Original article

Recombinant variant fibrinogens substituted at residues γ326Cys and γ339Cys demonstrated markedly impaired secretion of assembled fibrinogen

Ayumi Haneishi[1], Fumiko Terasawa[2], Noriko Fujihara[3], Kazuyoshi Yamauchi[3], Nobuo Okumura[2], Tsutomu Katsuyama[1]

[1]Department of Laboratory Medicine, Shinshu University School of Medicine, [2]Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, [3]Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan

Correspondence: Fumiko Terasawa, Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, 3-1-1 Asahi, Matsumoto, 390-8621 Japan. Tel.: +81 263 37 2387; fax: +81 263 37 2370; e-mail: fterasa@shinshu-u.ac.jp

Short running title: Impaired secretion of γ326Cys, γ339Cys variant fibrinogens

Word count (Text, References, Figure legends): 3799 (Abstract:199)
Number of figures: 3
Number of tables: 1
Number of references: 23

Abstract

Background: To study the functions of residues γ326Cys and γ339Cys in the assembly and/or secretion of fibrinogen, recombinant fibrinogens were synthesized to replicate naturally occurring γ326Tyr and γ326Ser variants, along with γ326Ala and γ339Ala variants.   *Methods*: A fibrinogen γ-chain expression vector was altered and transfected into Chinese hamster ovary (CHO) cells. Cell lysates and culture media of the established cell lines were subjected to ELISA and immunoblotting analysis. In addition, pulse-chase analysis was performed.   *Results*: The CHO cells synthesized mutant γ-chains and assembled these into fibrinogen in the cells, although these variant fibrinogens were barely secreted into the culture media. Pulse-chase analysis indicated that the rates of both assembly and secretion of the variant fibrinogens were lower than that of normal fibrinogen. *Conclusions*: The present study indicated that the 326-339 intrachain disulfide bond has a crucial role in maintaining the tertiary structure of the C-terminal domain of the γ-module, which is necessary for fibrinogen assembly and specifically secretion. A combination of the present results and observations from naturally occurring heterozygous cases of γ326Tyr and γ326Ser suggest that heterozygous fibrinogen molecules containing variant γ-chains might be secreted into plasma and show impaired fibrin polymerization, resulting in a phenotype of hypodysfibrinogenemia.

1. Introduction

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains, Aα, Bβ, and γ, which are stabilized by 29 disulfide bonds, including 12 intrachain and 17 interchain disulfide connections [1-4]. The fibrinogen molecule has a trinodular structure where 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. The regions are linked by two coiled-coil connectors, consisting of all three chains [5]. The chains are synthesized, assembled into a six-chain molecule in hepatocytes, secreted into blood, and circulated at 1.8-3.5 g/L. Genetic mutations in fibrinogen chain genes have been associated with either afibrinogenemia, hypofibrinogenemia, or dysfibrinogenemia, as listed in the fibrinogen variant data base (http://www.geth.org/databaseang/fibrinogen/), and the molecular bases for the genetic and/or post-translational changes causing dysfibrinogenemia or hypofibrinogenemia and afibrinogenemia are of substantial interest.

It was reported that hypofibrinogemia can be caused by the heterozygous missense mutation γ153C>R [6]. Expression analysis using Chinese hamster ovary (CHO) cells demonstrated that assembly of this variant fibrinogen was defective and its subsequent secretion was impaired. In addition, it was indicated that this variant γ-chain was defective in the formation of αγ and βγ complexes, which are essential intermediate forms of the hexameric fibrinogen molecule [7]. These finding suggested that the tertiary structure of the C-terminal domain of the γ-nodule formed by the disulfide bond between γ153C and γ182C is crucial for fibrinogen assembly. Furthermore, there is another disulfide bond

between γ326C and γ339C in the C-terminal domain of γ-nodule. Zhang *et al.* studied the assembly and secretion of variants at these residues and showed that variants substituted at residues γ326C and γ339C can assemble into fibrinogen normally in cells but are not secreted into the medium [8]. At residue γ326, 2 naturally occurring heterozygous substitutions (C>Y and C>S) have been reported in 3 families [9-11] and they showed somewhat different pathological conditions, namely, two family showed dysfibrinogenemia (the presence of variant fibrinogen in plasma) and the other showed hypofibrinogenemia (little or no presence of variant fibrinogen in plasma; secretion of variant fibrinogen is absent or very limited).

