Functional Analysis of Recombinant Bβ15C and Bβ15A Fibrinogens Demonstrates that Bβ15G Residue Plays Important Roles in FPB Release and in Lateral Aggregation of Protofibrils

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Short running title: Characterization of Bβ15Gly->Cys, Ala fibrinogens

Summary

Background and objectives: Analysis of dysfibrinogens has improved our understanding of molecular defects and their effects on the function of intact fibrinogen. To eliminate the influence of plasma heterozygous molecules, we synthesized and analyzed recombinant variant fibrinogens. *Methods:* We synthesized two recombinant variant fibrinogens with a single amino-acid substitution at the 15Gly residue in the Bβ-chain; namely, Bβ15Cys and Bβ15Ala. *Results:* Western blotting analysis of purified fibrinogen revealed the existence of a small amount of a dimeric form only for Bβ15Cys fibrinogen. For Bβ15Cys fibrinogen, functional analysis indicated (a) no thrombin-catalyzed fibrinopeptide B (FPB) release and (b) markedly impaired lateral aggregation in thrombin- and reptilase-catalyzed fibrin polymerizations. For Bβ15Ala fibrinogen, such analysis indicated slight impairments of both thrombin-catalyzed FPB release and lateral aggregation in thrombin-catalyzed fibrin polymerization, but nearly normal lateral aggregation in reptilase-catalyzed fibrin polymerization. These impaired lateral aggregations were accompanied by thinner fibrin fiber diameters (determined by scanning electron microscopy of the corresponding fibrin clots). *Conclusion:* We conclude that a region adjacent to Bβ15Gly plays important roles in lateral aggregation not only in desA fibrin polymerization, but also in desAB fibrin polymerization, and we speculate that the marked functional differences between Bβ15A and Bβ15C fibrinogens in FPB release and fibrin polymerization might be due not only to the presence of a substituted cysteine residue in Bβ15C fibrinogen, but also to the existence of disulfide-bonded forms. Finally, our data indicate that the Bβ15Gly residue plays important roles in FPB release and lateral aggregation of protofibrils.

Key Words: dysfibrinogen, fibrinopeptide B, lateral aggregation, thrombin, reptilase

Introduction

Fibrinogen is a dimeric plasma glycoprotein, each half of the dimer being composed of three polypeptide chains (Aα, Bβ, and γ), and it is expressed as a trinodular structure formed from two distal D nodules, a central E nodule, and two coiled-coil regions

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linking the D and E nodules [1]. During blood coagulation, fibrinogen is converted by thrombin into an insoluble fibrin clot in a two-step process [2]. In the first step, thrombin cleaves two fibrinopeptide A (FPA, Aα1-16 residues) from two Aα-chains and exposes two "A" polymerization sites in the E nodule. The "A" sites interact individually with the "a" polymerization site in the γC domain of the D nodule in two adjacent fibrinogen molecules. These "A:a" interactions (D:E binding) spontaneously lead to the formation of half-staggered, double-stranded protofibrils that cooperate with a so-called D:D interaction [3,4]. In the second step, these protofibrils grow in length, and thrombin cleaves fibrinopeptide B (FPB, Bβ1-14 residues), which exposes the "B" site and dissociates the two αC-domains from the E nodule [2,5]. Finally, these protofibrils assemble into thick, multi-stranded fibers that branch to form a fibrin network. This second step is usually called lateral aggregation.

Although the release of FPB and the subsequent interaction of the "B" site with the "b" polymerization site in the βC domain of another molecule [2,6-8] are known to enhance the lateral aggregation by which protofibrils form thick fibers, lateral aggregation is also supported by other interactions, including intermolecular interactions between the αC domains of different fibrin molecules [9,10] and interactions between the two βC domains of different protofibrils, specifically involving residues β330-375 [11]. In contrast, the desA fibrin monomers (without "B:b" interactions) undergo lateral aggregation by association contacts between the γD regions (γ350-360 and γ370-380) of different protofibrils [11]. In addition, Moen *et al*. [12] demonstrated, using a recombinant Bβ14Arg->His variant fibrinogen, that residue Bβ14 is involved in desA polymerization and contributes to the lateral aggregation of desA protofibrils. These results supported previous observations [13-17].

