Differences in the function and secretion of congenital aberrant fibrinogenemia between heterozygous γD320G (Okayama II) and γΔN319-ΔD320 (Otsu I)

Saki Mukai,[a,b] Minami Ikeda[a,b] Yuka Takezawa,[b] Mitsutoshi Sugano,[b] Takayuki Honda,[c] Nobuo Okumura[a]

[a] Department of Health and Medical Sciences, Graduate School of Medicine, Shinshu University, Matsumoto, Japan
[b] Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan
[c] Department of Laboratory Medicine, Graduate School of Medicine, Shinshu University, Matsumoto, Japan

Address correspondence to:
Nobuo Okumura, Ph.D.
Laboratory of Clinical Chemistry
Department of Biomedical Laboratory Sciences
School of Health Sciences
Shinshu University
3-1-1 Asahi, Matsumoto 390-8621, Japan
Tel.: 81-263-37-2392
Fax: 81-263-37-2370
E-mail: nobuoku@shinshu-u.ac.jp

Word count: Abstract; 240
Manuscript; 4470 (excluding title page, Abstract, and References)
Tables: 0, Figures: 5, References: 28

Abstract

Background: We encountered two patients with hypodysfibrinogenemia and designated them as Okayama II and Otsu I. Although the affected residue(s) in Okayama II and Otsu I overlapped, functionally determined fibrinogen levels and the ratio of functionally to immunologically determined plasma fibrinogen levels were markedly different. *Methods*: DNA sequence and functional analyses were performed for purified plasma fibrinogen. A recombinant protein was synthesized in Chinese hamster ovary (CHO) cells to determine the secretion of variant fibrinogens. *Results*: A heterozygous A>G in *FGG*, resulting in γ320Asp>Gly for Okayama II, and a heterozygous deletion of AATGAT in *FGG*, resulting in the deletion of γAsn319 and γAsp320 (γΔN319-ΔD320) for Otsu I, were obtained. SDS-PAGE and Coomassie staining revealed that the variant γ-chain was not clear in Okayama II, but was clearly present in Otsu I. The lag period for the fibrin polymerization of Okayama II was slightly slower than that of the normal control, whereas Otsu I fibrinogen indicated no polymerization within 30 minutes. Both variant γ-chains were synthesized in CHO cells and assembled into fibrinogen; however, the fibrinogen concentration ratio of the medium/cell lysate of γ320Gly was six-fold lower than that of γΔN319-ΔD320. *Conclusions*: We concluded that the plasma fibrinogen of Okayama II, constituted by a lower ratio of the variant γ-chain, led to the almost normal functioning of fibrin polymerization. However, the plasma fibrinogen of Otsu I, with a higher ratio of the variant γ-chain, led to marked

reductions in fibrin polymerization.

Key words: fibrinogen, γ-chain, dysfibrinogenemia, hypofibrinogenemia, secretion

Abbreviations: APTT: activated partial thromboplastin time, CHO: Chinese hamster ovary, EDTA: ethylenediaminetetraacetic acid, ELISA: enzyme-linked immunosorbent assay, FpA: fibrinopeptide A, FpB: fibrinopeptide B, GPRP: Gly-Pro-Arg-Pro, HEPES: N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], PAGE: polyacrylamide gel electrophoresis, PCR: polymerase chain reaction, PT: prothrombin time, SDS: sodium dodecyl sulfate

1. Introduction

Fibrinogen is a 340 kDa plasma glycoprotein that is composed of two sets of three different polypeptide chains: Aα, Bβ, and γ, which are stabilized by 29 disulfide bonds, including intrachain and interchain connections and represented as $(Aα\text{-}Bβ\text{-}γ)_2$ [1]. The fibrinogen molecule has a trinodular structure in which the central E region contains the N-termini of all six chains and the external D regions contain the C-termini of the Bβ- and γ-chains and a short segment of the Aα-chains. The C-termini of the Bβ- and γ-chains are called the βC and γC modules, respectively. The E and two D regions are linked by two coiled-coil connectors,

consisting of all three chains [1]. The C-termini of the Aα-chains (αC domains) extend briefly through the D regions and fold back into coiled-coil connectors [1]. Three chains are coded by *FGA*, *FGB*, and *FGG* [2], and synthesized, assembled into a six-chain molecule in hepatocytes, and secreted into blood.

During coagulation, thrombin cleaves fibrinogen, releasing two fibrinopeptide A (FpA) and two fibrinopeptide B (FpB) from one fibrinogen molecule, thereby converting it into a fibrin monomer [3]. Fibrin monomers spontaneously polymerize through a two-step process, and finally make fibers and coalesce to form a fibrin clot. In the first step, the release of FpA exposes a hidden knob 'A' that binds to hole 'a' in the γ-module of another fibrin molecule. These A:a interactions and subsequent D:D interactions (end-to-end junction) mediate the formation of half-staggered, double-stranded fibrin oligomers [3]. In the second step, these fibrin oligomers grow in length (20 to 25 half-staggered fibrin monomers; so-called protofibrils) and thrombin cleaves FpB, which exposes a hidden knob 'B', dissociates the αC regions from the central E region, and forms αC-polymers (so-called lateral aggregation). Lateral aggregation then makes thicker fibers and coalesces to form a complex, branching fibrin clot network [3]. Several important functional sites for fibrin polymerization and the interaction site for platelet thrombus formation have been defined in the γC module; hole 'a' (Q329, D330, and D364) [4, 5], high affinity calcium-binding site (D318, D320, F322, and G324) [6], D:D interaction site (γ275-300) [5], lateral aggregation sites (γ350-360 and

γ370-380) [7], FXIIIa-catalyzed cross-linking site (E398 on one molecule and K406 on another molecule) [8], and platelet-binding site (the last four residues, γ408-411) [9].

