［総説］The Relationship between Physicochemical Properties of Bound Thrombin and Action Mode of Antithrombin Agent on Bound Thrombin

The Relationship between Physicochemical Properties of Bound Thrombin and Action Mode of Antithrombin Agent on Bound Thrombin

Mariko Nakamura[1], Masanori Sunagawa[1], Miwa Yoshioka[1], Hiroatsu Tengan[2]
Shigeru Takara[2], Kazunao Nakamura[1], Yasutaka Kimura[1], Makoto Motomura[1]
Minao Tamaki[3], Ken Uehara[3], Kiyohiko Kinjoh[4] and Tadayoshi Kosugi[1]

[1] *1st Department of Physiology, Unit of Physiological Science, School of Medicine, University of the Ryukyus*
[2] *Department of Physical Therapy, Okinawa College of Rehabilitation*
[3] *Department of Otorhinolaryngology-Head and Neck Surgery, School of Medicine, University of the Ryukyus*
[4] *Anesthesiology Research Laboratory, University of California, San Diego, La Jolla, USA*

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ABSTRACT

Procoagulant activity was known to increase after thrombolytic therapy. It was thought that the enhancement of procoagulant activity was dependent upon the new generation and development of novel kinds of thrombin after thrombolytic therapy. Such thrombin was also produced by percutaneous transluminal coronary angioplasty (PTCA) and percutaneous transluminal coronary reperfusion (PTCR), resulting in unexpected rethrombosis and vascular restenosis. That is, thrombin appeared again in the circulating blood after thrombolysis or mechanical destruction of the previously formed thrombus. Such the newly generated thrombin was termed postclotting thrombin (bound thrombin). The postclotting thrombins were divided into three classes: native, intact, and bound thrombin. It was emphasized in this review that the bound thrombin generated during the formation of fibrin or thrombus has the clotting activity like a native thrombin. The reliable control of the bound thrombin using synthetic antithrombin agents led the clinical doctors to reasonable haemostasis, tissue repair and regeneration, and prevention from atherosclerotic change of blood vessel. *Ryukyu Med. J., 25(3,4) 85～93, 2006*

Key words: bound thrombin, thrombolytic therapy, coronary intervention, anticoagulant agents, structure and function relationship

INTRODUCTION

It has been notionally expected that a procoagulant activity be increased after thrombolytic therapy due to a negative feedback mechanism to maintain physiological haemostasis. However, the mechanism for the development of the increased procoagulant activity after thrombolytic therapy has not been thoroughly investigated by substantial and experimental data. Effective thrombolytic therapies for thrombotic diseases depend upon the inhibition of increased procoagulant activity after thrombolytic therapy. It was thought that the enhancement of procoagulant activity was dependent upon the generation of novel kinds of thrombins after thrombolytic therapy. In addition, the generation of local thrombin after thrombolytic therapy by intravenous administration, percutaneous transluminal coronary angioplasty (PTCA), and percutaneous transluminal coronary reperfusion (PTCR) increased the activity of coagulation, the development of rethrombosis, and vascular restenosis[1,2]. If the newly generated thrombins have produced those pathological features, this indicates that thrombins in the preceding thrombus are released again in circulating blood following thrombolysis and mechanical destruction, and might form the thrombus and fibrin clot due to the remaining coagulant activity and multiple biological functions. The newly generated (or released) thrombins were termed post-

clotting thrombin. The post-clotting thrombin is the residual thrombin in clot, which is incorporated into fibrin clot by binding to fibrinogen during the formation of fibrin clot. In addition, it is known that post-clotting thrombin retains its clotting activity. We classified post-clotting thrombins into " intact thrombin" and "bound thrombin" based on the concept we have proposed. Intact thrombin refers to thrombin that transiently binds to fibrinogen but not cleave fibrinogen into fibrin. Bound thrombin refers to thrombin, which binds to fibrinogen to convert into fibrin and is liberated from clot by mechanical crush and by clot-lysate. We expected that the structure of the bound thrombin was different from that of native (wild) thrombin and that the bound thrombin existed in both circulating blood and impaired tissue. In addition, we paid a special attention on investigating whether the bound thrombin would be one of the possible notorious factors to develop the restenosis of blood vessel.

