Mutation Report

Analysis of plasmin generation and clot lysis of plasma fibrinogen purified from a heterozygous dysfibrinogenemia, BβGly15Cys (Hamamatsu II)

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Abstract
[***Objective***] We found a heterozygous dysfibrinogenemia caused by the substitution of BβGly15Cys and designated it fibrinogen Hamamatsu II (H-II). Although the propositus suffered an infarction of the medulla oblongata, other thrombotic risk factors, paradoxical cerebral infarction, and arterial dissection were not found. To determine whether the delayed lysis of fibrin clots or not in the context of the BβGly15Cys substitution, we examined the clot lysis and plasmin generation of propositus' fibrinogen. [***Methods***] Fibrinogen was purified from the propositus' and normal control plasma by immunoaffinity chromatography and was used for the following experiments: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fibrin polymerization, scanning electron microscopic observation of fibrin clot and fibers, clot lysis, and tissue-type plasminogen activator (tPA)-mediated plasminogen activation. [***Results and Conclusion***] The H-II plasma fibrinogen showed the presence of albumin-binding variant forms, a dimeric molecule of variant fibrinogen, and impairment of lateral aggregation during fibrin polymerization. The H-II fibrin clot showed lower density of bundles and thinner diameters of fibers than in the normal fibrin clot. In the clot lysis experiments with overlaid plasmin, H-II fibrin showed a similar lysis period and lysis rate to the normal control. Moreover, plasmin generation from a mixture of thrombin, tPA, plasminogen, and H-II fibrinogen also showed a similar rate to normal fibrinogen**.** Although the propositus suffered an infarction, the present study did not observe delayed clot lysis; i.e., the clot was not resistant to plasmin degradation. Therefore, we did not clarify an association between the BβGly15Cys dysfibrinogenemia and arterial thrombosis.

Introduction

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ), and it is expressed as $(A\alpha, B\beta, \gamma)_2$ [1,2]. Each chain is synthesized, assembled into a six-chain molecule in hepatocytes, secreted into the blood, and fibrinogen circulates at 1.8-3.5 g/l. During blood coagulation, thrombin cleaves four short peptides from fibrinogen to form fibrin monomers. The peptides are released from the N-termini of Aα- and Bβ-chains and are designated fibrinopeptide A (FPA) and fibrinopeptide B (FPB), respectively. The fibrin monomers spontaneously polymerize in an ordered fashion [3]. In the first step, so-called D-E and D-D interactions form double-stranded protofibrils and the second step of polymerization, termed lateral aggregation, occurs. The final product is an insoluble fibrin network consisting of multi-stranded, branched fibers [3].

As many as 234 families with dysfunctional fibrinogens have been analyzed genetically and/or structurally. These are listed on the GEHT homepage [4] (updated on 16/09/2008, http://www.geht.org/databaseang/fibrinogen/). Most of these variants are present in either the Aα-chain (123 families) or the γ-chain (80 families), while variants of the Bβ-chain have been found in no more than 31 families throughout the world. Furthermore, two clinical manifestations, a tendency towards bleeding (25.6%) and thrombosis (21.8 %), are associated with dysfibrinogenemia; whereas, 52.6% of carriers are asymptomatic. However, it has been difficult to demonstrate that fibrinogen mutations directly cause thrombosis [5].

In 2003, we reported two BβGly15Cys variants, termed Kosai and Ogasa, which are characterized by the presence of albumin-binding variant fibrinogens and by impairments in both FPB release and lateral aggregation during fibrin polymerization [6]. To remove the complication of normal and/or normal-variant heterodimer molecules when assessing the function of plasma fibrinogen, we synthesized the recombinant variant fibrinogens Bβ15Cys (Bβ15C) and Bβ15Ala and examined their functions in relation to the changes in their primary structure [7]. The findings indicated that the Bβ15Gly residue plays important roles in FPB release and the lateral aggregation of protofibrils.