More than 240 families with dysfunctional fibrinogens have been analyzed genetically and/or structurally. These are listed in the homepage (http://www.geht.org/pages/databaseang/fibrinogen). Most of these variants display amino-acid substitution either in the Aα-chain or in the γ-chain, while variants with substituted residues in the Bβ-chain number no more than 30 families throughout the world. We have already reported two Bβ15Gly->Cys variants, designated Kosai and Ogasa, that are characterized by the presence of albumin-binding variant fibrinogens

and impairments in both FPB release and lateral aggregation during fibrin polymerization [18]. Our data suggested (a) that the impaired functions in these variants may be due to the substitution of Cys for Bβ15Gly, together with the existence of some additional disulfide-bonded forms, and (b) that whether or not FPB is released, the "B" site of the fibrinogen Bβ-chain plays important roles in the lateral aggregation of normal protofibrils.

As is the case with most dysfibrinogens, these substitutions occur in individuals who are heterozygous for the mutation. This complicates functional analysis because the fibrinogen in these individuals is a complex mixture of normal homodimers, heterodimers, and variant homodimers [19,20]. We have addressed this complication by synthesizing recombinant fibrinogens variant in the Bβ-chain [namely, Bβ15Cys (Bβ15C) and Bβ15Ala (Bβ15A)], and we have examined the changes in function associated with changes in primary structure. The present data indicate that this residue plays important roles not only in FPB release, but also in the lateral aggregation of protofibrils.

Materials and Methods

Preparation of recombinant variant fibrinogens

Preparation of recombinant variant fibrinogens was performed according to the previously described procedure [21]. To change Bβ15Gly to Cys or Ala, the fibrinogen Bβ-chain expression vector, pMLP-Bβ (kindly provided by Dr. S.T. Lord, University of North Carolina), was altered by oligonucleotide-directed mutagenesis using the mutagenic primers 5'-CAGTGCCCGT<u>TGT</u>CATCGACC, 5'-CAGTGCCCGT<u>GC</u>TCATCGACC, respectively (the altered bases are underlined). Each of the variant expression plasmids was cotransfected with a histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells expressing normal human fibrinogen Aα- and γ-chains (kindly provided by Dr. S.T. Lord). Colonies were selected on both G418 (Gibco BRL, Rockville, MD) and histidinol (Aldrich Chemical, Milwaukee, WI). Selected clones were cultured either in Dulbecco modified Eagle medium Ham's nutrient mixture F12 (DMEM/F12) supplemented with 10μg/ml each of insulin, sodium selenite, and transferrin (Roche Diagnostics GmbH, Mannheim,

Germany) (termed serum-free medium) or in DMEM/F12 supplemented with 5%
bovine calf serum (HyClone Laboratories, Logan, UT)/ 5% Nu-serum (BD
Biosciences, Bedford, MA) (termed serum-containing medium) using 850 cm^2
roller-bottles.

Recombinant fibrinogen was purified from the harvested culture medium by
ammonium sulfate precipitation and subsequent immunoaffinity chromatography,
utilizing a calcium-dependent monoclonal antibody [18], and the concentration was
determined from the A_{280} value, as previously described [18]. The purity and
characterization of the proteins was determined by sodium dodecyl sulfate
(SDS)-polyacrylamide gel electrophoresis (PAGE), run under either nonreducing (5%
polyacrylamide gel) or reducing (10% polyacrylamide gel) conditions. This was
followed by Coomassie Brilliant Blue R-250 staining or by immunoblot analysis and
development either with a rabbit anti-human fibrinogen antibody (Dako, Carpinteria,
CA) or with a rabbit anti-human albumin antibody (Dako). Reacting species were
visualized with the aid of horseradish peroxidase-conjugated goat anti-rabbit IgG
(Medical and Biological Laboratories, Nagoya, Japan) and enhanced
chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech,
Buckinghamshire, UK) [21]. Proteins resolved by SDS-PAGE were electro-transferred
to a nitrocellulose sheet by the semidry technique, using a transfer buffer not
containing methanol.

