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Recombinant γT305A fibrinogen indicates severely impaired fibrin polymerization due to the aberrant function of hole 'a' and calcium binding sites

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Abstract

Introduction: We examined a 6-month-old girl with inherited fibrinogen abnormality and no history of bleeding or thrombosis. Routine coagulation screening tests showed a markedly low level of plasma fibrinogen determined by functional measurement and also a low level by antigenic measurement (functional/antigenic ratio = 0.295), suggesting hypodysfibrinogenemia. ***Materials and methods:*** DNA sequence analysis was performed, and γT305A fibrinogen was synthesized in Chinese hamster ovary cells based on the results. We then functionally analyzed and compared with that of nearby recombinant γN308K fibrinogen. ***Results:*** DNA sequence analysis revealed a heterozygous γT305A substitution (mature protein residue number). The γT305A fibrinogen indicated markedly impaired thrombin-catalyzed fibrin polymerization both in the presence or absence of 1 mM calcium ion compared with that of γN308K fibrinogen. Protection of plasmin degradation in the presence of calcium ion or Gly-Pro-Arg-Pro peptide (analogue for so-called knob 'A') and factor XIIIa-catalyzed fibrinogen crosslinking demonstrated that the calcium binding sites, hole 'a' and D:D interaction sites were all markedly impaired, whereas γN308Kwas impaired at the latter two sites. Molecular modeling demonstrated that γT305 is localized at a shorter distance than γN308 from the high affinity calcium binding site and hole 'a'. ***Conclusion:*** Our

findings suggest that γT305 might be important for construction of the overall structure of the γ module of fibrinogen. Substitution of γT305A leads to both dysfibrinogenemic and hypofibrinogenemic characterization, namely hypodysfibrinogenemia. We have already reported that recombinant γT305A fibrinogen was synthesized normally and secreted slightly, but was significantly reduced.

Key Words: hypodysfibrinogenemia, fibrin polymerization, high affinity calcium binding sites, hole 'a', D:D interaction sites

Abbreviations: APTT: activated partial thromboplastin time, CHO: Chinese hamster ovary, EDTA: ethylenediaminetetraacetic acid, FpA: fibrinopeptide A, FpB: fibrinopeptide B, GPRP: Gly-Pro-Arg-Pro, HBS: N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], pH 7.4, 0.12 M NaCl, HEPES: N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], PAGE: polyacrylamide gel electrophoresis, PT: prothrombin time, SDS: sodium dodecyl sulfate, SEM: scanning electron microscopy.

Introduction

Fibrinogen is a 340 kDa plasma hexameric glycoprotein composed of two sets of three different polypeptide chains (Aα: 610, Bβ: 461, and γ: 411 residues) [1], and encoded by three genes, *FGA*, *FGB*, and *FGG*, respectively. Each chain is synthesized, assembled into a three-chain monomer, (Aα-Bβ-γ), by disulfide bonds, further held together into a six-chain dimer, $(Aα\text{-}Bβ\text{-}γ)_2$, by disulfide bonds in the hepatocytes [2], finally secreted into the blood and is present at 1.8-3.5 g/l in plasma. The six chains are arranged into three globular nodules. The central E region contains the N-termini of all chains and the distal D regions contain the C-termini of the Bβ, γ and a short segment of the Aα chains. The C-termini of the Aα chains (αC domains) extend briefly through the D regions and fold back into coiled-coil connectors. Coiled-coil connectors composed of all three chains link the E and D nodules [3].

During thrombin-catalyzed fibrin polymerization, thrombin cleaves fibrinogen, releasing fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from the N-termini of the Aα and Bβ chains, respectively, and converting fibrinogen to fibrin monomers [4]. Fibrin monomers polymerize spontaneously through a two-step process. In the first step, the release of FpA exposes a new N-terminal segment, knob 'A', which starts with sequence Gly-Pro-Arg-, and binds to hole 'a' in the γ module of another fibrin molecule.

These A:a interactions mediate the formation of double-stranded protofibrils with half-staggered overlap between molecules in different strands [4]. The end-to-end alignment of monomers in each protofibril strand requires so-called D:D interaction, which abuts the γ-chain of two adjacent molecules [5]. In the second step, these protofibrils grow in length and thrombin cleaves FpB, which exposes a new N-terminal segment, knob 'B', which likely interacts with hole 'b' in the β module of the D region of another molecule to promote lateral aggregation of the protofibrils [6], resulting in the formation of thicker fibers and finally an insoluble fibrin clot consisting of a multi-stranded and branched fiber network [7].

Inherited fibrinogen disorders causing quantitative or qualitative alterations of this molecule have been phenotypically as hypofibrinogenemia, afibrinogenemia, dysfibrinogenemia. As many as 400 families with inherited fibrinogen disorders have been analyzed genetically and/or structurally. These are listed on the GEHT homepage [8] (updated on 26/01/2012) (http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html). Crystallographic studies have provided high-resolution structures of the γ module, which has several important sites relating to fibrin polymerization, including the high affinity calcium

binding site, hole ‘a’ and the D:D interaction site [9,10]. Many amino acid substitutions in the fibrinogen γ module lead to reduced fibrin polymerization, namely dysfibrinogenemia [8] and some substitutions lead to impaired synthesis and/or secretion of fibrinogen in hepatocytes, namely hypofibrinogenemia caused by heterozygotes [8,11], but no substitutions lead to afibrinogenemia caused by homozygotes [8]. Amino acid substitutions in the γ module rarely show both characteristics of dysfibrinogenemia and hypofibrinogenemia, so-called hypodysfibrinogenemia [12, 13]. The analysis of bases of dysfibrinogenemia and hypofibrinogenemia has provided useful information for understanding the molecular details of the mechanisms of thrombin-catalyzed fibrin polymerization [14,15] and fibrinogen synthesis and/or secretion of hepatocytes [11,16,17], respectively.

