Bound Thrombin from Crushed Clots Is Composed of α -Thrombin and the N-Terminal Regions of α - and γ -Chains of Fibrinogen

Kiyohiko Kinjoh^a Mariko Nakamura^a Zeng Gang^a Masanori Sunagawa^a
Yukinori Eguchi^b Tadayoshi Kosugi^a

^a1st Department of Physiology and ^bThe Research Laboratory Center, School of Medicine, University of the Ryukyus, Okinawa, Japan

Key Words

Bound thrombin · Mechanical operation · Bovine thrombin · Rabbit fibrinogen

Abstract

We aimed at clarifying the structural characteristics of the bound thrombin that is liberated by mechanical breakdown of fibrin clots. Fibrin clots were prepared with bovine thrombin and rabbit fibrinogen, and were crushed mechanically with a glass rod. The supernatant of the crushed clots was subjected to immunoaffinity chromatography to isolate the bound thrombin. Western blotting analysis revealed that the bound thrombin could be reacted with both antithrombin and antifibrinogen under unreduced conditions. SDS-PAGE under reduced conditions revealed that there were three bands, two of which were found to be the N-terminal fragments of the α - and γ -chains of fibrinogen. The bound thrombin could be dissociated into three distinct fibrin fragments and bovine α -thrombin when denatured by 8 M urea. Thus, the bound thrombin liberated from crushed clots is a stable complex between bovine α -thrombin and fibrin fragments of the N-terminal regions of rabbit α - and γ -chains.

Copyright © 2002 S. Karger AG, Basel

Introduction

In the clinical field, it has been reported that rethrombosis and reocclusion often occur following successful balloon angioplasty [1, 2]. In patients developing rethrombosis following coronary interventions, the levels of fibrinopeptide A (FPA), thrombin-antithrombin III complex and prothrombin fragment 1+2 were significantly increased [3, 4]. Bound thrombin might be generated by mechanical operations, and the liberated bound thrombin might be pathophysiologically involved in rethrombosis and reocclusion. We postulated that the activation of blood coagulation and proliferation of vascular smooth muscle cells may be initiated by the bound thrombin generated by the breakdown of blood clots. We then attempted to investigate the biological activity of bound thrombin in animal experiments.

Experiments with rabbits have contributed to clarify the pathological processes of vascular restenosis induced by blood coagulation and/or intimal hyperplasia after transluminal angioplasty [5–7]. Hatton et al. [8] demonstrated that thrombin converted fibrinogen to fibrin, and that fibrin-bound thrombin was deposited on the exposed subendothelium after balloon-catheter injury. Speidel et

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2002 S. Karger AG, Basel
1424–8832/02/0324–0165\$18.50/0

Accessible online at:
www.karger.com/pht

Kiyohiko Kinjoh
1st Department of Physiology, School of Medicine
University of the Ryukyus, 207 Uehara, Nishihara
Okinawa 903-0215 (Japan)
Tel. +81 98 895 1108, Fax +81 98 895 1402, E-Mail physiol1@med.u-ryukyu.ac.jp

al. [9] demonstrated that thrombin binding to the injured aortic wall retained its FPA-releasing activity after balloon hyperinflation. In our previous study, we found that bound thrombin in the supernatant of crushed clots induced platelet aggregation with increased cytosolic Ca^{2+} levels, which was not significantly different from that induced by native thrombin [10]. We also demonstrated that argatroban inhibited the activation of platelets induced by bound thrombin and that the inhibitory effect of argatroban on bound thrombin was equivalent to that on native thrombin [10]. Thus, the possibility of the generation of bound thrombin and its pathological contribution to rethrombosis and reocclusion has become apparent in previous experiments.

However, the physicochemical features of the bound thrombin liberated by mechanical operations have not been specified. We thus considered that a basic *in vitro* study on bound thrombin generated using purified thrombin and fibrinogen was needed in order to clarify the mechanism of its biological action and pathophysiological role. In the present study, therefore, we attempted to isolate the bound thrombin liberated *in vitro* from fibrin clots by mechanical crushing to clarify the structural characteristics of the bound thrombin.

