation under aseptic conditions(4). The activity was detected scarcely in the spleen of normal mice, but it increased in the sarcoma 180 inoculated mice(5). In these two reports, the fibrinolytic activity was induced with the increase

of plasma fibrinogen and leukocyte number in the circulating blood. Recently, in the case of turpentine inflammation, the increased number of leukocytes was reported in germfree mice(6). These accumulated results suggest the correlation between the variation of spleen fibrinolytic activity and fibrin(ogen) degradation by leukocytes. The aim of this paper is to know the variation of fibrinolytic activity in the spleen and parameters of blood coagulation and fibrinolysis in mice with an inflammation induced by carrageenan. Furthermore, the properties of the spleen fibrinolytic enzyme were also studied.

Materials and Methods

Experimental design: Male Jcl-ICR mice weighting around 30g at the beginning were used in this experiment. The method of Tsurufuji et al. (7) were adopted to induce a carrageenan air-pouch inflammation. The mice anesthetized with ether were subcutaneously injected 2ml of air at their dorsum to make an air-pouch. After 24 hrs, 0.8ml of 2% carrageenan(type Ⅳ Lambda Carrageenan, Sigma Chem. Co., St. Louis) solution in saline was injected into the pouch under ether anesthesia. At the 6 and 12 hr, 1st, 3rd, 6th, 10th day after the carrageenan injection, whole blood withdrawn from the inferior vena cava for blood examinations, and the spleen were excised immediately after sacrifice and kept at −20℃ until use.

Blood examination: Concentration of plasma fibrinogen was measured by the method of Quick(8) using citrated-plasma. The prothrombin time(PT)(9) was measured according to Quick's one step method: 100μl of preincubated citrated-plasma for 3 min at 37℃ was mixed with 200μl of prewarmed thromboplastin(Thromboplastin C, Dade, Aguada). The activated partial thromboplastin time (APTT)(10) was measured using activated cephaloplastin including ellagic acid(Actin, Dade): 100μl of citrated-plasma and 100μl of Actin were mixed and preincubated for 5 min at 37℃, then to the mixture was added 100μl of prewarmed 0.025M $CaCl_2$ at 37℃. The diluted blood clot lysis(DBCL) was measured by slightly modification of Gallimore et al. (11): the weight of remaining clot after incubation for 2 hrs at 37℃ was estimated instead of measuring the clot lysis time. The number of leukocyte was counted by Büker-Türk style calculating well using the citreted blood diluted with Türk solution by melangeur for leukocyte. Concentrations of fibrin and fibrinogen degradation products(FDP) were measured by Staphylococcus(NewmanD_2C, Sigma) clumping test(12).

Extraction of proteinase from the spleen or leukocytes: Extraction of proteinase from tissue; a frozen tissue was thawed at room temperature, homogenized with 10 volumes of physiological saline, and centrifuged at 10,000xg for 30 min to remove the contamination of blood. These procedures were repeated. The precipitate obtained was weighed and extracted with 10 volumes of 2M potassium thiocyanate(KSCN). After extraction at 4℃ for 2 hrs the homogenate was centrifuged at 10,000xg for 30 min. The clear supernatant obtained was used for fibrinolytic assay using fibrin plates(3). Extraction of proteinase from leukocytes; leukocytes were separated from citrated blood by using 6% dextran and sodium metrizoate, and were collected by centrifugation(12). The leukocytes precipitated were suspended in distilled water in order to remove the remaining erythrocytes, and centrifuged for 30 min at 10,000xg. The precipitate of $2x10^7$ leukocytes obtained was suspended with lml of 2M $NaClO_4$, extracted for 2 hrs at 4℃, and centrifuged for 30 min at 10,000xg. The supernatant was used for fibrinolytic assay.

Assay for enzyme activity: Fibrinolytic activity was measured by employing plasminogen-free fibrin plate containing bovine fibrinogen(Povite, Amsterdam) as described previously(3), and the activity was express as inhibitory units calculating from a standard curve between casein unit(cu) of plasmin (AB Kabi, Stockholm) and lysis areas(mm^2) on plasminogen-free fibrin plates after incubation at 37℃ for 20 hrs. Amidolytic activity was calculated by measuring the releasing pNA from substrates at 405nm. Two types of specific substrates

were used; suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA for an elastase-like proteinase(13), and suc-L-Tyr-L-Leu-L-Phe-pNA for a chymotrypsin-like proteinase(14). These substrates were synthesized in our laboratories.