We examined a girl with a low plasma fibrinogen level determined by both functional and antigenic measurement but each level was incompatible (functional/antigenic ratio = 0.295), suggesting hypodysfibrinogenemia. DNA sequence analysis revealed the heterozygous substitution of Ala for Thr at γ305 residue (mature protein; γT305A). We designated this variant as fibrinogen Nagakute. Since we had already reported the synthesis and/or secretion of γT305A fibrinogen [18], in this report, we purified the recombinant γT305A fibrinogen and analyzed the markedly impaired

function of fibrin polymerization, and compared it with a nearby recombinant variant fibrinogen, γN308K [19], which was synthesized based on dysfibrinogenemia [20].

Materials and methods

This study was approved by the Ethics Review Board of Shinshu University School of Medicine. After informed consent had been obtained from the patients, blood samples were collected for biochemical and genetic analyses.

Patients and coagulation screening tests

The proposita of Nagakute was a 6-month-old girl with suspected Kawasaki disease who had no history of bleeding or thrombosis. Nine volumes of blood were collected from intermediate cephalic vein into plastic tubes containing one volume of 3.2% trisodium citrate. Plasma was separated by centrifugation at 1500 x g for 10 minutes at 4 °C. Routine coagulation screening tests showed a markedly low concentration of plasma fibrinogen.

Prothrombin time (PT), activated partial thromboplastin time (APTT), and the fibrinogen concentration, which were determined by the thrombin time method, were measured with an automated analyzer, Coagrex-800 (Sekisui Medical Co., Tokyo,

Japan). The immunological fibrinogen concentration was determined by a latex photometric immunoassay using anti-fibrinogen antibody-coated latex particles (Mitsubishi Chemical Medience Co., Tokyo, Japan) [21].

Polymerase chain reaction (PCR) and DNA sequence analysis

Genomic DNA was extracted from white blood cells using a DNA Extraction WB Kit (Wako Pure Chemical Ltd, Osaka, Japan), according to the manufacturer's instructions. To analyze all exons and exon–intron boundaries of the fibrinogen genes, long-range PCR for *FGA*, *FGB* and *FGG* was performed using the TaKaRa LA Taq (Takara Bio Inc., Otsu, Japan) and the three pair of primers, as described elsewhere [17]. The purified PCR products were directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 3100 Genetic analyzer (both from Applied Biosystems, Foster City, CA) and 28 pairs of primers.

Preparation of recombinant variant fibrinogens

Recombinant variant fibrinogen was prepared as previously described. Briefly, the variant fibrinogen γ-chain expression vector, pMLP-γT305A, was altered from pMLP-γ [18] at codon 305 ACA (Thr) to GCA (Ala) by oligonucleotide-directed mutagenesis

using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The resultant expression vector γT305A was cotransfected with the histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Aα and Bβ chains (AαBβ CHO cells), using a standard calcium-phosphate co-precipitation method [11]. A selected and cloned cell line was cultured in serum-free medium using the roller bottle culture system. Recombinant fibrinogen, γT305A was purified from harvested culture medium by immunoaffinity chromatography, utilizing a calcium-dependent monoclonal antibody (IF-1; Iatron Laboratories, Tokyo, Japan) [14]. The fibrinogen concentration was determined from A280-320, assuming that a 1 mg/mL solution has an absorbance of 1.51. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reduced conditions (10% polyacrylamide gel). Purified γN308K fibrinogen, the neighboring residue at γT305, and wild-type (normal) fibrinogen were used as control fibrinogen [19].

Thrombin-catalyzed fibrin polymerization and clottability

Polymerization was followed by measuring the change in turbidity at 350nm at ambient temperature using a UV-140-02 spectrophotometer (Shimadzu, Tokyo, Japan).

Reactions were performed in a final volume of 100 μl, as described elsewhere [15]. Briefly, fibrinogen (90 μl at 0.44 or 0.22 mg/mL) in 20 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (HBS buffer) was mixed with human α-thrombin (Enzyme Research Laboratories, South Bend, MA, USA, 10 μl at 0.5 U/mL). Three parameters, lag time, the maximum slope, and the absorbance change (ΔOD) for 30 min, were obtained from the turbidity curves, as previously described [14]. The reactions were performed in triplicate for each sample.

The clottability of purified fibrinogens was determined essentially as described before [14], human α-thrombin (final concentration, 0.05 U/mL) being mixed with fibrinogen (final concentration, 0.4 mg/mL) in HBS buffer. Samples were incubated overnight at ambient temperature. After centrifugation at 15 000 rpm for 10 minutes, the fibrin(ogen) not incorporated into the fibrin gel was determined from the A_{280} value of the supernatant, and clottability was calculated as (Abs_{280} at zero time × 0.9 – Abs_{280} of the supernatant) / (Abs_{280} at zero time × 0.9) × 100 (%).