The establishment of an effective anticoagulant therapy to inhibit the activity of bound thrombin would be a successful strategy for the prevention from restenosis and rethrombosis after thrombolytic therapy, PTCA, and PTCR. To select and discover an available antithrombin agent for the bound thrombin, we must clarify the characteristics of its structure and behavior to some antithrombin agents. In this review paper, we summarized the data from literatures and the experimental results of our laboratory in relation to the structure of bound thrombin and inhibitory efficacy by some antithrombin agents.

THROMBOLYTIC THERAPY OF MYOCARDIAL INFARCTION (MI)

Dietrich et al investigated the relationship between the change of the haemostatic data and the prognosis based on the outcomes of the treatment for MI[3]. The coronary angioplasty was procedured to 55 patients with MI at 90 min after the administration of pro-urokinase or tissue type-plasminogen activator (t-PA), and the thrombolytic therapy continued for 24 to 36 hours. They reported that the patients with the increased level of thrombin-antithrombin III (TAT) had the poor prognosis[3]. They concluded that in order to succeed in a thrombolytic therapy for MI, it is necessary to

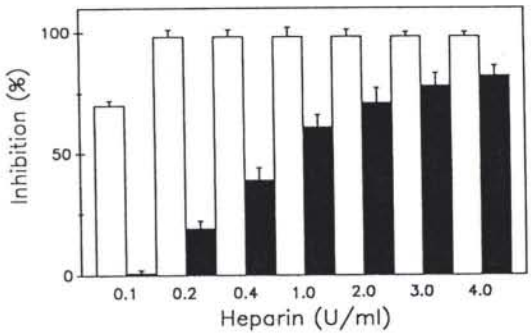

Fig. 1 Comparison of the inhibitory effect of heparin against fluid-phase (open bars) and clot-bound thrombin activity (*solid bars*).
Each bar represents the mean of three separate experiments (each done in duplicate), while the lines above the bars represent the SD. This figure was cited from reference 4.

decrease TAT level. In addition, it was determined that the dosage of thrombolytic agents and inhibition of newly generated thrombin (bound thrombin) were two keys to success in the treatment of MI. On the other hand, it has been reported that the rethrombosis of coronary artery and venous thrombosis have been frequently observed. Unexpectedly, the development of such a thrombosis could not be prevented and inhibited by the customary antithrombin agent, heparin. Consequently, the need of novel treatments different from the usual one became pressing for the control of the increased activity of coagulation after PTCA, PTCR and thrombolytic therapy. The efficacy of anticoagulant drug for the activity of thrombin under liquid condition is different from that under solid state. It has been already reported that heparin did not have a high effectiveness for the activity of clot-bound thrombin (the thrombin under solid condition) (Fig. 1)[4]. The clot-bound thrombin, however, was susceptible to inactivation by antithrombin III independent inhibitors[4]. On the other hand, there were other results inconsistent with the above-mentioned results. The administration of heparin, immediately after the infusion of recombinant tissue-type plasminogen activator (rt-PA) for coronary thrombolysis, decreased the level of prothrombin fragment 1, 2 in circulating blood, whereas the non-administration of heparin did not[5]. Therefore, heparin was available to inhibit the development of rethrombosis. The amount of prothrombin fragment 1, 2 in circulating blood

reflects the generation of α-thrombin. Prothrombin fragment 1, 2 does not contain protease activity, which is produced when prothrombinase complex (Xa, Va, Ca^{2+}, and phospholipids) activates prothrombin by cleaving the bond between Arg^{271} and Thr^{272}. Although the activity of the thrombin was reduced by heparin, prothrombin fragment 1, 2 (generation of α-thrombin) was not decreased, indicating that heparin could not prevent the generation of α-thrombin[6]. In addition, it has been known that streptokinase, one of thrombolytic agents, activate directly or indirectly the prothrombinase complex in the manner of plasminogen-independent mechanism, resulting in the generation of α-thrombin[7].