Inhibition studies: One hundred μl of KSCN extract from the spleen(at the 10th day after carrageenan injection) was incubated at 37℃ for 10 min with 100μl of various inhibitors; phenylmethylsulfonyl fluoride (PMSF, Sigma), diisopropyl fluorophosphate (DFP, Sigma), p-nitrophenyl p-guanidinobenzoate hydrochloride(NPGB, Sigma), p-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride(TLCK, Nacalai tesque, Kyoto) or p-toluene sulfonyl-L phenylalanine chloromethyl ketone(TPCK, Nacalai tesque) and 30ml of the mixture was dropped on plasminogen-free fibrin plate. While tranexamic acid(t-AMCHA, Dai-ichi seiyaku Co, Tokyo), aprotinin(Bayer yakuhin Ltd, Osaka), soybean trypsin inhibitor type IS(SBTI, Sigma), limabean trypsin inhibitor(LBTI, Cooper biomedical, Malvern) or chicken ovoinhibitor type Ⅲ-O(COI, Sigma) was dissolved in the fibrinogen solution before adding thrombin to make fibrin plate. Thirty μl of the extract was dropped on these fibrin plate, and the fibrinolysis with or without inhibitors were compared.

Results

Variations of blood parameters after carrageenan injection: Variations of leukocyte count and fibrinogen level after the carrageenan injection are shown in Fig. 1. The numbers of leukocyte were significantly decreased at the 12 hrs, but increased on the 6th to 15th day after the carrageenan injection. On the 3rd day after the carrageenan injection. The concentration of fibrinogen was obviously increased from the 12 hrs to 6th day after the carrageenan injection. On the 3rd day after the carrageenan injection, the concentration of fibrinogen came to three time against the control value(air-alone). Variations of PT and APTT after the carrageenan injection are illustrated in Fig. 2. No change of PT was observed at the all ranges. The carrageenan injection markedly prolonged APTT at the early stage from the 6 to 12hr. Fig. 3 shows the weight of remaining diluted blood clot(DBCL) after incubation for 2 hrs at 37℃. A peak of remaining clot was seen on the first day after the carrageenan injection, although no variation within 12 hrs. After the 3rd day, the remaining clot was steady closed to the control. Variation of FDP level after the carrageenan

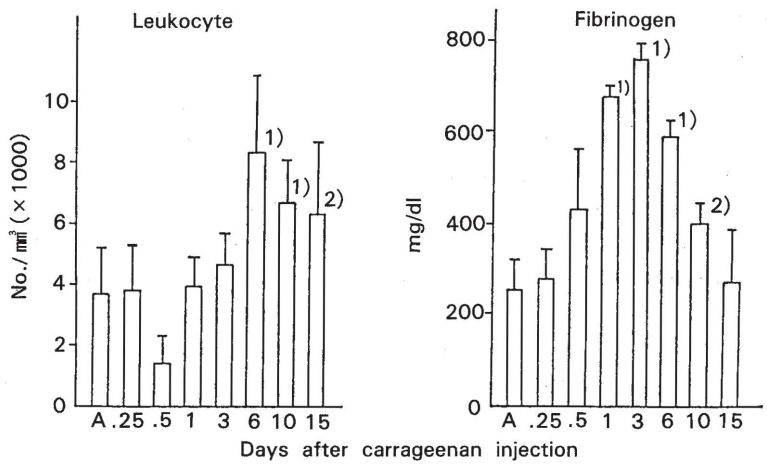

Fig. 1. Variations of leukocyte count(left) and plasma fibrinogen level(right) induced by the carrageenan injection. The results were expressed as mean±SD of 10 to 14 animals. Statistical significance were determined by the unpaired Student's t-test, 1):$p<0.001$, 2):$p<0.01$. These values were compared with control(A:air-alone).