Plasmin protection assay

Fibrinogens (0.30 mg/mL) in HBS buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 1 or 5 mM $CaCl_2$, and 1 or 5 mM GPRP (the synthetic peptides

Gly-Pro-Arg-Pro acetate salt, purity >97%; Sigma-Aldrich, St. Louis, MO), were incubated with 0.18 U/mL plasmin (Chromogenix AB, Molngal, Sweden) for 2 h at 37 °C. The reaction was stopped by adding SDS sample buffer and heating at 100 °C for 5 minutes. The plasmin digests were then analyzed on 10% gels that were stained with Coomassie brilliant blue R-250 [22].

Factor(F)XIIIa-catalyzed cross-linking of fibrin or fibrinogen

Factor (F) XIIIa (800 U/mL; Enzyme Research Laboratories) was activated with human α-thrombin (2 U/mL) for 60 minutes at 37 °C in HBS buffer with 5 mM $CaCl_2$. To examine the cross-linking of fibrin, fibrinogen (final concentration, 0.47 mg/mL) was incubated at 37 °C with FXIIIa (final concentration, 3.3 U/mL) and human α-thrombin (final concentration, 0.07 U/mL) in HBS buffer with 0.67 mM $CaCl_2$. To examine the cross-linking of fibrinogen, hirudin (10 U/mL) was added to thrombin-activated FXIIIa (final concentration, 8.3 U/mL) prior to incubation with fibrinogen (final concentration, 0.47 mg/mL) in HBS buffer with 0.67 mM $CaCl_2$. The reactions were stopped at various times by addition of an equal volume of SDS sample buffer with 2-mercaptoethanol and incubation at 100 °C for 5 minutes. Samples equivalent to 4.7 μg fibrinogen were separated on 8% SDS-PAGE and stained with Coomassie brilliant blue R-250 [15].

Scanning electron microscopy (SEM)

The SEM preparation was performed as described previously [15]. Thrombin (10 μl, 0.5 U/mL) was added to fibrinogen solution (40 μl, 0.4 mg /mL) and mixed by repeated pipetting. Polymerization proceeded overnight at 37 °C, the clots were then fixed in 2.5% glutaraldehyde overnight, stained with 1% osmium tetroxide, mounted, osmium plasma-coated at 5-nm thickness in an Neo-AN (Meiwafosis Co. Ltd, Tokyo, Japan), and viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan). An image was taken at 3000x or 20 000x with 5-kV accelerating voltage. Fiber diameters were measured using a Vernier clipper on a 300% enlargement of a photograph at 20 000x magnification.

Molecular modeling

We generated the model of γ module of fibrinogen using the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) 2FIB [9] and Swiss-Pdb Viewer 4.0.1 (http://spdbv.vital-it.ch/).

Statistical analysis.

The statistical significance of differences between wild-type and variant fibrinogen was determined using one-way ANOVA (analysis of variance) and following Tukey-Kramer tests. A difference was considered significant when $p < 0.05$.

Results

Coagulation screening tests and DNA sequence analysis

The patient's PT and APTT were 13.4 and 37.1 seconds (normal range 10.8 to 13.2 and 23.0 to 38.0 seconds), respectively. The plasma fibrinogen concentration determined by the thrombin time method was 0.33 g/L and that determined by the immunological method was 1.12 g/L (normal range: 1.80 to 3.50 g/L). DNA sequence analysis revealed a novel heterozygous mutation of A>G in *FGG* exon 8 (at c.1132A>G; NCBI NM 000509.4), resulting in substitution of Ala (GCA) for Thr (ACA) at γ305 residue (mature protein) or γ331 residue (native protein) (γT305A).

Synthesis and characterization of recombinant fibrinogen

We synthesized recombinant γT305A fibrinogen and purified as described in Materials and methods. SDS-PAGE performed under reduced conditions indicated that the γT305A fibrinogen was pure and that the usual pattern of 3 bands corresponded to the

Aα, Bβ and γ chain in (Figure 1), whereas the migration of the γ-chain band in γN308K was faster than normal, as previously reported [19] [20].

Thrombin-catalyzed fibrin polymerization and clottability

Thrombin-catalyzed fibrin polymerization was performed using the procedure described in Materials and methods. Under conditions with 0.4 mg/mL fibrinogen in the presence of 1.0 mM $CaCl_2$, polymerization of γT305A fibrinogen was markedly reduced compared with that of γN308K fibrinogen (Figure 2A, 2B and Table 1). Namely, the lag time of γT305A fibrinogen was 16-fold and 4.6-fold longer than normal and γN308K fibrinogen, respectively, and the maximum slope was 30-fold and 11-fold slower than normal and γN308K fibrinogen, respectively. Furthermore, in the absence of $CaCl_2$, the slight turbidity increase of γT305A started 140 minutes after the addition of thrombin and the absorbance reached only 0.016 ± 0.004 at 10 hours (Table 1).