Two identical variants have already been reported as fibrinogens Ise [8] and Fukuoka II [9]. It is interesting that no BβGly15Cys variant has ever been reported outside of Japan. Only the Kosai propositus out of the four heterozygous BβGly15Cys variants suffered from arteriosclerosis obliterans, and no history of thrombosis or bleeding tendency was reported among the members of the four families; whereas, in 6 out of the 8 families with the BβArg14Cys variant, which is the immediate vicinity of BβGly15 residue and with a potential albumin binding, severe thrombosis (such as deep venous thrombosis, pulmonary embolism, or cerebral infarction) has been reported [4, 10-16]. Recently, we also found a new heterozygous dysfibrinogenemia, designated as Hamamatsu II, which is a BβGly15Cys variant in a patient who suffered from an infarction of the medulla oblongata (Wallenberg syndrome). In this report, to examine the impaired fibrinolysis of clots in the context of the BβGly15Cys substitution, we compared the tissue-type plasminogen activator (tPA)-mediated plasminogen activation and clot lysis of the propositus' plasma fibrin (ogen) to that of a normal control.

Materials and Methods

Patient data relating to fibrinogen Hamamatsu II

The propositus of Hamamatsu-II (H-II) was a 9-year-old male who was hospitalized with headaches, oculogyric illusions, truncal ataxia, sensory impairment of an arm and leg, and Horner signs and was finally diagnosed as having suffered an infarction of the left medulla oblongata by magnetic resonance imaging. Additional magnetic resonance angiography and ultra sound imaging of his heart eliminated arterial dissection and paradoxical cerebral infarction. Furthermore, no other thrombotic risk factors; i.e., protein C, protein S, antithrombin, antiphospholipid syndrome, factor V Leiden mutation, prothrombin G20210A polymorphism, homozygous methyltetrahydrofolate reductase Ala222Val, and hyperhomocysteinaemia were found with biochemical or coagulation tests, or genetic analyses. Blood was collected from the propositus with informed consent for biochemical and genetic analyses. Nine volumes of blood were collected into plastic tubes containing 1 volume of 3.2% trisodium citrate. Separated plasma was used for coagulation tests and purification of fibrinogen, and the buffy coat cells were extracted to prepare genomic DNA. This analysis was approved by the Ethical Committees for Genetic Analysis of Shinshu University School of Medicine. Unfortunately, we did not analyze the presence of dysfibrinogenemia in his family; however, they displayed no thrombotic complications.

Polymerase chain reaction (PCR)-amplification of the fibrinogen gene and DNA sequencing

To amplify all exons and exon-intron boundaries in the Aα-chain, Bβ-chain, and γ-chain genes, 32 PCR primers were designed, and DNA was amplified by PCR as described elsewhere [17]. The PCR products were purified from agarose gels, and the purified PCR products were directly sequenced using a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (v1.1) and an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA).

Coagulation tests and purification of plasma fibrinogen

The blood coagulation tests were performed on the propositus, and the immunologically determined fibrinogen concentration was measured by an automated latex photometric immunoassay [6]. Fibrinogen was purified from citrated plasma obtained from the propositus and from a normal control subject (NC) with informed consent. Purification and measurement of the fibrinogen concentration was performed as described [6]. The purity and characterization of the proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing (5% polyacrylamide gel) or reducing conditions (10% polyacrylamide gel) followed by immunoblot analysis and using with a rabbit antihuman fibrinogen antibody (Dako, Carpinteria, CA, USA) or a rabbit antihuman albumin antibody (Dako) with the reacting species being visualized with the aid of alkaline phosphatase-conjugated goat antirabbit IgG antibody (EY Laboratories Inc., San Mateo, CA USA).