Kinetics of FPA- and FPB- release
Fibrinopeptide release was examined as previously described [18], with modifications.
Fibrinogen (225 μl at 0.16 mg/ml) was added to human α-thrombin (Enzyme Research
Laboratories, South Bend, IN; 14 μl at 0.17 unit/ml, 3265 unit/mg) and incubated at
25˚C for various incubation periods, with subsequent boiling for 3 min. To prepare an
infinite time-point, 14 μl of thrombin (10 unit/ml) was added to 225 μl of fibrinogen,
and incubation allowed to proceed at 37˚C for 3h. After centrifugation, the
supernatants were analyzed by high-performance liquid chromatography (HPLC)
using a Cosmocil 5C18P column (Nacalai Tesque, Inc, Kyoto, Japan; 4.5 x 150 mm),
as previously described [18]. The percentage release was calculated by taking the

amount of FPA (A peptide) or FPB (the des-Arg-B and B peptides) released at an infinite time-point as 100%. FPA data were fitted with a simple first-order reaction, while FPB data were fitted to a standard equation describing two first-order processes, as described elsewhere [22], and the curves were plotted using DeltaGraph (DeltaPoint Inc., Monterey, CA). The specificity constant, k_{cat}/K_m, was determined as previously described [22].

Thrombin- or reptilase-catalyzed fibrin polymerization

Polymerization was followed by a monitoring of turbidity at 350 nm, as described elsewhere [18]. Briefly, fibrinogen (90 µl at 0.4 mg/ml) in 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl, with added 1 mM $CaCl_2$, was mixed either with human α-thrombin (10 µl at 0.5 unit/ml) or with reptilase-R (snake venom from *Bothrops atrox*; Pentapharm Ltd., Basel, Switzerland; 10 µl at 0.5 unit/ml), and changes in turbidity were monitored at ambient temperature. We measured the lag period, which reflects the rate of protofibril formation, the maximum slope (Vmax), which reflects the lateral aggregation rate of protofibrils, and the Δabsorbance over 30 min, as described elsewhere [18]. The reactions were performed in triplicate for each sample.

Fibrinogen clottability and fibrin monomer polymerization

The clottability of the purified fibrinogens was determined essentially as described before [18], human α-thrombin (final concentration, 0.05 unit/ml) being mixed with fibrinogen (final concentration, 0.36 mg/ml), then incubated for 3 h at 37 ˚C, followed by an overnight incubation at 4˚C. After centrifugation at 15,000 rpm for 15 min, clottability was calculated as described elsewhere [18].

   The fibrin clots harvested during the clottability tests were washed three times and dried on filter paper. Then, fibrin monomers were obtained by dissolving the clot in 0.02 M acetic acid. Ten microliters of fibrin monomer solution (0.007 mg) was diluted 10-fold in 20 mM HEPES, pH 7.4, containing 0.12 M NaCl, and polymerization was examined and estimated as described above.

Scanning electron microscopy

The scanning electron microscopy preparation was performed as previously described [18], with a few minor modifications. Briefly, 5 μl of either thrombin or reptilase (1.0 unit/ml) was added to 45 μl of fibrinogen solution (0.4 mg/ml), and polymerization allowed to proceed for 6 h at 37°C. The clots were then fixed, stained, critical-point dried, mounted, and osmium plasma-coated at 5-nm thickness, then viewed. Images were taken at 3000x or 20000x, and fiber diameters were measured using a vernier caliper on a 300% enlargement from a photograph of a 20000x observation.

Statistical analysis

Statistical values comparing normal recombinant and variant fibrinogen were determined using unpaired *t*-tests. A difference is significant when the *p* value is <0.05.

Results

Characterization of recombinant fibrinogen

When SDS-PAGE was run under reducing conditions and stained using Coomassie brilliant blue R-250, it was revealed that the Bβ15A, Bβ15C (including Bβ15C purified from serum-containing medium), and wild type [Bβ15Gly (Bβ15G)] fibrinogens, purified from culture media, all had the normal Aα, Bβ, and γ-chain composition (Fig.1A). However, gel resolved under non-reducing conditions and stained using Coomassie revealed that Bβ15C fibrinogen had an extra band (638 kDa) besides the high molecular weight (HMW) and low molecular weight (LMW) fibrinogens [23] (Fig.1B). Densitometric analysis indicated that the amounts of extra band in lanes 3 and 4 (Bβ15C fibrinogen) in Fig.1B represented 1.9 % and 9.2% of total fibrinogen, respectively. The extra band reacted weakly with a rabbit antihuman fibrinogen antibody (Fig.1C), but not at all with a rabbit antihuman albumin antibody (data not shown).