To determine whether variant fibrinogens inhibit or participate in fibrin fiber formation of normal fibrinogen, mixing experiments of variant fibrinogens with normal fibrinogen were performed, and these polymerization curves were compared to those generated from half-concentrations of normal fibrinogen. The turbidity observed for an equimolar mixture (0.1 mg/mL) of γT305A with normal fibrinogen neither increased

nor decreased (Figure 2C). In contrast, the turbidity observed for γN308K with normal fibrinogen polymerized with a 2.7-fold shorter lag time and 2.4-fold maximum slope than γN308K fibrinogen at 0.1 mg/mL and also with a 1.3-fold longer lag time and 1.4-fold maximum slope than normal fibrinogen at 0.1 mg/mL (Figure 2D).

Next, to determine whether variants monomers were incorporated into fiber, we examined clottability in duplicate, as described in Materials and methods. The values obtained for the percentage of fibrin(ogen) incorporated into fibrin gels were smaller: 62.1 ± 0.8 for γT305A fibrinogen (p <0.01), and 80.3 ± 0.3 for γN308K fibrinogen (p <0.01), compared with 93.9 ± 1.1 for normal fibrinogen.

Plasmin protection assay

To assess the impaired function of hole ’a’ and calcium binding sites, we performed a plasmin protection assay, as described in Materials and methods. In the presence of only EDTA, the absence of GPRP or $CaCl_2$, D_1 fragments derived from normal were cleaved to the smaller fragments D_2 and D_3, indicating no protection from plasmin digestion. In the presence of 1 mM $CaCl_2$ or 5 mM GPRP, D_1 fragments derived from normal fibrinogen were not cleaved to fragment D_2 and D_3, indicating protection from plasmin digestion (Figure 3A). D_1 fragments derived from γT305A were digested into fragments

D_2 and D_3 in the presence of 5 mM $CaCl_2$ or 5 mM GPRP, suggesting no protection from plasmin digestion (Figure 3B). In contrast, D_1 fragment derived from γN308K was not digested in the presence of 5 mM $CaCl_2$ but was digested in the presence of 5 mM GPRP (Figure 3C). These results indicate that the functions of both hole 'a' and calcium binding sites were impaired in γT305A, and only the function of hole 'a' was impaired in γN308K.

FXIIIa-catalyzed cross-linking of fibrin or fibrinogen

Cross-linking of fibrin α and γ chains was performed in the presence of FXIIIa and thrombin, and the reaction products were analyzed by SDS-PAGE, as described in Materials and methods. With normal fibrin, γ-γ dimer bands were evident after 5 minutes, and two α-polymer bands appeared, weakly evident at 5 minutes. With longer incubation, the intensity of γ-γ dimer and α-polymer bands increased, whereas the intensity of α- and γ-chain bands decreased (Figure 4A). With γT305A, the γ-γ dimer band appeared markedly slower and the α-polymer band appeared slightly slower than normal fibrin; namely these bands were faintly evident after 20 and 10 minutes, respectively, and the increase of the intensity of these bands was slower than normal (Figure 4B). With γN308K, γ-γ dimer and α-polymer bands also appeared slower than

normal fibrin; these bands were weakly evident after 5 and 10 minutes, respectively (Figure 4C). Less impaired γ-γ dimer formation of γT305A fibrin than that of γN308K fibrin reflected the degree of impairment of thrombin-catalyzed fibrin polymerization.

To confirm the difference in D:D interactions between γT305A and γN308K, we examined the F XIIIa-catalyzed cross-linking of fibrinogen. After F XIII had been activated with thrombin, hirudin was added to inhibit thrombin, which catalyzes fibrinopeptide release and fibrin polymerization. After a 30-minute reaction, γ-γ dimer bands were clearly evident for normal fibrinogen and weakly evident for both variant fibrinogens (Figure 4D-F). With normal fibrinogen, the intensity of γ-γ dimer bands increased with longer incubation (Figure 4D). In contrast, for both variant fibrinogens, the increase of the intensity of γ-γ dimer bands was markedly slower than normal, and γN308K fibrinogen was still slower than γT305A fibrinogen (Figure 4E, 4F). These results reflected the degree of impairment of D:D interaction. Therefore, α-polymer formation was not impaired and an α-polymer band was evident after 30 minutes for normal and both variant fibrinogens.

Scanning electron microscopy (SEM)

To analyze the difference in the ultrastructure of fibrin clots among normal, γT305A and

γN308K fibrinogen, we observed fibrin clots under an SEM (Figure 5). A clot prepared from γT305A fibrinogen had less density than the bundle of fibrin fibers (Figure 5B), and had larger pores than normal fibrinogen (Figure 5A) and γN308K fibrinogen (Figure 5C). Measurement of the fiber diameters showed that the variant fibers were thicker: 247 ± 80 nm for γT305A fibrinogen (n = 23, p <0.01), and 171 ± 45 nm for γN308K fibrinogen (n = 29, no significance), compared with 112 ± 11 nm for normal fibrinogen (n = 30). In addition, fibers derived from γT305A fibrinogen (Figure 5E) showed more twisted fibers than normal (Figure 5D) and γN308K fibrinogen (Figure 5F).