Almost 300 species of genetic mutations in the fibrinogen genes, *FGA*, *FGB*, and *FGG*, have been associated with either the phenotype of afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, or renal amyloidosis, as listed in the fibrinogen variant data base [10], and the function and genetic and/or post-translational changes causing these phenotypes have been analyzed by molecular bases. In addition, hypodysfibrinogenemia, which has the characteristics of dysfibrinogenemia and hypofibrinogenemia, has mainly been reported in *FGB* or *FGG*, caused by a heterozygous missense or one or two residue deletion mutations [10]. The residue at γN319 or γD320, heterozygous deletion or substitution variants, have been reported as dysfibrinogenemia or hypodysfibrinogenemia; γΔN319 [10], γN319K [10], γΔD320 [11,12], γD320E [13], γD320G [14], and the two residue deletions of γN319 and γD320 [15,16], and many of these cases have manifested as a history of bleeding and/or thrombosis.

We encountered two patients with hypodysfibrinogenemia and identified γD320G, called Okayama II, and γΔN319-ΔD320, called Otsu I, in the γC module. Although the affected residues in Okayama II and Otsu I overlapped, functionally determined fibrinogen levels and the fibrinogen concentration ratio (ratio of functionally to immunologically determined plasma fibrinogen concentrations) were markedly different. In the present study, fibrinogen

concentrations, the assembly process, and secretion of variant fibrinogens were analyzed using recombinant fibrinogen-expressing Chinese hamster ovary (CHO) cells in an attempt to clarify differences between Okayama II and Otsu I.

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 patients, blood samples were collected for biochemical and genetic analyses.

Patient and coagulation tests

The proposita of Okayama II was a 29-year-old woman who had no history of bleeding or thrombosis. Preoperative coagulation screening tests for cervix uteri carcinoma showed a lower level of plasma fibrinogen. The proposita of Otsu I was a 37-year-old woman who also had no history of bleeding or thrombosis. At 36 weeks of gestation, her fetus had been in the breech presentation. Prior to Caesarean section, her coagulation screening tests also revealed a lower level of plasma fibrinogen. Blood collection and plasma separation were performed as described elsewhere [17].

Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentrations, which were determined by the thrombin time method, were measured with the

automated analyzer, Coapresta 2000 (Sekisui Medical Co., Tokyo, Japan). Immunological fibrinogen concentrations were determined by a latex photometric immunoassay using anti-fibrinogen antibody-coated latex particles (Mitsubishi Chemical Medicine Co., Tokyo, Japan) [18].

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. In order to analyze all exons and exon-intron boundaries of the fibrinogen genes, long-range polymerase chain reaction (PCR) for *FGA*, *FGB,* and *FGG* and direct sequencing were performed as described elsewhere [19].

Purification and characterization of plasma fibrinogen

Fibrinogen was purified from citrated plasma obtained from the propositas of Okayama II and Otsu I and from a normal control (NC) subject (Japanese woman). Purification was performed by immunoaffinity chromatography, utilizing a calcium-dependent monoclonal antibody (IF-1; Iatron Laboratories, Tokyo, Japan), as described [19]. Eluted fractions were pooled and dialyzed at 4°C against 20 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (polymerization buffer). Fibrinogen

concentrations were determined at 280 nm, assuming that a 1 mg/mL solution had an absorbance of 1.51. The purity and characterization of proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-reduced conditions (7% polyacrylamide gel) and reduced conditions (10% polyacrylamide gel), followed by Coomassie Brilliant Blue R-250 staining.

After the separation of purified fibrinogen or plasma protein by a SDS-10% polyacrylamide gel under reduced conditions, immunoblots were developed with a rabbit anti-human fibrinogen antibody (DAKO, Carpinteria, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Medical and Biological Laboratories Ltd, Nagoya, Japan), or a mouse monoclonal antibody against the human fibrinogen γ-chain (2G10, specific for γ15-35; Accurate Chemical and Scientific, Westbury, NY, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Medical and Biological Laboratories Ltd), and enhanced with chemiluminescence detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on Hyperfilm-ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Thrombin-catalyzed fibrin polymerization and clottability

Thrombin-catalyzed fibrin polymerization was followed by measuring changes in turbidity at 350 nm at ambient temperature using an UVmini-1240 spectrophotometer (Shimazu, Tokyo,

Japan), as described elsewhere [19]. Three parameters: the lag time, maximum slope, and absorbance change (ΔAbs) for 30 minutes, were obtained from the turbidity curves, as previously described [19]. The reactions were performed in triplicate for each sample.

The clottability (percentage of fibrinogen incorporated into a fibrin gel) of purified fibrinogens was determined as described before [20].

Protection assay of the plasmin digestion of fibrinogen

The protection of the plasmin digestion of fibrinogen in the presence of 1 or 5 mM $CaCl_2$, and GPRP (the synthetic peptides Gly-Pro-Arg-Pro acetate salt, purity >97%; Sigma-Aldrich, St. Louis, MO), was analyzed by SDS-PAGE under non-reduced conditions (10% polyacrylamide gel) and followed by Coomassie brilliant blue R-250 staining, as described before [19].

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

Factor (F) XIIIa was activated with human α-thrombin, and, in order to examine the cross-linking of fibrinogen, hirudin was added to thrombin-activated FXIIIa prior to its incubation with fibrinogen. The reactions were stopped at various times by the addition of an equal volume of SDS sample buffer with 2-mercaptoethanol and boiling. Samples were

separated on 8% SDS-PAGE and stained with Coomassie blue R-250, as described previously [19].