Thrombin-catalyzed fibrin polymerization

Polymerization was performed in a micro-quartz cell and followed by measurement of the turbidity change with time at 350 nm using a UV-110-02 spectrophotometer (Shimadzu Co., Tokyo, Japan). The reactions were performed in a final volume of 100 μl as described elsewhere [6]. Briefly, fibrinogen (90 μl at 0.2 mg/ml) in *N*-[2-hydroxy-ethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES) at pH 7.4 and 0.12M NaCl (referred to from now on as polymerization buffer) containing 1.0 mM $CaCl_2$ were

mixed with human α-thrombin (10 μl at 0.5 unit/ml) (Enzyme Research Laboratories, South Bend, IN, USA). The final concentrations of fibrinogen and thrombin were 0.18 mg/ml and 0.05 U/ml, respectively. The changes in turbidity were monitored at ambient temperature, and three parameters were analyzed as described previously [6]. The reactions were performed in triplicate for each sample.

Clot lysis assay

The clot lysis assay was performed with plasmin or a mixture of two-chain tissue type-plasminogen activator (tPA) and plasminogen [18]. Briefly, 100 μl of purified fibrinogen was polymerized in a micro-quartz cell with 1.0 U/ml of human α-thrombin in polymerization buffer containing 1.0 mM $CaCl_2$ and was then incubated for 2 h at 37˚C. The final concentrations of fibrinogen and thrombin were 0.45 mg/ml and 0.1 U/ml, respectively. After the completion of polymerization, 100 μl of plasmin (Chromogenix AB, Molngal, Sweden) or a mixture of a tPA (Genentech, South San Francisco, CA) and plasminogen (Roche Diagnostics GmbH, Mannheim, Germany) (tPA/plasminogen mixture) was overlaid onto the clot (final concentration: plasmin: 0.25 U/ml, tPA: 1000 U/ml, and plasminogen: 1.0 U/ml), and clot lysis was monitored as the decrease in turbidity at 350 nm. Three parameters, lag period [the period until maximum absorbance (as 100 %) decreases to 98 %], the maximum lysis rate (L_{max}), and the 50 % lysis-time [the period until maximum absorbance decreases to 50 %], were obtained from the turbidity curves. All reactions were performed in duplicate.

Plasmin generation assay

The generation of plasmin from plasminogen by activation with tPA was performed in 2 sets of experimental conditions as described previously [18]. In the first set of experiments, 100 μl of purified fibrinogen (final concentration: 0.45 mg/ml) was polymerized in a 96-well MaxiSorp-treated polystyrene micro-plate (NUNC Immuno 96 Microwell Plate, Thermofisher Scientific Co, Yokohama, Japan) with 1 U/ml of human α-thrombin in polymerization buffer containing 1.0 mM $CaCl_2$ in the wells of a microtiter plate and then incubated for 2 h at 37˚C. After the completion of polymerization, 100 μl of a mixture of tPA, plasminogen, and the chromogenic substrate S-2251 (H-D-valine-leucine-lysine-p-nitroanilide, Chromogenix AB, Molngal, Sweden) in polymerization buffer containing 1.0 mM $CaCl_2$ was poured onto each clot (final concentration: tPA: 200 U/ml, plasminogen: 2.0 U/ml, S-2251: 0.44 mM), and the generation of plasmin was monitored as the increase in absorbance at 405 nm. For the second set of experiments, 20 μl of human α-thrombin (final concentration: 1.0 U/ml) was added to a 180 μl mixture of the fibrinogens (final concentration: 0.23 mg/ml), tPA (final concentration: 100 U/ml), plasminogen (final concentration: 0.2 U/ml), and S-2251 (final concentration: 0.44 mM) in polymerization buffer containing 1.0 mM $CaCl_2$. The plasmin-specific changes in absorbance at 405 nm were obtained by subtraction of the absorbance change due to the turbidity of fibrin generation or degradation of the S-2251.