Material and Methods

Rabbits

For purification of fibrinogen, blood was drawn from anesthetized rabbits (Japanese white, 2.5–3.0 kg) which were purchased from Kyudo Co. (Kumamoto, Japan). Animal care and management was in compliance with the 'Standard Relating to the Care and Management of Experimental Animals' (Notification No. 6, March 27, 1980, from the Prime Minister's Office, Tokyo, Japan) and 'the Guide for Animal Experiments of the University of the Ryukyus for the care and use of the animals'. The animal experiment in this study was approved by the Committee on Animal Experiments of the University of the Ryukyus.

Reagents

Rabbit fibrinogen was purified from citrated rabbit plasma by the technique of Doolittle et al. [11]. Bovine thrombin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). A mouse monoclonal antibody against bovine thrombin (MAb_{CC2}) was purified in a protein A-Sepharose CL-4B column, as described previously [12]. Rat polyclonal antibodies against rabbit fibrinogen and bovine thrombin were purified using a protein G column followed by hydroxyapatite column chromatography [13, 14]. Protein A-Sepharose CL-4B, HiTrap Protein G, CNBr-activated Sepharose 4B, and LMW/HMW electrophoresis calibration kits were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Hydroxyapatite Bio-Gel HT, urea (electrophoresis purity), peroxidase (POD)-conjugated anti-rat IgG, and polyvinylidene difluoride (PVDF)

membranes (Trans-Blot®) were purchased from Bio-Rad Laboratories (Richmond, Calif., USA). 4-Chloro-1-naphthol was purchased from E. Merck AG (Darmstadt, FRG). SDS, Silver Stain II Kit Wako, trifluoroacetic acid (TFA), acetonitrile, and reagents for protein sequencing were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Unless otherwise stated, the other reagents were of analytical grade.

Preparation of Clot

200 μl of rabbit fibrinogen (1.5 mg/ml) was mixed with 200 μl of bovine thrombin (16 units/ml) and incubated at 37°C for 5 min. 400 μl of cold ovalbumin solution (1 mg/ml in PBS) was added to fibrin clots, followed by centrifugation at 3,000 rpm for 15 min at 4°C. After removal of the supernatant, the clots were placed in ice. 400 μl of cold ovalbumin solution was added to the residual fibrin clots. The clots were manually crushed using a glass rod with about 120 strokes/min for 5 min at which time the clot had the appearance of white grains. This solution was centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was collected and subjected to affinity chromatography.

Preparation of Immunoaffinity Columns

Immunoaffinity columns were prepared by immobilizing monoclonal antibody against bovine thrombin and rat antirabbit fibrinogen antibody onto CNBr-activated Sepharose 4B, respectively [15]. In brief, 5 mg of purified antibodies were added to 2.5 ml of CNBr-activated Sepharose 4B in 0.1 M NaHCO₃, 0.5 M NaCl and the mixture was gently agitated at room temperature for 3 h. After the conjugate reaction, free active groups on gels were blocked with 1 M monoethanolamine (pH 8.0) at 4°C overnight. After blocking, the conjugated gel was washed with 0.1 M sodium acetate and 0.5 M NaCl at pH 4.0 or 0.1 M NaHCO₃ and 0.05 M NaCl, alternately. The antibody-immobilized gels were packed into columns (0.9 $\times$ 5 cm). The columns were then equilibrated with Dulbecco's PBS (D-PBS).

Affinity Chromatography

Pooled supernatant of crushed clots (20 ml) was passed through the antbovine thrombin-immobilized column at a flow rate of 10 ml/h. The absorbed proteins were eluted with 0.36 M NaCl in D-PBS. Fractions containing proteins were pooled and dialyzed against D-PBS. The dialyzed sample (10 ml) was then passed through the antirabbit fibrinogen-immobilized column at a flow rate of 10 ml/h. After washing with 0.1 M phosphate at pH 7.0, the absorbed proteins were eluted with a linear gradient elution to a limiting buffer: 0.1 M sodium glutamate-phosphate and 0.5 M NaCl at pH 2.2. The eluted fractions containing proteins were pooled, dialyzed against distilled water, and then lyophilized. The prepared sample was referred to as bound thrombin.

Analysis of Bound Thrombin by SDS-PAGE and Western Blotting

Electrophoresis was performed according to the method of Laemmli [16]. Five micrograms of the bound thrombin was dissolved in 50 μl of 0.125 M Tris-HCl, 2.5% SDS, 10% glycerol at pH 6.8 with or without 2-mercaptoethanol (5%). A 10–18% gradient polyacrylamide slab gels or 12 and 18% homogeneous polyacrylamide gels (110 $\times$ 70 $\times$ 1 mm) with 4.5% polyacrylamide stacking gel (25 $\times$ 70 $\times$ 1 mm) were used to separate the bound thrombin with a gel electrophoresis apparatus GE 2/4 (Pharmacia Fine Chemicals, Uppsala, Sweden) at a constant current of 15 mA. The separated

proteins in the gels were stained with silver staining or electroblotted onto PVDF membranes.