Expression of recombinant variant fibrinogens

Recombinant variant fibrinogens were prepared as previously described [17]. Briefly, the variant fibrinogen γ-chain expression vectors, pMLP-γD320G, pMLP-γD320E, pMLP-γΔD320, and pMLP-γΔN319-ΔD320, were altered from the pMLP-γ plasmid, which contained the wild-type γ-chain cDNA, by oligonucleotide-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following primer pairs (the altered base is underlined);

5’-CCTGGGACAATGACAATGGTAAGTTTGAAGGCAAC-3’ (sense) and

5’-GTTGCCTTCAAACTTACCATTGTCATTGTCCCAGG-3’ (antisense) for γD320G,

5’-CCTGGGACAATGACAATGAGAAGTTTGAAGGCAAC-3’ (sense) and

5’-GTTGCCTTCAAACTTCTCATTGTCATTGTCCCAGG-3’ (antisense) for γD320E,

5’-CTGGGACAATGACAAT AAGTTTGAAGGCAACTGTG-3’ (sense) and

5’-CACAGTTGCCTTCAAACTT ATTGTCATTGTCCCA G-3’ (antisense) for γΔD320. The expression vector, pMLP-γΔN319-ΔD320, was used, as previously established [21].

The resultant expression vectors were co-transfected 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. The cell lines were designated as γD320G-, γD320E-, γΔD320-, γΔN319-ΔD320-, and normal (N)-CHO cells, respectively. Cells were cultured and colonies were selected on histidinol (Aldrich Chem. Co. Milwaukee, WI, USA), as described elsewhere [22]. Fibrinogen concentrations in cell lysates or culture media from the selected clones were measured by the enzyme-linked immunosorbent assay (ELISA), and the assembly of fibrinogen and/or synthesis of three polypeptide chains in the cell lysate was analyzed by SDS-PAGE and immunoblotting as described previously [22].

Statistical analysis

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

Result

Coagulation screening tests, DNA sequence analysis, and molecular characterization of variant fibrinogens.

PT and APTT of the Okayama II patient were 14.4 (normal range: 10.0 to 13.0 seconds) and 33.3 seconds (normal range: 23.0 to 38.0 seconds), respectively. Her plasma fibrinogen

concentration, determined by the thrombin time method, was 0.41 g/L and that determined by the immunological method was 0.85 g/L (normal range: 1.80 to 3.50 g/L). A DNA sequence analysis revealed a heterozygous mutation of A>G in *FGG* exon 8 (at c.1037A>G; NCBI NM_000509.4), resulting in the substitution of Gly (GGT) for Asp (GAT) at the γ320 residue (mature protein) or γ346 residue (native protein) (γD320G). We designated this patient as Okayama II (p.γD320G) according to the patient's residence.

PT and APTT of the Otsu I patient were 14.8 and 42.2 seconds, respectively. Her plasma fibrinogen concentration, determined by the thrombin time method, was 0.09 g/L and that determined by the immunological method was 1.43 g/L. A DNA sequence analysis revealed a heterozygous deletion of AATGAT in *FGG* exon 8 (c.1033-1038; NCBI NM 000509.4), resulting in the deletion of the γAsn319 (AAT) and γAsp320 (GAT) (mature protein) or γAsn345 and γAsp346 residues (native protein) (γΔN319-ΔD320). The patient's residence was Otsu city and the variant was same as Otsu I which we previously reported [16], therefore we designated this patient as Otsu I.

We purified plasma fibrinogens, as described in the Materials and methods section, and analyzed them using SDS-PAGE. Coomassie staining indicated that Okayama II fibrinogen was pure, the pattern of the fibrinogen band was normal under non-reduced conditions (Fig. 1A), and three bands corresponded to the Aα-, Bβ-, and γ-chains under reduced conditions (γ-chain was broader than normal control γ-chain; Fig. 1B). An additional variant γ-chain that

migrated faster than the normal γ-chain was detected in Otsu I fibrinogen (Fig. 1B). An immunoblot analysis was performed for purified plasma fibrinogen in order to visualize the presence of the variant γ-chain of Okayama II fibrinogen. As shown in Figure 1C with an anti-human fibrinogen antibody, the variant γ-chain was not clear (the γ-chain was broader than normal control γ-chain) in Okayama II fibrinogen, but was clearly present in Otsu I fibrinogen. However, an anti-fibrinogen γ-chain antibody detected a faint variant γ-chain in Okayama II fibrinogen and indicated that migration was slower than that of the variant γ-chain in Otsu I fibrinogen (Fig. 1D).

Thrombin-catalyzed fibrin polymerization and clottability

Thrombin-catalyzed fibrin polymerization was performed using the procedure described in the Materials and methods section. Under the conditions of 0.18 mg/mL fibrinogen in the presence of 1.0 mM $CaCl_2$, the polymerization of Okayama II fibrinogen was similar to that of the normal control for ΔAbs for 30minutes (0.385 ± 0.021 for Okayama II and 0.374 ± 0.001 for normal control) and the maximum slope (1.05 ± 0.04 Abs x 10^{-3}/sec for Okayama II and 1.15 ± 0.11 Abs x 10^{-3}/sec for normal control), whereas the lag period (5.0 ± 0.0 minutes) was slightly slower than that of the normal control (4.0 ± 0.0 minutes) (Fig. 2). The polymerization of Otsu I fibrinogen was markedly less than that of the normal control and Okayama II; namely, an increase in the turbidity of Otsu I was not observed 30 minutes after

the addition of thrombin.