Discussion

We identified a novel heterozygous hypodysfibrinogenemia, Nagakute, γT305A (p.γT331A). Coagulation screening test suggested that the proposita had hypodysfibrinogenemia, because the functionally and immunologically determined plasma fibrinogen concentration was 0.33 and 1.12 g/L, both lower than the normal range but markedly different from each other. However, there is a possibility that the plasma fibrinogen concentration of newborn infant is lower than that of adults. Therefore, we examined recombinant variant fibrinogen production of γT305A using

CHO cells, resulting in mildly but significantly reduced secretion of fibrinogen compared with normal fibrinogen-synthesizing cell lines, as already described elsewhere [18]. Although there is a possibility that characterization of fibrinogen synthesis and secretion are not coincident between human hepatocytes and CHO cells, we surmised that γT305A variant fibrinogen might be a mild hypofibrinogenemic feature. Because heterozygous γH307Y variant fibrinogen, which is a neighboring residue of γT305, was reported as hypofibrinogenemia [23], and markedly reduced secretion of the recombinant variant fibrinogen was demonstrated using yeast, *Pichia pastoris* [24].

In this report, we purified recombinant γT305A fibrinogen, analyzed its function and compared with that of recombinant γN308K fibrinogen, because we did not obtain enough of the proposita's blood to purify plasma fibrinogen as she was a newborn. The γT305A fibrinogen indicated markedly impaired thrombin-catalyzed fibrin polymerization in the presence or absence of 1 mM calcium ion and was clearly different from that of γN308K fibrinogen. Plasmin protection assay revealed that the functions of calcium binding sites and hole 'a' were markedly impaired for γT305A fibrinogen, whereas γN308K fibrinogen had almost normal function of calcium binding sites and markedly impaired function of hole 'a', which was mildly impaired compared

with that of γT305A fibrinogen. Furthermore, the function of the D:D interaction of both γT305A and γN308K fibrinogen, which was determined by FXIIIa-catalyzed cross-linking of fibrinogen γ-chain (γ-γ dimer formation), was markedly reduced compared with that of normal fibrinogen, but γT305A fibrinogen was less impaired than γN308K fibrinogen. The greater impairment of the D:D interaction of γN308K fibrinogen might have been caused by the charge repulsion with γK321 [25]. On the other hand, FXIIIa-catalyzed cross-linking of fibrinogen α-chain (α-polymer formation) was not different among normal and both variant fibrinogens. These results indicate that α-polymer formation of fibrinogen was not influenced by the impairment of the D:D interaction, because α-polymer is formed between two flexible αC domains in different molecules [26].

To demonstrate the markedly aberrant function of the γ module of γT305A, a tertiary structure was depicted and compared with that of γN308K fibrinogen (Figure 6A). Residue γT305 is localized inside the γ module, whereas residue γN308 is localized outside the γ module. Furthermore, γT305 was in the vicinity of the high affinity calcium binding site and hole ‘a’, both at a shorter distance than γN308. Four hydrogen bonds are formed between γT305 and γD301 or γF293, whereas only one hydrogen bond is formed between variant γA305 and γD301 (Figure 6B and C). For γN308K, the

three hydrogen bonds between γN308 and γY278 or γG309 are reduced to only one hydrogen bond between γK308 and γY278 (Figure 6D and E). Altogether, substitution of Ala for Thr at γ305 leads to the abolition of three hydrogen bonds inside the γ module, which might induce a marked conformational change of the tertiary structure of the high affinity calcium binding site, hole 'a' and the D:D interaction site [9,10], resulting in markedly impaired fibrin polymerization compared with γN308K.

Heterozygous γN308K, γN308I, γG309D, and γM310T variant fibrinogens [8] near the γT305 residue were reported as dysfibrinogenemias and their thrombin-catalyzed fibrin polymerization was impaired, but to a lesser degree than γT305A [20, 27-29]. Furthermore, Park demonstrated that recombinant γA341D and γA341V fibrinogens showed markedly impaired thrombin-catalyzed fibrin polymerization, causing aberrant calcium binding sites and hole 'a' [22]. Of interest, molecular modeling of the γ module of γA341D fibrinogen demonstrated that the alteration of residue γA341 to γD341 led to a clash between the side chain of γD341 and the backbone of γT305, resulting in aberrant hole 'a' conformation and impaired fibrin polymerization [22]. Polymerization at the same fibrinogen concentration as 0.2 mg/mL indicated that the lag time of γT305A fibrinogen was 90 minutes and 1.8 fold longer than that of γA341D fibrinogen [22]; namely, γT305A was still more impaired than γA341D.

In conclusion, a novel heterozygous hypodysfibrinogenemia, γT305A, indicated that markedly impaired thrombin-catalyzed fibrin polymerization was caused by the marked conformational change of the tertiary structure of the high affinity calcium binding site, hole 'a' and D:D interaction site. These findings suggest that γT305 might be important for the construction of the overall structure of the γ module of fibrinogen.

Authorship

M. Ikeda performed all experiments, analyzed data, and wrote the paper. T. Kobayashi established CHO cell lines and performed the secretion experiment. Y. Takezawa performed polymerization, clottability, and scanning electron microscopy. S. Arai, S. Mukai, F. Terasawa, and N. Okumura designed the research and discussed the data. N. Okumura reviewed the paper.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

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Table legend

Table 1. Three parameters of thrombin-catalyzed fibrin polymerization.