Electroblotting was performed by the method of Hirano and Watanabe [17], as described previously [18]. The protein-blotted membranes were blocked with 1% milk (dried nonfat milk dissolved in D-PBS) overnight at 4°C, and then reacted with rat antirabbit fibrinogen and rat antibovine thrombin (500 µg/ml) for 24 h at 4°C. Following the incubation with POD-conjugated antirat IgG ($\times 1/1,000$), the membranes were soaked in 4-chloro-1-naphthol solution, and the bands were visualized by ultraviolet light according to the method of Domingo and Marco [19].

N-Terminal Sequence Analysis of Bound Thrombin

About 30 µg of the bound thrombin was dissolved in the mixture of 10 µl of acetonitrile with 30 µl of electrophoresis buffer (0.125 M Tris-HCl, 6% SDS, 12% 2-mercaptoethanol, 20% glycerol, pH 6.8), and then reduced for 90 min at room temperature. Protein components were then resolved by SDS-PAGE using an 18% homogeneous polyacrylamide gel, and were transferred electrophoretically onto a PVDF membrane, as described above. Rabbit fibrinogen (2 mg) was also dissolved in 100 µl of the electrophoresis buffer and subjected to SDS-PAGE using a 12% polyacrylamide gel, and then electroblotted onto a PVDF membrane. The blotted proteins on PVDF membranes were subjected to N-terminal sequence analysis, using a Shimadzu PSQ-1 gas-phase sequencer system (Shimadzu Co., Kyoto, Japan), as described previously [18, 20].

Analysis of Bound Thrombin by High-Performance Liquid Chromatography

About 5 µg of the bound thrombin was dissolved in 200 µl of 4 M, 6 M or 8 M urea in 0.4% SDS, 10% acetonitrile, 0.1% TFA, and 100 µl of the denatured samples were analyzed by Hitachi 6210/6010 high-performance liquid chromatograph equipped with a Model AS-4000 sample injector and a Model D-2500 integrator, all from Hitachi Ltd. (Tokyo, Japan). A C4 reverse-phase column (Cosmosil 5C4-AR-300, 4.6 $\times$ 150 mm, Nacalai Tesque, Kyoto, Japan), which was equilibrated with 0.05% TFA, was used. HPLC analysis was performed with a 0–60% acetonitrile gradient during 60 min. The flow rate was adjusted to 0.5 ml/min, and polypeptide materials were monitored at 210 nm employing Hitachi L-4000 UV detector. Eluates of each peak on HPLC were lyophilized, dissolved into 20 µl of 0.05% TFA, and then subjected to N-terminal sequence analysis.

Results

Profile of Immunoaffinity Column Chromatography

The supernatant of crushed fibrin clots was subjected to immunoaffinity chromatography using an antibovine thrombin-immobilized Sepharose 4B column. As shown in figure 1A, fractions 43–46 (indicated by a thick horizontal bar) were pooled, then dialyzed against D-PBS. Isolated protein material was further purified using an anti-rabbit fibrinogen-immobilized Sepharose 4B column (fig. 1B). In a gradient elution, the baseline rose gradually because of high absorbance of the limiting buffer (A_{280} : 0.01). Fractions 30–33 containing proteins, indicated by a

thick horizontal bar, were pooled. The pooled eluate was dialyzed against distilled water, and lyophilized.

Electrophoresis under Nonreduced Conditions

The lyophilized material obtained by immunoaffinity chromatography was analyzed by SDS-PAGE under non-reduced conditions. This material was resolved into several bands, and a band of 170,000 D was the most clearly seen. In addition, a fine band of about 220,000 D was also observed (arrow, lane 1 in fig. 2A). The former band was reacted with both polyclonal antibodies against rabbit fibrinogen and bovine thrombin (lanes 1 and 4 in fig. 2B), indicating that the material obtained by immunoaffinity chromatography was a complex consisting of the fragments of fibrin(ogen) and the thrombin molecule. Thus, the material obtained by immunoaffinity chromatography proved to be the bound thrombin.