ACTION MODE OF ANTICOAGULANT AGENTS ON THE POSTCLOTTING THROMBIN

Postclotting thrombins were divided into 3 groups on the basis of the results from *in vitro* experiments: native thrombin, intact thrombin and clot-bound thrombin (bound thrombin)[8,9]. The insusceptibility of bound thrombin to inhibition by heparin is dependent upon the following mechanisms[10]. The inhibition of the interaction of thrombin with fibrinogen or fibrin resulted from the occupation or the conformational change of the heparin-binding site of thrombin by heparin. Heparin, however, could not occupy or induce conformational change of the heparin-binding site, thereby inhibiting insufficiently the activity of bound thrombin. On the other hand, it was thought that the binding of fibrinogen to thrombin did not structurally change the binding site of antithrombin III-independent inhibitors of thrombin. Actually, it has not been determined what kinds of anticoagulant drugs are most useful for the inhibition of the activity of thrombolytic therapy-induced bound thrombin. From the above-mentioned background, it was stressed that bound thrombin-induced rethrombosis and restenosis could be prevented by the sufficient dose of thrombolytic agent, based on the assumption that the thrombolytic agents could digest the bound thrombin into fragments with the low molecular weight without the clotting activity. As a consequence, it would be a therapeutic strategy for inhibition of bound thrombin activity by proteolysis of α-thrombin by urokinase[11]. The mechanism by which heparin could not inhibit the activity of bound thrombin has been analysed using the heparin/serpine complex from the aspect of molecular interaction between heparin with thrombin[12]. It was clarified that heparin coexisting with antithrombin III could inhibit the activity of the native (wild) thrombin, but not inhibit the activity of bound thrombin. This is probably because they cannot access the active site of bound thrombin. On the other hand, it was shown that the heparinoid and heparin analogue interacting with heparin cofactor II could inhibit the activity of bound thrombin. Furthermore, it was evidently demonstrated that the synthetic antithrombin agent with a low molecular weight, argatroban, could inhibit the activity of bound thrombin[9,10,13].

THE ROLE AND SIGNIFICANCE OF THE BOUND THROMBIN IN COAGULATION SYSTEM AND PLATELET FUNCTION

To study the bound thrombin-induced activation of coagulation factor, two kinds of fibrin clots, that is, the clot induced by thrombin-like enzyme derived from the snake venom and clot induced by native (wild) thrombin were formed under liquid condition. It was elucidated that the bound thrombin could activate Factor V, VIII and platelet as well as native (wild) thrombin. In addition, it was shown that the fibrin alone could enhance the procoagulant action of platelet[14]. The quantity of the generated bound thrombin and native thrombin was determined and influenced by which coagulation pathway was stimulated. In physiological environment, the pathways for the thrombin generation are classified into two routes. Accordingly, the differences in the structure of fibrin clot and susceptibility of fibrin clot to fibrinolysis are determined by which pathway for thrombin generation was stimulated. The fibrin clot formed by the thrombin that was generated via pathway through the activation of contact phase and factor XIa was highly resistant to fibrinolysis induced by the fibrinolytic agents. It has been reported that the development of the therapy-resistant haemostatic plug related to the characteristics of thrombin in the preceding fibrin clot and that the thrombin activation pathway modulated the susceptibility to lysis of human plasma clots[15]. Fibrin clot-associated thrombin that was prepared from the clot by incubating human fibrinogen with thrombin, could aggregate the