Scanning electron microscopic observation of fibrin clot and fibers

Samples for scanning electron microscopy were prepared as described previously [19]. Briefly, 10 μl of α-thrombin were added to 40 μl of fibrinogen solution from the NC or H-II and mixed by repeated pipetting. Polymerization proceeded in a moisture chamber at 37˚C for 4 h. The final concentrations of fibrinogen and human α-thrombin were 0.40 mg/ml and 0.1 U/ml, respectively. The clots were fixed in 2.5 % glutaraldehyde overnight, stained with 1.0 % osmium tetroxide, freeze-dried (JFD-310 freeze drying device; Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan), mounted, osmium plasma-coated at 5 nm thickness in an

NL-OPC40 (Nippon Laser and Electronics Laboratory, Nagoya, Japan), and finally viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd). Images were taken at 3,000× or 20,000× with a 15.0 kV accelerating voltage. The fiber diameters were measured using a vernier caliper on a 300 % enlargement of a photograph taken at a magnification of 20,000× (n=50).

Results

DNA sequence analysis of the fibrinogen gene of the propositus

Mutation analysis indicated the missense mutation g.2807G>T in the Bβ-chain gene in a heterozygous state, causing amino acid exchange Gly15Cys in the Bβ-chain.

Coagulation tests and characterization of purified fibrinogen

The propositus' plasma fibrinogen concentration was much lower when determined by the thrombin-time method (1.07 g/l) than when determined by the immunological method (2.54 g/l). The propositus' prothrombin time and activated partial thromboplastin time were each within their reference range (11.8 sec and 31.0 sec, respectively). Plasma fibrinogen was purified by immunoaffinity chromatography with the aid of a Ca ion dependent monoclonal antibody, IF-1 [20], and the purified fibrinogen was analyzed by SDS-PAGE.

When the SDS-PAGE was run under reducing conditions, it was revealed that the purified fibrinogens from the propositus and the NC were high purity and that the propositus had normal Aα-, Bβ-, and γ-chain components (Figure 1A). However, the gel resolved under non-reducing conditions and stained by Coomassie revealed that the normal pattern shows three bands, corresponding to high molecular weight (340 kDa; HMW)-, low molecular weight (305 kDa; LMW)-, and low molecular weight prime (270 kDa; LMW')-fibrinogen fractions [21], and a high molecular weight band (Fig 1B, band*) higher than HMW-fibrinogen, while the propositus' fibrinogen had four minor extra bands larger than the HWM-fibrinogen fraction (Figure 1B; numbered as Band-1 – Band-4, respectively).

To identify these bands, SDS-PAGE was run under non-reducing conditions followed by immunoblot analysis using a rabbit antihuman fibrinogen antibody (Figure 1C) or a rabbit antihuman albumin antibody (Figure 1D). Since there is no appropriate higher molecular weight markers than 250 kDa, precise relative molecular mass of band is not estimated. However, we speculate that the three bands reacted with the antihuman albumin antibody are follows by reactivity against antihuman fibrinogen antibody; Band-2 – Band-4 are corresponding to any of following six variant fibrinogen-albumin complexes, variant HMW-fibrinogen-albumin-albumin, variant HMW-fibrinogen-albumin, variant LMW-fibrinogen-albumin-albumin, variant LMW fibrinogen-albumin, variant LMW'-fibrinogen-albumin-albumin, variant LMW'-fibrinogen-albumin. Band-1 only reacted with the rabbit antihuman fibrinogen antibody, and we speculate that this band is a dimeric molecule of variant fibrinogen (Figures 1C and 1D). The residual band that was only present in the normal control and was stained by Coomassie (Figure 1B, band*) did not react with either antifibrinogen or antialbumin antibody, and so it was not identified.

Thrombin-catalyzed fibrin polymerization.

Representative curves of thrombin-catalyzed fibrin polymerization are shown in Figure 2. We found that polymerization of the H-II fibrinogen was impaired compared with that of NC; namely, it demonstrated a longer lag period (5.1 ± 0.1 min), a slower maximum slope (1.90 ± 0.03 Abs x 10^{-2} /min), and a smaller 30 min-Δabsorbance (0.235 ± 0.010 Abs) than NC (3.5 ± 0.1 min, 5.60 ± 0.35 Abs x 10^{-2} /min, and 0.450 ± 0.027 Abs, respectively). In addition, the clottability of H-II fibrinogen; i.e., the percentage of fibrinogen used for clot formation, was 93.1 ± 0.5 %, which was not significantly different from that of the normal control (93 .4 ± 0.4 %).