Three parameters, lag time, the maximum slope, and the absorbance change (ΔOD), were obtained from the turbidity curves and the data are the mean ± standard deviation of triplicate experiments. ΔOD for normal or γN308K fibrinogen was obtained at 30 minutes, whereas ΔOD for γT305A fibrinogen was obtained at 10 hours. ND: not determined.

Figure legends

Fig. 1. Characterization of recombinant fibrinogens. Purified fibrinogen was resolved on 10% SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue R-250. Lane 1: wild type (normal), lane 2: γT305A, lane 3: γN308K fibrinogen, lane M: molecular marker.

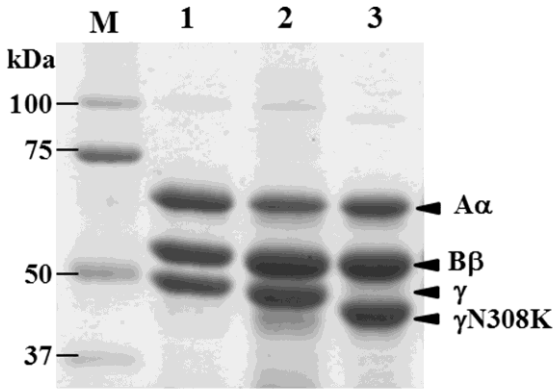


Fig. 2. Thrombin-catalyzed fibrin polymerization. Polymerization of fibrinogen (0.4 mg/mL) (A and B) was initiated with thrombin (0.05 U/mL), and the change in turbidity with time was followed at 350 nm for normal (1.0 mM $CaCl_2$; ●, 0.0 mM $CaCl_2$; ○), γT305A (1.0 mM $CaCl_2$; ■, 0.0 mM $CaCl_2$; □) and γN308K (1.0 mM $CaCl_2$; ▲, 0.0 mM $CaCl_2$; △) fibrinogen in HBS buffer. Polymerization was performed with 0.1 mg/mL normal (○), 0.2 mg/mL normal (●) or γA305 fibrinogen (■), and an equimolar (0.1 mg/mL) mixture of normal and γT305A fibrinogen (□) (C), and polymerization was performed with 0.1 mg/mL normal (○), 0.2 mg/mL normal (●) or γN308K fibrinogen (▲), and an equimolar (0.1 mg/mL) mixture of normal and γN308K fibrinogen (△) (D).

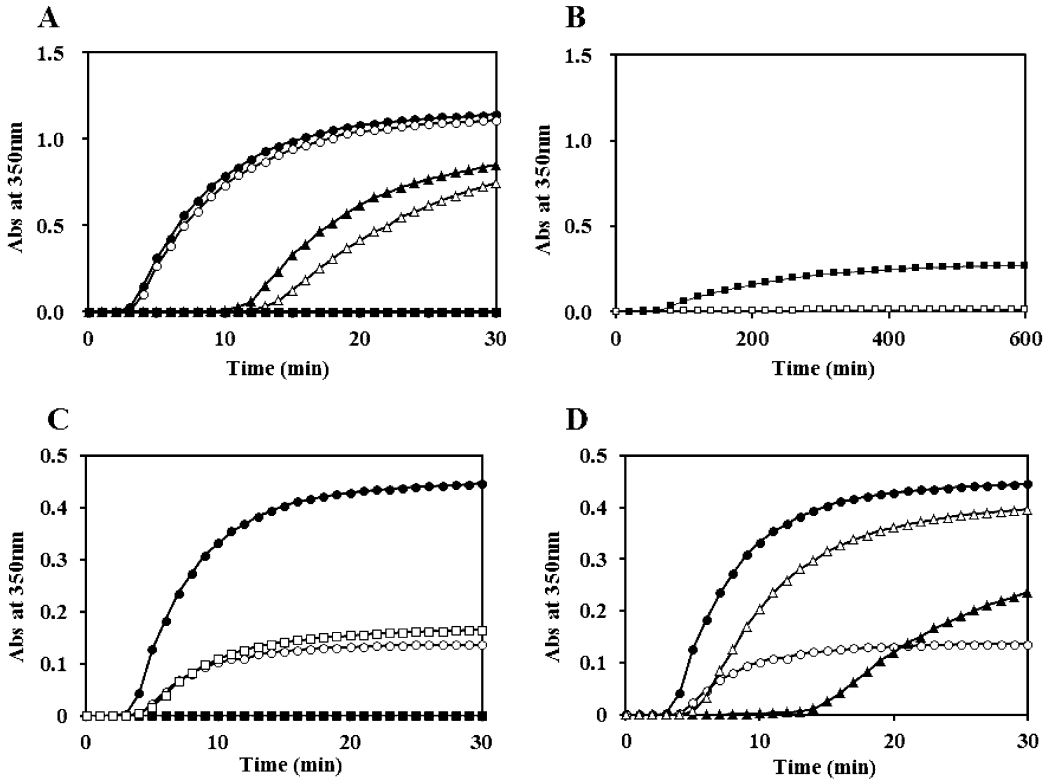

A
B
C
D
Abs at 350nm
Time (min)
1.5
1.0
0.5
0.0
0.4
0.3
0.2
0.1
0
10
20
30
200
400
600

Fig. 3. Plasmin protection assay. Fibrinogen (0.30 mg/mL) in HBS buffer containing 5 mM EDTA (lane 1), 1 (lane 2) or 5 mM $CaCl_2$ (lane 3), and 1 (lane 4) or 5 mM GPRP (lane 5) was incubated with 0.18 U/mL plasmin for 2 h at 37 °C. The digests were analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue. The degradation products are indicated as D1, D2, and D3 on the left side of the gel. Lane M: molecular marker.