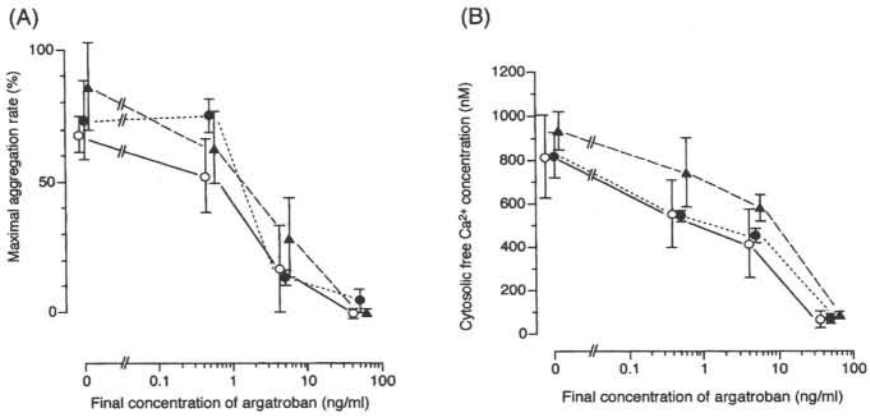

Fig. 2 Effects of argatroban on platelet aggregation (A) and cytosolic Ca^{2+} concentration (B) induced by native (●), intact (▲) or bound (○) thrombin. Results are expressed as the mean ± S.E. of 3 experiments. This figure was cited from reference 8.

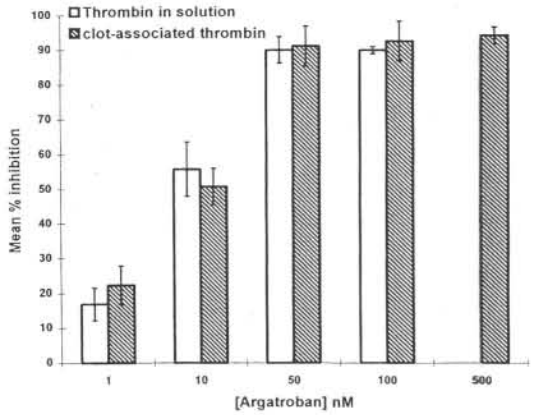

Fig. 3 Effects of argatroban on platelet aggregation induced by thrombin in solution (open bars) and plasma clots (hatched bars).
The results are expressed as the mean (± s.e.m.) percent inhibition from 5-7 determinations, where percent inhibition was calculated using the decrease in single platelets 3 min after the addition of washed platelets to the two thrombin preparations in each separate experiment. This figure was cited from reference 18.

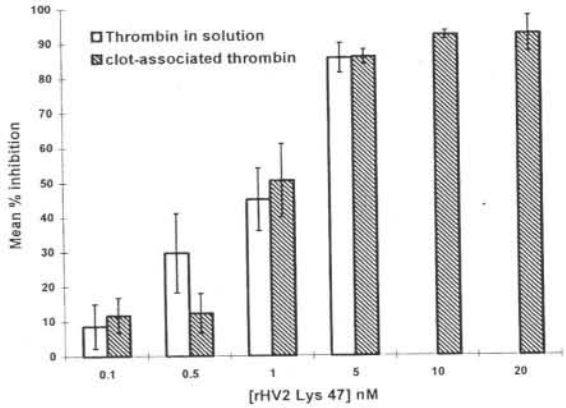

Fig. 4 Effects of rHV2Lys47 on platelet aggregation induced by thrombin in solution (open bars) and plasma clots (hatched bars).
The results are expressed as the mean (± s.e.m.) percent inhibition from 4-6 determinations, where percent inhibition was calculated using the decrease in single platelets 3 min after the addition of washed platelets to the two thrombin preparations in each separate experiment. This figure was cited from reference 18.

washed platelet of rabbits. In addition, argatroban, specific thrombin inhibitor, was more available for the inhibition of thrombus formation induced by platelet rich plasma, as compared with the inhibition by heparin[16]. Furthermore, argatroban inhibited the platelet aggregation of the washed platelet of rabbits induced by the bound thrombin that was prepared from the clot by incubating rabbit fibrinogen with bovine thrombin (Fig. 2)[8].