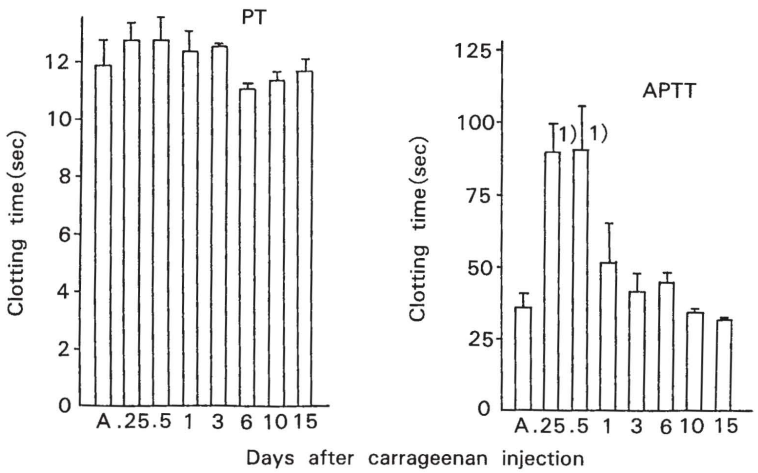

Fig. 2. Effects of the carrageenan injection on prothrombin time (PT, left) and activated partial thromboplastin time (APTT, right). The results were expressed as mean ± SD of 10 to 14 animals. Statistical significance were determined by the unpaired Student's t-test, 1): $p<0.001$. These values were compared with control (A: air-alone).

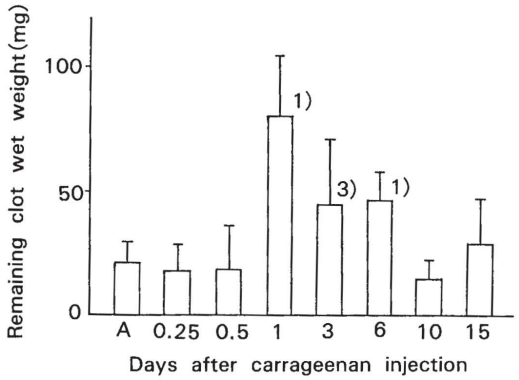

Fig. 3. Effect of the carrageenan injection on diluted blood clot lysis. The results were expressed as mean ± SD of 10 to 14 animals. Statistical significance were determined by the unpaired Student's t-test, 1): $p<0.001$, 3) : $p<0.02$. These values were compared with control (A : air-alone)

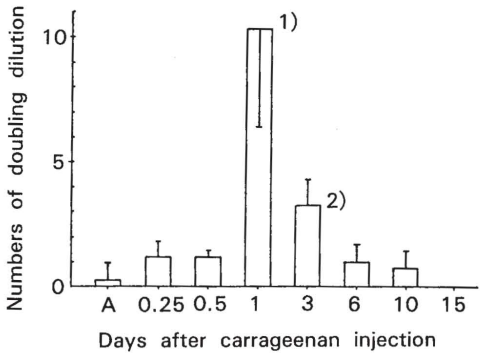

Fig. 4. Variation of FDP levels in serum after the carrageenan injection. The results were expressed as mean ± SD of 7 to 8 animals. Statistical significance were determined by the unpaired Student's t-test, 1) : $p<0.001$, 2) : $p<0.02$. These values were compared with control (A : air-alone).

injection is shown in Fig. 4. A sharp peak of FDP level was observed on the 1st day after carrageenan injection.

Variations of weight and fibrinolytic activity of the spleen after carrageenan injection: As shown in Fig. 5, the carrageenan injection increased in the weight or fibrinolytic activity of the spleen with a peak on the 10th day. No alteration was shown until the 3rd day after the carrageenan injection in either the weight or the activity. Plasminogen activator activity in the spleen extract was no significantly shagged in all ranges.