N-Terminal Sequences of Fibrinogen Fragments Bound to a Thrombin Molecule

SDS-PAGE analysis revealed that the reduced bound thrombin was resolved into three low-molecular components (indicated by arrows in fig. 3). In order to identify the low-molecular components, these components were subjected to immunoblotting analysis (fig. 4A, a). Immunoblotting analysis demonstrated that three distinct bands (molecular weights of 23,000, 17,500, and 15,000) reacted with antirabbit fibrinogen antibody (indicated by arrows in fig. 4A, a). The three bands were then subjected to N-terminal sequence analysis. The reduced rabbit fibrinogen was also subjected to N-terminal sequence analysis for reference (fig. 4A, b). As shown in figure 4B, the N-terminal sequence of one component of the fibrin fragment (fragment 1) was YVATR that was identical to the amino acid sequence γ 1–5 of the rabbit γ -chain; the sequence of another component (fragment 2) was GPRVV that was identical to the amino acid sequence α 17–21 of the rabbit α -chain. However, the sequence of the other component (fragment 3) turned out to be HVEG, the origin of which could not be identified.

N-Terminal Sequence Analysis of the Denatured Bound Thrombin

HPLC analyses revealed that the bound thrombin was resolved into several peaks (peaks RT 28, 34, 45, and 50 in fig. 5A) when denatured with 8 M urea. The peak RT 50 was also observed when native thrombin (bovine thrombin) was analyzed (fig. 5D). The proteins in those peaks were subjected to N-terminal sequence analysis. Consequently, the N-terminal sequence of peak RT 34 of

Fig. 1. Profile of serial affinity chromatograms. The supernatant of fibrin clots was subjected to affinity chromatography on an antbovine thrombin antibody-immobilized Sepharose 4B column (**A**). Fractions indicated by a thick horizontal bar were pooled, and the pooled eluate was further purified using a column-immobilizing antibody against rabbit fibrinogen (**B**). Fractions containing proteins (indicated by thick horizontal bar) were collected, dialyzed against distilled water, and then lyophilized.

the bound thrombin was identical to that of the fibrin fragment consisting of fragments 1, 2, and 3. The N-terminal sequences of peaks RT 28 and RT 45 partially corresponded to those of the fibrin fragment (table 1). Both peaks RT 50 of the bound thrombin and native thrombin were identical to the N-terminal sequence of the bovine α -thrombin [21].

Discussion

In the present study, we prepared bound thrombin using bovine thrombin and rabbit fibrinogen. On the basis of the amino acid sequence of rabbit prothrombin determined by Seiffert et al. [22], we found that the rabbit sequence of the region corresponding to exosite 1 on

Fig. 2. SDS-PAGE and immunoblotting. **A** Lyophilized fractions obtained by affinity chromatography were analyzed by electrophoresis using a 10–18% polyacrylamide gel under nonreduced conditions. Lane 1: material obtained by affinity chromatography; lane 2: native thrombin; lane 3: rabbit fibrinogen; lane 4: low-molecular marker proteins; lane 5: high-molecular marker proteins. The gel was stained with silver staining. **B** Proteins blotted onto a PVDF membrane were subjected to immunostaining with antibody against bovine thrombin (**a**) and with antibody against rabbit fibrinogen (**b**). Lanes 1, 4: material obtained by affinity chromatography; lanes 2, 5: native thrombin; lanes 3, 6: rabbit fibrinogen. The blot of low-molecular marker proteins (lane 7) was stained with 0.025% Coomassie brilliant blue R-250.

human and bovine thrombins was highly homologous between them. Exosite 1 is the fibrin(ogen) binding site on thrombin [23, 24]. Consequently, we considered that the binding properties of rabbit thrombin to fibrin fragment might be equal to those of human or bovine thrombin. On the other hand, we determined the N-terminal sequence of rabbit fibrinogen in this study. In the rabbit sequence of the N-terminal region corresponding to FPA (A α 1–16) or fibrinopeptide B (FPB; B β 1–13), only a few amino acid residues (e.g., Phe⁸ and Arg¹⁶ in FPA, Arg¹³ in FPB) were homologous between rabbit and human or bovine sequences. The variability of FPA and FPB sequences among mammalian species had been reported by Blombäck et al. [25, 26]. On the contrary, the N-termini of fibrin α -, β -, and γ -chains were highly homologous between rabbit and human or bovine fibrins, suggesting that the structure of N-terminal fragments of rabbit fibrin may be analogous to human and bovine fibrin. From these results, we considered that the use of bovine thrombin and rabbit fibrinogen may not create any discrepancies compared to the findings with the pure rabbit protein system and the pure human protein system.