THE INTERACTION OF THROMBIN WITH FIBRINOGEN OR FIBRIN DERIVATIVES

Thrombin could bind to the soluble fibrin degradation product (FDP). The (DD)E and E fragment of FDP could bind to thrombin and the E fragment also had a thrombin-binding site[17]. Clot-associated thrombin derived from plasma of rabbit could bind to FDP and activate the platelet of rabbits. Additionally, thrombin inhibitors such as argatroban or rHV2Lys47 could inhibit the activation of platelet induced by clot-associated thrombin (Figs. 3 ,4)[18]. Bovine thrombin could bind to the F8Y peptide (Phe^8 replacing Tyr), corresponding N-terminal residues 1-23 of the fibrinogen Aα-peptide. When Phe^8 of Aα in fibrinogen is substituted with Tyr, the regular cleavage of the peptide bond at Arg^{16} in Aα induced by thrombin was

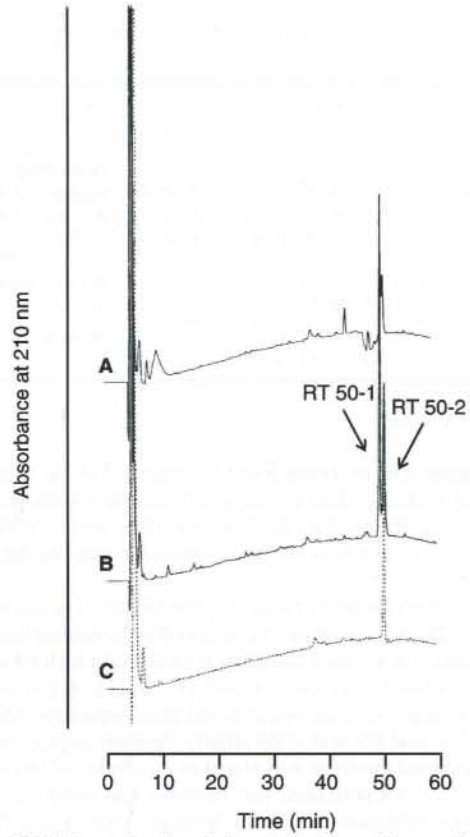

Fig. 5 HPLC analysis of bovine thrombin and material isolated from clot lysate denatured with 4 M urea. Isolated material on the affinity chromatography was dissolved in 200 μl of denaturing solution containing 4 M urea, and 100 μl of this solution was subjected to HPLC analysis (B). Crude lysate of rabbit fibrinogen (A) and bovine thrombin (native thrombin) (C) were also subjected to HPLC analysis. The eluates of two peaks of isolated material (peaks RT 50-1 and RT 50-2) were subjected to N-terminal sequence analysis. This figure was cited from reference 22.

strongly inhibited. From this result, it was shown that

Phe[8] in Aα chain of fibrinogen was essential for the interaction of thrombin with fibrinogen[19]. In addition, it was demonstrated by the analysis of amino acids at positions from 1 to 16 in Aα chain of fibrinogen that fibrinopeptide A (FPA) exhibits a strand-turn-strand motif, with a β-turn centered at residues Gly[12] and Gly[11]. The rotation of the structure in FPA was important and specific for the binding to thrombin[20]. Alternatively, its rotation was very important for the development of substrate specificity. The stepwise process of fibrin formation by the interaction of thrombin with fibrinogen must be kept in mind to understand the binding mode of thrombin to fibrinogen or fibrin derivatives. At the first step, the clot was composed of fibrin I (Fn-I) and fibrinogen. Next, γ - γ cross linking of fibrinogen developed before the formation of Fn-II, and Bβ chain in Fn-II at this time did not react to another chain of fibrinogen thereby remaining to be intact[21]. Alternatively, although Aα chain and γ chain of fibrinogen did respectively react with those of the other fibrinogen in the initial phase of the interaction of thrombin with fibrinogen Bβ chain did not react at all. It was suggested that after nearly simultaneous completion of the interaction of Aα with γ chain in fibrinogen, Bβ-chain initiated to react with any of it of another fibrinogen. In addition, it was elucidated from the results of structural analysis of bound thrombin that the derivatives of fibrinogen bound to thrombin were dif-