Observation of fibrin clots and fibers by scanning electron microscopy

To clarify the differences in the ultrastructures of the fibrin clots between the NC and H-II fibrinogen, we observed fibrin clots under scanning electron microscopy (Figure 3). The

density of the bundles of fibrin fibers was lower, and the fiber diameters were significantly thinner in the H-II clot (66.6 ± 18.8 nm) than in the NC clot (82.4 ± 15.3 nm) (Student's *t*-test, $p < 0.001$)

Fibrin clot lysis.

Lysis curves for the 2 sets of experimental conditions using plasma fibrinogen are shown in Figures 4A and B. From the curves, we measured the lag period, the L_{max}, and the 50 % lysis time (Table 1). For clot lysis initiated with the addition of plasmin, the turbidity of H-II decreased after 11.5 ± 0.7 min, and the L_{max} and 50 % lysis time of H-II were 1.30 ± 0.00 Abs x 10^{-2}/min and 65.0 ± 0.0 min, respectively, and no parameters were significantly different from those of the NC (Figure 4A). For clot lysis initiated with the tPA/plasminogen mixture, the lag period of H-II was 43.0 ± 4.2 min, and the L_{max} and 50 % lysis time of H-II were 1.75 ± 0.07 Abs x 10^{-2}/min and 80.0 ± 0.0 min, respectively, and no parameters were significantly different from those of the NC (Figure 4B).

Plasmin generation by t-PA.

We analyzed the tPA-catalyzed plasmin generation from plasminogen during fibrin formation. For the first set of mixtures of tPA and plasminogen overlaid on the fibrin clot, H-II and NC showed almost the same plasmin generation curve as shown in Figure 5A. In addition, for the second set of experiments, thrombin, fibrinogen, tPA, and plasminogen were mixed simultaneously and H-II and NC showed similar plasmin generation curves (Figure 5B).

Discussion

In this report, we identified a heterozygous dysfibrinogenemia, BβGly15Cys, and designated it Hamamatsu II, according to the place of residence of the propositus. Hamamatsu city is near Kosai city and Ogasa prefecture, where identical variants have been reported [6,7]; however, the consanguinity of the individuals involved is not known. Plasma fibrinogen from H-II showed the presence of albumin-binding variant fibrinogens and a dimeric molecule of variant fibrinogens identical with the Kosai and Ogasa fibrinogens [6,7]. In addition, impairment of lateral aggregation during fibrin polymerization and thinner fiber diameter were also observed in this case [6]. Although the propositus of H-II suffered from an infarction of the medulla oblongata (Wallenberg syndrome), other causes than dysfibrinogenemia were excluded. Therefore, in this report, we analyzed the plasmin generation and the clot lysis for plasma fibrinogen purified from H-II propositus.

From observation of the fibrin clot structures under scanning electron microscopy, the fiber diameter of H-II was found to be much thinner than that of NC, and the density of the bundles of fibrin fibers of H-II was lower and more porous than that of NC. We considered that for the H-II clot, the penetration of tPA/plasminogen or plasmin solution was much faster than for the NC clot; whereas, previous studies have suggested that clots with thinner fibers lyse at lower rates than clots with thicker fibers [22-24]. Therefore, the reasons why clots formed from H-II fibrin lyse faster or slower than NC clots are complex. When plasmin or the mixture of t-PA and plasminogen was overlaid onto a fibrin clot, the clots made from H-II showed similar lysis rates to those from the NC in both experimental conditions. Moreover, the plasmin generation from a thrombin-stimulated mixture of t-PA, plasminogen, and H-II fibrinogen was similar to that of normal fibrinogen**;** namely, H-II fibrinogen acts normally with regard to tPA-mediated plasminogen activation during fibrin clot formation. Doolittle *et al.* demonstrated that B-b binding locks and prevents tPA and plasminogen binding to the binding site at α148-160 and finally delays clot lysis; i.e., the clot is resistant to fibrinolysis by plasmin [25]. Bβ15Cys fibrinogen can not release fibrinopeptide B and expose the" B" site,