Functional analysis of recombinant fibrinogens

The kinetics of FPA- and FPB- release were analyzed by HPLC. With induction by thrombin, the release of FPA from recombinant fibrinogen did not differ substantially

among the Bβ15A, Bβ15C, and Bβ15G fibrinogens (Fig.2). Indeed, the specificity constants, k_{cat}/K_m, obtained for FPA were 9.92 ± 0.57 (10^6 M^{-1} sec^{-1}) for Bβ15G, 7.01 ± 0.49 for Bβ15A, and 8.14 ± 0.11 for Bβ15C fibrinogen. As illustrated in Fig.3, at an infinite time-point the absolute FPB release from Bβ15C fibrinogen (Fig.3c) was only 6.7% of the release from Bβ15G (Fig.3a). Therefore, for subsequent calculations of the percentage FPB release from Bβ15C fibrinogen, we took the amount of FPB released from Bβ15G fibrinogen at an infinite-time point as 100%. FPB release from Bβ15C fibrinogen was not observed at all throughout the 3h incubation period at room temperature (Fig.2). As illustrated in Fig.3, at an infinite time-point the absolute thrombin-induced FPB release from Bβ15A fibrinogen (Fig.3b) was 91.9% of the release from Bβ15G (Fig.3a). Although the FPB release from Bβ15A fibrinogen was slower than that from Bβ15G fibrinogen, the k_{cat}/K_m values obtained for FPB were not statistically different between Bβ15A fibrinogen [3.21 ± 0.30 (10^6 M^{-1} sec^{-1})] and Bβ15G fibrinogen (5.20 ± 0.59) (Fig.2).

Thrombin-catalyzed fibrin polymerization was monitored as the change in turbidity at 350 nm. Representative turbidity curves for Bβ15G, Bβ15A, and Bβ15C fibrinogens are shown in Fig. 4(A and B). Bβ15C fibrinogen showed a markedly impaired polymerization (Fig.4A). In addition, the lag period was longer, and Vmax and Δabsorbance were slower and lower, respectively, for Bβ15C fibrinogen than for Bβ15G fibrinogen (Table 1). Bβ15A fibrinogen showed slightly impaired polymerization compared with that obtained for Bβ15G fibrinogen (Fig.4A and Table 1). Since the turbidity measurements for Bβ15C fibrinogen indicated that thrombin-catalyzed fibrin polymerization was markedly impaired, we measured clottability to test how much fibrinogen was being converted to fibrin polymers. As is typically found, the clottability with thrombin was 98.6% for Bβ15G fibrinogen. In the case of the Bβ15A and Bβ15C fibrinogens, the corresponding values were 91.8% and 68.9%, respectively. In view of the lower clottability for Bβ15C than for Bβ15G and Bβ15A fibrinogens, we also performed thrombin-catalyzed fibrin polymerization at a 1.4-fold concentration (0.50 mg/ml) of Bβ15C fibrinogen. Although Vmax and Δabsorbance were faster and higher, respectively, at this high concentration than at the

low concentration of Bβ15C fibrinogen, they were still slower and lower, respectively, than those for the Bβ15G and Bβ15A fibrinogens (Fig.4A and Table 1).

To investigate the polymerization of desA fibrin, reptilase-catalyzed fibrin polymerization was examined. Bβ15C fibrinogen again showed markedly impaired polymerization by comparison with Bβ15G fibrinogen (Fig.4B and Table 1), whereas the polymerization of Bβ15A fibrinogen was quite similar to that of Bβ15G fibrinogen (Fig.4B and Table 1). To eliminate the impairment of polymerization at the FPB-release step, and to determine if an alteration in polymerization was caused by the substitution of Bβ15G by A *per se,* we prepared desAB monomers from both Bβ15G and Bβ15A fibrinogens, and simultaneously obtained desA monomers from Bβ15C fibrinogen by the use of thrombin. The polymerization curve obtained for Bβ15C fibrinogen showed a markedly slow, steady increase in turbidity (Fig.4C), while the desAB fibrin monomer polymerizations of the Bβ15G and Bβ15A fibrinogens exhibited a lag time followed by a fairly abrupt increase in turbidity. The polymerization curves for Bβ15A and Bβ15G fibrinogens displayed similar lag periods, but Vmax and Δabsorbance were slower and lower, respectively, for Bβ15A than for Bβ15G fibrinogen (Fig.4C and Table 1).