In order to determine whether variant fibrin monomers were incorporated into fibers, we examined clottability in duplicate, as described in the Materials and Methods section. The value obtained for Okayama II fibrinogen was 91.8 ± 2.1% and was not significantly different from that of the normal control (92.5 ± 0.6%). The value obtained for Otsu I fibrinogen was 30.0 ± 3.9% and was markedly smaller than those of the normal and Okayama II fibrinogen ($p < 0.01$).

Protection assay of the plasmin digestion of fibrinogen

We performed a protection assay of plasmin digestion to assess the impaired function of hole 'a' and high affinity calcium binding sites, as described in Materials and Methods section. In the presence of 5 mM ethylenediaminetetraacetic acid (EDTA) and the absence of GPRP or $CaCl_2$, D_1 fragments derived from normal fibrinogen were cleaved to the smaller fragments D_2 and D_3, indicating no protection from plasmin digestion. In the presence of 1 or 5mM of $CaCl_2$ or GPRP, D_1 fragments derived from normal fibrinogen were not cleaved to the fragments D_2 and D_3, indicating strong protection against plasmin digestion (Fig. 3A). The D_1 fragments derived from Okayama II and Otsu I fibrinogen were mildly digested into the fragments D_2 and D_3 in the presence of 1 mM $CaCl_2$ or 1 mM GPRP, suggesting weak protection against plasmin digestion (Fig. 3B and C). These results indicated that the

functions of hole ‘a’ and high affinity calcium binding sites in Okayama II or Otsu I fibrinogen were weaker than those in normal fibrinogen.

FXIIIa-catalyzed cross-linking of fibrinogen

We examined the FXIIIa-catalyzed cross-linking of fibrinogen to confirm differences in the functions of D:D interactions between Okayama II and Otsu I fibrinogen. After FXIII 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, and the intensity of the γ-γ dimer bands increased with a longer incubation for normal fibrinogen and both variant fibrinogens (data not shown). These results demonstrated that the functions of D:D interactions of Okayama II and Otsu I fibrinogen were similar to those of the normal control.

Synthesis and secretion of recombinant variant fibrinogens in CHO cells

We established γD320G-, γΔN319-ΔD320-, and N-fibrinogen-expressing CHO cells together with γD320E-, γΔD320-fibrinogen-expressing CHO cells. The latter two variants were produced according to naturally occurring mutations [11-13]. Fibrinogen concentrations in the culture media and cell lysates of each of the 10 to 12 fibrinogen-synthesizing cell lines were measured by ELISA and the results are shown in Figure 4. Normal fibrinogen

concentrations (mean ± SD, n=10) were 1043 ± 146 ng/mL for cell lysates and 1345 ± 119 ng/mL for the culture media. The fibrinogen concentration ratio of the medium/cell lysate was 1.31 ± 0.19. The concentrations in the cell lysates for each of the variant fibrinogen-expressing cell lines were significantly higher than those of normal cells: γD320G; 6525 ± 2323 ng/mL (6.3-fold), γD320E; 11670 ± 9587 ng/mL (11.2-fold), and γΔD320; 4322 ± 1803 ng/mL (4.1-fold), respectively. Concentrations in the culture media of the variant fibrinogen-expressing cell lines were lower than those of normal cells: γD320G; 696 ± 212 ng/mL (0.52-fold), γD320E; 590 ± 291 ng/mL (0.44-fold), and γΔD320; 362 ± 59 ng/mL (0.27-fold), respectively. The fibrinogen concentration ratios of the medium/cell lysate of the variant cell lines were markedly lower than those of the normal cells: γD320G; 0.11 ± 0.02 (0.08-fold), γD320E; 0.06 ± 0.03 (0.05-fold), and γΔD320; 0.09 ± 0.03 (0.07-fold), respectively. These ratios were significantly different from those of the normal control ($p < 0.001$). In the γΔN319-ΔD320 fibrinogen-expressing cell lines, fibrinogen concentrations were 1522 ± 885 ng/mL for cell lysates and 923 ± 402 ng/mL for the culture media, and the fibrinogen concentration ratio of the medium/cell lysate was 0.66 ± 0.18. The ratio was significantly different from that of the normal control ($p < 0.001$). Furthermore, the ratios of the medium/cell lysate of the γD320G-, γD320-E, and γΔD320-CHO cell lines were also significantly different from that of the γΔN319-ΔD320-CHO cell line ($p < 0.001$).

In the selected cell lines for each variant γ-chain transfected cell, cell lysates were analyzed

by SDS-PAGE and immunoblotting under non-reducing or reducing conditions to confirm the assembly of fibrinogen and/or synthesis of three polypeptide chains inside the cells, as described in the Materials and Methods section. All cell lines synthesized variant γ-chains as well as Aα- and Bβ-chains, as shown in Figure 5B (under reducing conditions). Furthermore, variant γ-chains were assembled into fibrinogens inside the cells, as shown in Figure 5A (under non-reducing conditions). A fibrinogen band at 340kDa, each of the non-assembled Aα- Bβ-, and γ-chains, and some intermediate complexes of the fibrinogen assembly process were observed in all variant cell lines as well as the normal fibrinogen-synthesizing cell line. Variant γ-chains derived from γD320G-, γΔD320-, and γΔN319-ΔD320-CHO cells migrated faster than those derived from γD320E and normal γ-chains. The faster migrating band observed in γD320E-CHO cells was speculated to be the degraded γ-chain of γD320E (Fig. 5B). Although the variant γ-chains derived from γD320G-, γΔD320-, and γΔN319-ΔD320-CHO cells migrated at a similar speed to the normal γ-chain under non-reduced conditions, the γ-chain of γD320E migrated slower than the others, as shown in Figure 5A.