so no B-b binding occurs after thrombin stimulation [6.7]. Our results showed that clot lysis and t-PA induced plasmin generation of BβGly15Cys were normal; i.e., contrary to the report of Doolittle *et al.*, the variant clot is not resistant to fibrinolysis by plasmin. Finally, our *in vitro* observations suggest the following possible pathogenesis of the infarction of the medulla oblongata in H-II patient: (a) thrombus or fibrin clot formed by H-II fibrinogen is resistant to plasmin catalyzation *in vivo*, (b) thrombin generation is elevated *in vivo,* or (c) plasmin generation is reduced and/or plasmin inhibition is elevated *in vivo.*

Approximately 20 % of the reported dysfibrinogens are associated with thrombosis [4], but to demonstrate that a mutation found in a fibrinogen directly causes this phenotype has been difficult. Several dysfibrinogenemias involving thrombosis have been reported [4]; however, thrombosis is often found in only one family with a few members or in several dysfibrinogenemias with the same variants or mutation sites. To date, the best evidence linking dysfibrinogens with thrombophilia is the families with the AαArg55Cys mutation (AαR554C). Five independent families with AαR554C-albumin binding have been identified and, strikingly, all propositi have presented with thrombosis [26]. Additionally, *in vitro* studies with the AαR554C fibrinogen have demonstrated that impaired fibrinolysis, thinner and many branched fibers, increased clot stiffness, and an increased cross-linking potential probably explain the thrombosis and embolism seen in the families afflicted [22, 27-30]. It is interesting that for the BβArg14Cys variants, which are the immediate vicinity of BβGly15 residue and with a potential albumin binding (demonstrated in the propositus of Ijmuiden [12]; however, no others have been analyzed), severe thrombosis such as deep venous thrombosis, pulmonary embolism, or cerebral infarction has been reported in 6 out of the 8 families [4, 10-16]. Of the four previously found heterozygous BβGly15Cys variants, which were positive for the albumin binding form, only the Kosai propositus [6,7] suffered from arteriosclerosis obliterans, and no history of thrombosis was reported among the members of the Ogasa [6,7], Ise [8], or Fukuoka II [9] families.

Finally, fibrinogen H-II is a dysfunctional fibrinogen characterized by the substitution of Cys for Bβ15Gly, the existence of additional disulfide-bonded forms, and impairment of lateral aggregation during fibrin polymerization. The propositus suffered from an infarction of the medulla oblongata; however, the present study did not observe delayed clot lysis under restricted *in vitro* conditions. In conclusion, we did not clarify an association between the BβGly15Cys dysfibrinogenemia and the arterial thrombosis.

Disclosure of Conflict of Interests

The authors state that they have no conflicts of interest.

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Table 1. The three parameters obtained by analysis of the clot lysis curves

Fibrinogen	Plasmin laid onto clot			tPA/Plasminogen laid onto clot		
	Lag period (min)	L_{max} (Abs x 10^{-2}/min)	50% lysis time (min)	Lag period (min)	L_{max} (Abs x 10^{-2}/min)	50% lysis time (min)
NC	12.5 ± 3.5	1.45 ± 0.07	64.0 ± 2.8	51.5 ± 9.2	1.60 ± 0.14	97. 5± 10.6
H-II	11.5 ± 0.7	1.30 ± 0.00	65.0 ± 0.0	43.0 ± 4.2	1.75 ± 0.07	80.0 ± 0.0

Abbreviations: NC: plasma normal control, H-II: Hamamatsu II. The three parameters were obtained as described in Materials and Methods.

Figures and Figure legends

Fig. 1.