It has been reported that thrombin could form a complex with (DD)E and fragment E, in which the N-terminal region of the A α -chain proved to be essential for the bind-

Fig. 3. SDS-PAGE of bound thrombin under reduced conditions. The reduced bound thrombin was resolved by SDS-PAGE on a 10–18% polyacrylamide gel. Lane 1: bound thrombin; lane 2: native thrombin; lane 3: rabbit fibrinogen; lane 4: low-molecular protein markers; lane 5: high-molecular marker proteins.

Fig. 4. N-terminal sequence analysis of the fibrin 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.

Table 1. 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	$\times$	$\times$	$\times$	fibrinogen fragments (from fig. 4B) 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	$\times$	$\times$	
	RT 45	Y G	$\times$	$\times$	$\times$	$\times$	
	RT 50	T I	F V	G E	G	$\times$	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.

Fig. 5. HPLC pattern of native thrombin and denatured bound thrombin. The purified bound thrombin (about 5 μ g) was dissolved in 200 μ l of 0.4% SDS, 10% acetonitrile, 0.1% TFA containing 8 M urea (**A**), 6 M urea (**B**), or 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.

ing between thrombin and fibrin(ogen) [27–29]. Malkowski et al. [30] demonstrated that the thrombin-cleavage at Arg¹⁶ of fibrinogen A α was strongly inhibited when Phe⁸ was mutated to Tyr⁸, indicating that thrombin exerts its enzymatic activity through binding to fibrinogen 1–23. Furthermore, thrombin forms fibrin clots in a stepwise manner: (1) two-stranded fibrin protofibril, (2) two- or three-dimensional fibrin gel [31, 32]. FPA is cleaved and released during the formation of the fibrin protofibril, which results in the formation of a complex of type I fibrin with fibrinogen, while the fibrinogen B β -chain remains intact. It is well known that the release of FPB is delayed and lessened as compared with that of FPA after rabbit fibrin clot formation by thrombin [32, 33]. Since the β -chain of fibrin could not be found in the bound thrombin, the bound thrombin might be generated during the formation of fibrin protofibril. That is, the bound thrombin likely complexed with desAA type I fibrin.

In the present study, since the bound thrombin was prepared by crushed clots, we could not directly compare its characteristics with those reported in previous studies (on the bound thrombin released by fibrinolytic enzymes). Our data demonstrated that the bound thrombin complexed with at least two fibrin(ogen) derivatives, the N-terminal amino acids which were identical to α - and γ -chains of rabbit fibrin (fig. 4). Furthermore, HPLC analysis revealed that at least three distinct bound thrombins might have been generated in our preparation (table 1). The three bound thrombins appear to complex with the fibrinogen derivatives including N-terminal regions of α - and γ -chains, which are consistent with the data of figure 4. Taken together, the bound thrombin may complex with various fibrin(ogen) derivatives of fibrinogen subunits (A α , B β , and γ).

It was reported that the binding between thrombin and fibrin fragment (or its α -chain fragment) was labile under high ionic strength (e.g., 0.2–0.3 M NaCl) or in the presence of SDS [27–29, 34]. However, in this study, the bound thrombin proved to be a complex of thrombin and fibrin fragment on SDS-PAGE after elution by 0.36 M NaCl and acid gradient in the affinity chromatography system. We then inferred that the thrombin and fibrin fragment in the bound thrombin isolated in this study might be linked with multiple chemical bonds such as ionic, hydrogen, and/or hydrophobic bonds. This multiple linkage might make the bound thrombin stable in high ionic strength and acidic elution.

For the correlation between clot age and the structural characteristics of bound thrombin, we have no data for the time being. However, liberated bound thrombin could

be different from that isolated in this experiment if fibrin clots were prepared in a different manner. When we isolated the bound thrombin from clot lysates prepared with urokinase, the bound thrombin was dissolved into thrombin and fibrin fragment by treatment with 4 M urea, and sequence analysis revealed that the thrombin molecule remained as α -thrombin and the fibrin fragment contained the N-terminus of the β -chain in addition to those of the α - and γ -chains [35]. Thus, we now consider that the bound thrombin is not a homogeneous complex, and that the variability of bound thrombin may be due to differences in the structure of the fibrin fragment.