Discussion

We encountered two patients with asymptomatic heterozygous hypodysfibrinogenemia, Okayama II; γD320G and Otsu I; γΔN319-ΔD320, and found that the affected residues

overlapped each other in the γC module. However, the fibrinogen concentration ratio (ratio of functionally to immunologically determined plasma fibrinogen concentrations) of the Otsu I proposita was 0.063 and markedly lower than that of the Okayama II proposita (0.482). Therefore, we purified fibrinogen from plasma samples of the propositas and analyzed them by SDS-PAGE and Western blotting. The results obtained showed that the proportion of the variant γ-chain of Okayama II was less than that of Otsu I. In contrast, thrombin-catalyzed fibrin polymerization for Otsu I fibrinogen was markedly weaker than that for Okayama II fibrinogen, namely, the function of Okayama II fibrinogen was slightly weaker than that of the normal control. Therefore, we speculated that some fibrinogen functions may have been markedly impaired for both variant fibrinogens from Otsu I and Okayama II, whereas the proportion of variant fibrinogen to normal fibrinogen in plasma was markedly different between Otsu I and Okayama II (Otsu I > Okayama II). In order to investigate our speculation regarding the characterization of the variant fibrinogens, Okayama II and Otsu I, we produced and analyzed the corresponding recombinant fibrinogen-expressing CHO cells.

Heterozygous variant γD320G had already been reported in a Chinese patient [14]. Although there was no personal or family history of bleeding or thrombosis, the plasma fibrinogen concentration determined by the thrombin time method was 0.49 g/L while that determined by the immunological method was 0.90 g/L, and the fibrinogen concentration ratio was 0.544 [14]. These values were consistent with those obtained for Okayama II.

However, the proportion of the variant fibrinogen in plasma and the electrophoretic mobility of the variant γ-chains were not discussed. On the other hand, the heterozygous variant γΔN319-ΔD320 was also reported as Vlissingen/ Frankfurt IV [15] and Otsu I [16]. The former was reported as a thrombotic manifestation and the latter as an asymptomatic case. Plasma fibrinogen concentrations determined by the thrombin time method were 0.40 g/L and 0.27 g/L while those determined by the immunological method were 3.10 g/L and 1.77 g/L, respectively. The fibrinogen concentration ratios were 0.129 and 0.153, respectively. Moreover, at the γN319 or γD320 residues, heterozygous patients with γN319K [10], γΔN319 [10], γD320E [13], and γΔD320 [11,12] were reported to have dysfibrinogenemia or hypodysfibrinogenemia. Patients with γD320E and γΔD320 exhibited a mild bleeding tendency and bleeding plus thrombotic complications, respectively. Furthermore, the proportion of variant fibrinogen in plasma was reported to be 5 to 10% of all fibrinogen in patients with γD320E [13] and two γΔD320 [11,12], and the electrophoretic mobilities of the variant γ-chains were larger than that of the normal control. Therefore, the residues at γN319 and/or γD320 may have crucial roles in the synthesis and functions of fibrinogen.

In order to clarify the reduced synthesis of variant γ-chains, we established the CHO cell lines producing variant fibrinogen identified as Otsu I (γΔN319-ΔD320), Okayama II (γD320G), or others (γD320E, γΔD320) at γD320 residue. The fibrinogen concentration ratios of the culture media to cell lysates of γD320G-, γD320E-, and γΔD320 fibrinogen-expressing

cell lines were markedly lower than those of the normal- and γΔN319-ΔD320 fibrinogen-expressing cell lines. The fibrinogen concentration ratios of the γΔN319-ΔD320 fibrinogen-expressing cell lines were also significantly lower than that of the normal fibrinogen-expressing cell line. Furthermore, the weakly reduced secretion of variant fibrinogen was also demonstrated using CHO cells [21] and transgenic mice [23]. A substitution or deletion at residues γN319 and/or γD320 induced aberrant secretion and not the synthesis of fibrinogen, and our recombinant experiments demonstrated the markedly different secretion rate of variant fibrinogen between Otsu I and Okayama II.

γN319 and/or γD320 have been identified as important residues for the function of fibrinogen because high affinity calcium binding sites are composed of γD318, γD320, γF322, and γG324 [6]. Yakovlev S *et al*. [24] demonstrated that one of two t-PA binding sites were located in γ312-324 residues, which were exposed after fibrinogen-fibrin transition, namely, after binding of the so-called knob 'A' into the hole 'a'. The functions of recombinant γΔN319-ΔD320 fibrinogen have been extensively examined [25] and thrombin-catalyzed fibrin polymerization did not occur more than 24 hours after the addition of thrombin. In brief, the functions of hole 'a', the Ca ion binding site, and factor XIII cross linking were shown to be markedly affected. These findings prompted us to speculate that the functions of variant fibrinogens with a substitution or deletion at residues γN319 and/or γD320 were markedly reduced, namely, the function of γD320G fibrinogen may be similar to γΔN319-ΔD320

fibrinogen and markedly affected. The residues at γN319 and/or γD320 may have crucial roles in the functions of fibrinogen, and variants at these residues manifest as bleeding and/or thrombotic complications.

We concluded that the plasma fibrinogen of Okayama II was constituted by a higher proportion of normal fibrinogen and less of the variant fibrinogen, resulting in almost normal functions for fibrin polymerization. However, Otsu I plasma fibrinogen showed markedly reduced thrombin-catalyzed fibrin polymerization due to the higher component ratio of variant fibrinogen.