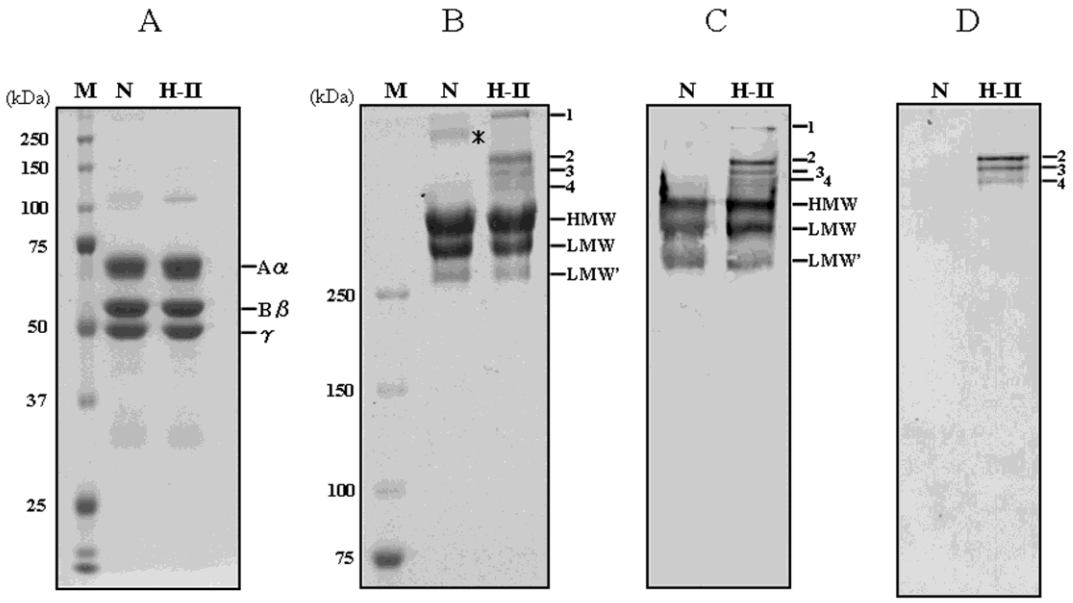

Figure 1. SDS-PAGE analysis and Western blot analysis of Hamamatsu II fibrinogen. **(A)** Coomassie brilliant blue-stained 10% SDS-PAGE run under reducing conditions and **(B)** 5% SDS- PAGE run under non-reducing conditions. The proteins that were transferred to a nitrocellulose sheet from 5% SDS- PAGE run under non-reducing conditions were visualized with a rabbit antihuman fibrinogen antibody **(C)** or a rabbit antihuman albumin antibody **(D).** HMW, LMW, and LMW' are the high molecular weight-, low molecular weight-, and low molecular weight prime-fibrinogen fractions, respectively. The extra bands observed for Hamamatsu II are designated as Band-1 to Band-4. The lanes are for normal control fibrinogen (NC), Hamamatsu II (H-II), and the molecular markers (M).

Fig. 2.

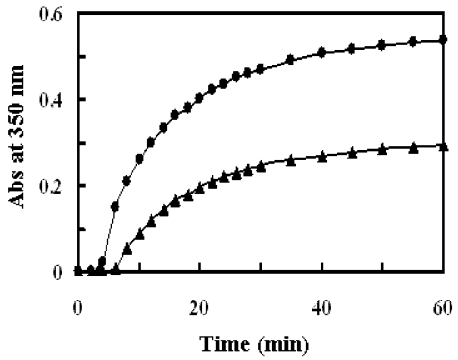

Figure 2. Thrombin-catalyzed fibrin polymerization. Thrombin-catalyzed fibrin polymerization was monitored at ambient temperature. Normal control (●) and Hamamatsu II (▲).