Table 1 N-Terminal sequence analysis of the fibrin fragments and thrombin recovered from the bound thrombin in clot-lysis lysate

Samples	Peak	Amino acids										Reference sequence	
		1	2	3	4	5	6	7	8	9	10*		
												rabbit fibrin	
Bound thrombin	RT 50-1	G	P	R	V	V	D	K	P	P	x	α-chain	GPRVVDKPPS-
from clot-lysis		G	H	R	P	I	D	x	K	x	x	β-chain	GHRPIDRKRE-
lysate		Y	V	A	T	R	E	N	x	x	x	γ-chain	YVATRENxxx-
	RT 50-2	T	F	G	A	G						bovine α-thrombin	
		I	V	E	G	x						A-chain	TFGAGEA-
Native thrombin		T	F	G	A	G						B-chain	IVEGNDA-
		I	V	E	G	N							

x: not determined.
*: cycle of Edman degradation.

RT 50-1, RT 50-2 in this table were indicated in Fig. 5.
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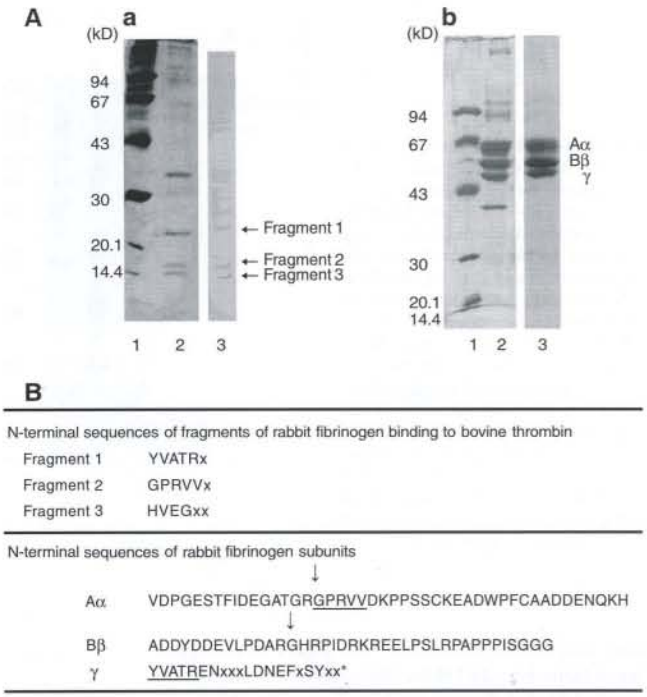

Fig. 6 N-terminal sequence analysis of the thrombin fragment incorporated in the bound thrombin and the rabbit fibrinogen.
A, a) SDS-PAGE and immunoblotting analysis of the reduced bound thrombin. Lane 1: low-molecular marker proteins; lane 2: bound thrombin; lane 3: bound thrombin blotted onto a PVDF membrane, which was immunologically stained with antirabbit fibrinogen antibody. The three bands (indicated by arrows) were subjected to N-terminal sequence analysis.
b) SDS-PAGE and electroblotting of reduced rabbit fibrinogen. Lane 1: low-molecular marker proteins; lane 2: reduced rabbit fibrinogen; lane 3: reduced rabbit fibrinogen blotted onto a PVDF membrane. Each band of three subunits was subjected to N-terminal sequence analysis. B N-terminal sequence analysis of the fragments of rabbit fibrinogen binding to bovine thrombin (upper) and rabbit fibrinogen subunits (lower). ×denotes undetermined residues. Arrows on the sequence of fibrinogen subunits (Aα and Bβ) indicate the cleavage site by thrombin. This figure was cited from reference 23.