We speculated that marked reductions in the fibrin polymerization of Otsu I fibrinogen was caused by marked decreases in lateral aggregation in addition to the aberrant protofibril formation. The precise mechanisms responsible for the lateral aggregation of protofibrils remain largely unknown [3]. The following portions of the fibrinogen structure have been shown to contribute to lateral aggregation: knobs ‘B’ [7], holes ‘b’ [7], γC modules [7], βC modules [7], αC regions [26], coiled coils [27], and carbohydrates at residues Bβ364Asn and γ52Asn [28]. γ350-360 and γ370-380 residues in the γC module are critical for lateral aggregation, and the deletion of γN319 and γD320 (Otsu I) fibrinogen induced disruptive conformational changes in these region, resulting in larger decreases in lateral aggregation than the substitution of γD320 (Okayama II) fibrinogen.

In conclusion, heterozygous variant fibrinogen, the deletion of the γN319 and γD320

residues (Otsu I) and substitution of γD320 by G (Okayama II), affected the overall structure of the γC module and lead to marked reductions in the function and secretion of fibrinogen, respectively. However, it is difficult to explain the paradoxical phenomenon in which the secretion of fibrinogen composed of the two-residue deletion of γN319 and γD320 in the γC module was only slightly less than that of normal fibrinogen, whereas that composed of the one residue substitution at γD320 was markedly less.

Authorship

Saki Mukai performed the research, analyzed the data, and wrote the manuscript. Minami Ikeda analyzed DNA sequences, Yuka Takezawa, Mitsutoshi Sugano, Takayuki Honda, and Nobuo Okumura designed the research and discussed the data, and N Okumura reviewed the manuscript.

Conflict of Interest Statement

The authors state that they have no conflicts of interest.

Acknowledgments

We gratefully acknowledge Dr. Eisei Kondo (Okayama University Graduate School of Medicine) for patient referral of Okayama II and Dr. Nobuyuki Kita (Shiga University of

Medical Science) for patient referral of Otsu I.

References

[1] Weisel JW. Fibrinogen and fibrin. Adv Protein Chem 2005;70:247-99.

[2] Kant JA, Fornace AJ Jr, Saxe D, Simon MI, McBride OW, Crabtree GR. Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. Proc Natl Acad Sci USA 1985;82:2344-8.

[3] Weisel JW, Litvinov RI. Mechanisms of fibrin polymerization and clinical implications. Blood 2013;121:1712-9.

[4] Pratt KP, Côté HCF, Chung DW, Stenkamp RE, Davie EW. The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal gamma chain fragment complexed with the peptide Gly-Pro-Arg-Pro. Proc Natl Acad Sci USA 1997;94:7176-81.

[5] Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. Nature 1997;389:455-62.

[6] Yee VC, Pratt KP, Côté HCF, Le Trong I, Chung DW, Davie EW, et al. Crystal structure of a 30 kDa C-terminal fragment from the γ chain of human fibrinogen. Structure 1997;5:125-38.

[7] Yang Z, Mochalkin I, Doolittle RF. A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. Proc Natl Acad Sci USA 2000;97:14156-61.

[8] Guthold M, Carlisle C. Single fibrin fiber experiments suggest longitudinal rather than transverse cross-linking: reply to a rebuttal. J Thromb Haemost 2010;8:2090-1.

[9] Rooney MM, Parise LV, Lord ST. Dissecting clot retraction and platelet aggregation. Clot retraction does not require an intact fibrinogen gamma chain C terminus. J Biol

Chem 1996;271:8553-5.

[10] Groupe d'Etude sur 1'Hémostase et la Thrombose. "Base de données des variants du Fibrinogène". GEHT Web site, 〈http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html〉, [updated on 30/10/2014].

[11] Brennan SO, Davis RL, Mosesson MW, Hernandez I, Lowen R, Alexander SJ. Congenital hypodysfibrinogenaemia (Fibrinogen Des Moines) due to a γ320Asp deletion at the Ca^{2+} binding site. Thromb Haemost 2007;98:467-9.

[12] Brennan SO, Chitlur M. Hypodysfibrinogenaemia and thrombosis in association with a new fibrinogen γ chain with two mutations (γ114Tyr→His, and γ320Asp deleted). Thromb Haemost 2013;109:1180-2.

[13] Brennan SO, Laurie A. Functionally compromised FGG variant (γ320Asp→Glu) expressed at low level in plasma fibrinogen. Thromb Res 2014;134:744-6.

[14] Castaman G, Giacomelli SH, Duga S, Rodeghiero F. Congenical hypofibrinogenemia associated with novel heterozygous fibrinogen Bβ and γ chain mutations. Haemophilia 2008;14:630-3.

[15] Koopman J, Haverkate F, Briët E, Lord ST. A congenitally abnormal fibrinogen (Vlissingen) with a 6-base deletion in the gamma-chain gene, causing defective calcium binding and impaired fibrin polymerization. J Biol Chem 1991;266:13456-61.

[16] Terasawa F, Hogan KA, Kani S, Hirose M, Eguchi Y, Noda Y, et al. Fibrinogen Otsu I:A γ Asn319,Asp320 deletion dysfibrinogen identified in an asymptomatic pregnant woman. Thromb Haemost 2003;90:757-8.

[17] Ikeda M, Arai S, Mukai S, Takezawa Y, Terasawa F, Okumura N. Novel heterozygous dysfibrinogenemia, Sumida (AαC472S), showed markedly impaired lateral aggregation of protofibrils and mildly lower functional fibrinogen levels. Thromb Res 2015;135:710-7.

[18] Okumura N, Furihata N, Terasawa F, Nakagoshi R, Ueno I, Katsuyama T. Fibrinogen Matsumoto I: a gamma 364Asp → His (GAT→CAT) substitution associated with defective fibrin polymerization. Thromb Haemost 1996;75:887-91.

[19] Ikeda M, Kobayashi T, Arai S, Mukai S, Takezawa Y, Terasawa F, et al. Recombinant γT305A fibrinogen indicates severely impaired fibrin polymerization due to the aberrant function of hole 'a' and calcium binding sites. Thromb Res 2014;134:518-25.