We have not clarified the structural characteristics of the bound thrombin generated by whole blood clotting

and in vivo clotting. As the quantity of bound thrombin generated in vivo would be too small to be isolated and identified. We tried to detect it using a monoclonal antibody [36].

In conclusion, bound thrombin can be generated from mechanically crushed clots. The bound thrombin complexes with fibrin(ogen) derivatives including the N-terminal regions of α - and γ -chains of fibrin. The further characterization and quantitation of bound thrombin may help to understand the mechanism of rethrombosis after interventional medical procedures and to develop methods for improving the clinical outcome in these patients.

References

- Manolis AS, Melita-Manolis H, Stefanadis C, Toutouzas P: Plasma level changes of fibrinopeptide A after uncomplicated coronary angioplasty. *Clin Cardiol* 1993;16:548–552.
- Marmura JD, Merlini PA, Sharma SK, Khaghan N, Torre SR, Israel DH, Ardissino D, Ambrose JA: Thrombin generation in human coronary arteries after percutaneous transluminal balloon angioplasty. *J Am Coll Cardiol* 1994; 24:1484–1491.
- Oltrona L, Eisenberg PR, Lasala JM, Sewall DJ, Shelton ME, Winters KJ: Association of heparin-resistant thrombin activity with acute ischemic complications of coronary interventions. *Circulation* 1996;94:2064–2071.
- Tschöpl M, Tsakiris DA, Marbet GA, Labs KH, Jäger K: Role of hemostatic risk factors for restenosis in peripheral arterial occlusive disease after transluminal angioplasty. *Arterioscler Thromb Vasc Biol* 1997;17:3208–3214.
- Faxon DP, Sanborn TA, Weber VJ, Haudenschild C, Gottsman SB, McGovern WA, Ryan TJ: Restenosis following transluminal angioplasty in experimental atherosclerosis. *Arteriosclerosis* 1984;4:189–195.
- Plate G, Stanson AW, Hollier LH, Dewanjee MK, Solis E, Kaye MP: Effect of platelet inhibitors on platelet and fibrin deposition following transluminal angioplasty of the atherosclerotic rabbit aorta. *Eur J Vasc Surg* 1989;3:127–133.
- Nobuyoshi M, Tanaka M, Fujii M, Asanuma A, Takimoto M: Thrombus formation on the aorta injured by angioplasty and its prevention with dilazep in atherosclerotic rabbits. *Thromb Res* 1989;54:561–572.
- Hatton MWC, Ross B, Southward SMR, DeResk M, Richardson M: Pretreatment of rabbits with either hirudin, ancrod, or warfarin significantly reduces the immediate uptake of fibrinogen and platelets by the deendothelialized aorta wall after balloon-catheter injury in vivo. *Arterioscler Thromb Vasc Biol* 1998;18: 816–824.
- Speidel CM, Eisenberg PR, Ruf W, Edgington TS, Abendschein DR: Tissue factor mediates prolonged procoagulant activity on the luminal surface of balloon-injured aortas in rabbits. *Circulation* 1995;92:3323–3330.
- Zeng G, Kinjoh K, Nakamura M, Kosugi T: The activity of postclotting thrombins on platelet activation was identical to that of native thrombin. *J Jpn Coll Angiol* 2002;42:25–31.
- Doolittle RF, Schubert D, Schwartz SA: Amino acid sequence studies on artiodactyl fibrinopeptides. I. Dromedary camel, mule deer, and cape buffalo. *Arch Biochem Biophys* 1967;118: 456–467.
- Kinjoh K, Nakamura M, Zeng G, Kosugi T: An antibody that binds to primary specific pocket-associated structure in the active site of bovine thrombin. *Hybrid Hybridomics* 2002;21:61–67.
- Monestier M, Debbas ME, Zhou SL: Rat monoclonal antibodies to murine IgM determinants: Application to antibody purification. *Hybridoma* 1989;8:631–637.
- Bukovsky J, Kennet RH: Simple and rapid purification of monoclonal antibodies from cell culture supernatants and ascites fluids by hydroxylapatite chromatography on analytical and preparative scales. *Hybridoma* 1987;6: 219–228.
- Harlow E, Lane D: Immunoaffinity purification; in *Antibodies: A Laboratory Manual*. New York, Cold Spring Harbor Laboratory, 1988, pp 511–552.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- Hirano H, Watanabe T: Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis: Application to an insoluble protein. *Electrophoresis* 1990;11:573–580.
- Kinjoh K, Kosugi T, Nakamura M, Hanashiro K, Sunagawa M, Tokeshi Y, Eguchi Y: Habutoxin splits the Arg¹⁶-Gly¹⁷ bond in the A α chain of rabbit fibrinogen. *Thromb Haemost* 1997; 77:1127–1128.
- Domingo A, Marco R: Visualization under ultraviolet light enhances 100-fold the sensitivity of peroxidase-stained blots. *Anal Biochem* 1989;182:176–181.
- Nejime T, Kinjoh K, Nakamura M, Hanashiro K, Sunagawa M, Eguchi Y, Kosugi T: Habutoxin recognizes Thr⁷ in the sequence of fibrinopeptide A of rabbit fibrinogen. *Toxicon* 2000; 38:1029–1041.
- Lundblad RL, Noyes CM, Mann KG, Kingdon HS: The covalent differences between bovine α - and β -thrombin. *J Biol Chem* 1979;254: 8524–8528.
- Seiffert D, Mitchell TJ, Wang Z, Knabb RM, Barbera F, Reilly TM, Thoolen M: Prothrombin activation in rabbits. *Thromb Res* 1999;93: 101–112.
- Fenton JW 2nd, Olson TA, Zabinski MP, Wilner GD: Anion-binding exosite of human α -thrombin and fibrin(ogen) recognition. *Biochemistry* 1988;27:7106–7112.
- Tsiang M, Jain AK, Dunn KE, Rojas ME, Leung LLK, Gibbs CS: Functional mapping of the surface residues of human thrombin. *J Biol Chem* 1995;270:16854–16863.
- Blombäck B, Blombäck M, Grondahl NJ: Studies on fibrinopeptides from mammals. *Acta Chem Scand* 1965;19:1789–1791.