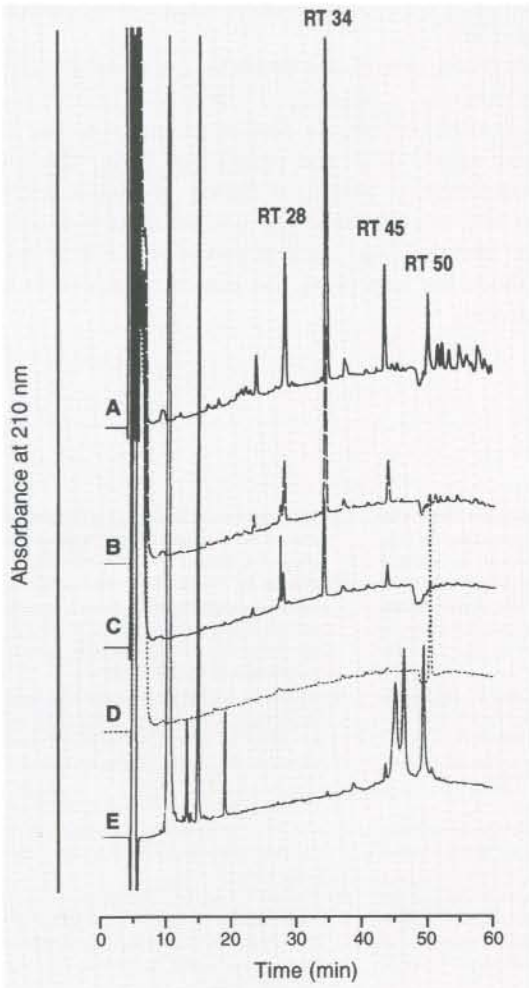

Fig. 7 HPLC pattern of native thrombin and denatured bound thrombin.
The purified bound thrombin (about 5 μg) was dissolved in 200 μl of 0.4% SDS, 10% acetonitrile, 0.1% TFA containing 8 M urea (A), 6 M urea (B), 4 M urea (C), then injected to C4 reverse-phase HPLC. As a control, native bovine thrombin (D) and S-pyridylethylated rabbit fibrinogen (E) were analyzed. The eluates of each peak (peak RT 28, RT 34, RT 45, and RT 50) of the bound thrombin and native thrombin (peak RT 50) were subjected to N-terminal sequence analysis. This figure was cited from reference 23.

ferent from the subunits (Aα, Bβ, γ chain) of fibrinogen (Figs. 5 ,6 and Tables 1 ,2)[22,23]. Reverse-phase HPLC demonstrated that bound thrombin isolated from clot lysate was a complex of α-thrombin (HPLC peak of RT50-1) with a fibrin fragments (HPLC peak of RT50-2) corresponding N-terminal regions of fibrinogen α-, β-, and γ-chains (Fig. 5 and Table 1), indicating thrombin molecule would bind to fibrin fragment consisting of N-terminal central domain. In addition, bound thrombin liberated from crushed clots is a stable complex between α-thrombin (HPLC peak of RT50) and fibrin fragments (Fragments 1 , 2 , and 3) (HPLC peaks of RT28, RT34, or RT45) of N-terminal region of fibrinogen α- and γ-chains (Fig. 6 and Table 2).

Three dimensional structural analysis was an important and useful method to show the structure of the portion being originated from the fibrinogen in bound thrombin. The recent advanced analysis

Table 2 N-terminal sequence analysis of the fibrin fragments and thrombin recovered from bound thrombin

Samples	Peak	Amino acids					Reference sequence
		1	2	3	4	5[1]	
Bound thrombin	RT 28	Y G H	V	×	×	×	fibrinogen fragments (from fig. 6B) fragment 1 Y-V-A-T-R- fragment 2 G-P-R-V-V- fragment 3 H-V-E-G-
	RT 34	Y G H	V P	A R E	×	×	
	RT 45	Y G	×	×	×	×	
	RT 50	T I	F V	G E	G	×	bovine α-thrombin [18] A-chain T-F-G-A-G-E-A- B-chain I-V-E-G-N-D-A-
Native thrombin	RT 50	T I	F V	G E	A G	G N	

× = Not determined.
[1] Cycle of Edman degradation.