Scanning electron microscopy of fibrin clots and fibers

To clarify the differences in the ultrastructure of the fibrin clot among the Bβ15A, Bβ15C, and Bβ15G fibrinogens, we observed fibrin clots under the scanning electron microscope. The clots prepared from Bβ15C fibrinogen with the aid of thrombin differed in ultrastructure from those prepared from Bβ15G fibrinogen, the density of the bundles of fibrin fibers being less in the former than in the latter (Figs.5A and 5C), and the fiber diameter being significantly thinner in the former (89.3 ± 24.1 nm) than in the latter (131.3 ± 28.2 nm) ($p<0.001$). The fibrin clots prepared from Bβ15A fibrinogen were of an intermediate size (101.8 ± 25.9 nm), and fiber diameter was

Table1. Three parameters characterizing fibrin polymerization

recombinant fibrinogen concentration (mg/ml)	thrombin-catalyzed fibrin polymerization			reptilase-catalyzed fibrin polymerization			desAB fibrin monomer polymerization		
	lag time (min)	Vmax $(\times 10^{-5}/\text{sec})$	$\triangle$OD (mAbs)	lag time (min)	Vmax $(\times 10^{-5}/\text{sec})$	$\triangle$OD (mAbs)	lag time (min)	Vmax $(\times 10^{-5}/\text{sec})$	$\triangle$OD (mAbs)
15G (0.36)	2.2±0.1	343.6±15.0	1300.4±29.2	2.0±0.1	350.0±23.6	972.5±29.0	1.8±0.2	31.4±0.4	63.5±2.1
15A (0.36)	2.7±0.1*	162.5±5.9**	1025.3±6.9**	1.8±0.1	279.2±5.9	876.5±34.6	1.7±0.2	20.0±0.1***	40.0±4.2*
15C (0.36)	4.6±0.3**	51.8±5.2**	382.7±31.4***	3.1±0.1**	78.5±5.0**	399.5±36.1**	ND	2.9±0.3***	17.0±3.3***
15C (0.50)	4.5±0.4**	7.7±5.5**	642.9±26.5**	NT	NT	NT	NT	NT	NT

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, unpaired *t*-test [15G vs.15A or 15G vs. 15C]. ND: not determined, NT: not tested.

significantly thinner for Bβ15A than for Bβ15G ($p<0.001$) (Fig.5B). The fiber diameter in fibrin bundles prepared with the aid of reptilase was 80.1 ± 11.3 nm for Bβ15C, 128.9 ± 18.6 nm for Bβ15A, and 143.0 ± 28.8 nm for Bβ15G fibrinogen (Figs.5D, E and F), the fiber diameter for Bβ15C fibrinogen being significantly thinner than that for Bβ15G fibrinogen ($p<0.001$).

Discussion

We synthesized two recombinant variant fibrinogens with a single-amino acid substitution at the 15Gly residue in the Bβ-chain, (Bβ15C and Bβ15A), the former being made as an analogue for the heterozygous dysfibrinogenemias we earlier reported as Kosai and Ogasa [18], while the latter was produced as a control variant. For Bβ15C, SDS-PAGE analysis with Commassie staining revealed the existence of a small amount of an extra band at 638 kDa. We identified this band as a dimeric form of Bβ15C (based on the molecular weight and antibody reactivity) that might be formed by one or two pairs of disulfide bonds occurring between free neo-cysteine residues in two Bβ15C fibrinogen molecules. The presence of a dimeric variant fibrinogen has already been observed by Kamura *et al.* in plasma from another dysfibrinogenemia, Fukuoka II [16]. Our results also indicate that no albumin-binding form of Bβ15C, which was observed in the Kosai and Ogasa probands' plasma fibrinogen [18], existed in culture media containing albumin. However, if Bβ15C were to be synthesized using albumin-producing cells, such as HepG2 cells, we might then observe an albumin-binding form of Bβ15C.