- 26 Blombäck B, Blombäck M, Grondahl NJ, Holmberg E: Structure of fibrinopeptides – its relation to enzyme specificity and phylogeny and classification of species. *Arkiv Kemi* 1966; 25:411–428.
- 27 Weitz JI, Leslie B, Hudoba M: Thrombin binds to soluble fibrin degradation products where it is protected from inhibition by heparin-anti-thrombin but susceptible to inactivation by antithrombin-independent inhibitors. *Circulation* 1998;97:544–552.
- 28 Vali Z, Scheraga HA: Localization of the binding site on fibrin for the secondary binding site of thrombin. *Biochemistry* 1988;27:1956–1963.
- 29 Binnie CG, Lord ST: A synthetic analog of fibrinogen- α 27–50 is an inhibitor of thrombin. *Thromb Haemost* 1991;65:165–168.
- 30 Malkowski MG, Martin PD, Lord ST, Edwards BF: Crystal structure of fibrinogen-A α peptide 1–23 (F8Y) bound to bovine thrombin explains why the mutation of Phe-8 to tyrosine strongly inhibits normal cleavage at Arg-16. *Biochem J* 1997;326:815–822.
- 31 Weisel JW, Veklich Y, Gorkum O: The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. *J Mol Biol* 1993;232:285–297.
- 32 Blombäck B, Hessel B, Hogg D, Therkildsen L: A two-step fibrinogen-fibrin transition in blood coagulation. *Nature* 1978;275:501–503.
- 33 Kinjoh K, Nakamura M, Kosugi T: Application of a monoclonal antibody to estimate rabbit fibrinopeptide A released by habutobin. *Toxicon* 1994;32:1413–1423.
- 34 Kaczmarek E, McDonagh J: Thrombin binding to the A α -, B β -, and γ -chains of fibrinogen and to their remnants contained in fragment E. *J Biol Chem* 1988;263:13896–13900.
- 35 Kinjoh K, Nakamura M, Sunagawa M, Kosugi T: Isolation of bound thrombin consisting of thrombin and fibrin N-terminal fragment from clot lysate. *Haematologia* 2003;32: in press.
- 36 Moriyama T, Nakamura M, Kinjoh K, Tanaka T, Kosugi T: A monoclonal antibody against bovine thrombin reacting to the C-terminal side of thrombin. *Hybrid Hybridomics* 2001; 20:397–403.