RT 28, RT 34, RT 45, RT 50 in this table were indicated in Fig. 7.
This table was cited from reference 23.

using X-ray crystallography showed the existence of core fragment in the structure of fibrinogen. The fragment D or double D in fibrinogen with dysfibrinogenemia showed the different structure from that in the intact fibrinogen[24]. Thus, it was clarified that core fragment in three dimensional structure of fibrinogen or fibrin existed in the D-domain. In addition, Mosesson et al, showed that C-terminal of γ chain in fibrinogen existed in the D domain of fibrinogen and fibrin[25]. This suggested that the binding site of fibrinogen to thrombin in the bound thrombin was situated at near the C-terminal region of γ chain of fibrinogen. From the analysis of physicochemical properties of the bound thrombin, we could know the origin of the bound thrombin. That is, whether the bound thrombin contained fibrinogen or fibrin derivatives determined the origin of the bound thrombin from the different kinds of clots. It has been reported that the monoclonal antibody against the epitope of fibrinogen Aα chain 529-539, could inhibit the cross linking of Aα chain induced by transglutaminase[26]. Since Aα 529-539 peptide existed in fibrinogen derivatives of bound thrombin, the bound thrombin was evidently derived from the clot, in which the Aα cross linking had already completed. In addition, the monoclonal antibody against hydrophobic 12 residues (Aα 487-498) of Aα chain located in αC domain was produced. From the investigation using this monoclonal antibody, Aα 487-498 peptide existed in the circulating blood with the resultant fibrinogenolysis[27]. Since Aα 487-498 of fibrinogen derivaties in the structure of bound thrombin could be detected, the bound thrombin was assumed to be originated from the lysed clots by fibrinolysis.

It has been shown that bound thrombin could activate the platelet, and α-thrombin in the bound thrombin played an important role in the activation of platelet. However, it could be expected that not only α-thrombin but also fibrinogen or fibrin derivatives could activate the platelet. It was proposed that Ala-Gly-Asp-Val in γ chain and Arg-Gly-Asp-Ser in Aα chain of fibrinogen were the principal peptides in the interaction between the platelet with fibrinogen-coated beads[28]. Therefore, it was expected that the peptides derived from Aα and γ chain in fibrinogen derivatives composed of the bound thrombin could bind to the platelet membrane thereby activating the platelets. It has been known that the fibrinogen was polymerized via cross-linking of γ chain and this polymerization was involved in the interaction with D: E fragment. The D: E fragment increased susceptibility of fibrin to thrombolysis, because the interaction with the D: E fragment developed polymerization and exposed the specific epitope to fibrin[29]. Consequently, the detection of D:

E fragment in the fibrinogen derivatives of bound thrombin suggested that the bound thrombin was originated from fibrinolysis-susceptible clot and was released from the clot digested by fibrinolysis.

SUMMARY

The postclotting thrombins produced by *in vitro* experiments were divided into three classes, that is: native thrombin, intact thrombin and bound thrombin. It was thought that the bound thrombins from mechanically crushed clots and from lysed clots existed in the circulating blood. It seems reasonable to emphasize that bound thrombins were classified on the basis of some different chemical structures but not of some different developments. Especially, the amino acid sequence analysis of fibrinogen and fibrin derivatives in bound thrombin was essential for the informative classification of the bound thrombin. Until the present time, unless otherwise noted, it has been generally accepted by clinical doctors that the physiological and pathophysiological roles of thrombin were restricted to the native thrombin, but not to the bound thrombin. We would like to emphasize in this review that the bound thrombin, which had so significant activity as the native thrombin, was increased after fibrin and thrombus was formed. Finally, it should be stressed that the reliable control of the bound thrombin using the synthetic antithrombin agent lead the clinical doctors to adequately control reasonable haemostasis, tissue repair, regeneration and prevention from atherosclerotic change of blood vessel[30,31,32].

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