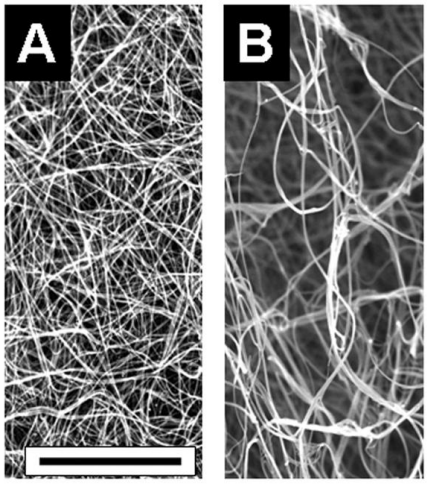

Figure 3. Scanning electron microscopic images of fibrin clots. The images were taken at 3,000× for **(A)** and **(B)**, with a 15.0 kV accelerating voltage. **A** shows normal control clots, and **B** shows the Hamamatsu II clots. The black bar represents 10 μm at 3,000.

Fig. 4.

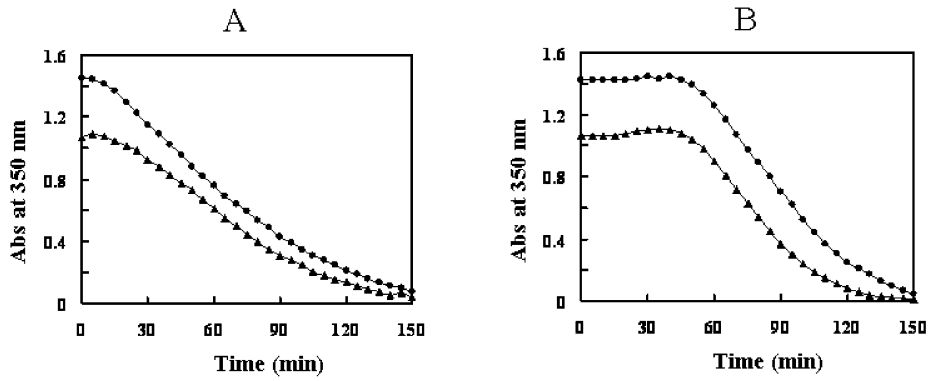

Figure 4. Lysis of clots made from plasma fibrinogen. After the completion of fibrin polymerization, either plasmin **(A)** or a mixture of tPA and plasminogen **(B)** was overlaid onto the clots. Lysis curves are shown for the normal control (●) and Hamamatsu II (▲).

Fig. 5

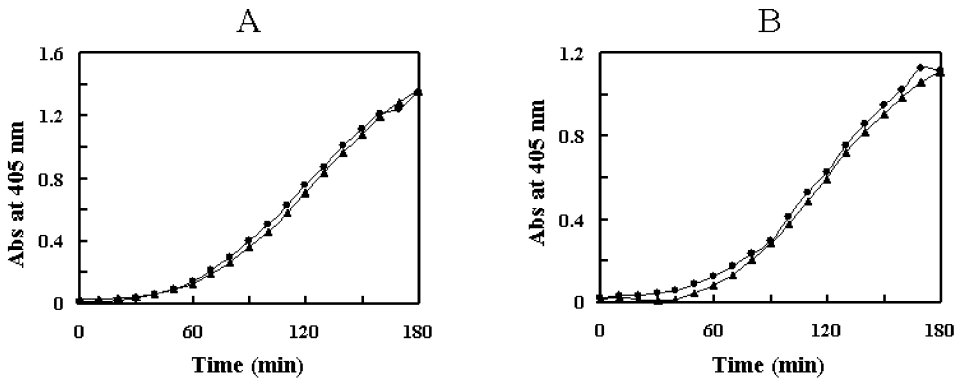

Figure 5. Plasmin generation from plasminogen with tPA. Purified fibrinogens were polymerized with thrombin. After the completion of polymerization, plasmin generation was initiated with a mixture of tPA, plasminogen, and S-2251 overlaid onto the clots **(A)** (the final concentration of fibrinogen was 0.23 mg/ml). Plasmin generation is shown for the co-addition of the fibrinogens, thrombin, plasminogen, tPA, and S-2251 **(B)** (the final concentration of the fibrinogen was 0.23 mg/ml). Mean absorbance curves from duplicate experiments are shown for the normal control (●) and Hamamatsu II (▲).