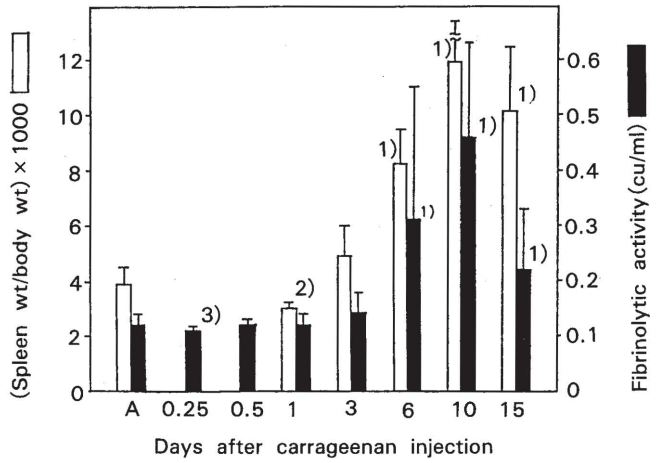

Fig. 5. Effects of the carrageenan injection on activity of spleen fibrinolytic proteinase and on weight of the spleen. The results were expressed as mean ± SD of 10 to 14 animals. Statistical significance were determined by the unpaired Student's t-test, 1) : $p<0.001$, 2) : $p<0.01$, 3) : $p<0.02$. These values were compared with control (A:air-alone).

Effects of various proteinase inhibitors on the spleen fibrinolytic activity: The extract with fibrinolytic activity was prepared from the spleen of carrageenan injecting mice as described before, and the effects of various proteinase inhibitors on the activity were examined. The results are illustrated in table 1. The activity was completely inhibited by DFP, PMSF, NPGB or SBTI, while was not by other inhibitors in the various concentrations shown in the table 1.

Comparisons of fibrinolytic and amidolytic activities between the spleen and leukocytes extracts: Fibrinolytic and amidolytic activities of the extracts of the spleen or leukocytes on the 10th day after the carrageenan injection are summarized in table 2. Suc-L-Tyr-L-Leu-L-Phe-pNA hydrolysis of leukocytes on the 10th day after the carrageenan injection was increased 11 times in comparison with the control(air-alone), but the spleen was only 3.5 times. Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA hydrolysis was not varied in extracts from the spleen induced by the carrageenan injection was increased about 2.3 times in comparison with the control, the activity of the spleen on the 10th day was furthermore strong of 16 times in comparison with the leukocytes.

Table 1. Effects of various proteinase inhibitors on the carrageenan-induced fibrinolytic proteinase in the spleen.

Compounds	Final Concentration	Remaining Activity(%)
DFP	10mM	0
PMSF	5mM	0
NPGB	10mM	0
TLCK	10mM	60.8
TPCK	10mM	0
t-AMCHA*	10mM	97.1
Aprotinin*	161KIE/ml	75.7
SBTI*	1mg/ml	1.8
LBTI*	1mg/ml	75.8
Ovoinhibitor*	1mg/ml	83.9
Antipain	1mM	82.6
Chymostatin	1mM	100
Leupeptin	1mM	61.4
Pepstatin A	1mM	73.2
Cysteine*	5mM	98.0
EDTA*	5mM	100

These values were a mean of 5 experiments of different spleen extracts.*:These inhibitors were directly added in fibrin plate.

Table 2. Comparison on amidolysis and fibrinolysis between the spleen and leukocyte.

	Amidolysis[1]				Fibrinolysis[2]	
	TLP[3]		ATLV[4]		Spleen[6]	Leukocyte[6]
	Spleen	Leukocyte	Spleen	Leukocyte		
Control n=31	1.26×10^{-3}[5] ± 1.37×10^{-3}	1.28×10^{-4} ± 2.86×10^{-4}	1.23×10^{-3} ± 7.07×10^{-4}	8.30×10^{-5} ± 1.86×10^{-4}	0.17 ± 0.27	0
Carrageenan (10th day) n=16	4.67×10^{-3}* ± 4.36×10^{-4}	1.41×10^{-3}* ± 8.70×10^{-4}	2.81×10^{-3}* ± 4.53×10^{-4}	9.60×10^{-5}** ± 1.12×10^{-4}	1.34* ± 0.63	0.079 ± 0.049

1) Amidolytic activity were expressed as the increasing of A_{405}/min.
2) Fibrinolytic activity were expressed as the activity(cu/ml) of plasmin.
3) TLP: suc-L-Tyr-L-Leu-L-Phe-pNA
4) ATLV: suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA
5) Mean±SD, statistical significance were determined by the unpaired Student's t-test, *: $p<0.001$, **: $p>0.2$. These values were compared with control(air-alone).
6) The spleen and leukocytes were extracted with 2M $NaClO_4$.

discussion

In the spleen of human, dog and rat, a fibrinolytic proteinase activity was detected(3). No activity in the spleen of germfree rat was at all, however, the activity in this rat was induced with elevation of fibrinogen by operation in aseptic condition(3,4). The spleen of mouse have weak fibrinolytic activity.