[20] Okumura N, Gorkun OV, Lord ST. Severely impaired polymerization of recombinant fibrinogen γ-364 Asp → His, the substitution discovered in a heterozygous individual. J Biol Chem 1997;272:29596-601.

[21] Kani S, Terasawa F, Lord ST, Tozuka M, Ota H, Okumura N, et al. In vitro expression demonstrates impaired secretion of the γ Asn319, Asp320 deletion variant fibrinogen. Thromb Haemost 2005;94:53-9.

[22] Okumura N, Terasawa F, Tanaka H, Hirota M, Ota H, Kitano K, et al. Analysis of fibrinogen γ-chain truncations shows the C-terminus, particularly γIle387, is essential for assembly and secretion of this multichain protein. Blood 2002;99:3654-60.

[23] Hogan KA, Merenbloom BK, Kim HS, Lord ST. Neonatal bleeding and decreased plasma fibrinogen levels in mice modeled after the dysfibrinogen Vlissingen/Frankfurt IV. J Thromb Haemost 2004;2:1484-7.

[24] Yakovlev S, Makogonenko E, Kurochkina N, Nieuwenhuizen W, Ingham K, Medved L. Conversion of fibrinogen to fibrin: mechanism of exposure of tPA- and plasminogen-binding sites. Biochemistry 2000;39:15730-41.

[25] Hogan KA, Gorkun OV, Lounes KC, Coates AI, Weisel JW, Hantgan RR, et al. Recombinant fibrinogen Vlissingen/Frankfurt IV. The deletion of residues 319 and 320 from the γ chain of fibrinogen alters calcium binding, fibrin polymerization, cross-linking, and platelet aggregation. J Biol Chem 2000;275:17778-85.

[26] Tsurupa G, Mahid A, Veklich Y, Weisel JW, Medved L. Structure, stability, and

interaction of fibrin αC-domain polymers. Biochemistry, 2011;50: 8028-37.

[27] Okumura N, Terasawa F, Hirota-Kawadobora M, Yamauchi K, Nakanishi K, Shiga S, et al. A novel variant fibrinogen, deletion of Bbata 111 Ser in coiled-coil region, affecting fibrin lateral aggregation. Clin Chim Acta 2006;365:160-7.

[28] Langer BG, Weisel JW, Dinauer PA, Nagaswami C, Bell WR. Deglycosylation of fibrinogen accelerates polymerization and increases lateral aggregation of fibrin fibers. J Biol Chem 1988;263:15056-63.

Figure legends

Figure 1. Characterization of purified fibrinogens.

Purified fibrinogens were resolved on 7% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under reducing conditions (B) and stained with Coomassie Brilliant Blue R-250. Resolved proteins on 10% SDS-PAGE under reducing conditions were electrically transferred to nitrocellulose sheets and reacted with an anti-fibrinogen antibody (C) or anti-fibrinogen γ-chain antibody (D), and reactive bands were detected by chemiluminescence, as described in the Material and Methods section. N: normal control, Ok: Okayama II, Ot: Otsu I, and M: molecular marker. ▷: variant γ-chain not observed, ▶: visualized variant γ-chain.

Fig.1

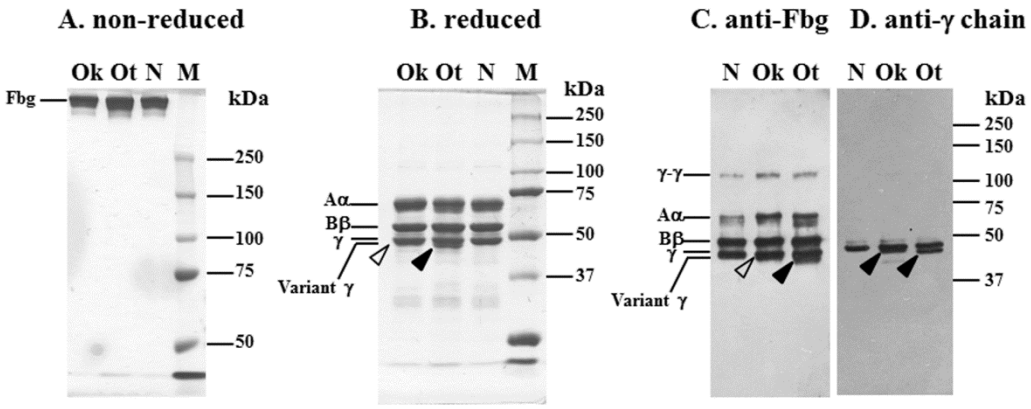

Figure 2. Thrombin-catalyzed fibrin polymerization.

The polymerization of fibrinogen (0.18 mg/mL) was initiated with thrombin (0.05 U/mL), and the change in turbidity with time was followed at 350 nm for normal (●), Okayama II (▲), and Otsu I (◆) in polymerization buffer.