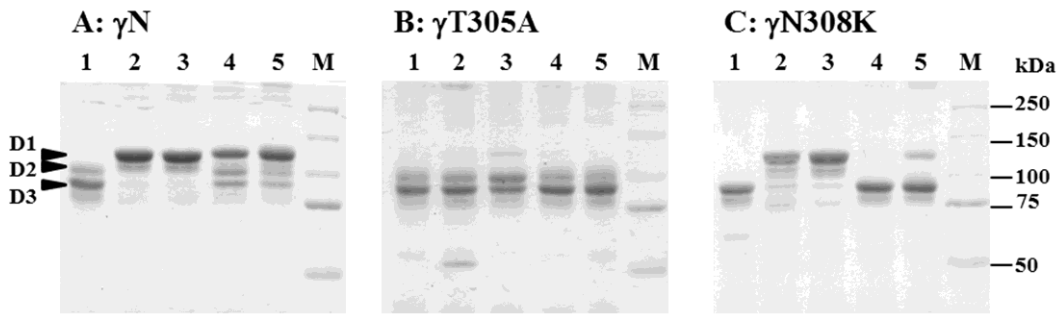


Fig. 4. F XIIIa-catalyzed cross-linking of fibrin or fibrinogen. Cross-linking of fibrin (Normal: A, γT305A: B, γN308K: C) or fibrinogen (Normal: D, γT305A: E, γN308K: F) by F XIIIa were examined by 8% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue R-250, as described in Materials and methods. The reduced fibrin or fibrinogen chains (Aα, Bβ, γ, cross-linked γ-γ dimer, and cross-linked α-chain polymers) are indicated on the left side of the gel.

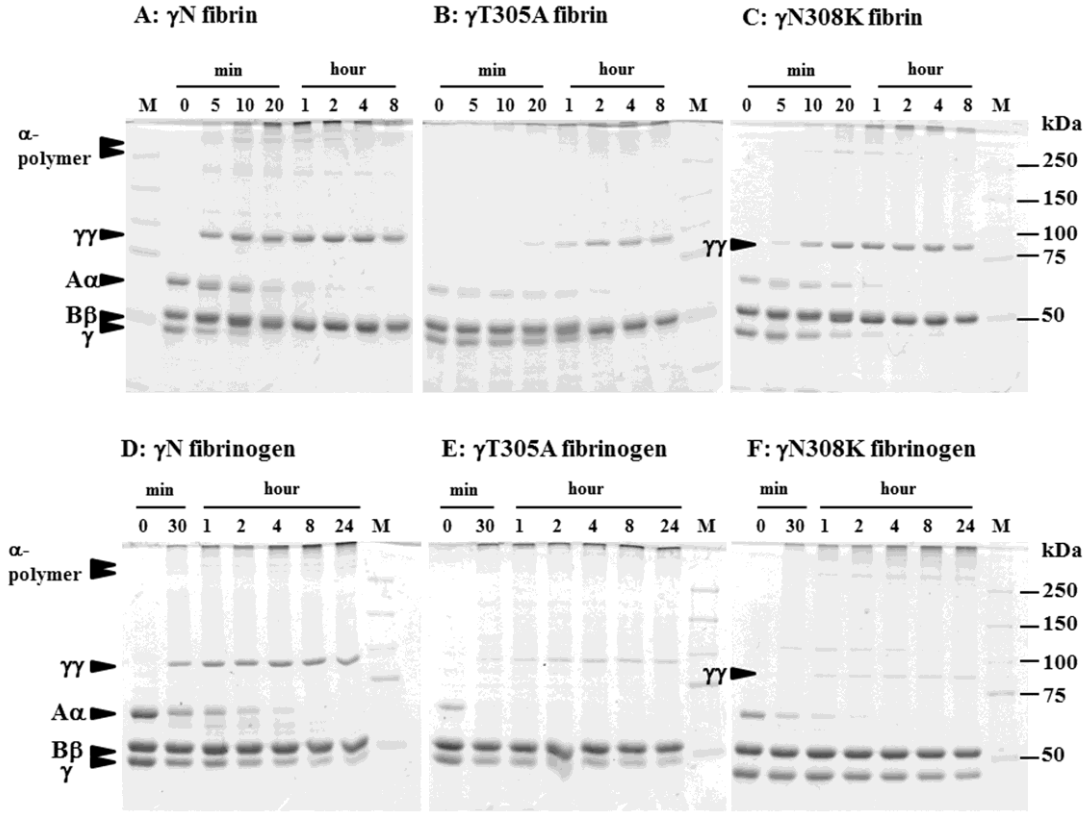


Fig. 5. Scanning electron microscopy (SEM) of fibrin clot. All samples were prepared as described in Materials and methods. Micrographs were taken at 3000x (Normal: A, γT305A: B, γN308K: C) and 20 000x (Normal: D, γT305A: E, γN308K: F). White bar represents 10 μm in A~C and 1.0 μm in D~F.