    We earlier observed that plasma fibrinogen samples purified from the Kosai and Ogasa probands exhibited small amounts of three extra bands, two being albumin-binding forms and one an undetermined band of 646 kDa [18]. At that time, higher molecular weight proteins could not be transferred to a nitrocellulose sheet from polyacrylamide gel using 20% methanol-containing transfer buffer. Therefore, we used a transfer buffer not including methanol, and we were then able to detect a weak band of the dimeric form of Bβ15C fibrinogen. We also identified the undetermined band observed in the Kosai and Ogasa probands' purified plasma fibrinogen as a dimeric form of Bβ15Gly->Cys fibrinogen (data not shown). To judge

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from the existence of an intermolecular dimeric form of recombinant variant fibrinogen and albumin-binding forms of the patients' plasma fibrinogen, it is possible that recombinant Bβ15C fibrinogen may have other disulfide-bonded forms involving cysteine and/or another mutated Bβ-chain (intramolecular disulfide bonds), but we cannot speculate as to how much free cysteine residue might be present.

Our examination of thrombin-catalyzed fibrinopeptide release from Bβ15C fibrinogen showed that FPA was released normally, but FPB release was almost completely lacking. A similar result has been reported for the recombinant Bβ14Arg->His variant [12]. In contrast, the thrombin-catalyzed fibrinopeptide release from Bβ15A fibrinogen showed an only slightly reduced FPB release compared to that from Bβ15G fibrinogen. These results demonstrate that at the concentrations usually employed, thrombin cannot cleave the 14Arg-15Cys bond in the Bβ-chain, but can cleave the 14Arg-15Ala bond to a considerable extent. However, we did not determine whether thrombin cannot cleave the 14Arg-15Cys bond *per se* or whether thrombin cannot bind to this site due to the presence of forms with disulfide-bonds involving neo-cysteine residues.

When thrombin-induced fibrin polymerization was examined, lateral aggregation was significantly weaker for Bβ15A than for Bβ15G fibrinogen. However, that induced with the aid of reptilase showed very similar lateral aggregations between these two fibrinogens. To eliminate the slightly impaired thrombin-catalyzed FPB release, desAB fibrin monomer polymerization was performed. Interestingly, the desAB fibrin monomer from Bβ15A fibrinogen polymerized with a similar lag period, a slower Vmax, and a lower Δabsorbance than that from the Bβ15G fibrinogen. These results indicate that (a) when FPB is released, Bβ15A perturbs the lateral aggregation of desAB protofibrils through an impaired "B:b" interaction and/or steric hindrance, whereas (b) when FPB is not released, Bβ15A plays nearly normal roles in the lateral aggregation of desA protofibrils. When we examined thrombin- and reptilase-induced fibrin polymerizations for Bβ15C fibrinogen, we found that each polymerization showed a markedly impaired lateral aggregation of protofibrils (versus Bβ15G fibrinogen). These results indicate that whether or not FPB is released, Bβ15C perturbs the lateral aggregation of protofibrils. These markedly impaired functions of Bβ15C

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versus Bβ15A fibrinogen and the inconsistency between the clottability of Bβ15C (68.9%) and the amount of the dimeric form of Bβ15C (1.9%) again lead to us speculate that recombinant Bβ15C fibrinogen might have several other disulfide-bonded forms.

A decade ago, Weisel and Nagaswami proposed a kinetic model of polymerization on the basis of a correlation between electron microscopic and turbidity observations [24]. This model involved a three-step mechanism (FPA release, protofibril formation, and lateral aggregation), and supposed that five kinetic constants would generate turbidity polymerization. The best fit of this model to Bβ15C (a normal FPA release, longer lag time, lower Vmax, and lower final turbidity) was observed when the rate of protofibril addition to the growing fiber was decreased. From this, we concluded that Bβ15C decreased the rate of fiber growth, or lateral aggregation, for both thrombin- and reptilase-catalyzed polymerization, whereas Bβ15A decreased (a) the rate of fiber growth, or lateral aggregation, for thrombin-catalyzed polymerization, and (b) the rate of lateral aggregation for reptilase-catalyzed polymerization. This evidence of impairments of lateral aggregation during polymerization for both Bβ15A and Bβ15C fibrinogens is consistent with our data as to fibrin fiber diameter (determined from the corresponding fibrin clots) and with the values obtained for clottability. That is to say, the fibrin fibers produced with the aid of thrombin were thinner in the order Bβ15G>Bβ15A>Bβ15C fibrinogen, and those produced with the aid of reptilase were thinner for Bβ15C than for Bβ15G fibrinogen. On the basis of the thrombin-catalyzed fibrin polymerization of Bβ15C fibrinogen at 0.50 mg/ml, we speculate that while the fibrin fiber diameter might be thicker at 0.50 mg/ml than at 0.36 mg/ml of Bβ15C fibrinogen, the former is still thinner than for Bβ15G and Bβ15A fibrinogen at 0.36 mg/ml [24].