Fig.2

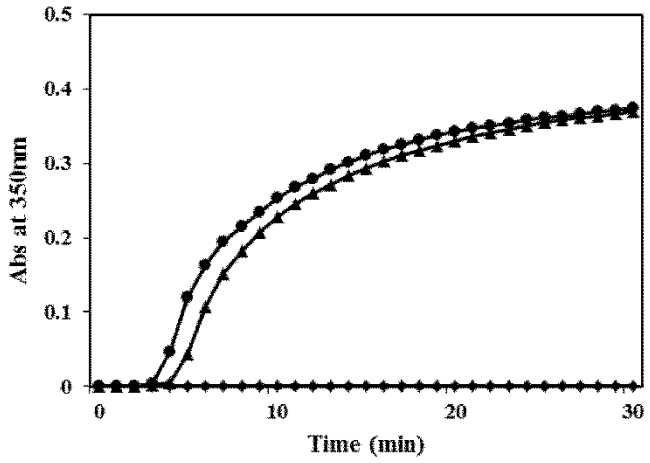

Fig.3

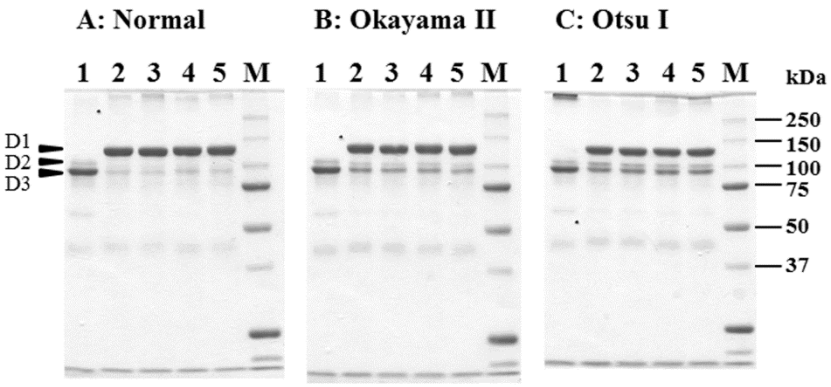

Figure 3. Protection assay of the plasmin digestion of fibrinogen.

Fibrinogen (0.30 mg/mL) in polymerization buffer containing 5 mM EDTA (lane 1), 1 mM (lane 2), or 5 mM $CaCl_2$ (lane 3), and 1 mM (lane 4) or 5 mM GPRP (lane 5) was incubated with 0.18 U/mL plasmin for 2 hours 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

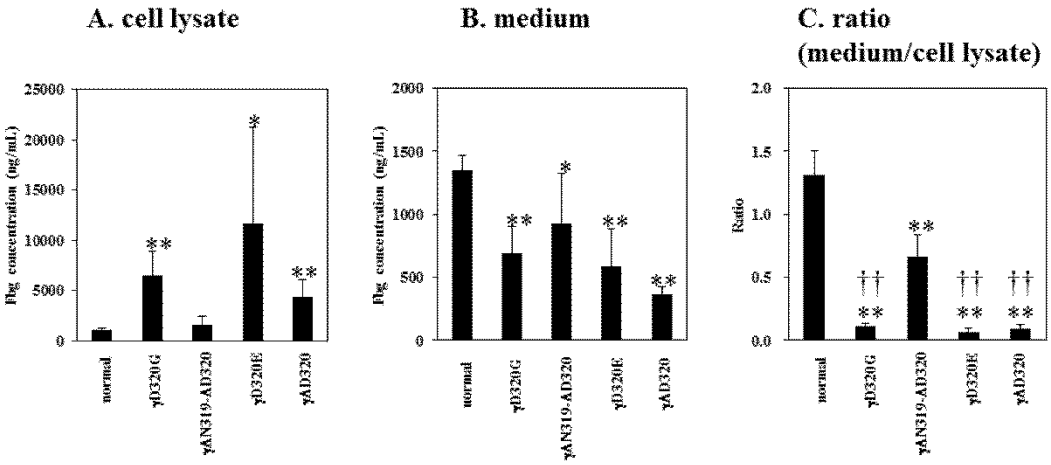

Figure 4. Synthesis of variant fibrinogens in transfected CHO cells.

Fibrinogen concentrations in cell lysates (A) and culture media (B) were determined by ELISA. The ratios of values of the culture medium to the cell lysate are shown in panel C. The mean values are presented with standard deviations indicated by error bars for normal: n=10; γD320G: n=12; γΔN319-D320: n=11; γD320E: n=10; γΔD320: n=11. Variants were significantly different from normal (*: $p < 0.01$, **: $p < 0.001$) and from γΔN319-ΔD320 (††: $p < 0.001$).

Fig.5

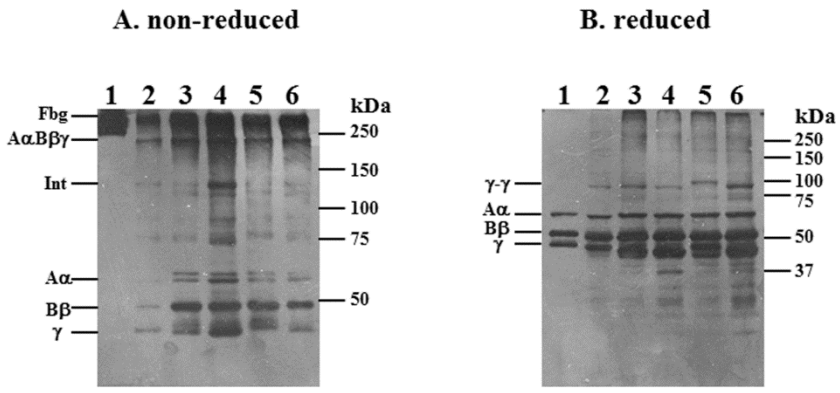

Figure 5. Immunoblotting analysis of cell lysates.

Samples from cell lysates were subjected to 8% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under reducing conditions (B). The blots were reacted with an anti-fibrinogen antibody and detected by chemiluminescence, as described in the Materials and Methods. Samples from individual CHO cell lines were electrophoresed in lanes 2: normal (N), 3: γD320G, 4: γΔN319-ΔD320, 5: γD320E, 6: γΔD320; Lane 1: purified fibrinogen from normal plasma. Int: intermediates of fibrinogen, Right sides of panels A and B show molecular size markers.