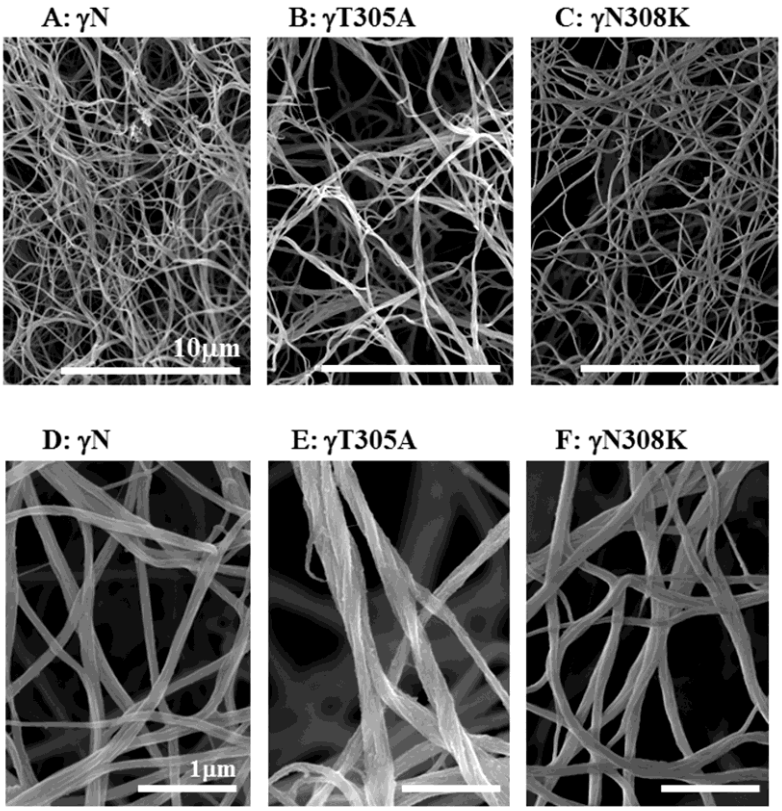
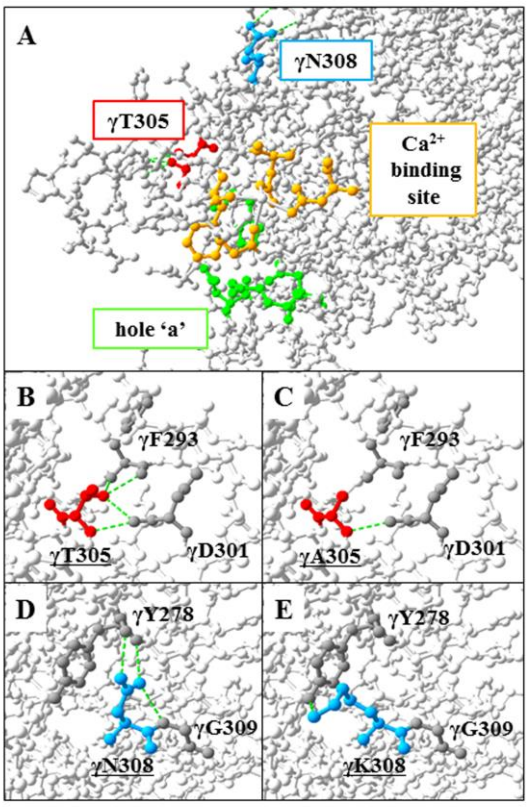


Fig. 6. Conformational models of normal and variant γ module. The γ module was depicted as a ball and stick model with Swiss-Pdb Viewer 4.0.1. Residue γT305 (red) is localized inside the γ module, whereas γN308 (blue) is localized of the surface of the γ module. Furthermore, γT305 is nearer the high affinity calcium binding site (yellow: γD318, γD320, γF322, γG324) and hole 'a' (green: γQ329, γD330, γH340, γD364) than γN308 (A). Four hydrogen bonds (shown as green dotted line) formed between γT305 and γD301 or γF293 (B), whereas only one hydrogen bond formed between γA305 and γD301 (C). Three hydrogen bonds between γN308 and γY278 or γG309 (D) were reduced to only one hydrogen bond between γK308 and γY278 (E).



Fibrinogen (mg/mL)	γN			γT305A			γN308K		
	0.18	0.36		0.18	0.36		0.18	0.36	
$CaCl_2$ (mM)	1.0		0.0	1.0		0.0	1.0		0.0
lag time (min)	3.5 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	90.0 ± 17.3	52.5 ± 3.5	ND	14.2 ± 1.2	11.4 ± 0.5	13.0 ± 0.0
maximum slop (x10^4/sec)	72.1 ± 6.4	95.1 ± 6.1	94.4 ± 2.5	0.5 ± 0.1	3.2 ± 0.0	ND	17.8 ± 1.1	34.0 ± 1.0	27.9 ± 0.9
⊿Abs	0.461 ± 0.021	1.141 ± 0.072	1.107 ± 0.144	0.076 ± 0.004	0.265 ± 0.008	0.016 ± 0.004	0.237 ± 0.012	0.847 ± 0.016	0.745 ± 0.005

Table1