Lateral aggregation is supported by multiple interactions, including the "B:b" interaction [7,8], intermolecular interactions between the αC domains of different fibrin molecules (αC:αC) [9,10], and interactions between the two βC domains of different protofibrils [11]. Although release of FPB results in an enhanced rate of lateral aggregation of protofibrils [2,6], desA fibrin monomers undergo lateral aggregation by association contacts between the γD regions, (γ350-360 and γ370-380)

of different protofibrils [11], without "B:b" and "αC:αC" interactions. Recently, Moen *et al.* [12] demonstrated, using a recombinant Bβ14Arg->His variant fibrinogen, that a residue of Bβ14 is involved in desA fibrin polymerization and that it contributes to the lateral aggregation of desA protofibrils, results that support previous observations [13-17]. Our result is consistent with that of Moen *et al*. In the present study, the substitution of the Bβ-chain residue Gly15 by Cys reduced, but its substitution by Ala did not reduce, the lateral aggregation of protofibrils when FPB release was absent. From this, we guess that the existence of a form with a disulfide bond(s) involving a substituted neo-cysteine residue(s) and its steric hindrance substantially influences lateral aggregation, as also does Cys or Ala substitution *per se*.

In conclusion, we found that compared to Bβ15G fibrinogen, Bβ15C fibrinogen exhibits (a) no thrombin-catalyzed FPB release, and (b) markedly impaired thrombin- and reptilase-catalyzed fibrin polymerizations. In contrast, Bβ15A fibrinogen exhibits slight impairments of both thrombin-catalyzed FPB release and thrombin-catalyzed fibrin polymerization, but nearly normal reptilase-catalyzed fibrin polymerization. These results demonstrate that a region adjacent to Bβ15G is important for lateral aggregation not only in desA fibrin polymerization, but also in desAB fibrin polymerization. We speculate that the functional differences in FPB release and fibrin polymerization between Bβ15A and Bβ15C fibrinogen may be due to the existence of a form with a disulfide bond(s) involving a neo-cysteine residue(s) in the latter fibrinogen.

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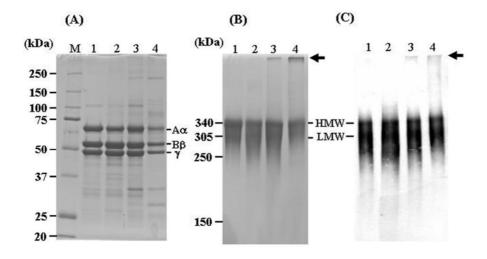
Figure 1. SDS-PAGE and immunoblot analysis of recombinant fibrinogens.