After carrageenan injection, fibrinogen level has already began increasing from the 12 hr and reached a maximum on the 1st day(Fig. 1-right). The result was similar to that of Koj(15) using rabbit turpentine inflammation. Even though leukocytes in blood(Fig. 1-left) was decreased at the 12 hr, the increasing of leukocyte was remarkably on the 1st, and a peak on the 6 day. The spleen fibrinolytic activity was obviously enhanced from the 3rd to 10th day(Fig. 5). These phenomena in the carrageenan induced-inflammation mice also appeared in both the injured germfree rats(3,4) and the sarcoma 180 inoculated mice(5), APTT were prolonged at the 6 and 12 hr after the carrageenan injection, but PT were not changed (Fig. 1-left&right). The contact system made progress within 12 hr after the carrageenan injection, but the concentrations of fibrinogen and prothrombin were not effected. These results were due to by the activation of Hageman-factor(16) by the carrageenan. DBCL were declined from the 1st to 6th day after the carrageenan injection(Fig. 3). The fibrinolytic system in plasma were apparently suppressed, because the increasing fibrinogen as substrate in the system induced the prolongation of the DBCL value. However, in spite of a peak of the fibrinogen level on the 3rd day, DBCL value which had a peak on the 1st day already decayed on the 3rd day. The facts show that the DBCL value does not depend on each fibrinogen level alone. FDP level in serum was significantly increased from the 1st to 3rd days after carrageenan injection(Fig. 4). The increasing pattern of the spleen weight was remarkably similar to that of the spleen fibrinolytic activity(Fig. 5). These rela-

tions indicate that the enhancement of the fibrinolytic activity in the spleen extract is depended on the inducing fibrinolytic activity in some spleen cell(macrophage), but is not on the increasing cells. According to Antoni et al. (17) the carrageenan treatment induced the increase of the spleen weight and the enhancement of [^{3}H] thymidine incorporation into the spleen cells. Imai et al. (18) showed that a fibrinolytic activity was detected in the spleen macrophage cells by Todd's method. The proteinase activity induced in the spleen specifically hydrolyzed plasminogen-free fibrin plate. The fibrinolytic proteinase is not plasmin judging from non-inhibition by t-AMCHA and aprotinin. The enzyme was inhibited by DFP, PMSF and NPGB indicating that the enzyme is a serine proteinase. TPCK completely inhibits the activity, but not TLCK. This results indicate that the proteinase is a chymotrypsin-like proteinase. These results indicate that the fibrinolytic proteinase is similar to a elastase or a cathepsin G(19, 20, 21). The enzyme activities of the spleen and leukocytes extracts with 2M $NaClO_4$ were compared with fibrin and synthetic substrates (Table 2). The fibrinolytic activity of the spleen extract was significantly enhanced with the carrageenan injection, but that of leukocyte was not. As Vassali et al. (22) pointed out, the weak fibrinolytic activity was detected in the leukocyte extract of the mice. On the other hand, in the hydrolytic activity for synthetic substrates, TLP(for cathepsin G) hydrolytic activity was enhanced only 3.5 times in the spleen extract and 11 times in the leukocyte by the carrageenan injection. ATLV(for elastase) hydrolytic activity was enhanced 2.5 times in the spleen, but only 1.1 times in the leukocyte. These results shows that a spleen fibrinolytic proteinase in the carrageenan-induced mouse is neither cathepsin G nor leukocyte elastase.

Our results show that the fibrinolytic activity in the spleen of mouse was significantly enhanced by the carrageenan injection. The fibrinolytic proteinase was a kind of serine proteinase and a chymotrypsin-like proteinase, but not plasmin, cathepsin G or leukocyte elastase. These results suggest that the proteinase in the spleen may play a possible fibrinolytic role in the organ. The proteinase must be inquired further for the physicochemical and pathophysiological properties in future.

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