Purified recombinant fibrinogen was resolved either on 10% acrylamide gel under reducing conditions (A) or on 5% acrylamide gel under non-reducing conditions (B and C). (A) and (B) were stained using Coomassie Brilliant Blue R-250, while (C) was followed by blotting and development using a rabbit antihuman fibrinogen antibody, with cross-reacting species being visualized with the aid of horseradish peroxidase-conjugated goat ant-rabbit IgG and enhanced chemiluminescence detection reagents. Bβ15G (lane 1), Bβ15A (lane 2), and Bβ15C fibrinogen (lane 3) as well as Bβ15C fibrinogen purified from serum-containing medium (lane 4) were analyzed; left side shows molecular size markers (A) or molecular weights of HMW-fibrinogen and LMW-fibrinogen (340 and 305 kDa, respectively) together with molecular size markers (150 and 250 kDa) (B). Arrow indicates 638 kDa band (B and C).
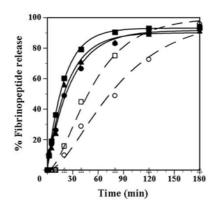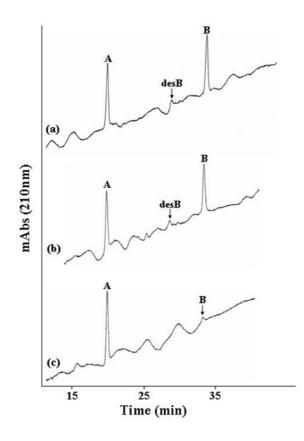
Figure 2. Thrombin-induced release of FPA and FPB from recombinant Bβ15G, Bβ15A, and Bβ15C fibrinogens. Fibrinogen (0.15mg/ml) was catalyzed using 0.01 U/ml of thrombin at 25˚C. When calculating the percentage release, the amount of FPA or FPB released at 3h of incubation time with a 59-fold concentration of thrombin at 37˚C (the infinite release) was taken as 100%. FPA and FPB data were fitted, and the curves plotted, as described in Materials and Methods. For the calculation of the percentage FPB release from Bβ15C fibrinogen, the infinite release from Bβ15G fibrinogen was taken as 100%. Square: Bβ15G fibrinogen; circle: Bβ15A fibrinogen; triangle: Bβ15C fibrinogen (solid symbol and solid line: FPA; open symbol and broken line: FPB).

Figure 3. Representative HPLC analyses of thrombin-induced release of FPA and FPB from various fibrinogens. Bβ15G (a), Bβ15A (b), and Bβ15C (c) fibrinogens were incubated with 0.59 U/ml of thrombin for 3h at 37˚C. Peptides were detected at 210nm, and the height of the FPB peak in (a) was about 4.5 mAbs. A,FPA; B, FPB.
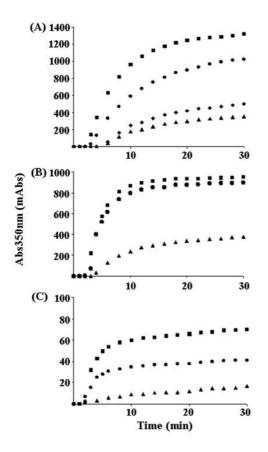
Figure 4. Thrombin- and reptilase-catalyzed fibrin polymerizations and fibrin monomer polymerization. Thrombin (0.05 U/ml, A) or reptilase (0.05 U/ml, B) was added at 0 min to fibrinogen (0.36 mg/ml) in 20 mM HEPES, pH 7.4, containing 0.12 M NaCl and 1 mM CaCl$_2$. The fibrin monomer (0.007 mg) generated by thrombin in 10 μl of 0.02M acetic acid was mixed with 90 μl of 20 mM HEPES, pH 7.4, containing 0.12 M NaCl (C). Change in turbidity with time was followed at 350 nm. Representative polymerization curves from triplicate experiments are shown for Bβ15G (square), Bβ15A (circle), and Bβ15C (triangle) fibrinogens. Diamond in panel A shows the representative polymerization curve obtained at a high concentration (0.50 mg/ml) of Bβ15C fibrinogen.

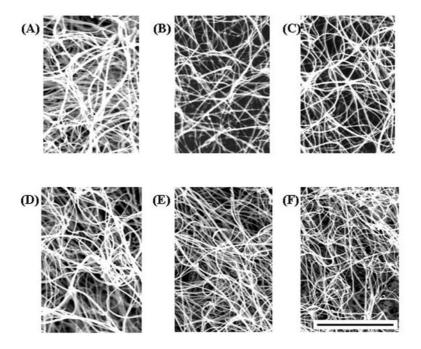**Figure 5**. **Scanning electron microscopy of fibrin clots formed using thrombin or reptilase.** All clots were polymerized, fixed, stained, critical-point dried, mounted, and osmium plasma-coated, then viewed. Fibrinogen (0.36 mg/ml) was polymerized using 0.1 U/ml of thrombin (A, B, C) or reptilase (D, E, F). All micrographs of fibrin clots made from Bβ15G (A, D), Bβ15A (B, E), and Bβ15C (C, F) fibrinogens were taken at the same magnification. Bar represents 10 μm.