In this study, to confirm the secretion of variant fibrinogen substituted at residues γ326 or γ339 into plasma, 2 naturally occurring variant fibrinogens (γ326Y and γ326S) and 2 control variants (γ326A and γ339A) were expressed in CHO cells, and the synthesis, assembly, and secretion of fibrinogen were analyzed *in vitro*.

2. Materials and methods

2.1. Construction of mutant expression vectors

The fibrinogen γ-chain expression vector pMLP-γ [12] was altered by oligonucleotide-directed mutagenesis using a Transformer Site-Directed Mutagenesis kit (Clontech Laboratories, Palo Alto, CA, USA). Briefly, single-stranded pMLP-γ was annealed to 5'-phosphorylated mutagenesis primers (γ326A; 5'-GAAGGCAAC<u>GCT</u>GCTGAACAG-3', γ326Y; 5'-GAAGGCAACT<u>AC</u>GCTGAACAG-3', γ326S; 5'-GAAGGCAACT<u>CC</u>GCTGAACAG-3', γ339A; 5'-GATGAACAA<u>GGC</u>TCACGCTGG-3', the altered bases are underlined) and a 5'-phosphorylated selection primer (5'-TCTAGGGCCCAGGCTTGTTTGC-3'), which had a deletion of a unique *Hind*III site in the vector. The second strand was synthesized using T4 DNA polymerase and ligated with T4 DNA ligase.

Plasmids lacking *Hind*III sites were sequenced using a BigDye[R] Terminator v1.1 Cycle Sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). To confirm the insertion of each mutation, the complete γ-chain cDNAs of the plasmids were sequenced using 2 forward and 2 reverse primers (Table 1).

2.2. Recombinant protein expression

CHO cell lines that expressed normal human fibrinogen Aα- and Bβ-chains, AαBβ-CHO cells, were obtained by cotransfecting the plasmids pMLP-Aα, pMLP-Bβ, and pRSV-neo into CHO cells. Each of the variant pMLP-γ vectors and the original pMLP-γ vector was cotransfected with the histidinol selection plasmid (pMSV-his) into the AαBβ-CHO cell line [6], using a standard calcium-phosphate co-precipitation method [13, 14]. Eight to fourteen colonies from fibrinogen-synthesizing CHO cells on both G418 (GIBCO BRL, Rockville, MD, USA) and histidinol (Aldrich Chemical, Milwaukee, WI, USA) were selected at random on medium containing both G418 and histidinol, and these cell lines were examined for fibrinogen synthesis as described previously [14]. The cell lines were designated as γ326A-, γ326Y-, γ326S-, γ339A-, and normal (N)-CHO cells,

respectively.

2.3. Immunoassays

Fibrinogen concentrations in the cell lysates or culture media were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously [15]. To analyze the assembly and secretion of fibrinogen and/or three polypeptide chains in the cell lysates, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis were performed as described previously [6, 7]. Samples for the immunological analyses were prepared as follows. For each of the cell lines, cells were grown to confluence in two 60-mm culture dishes (approximately 1.5– 2.0×10^6 cells), and the conditioned media from the first culture dishes were harvested 1 day after reaching confluence (6–8 days after seeding) for ELISA. Cells were harvested in trypsin-EDTA solution (Sigma, St Louis, MO, USA) from the same or second culture dishes, washed 3 times with PBS, and finally lysed in either 300 μL of 0.1% IGEPAL CA-630 (Sigma) and 10 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) in 50 mM Tris-HCl buffer pH 8.0 for ELISA, or 70 μL of Laemmli sample buffer for SDS-PAGE and immunoblotting analysis of cell lysates. Immunoblots were probed with a rabbit anti-human fibrinogen antibody (Dako, Carpinteria, CA, USA) and the reacting species were visualized using horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (Medical and Biological Laboratories, Nagoya, Japan) and enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on Hyperfilm-ECL (Amersham Pharmacia Biotech).

2.4. Pulse-chase analysis of protein synthesis using [^{35}S]-methionine

Pulse-chase studies were performed for selected cell lines, N-20 and γ326A-57, which had the most simple residues for the three types amino acid A, Y, S, and highest level of secreted fibrinogen into medium among all established cell lines. In addition, γ153A-10 cell line was used as a control of abolished fibrinogen assembly and secretion, as described previously [6, 16]. In brief, the medium was replaced with 1 mL of methionine-free DMEM supplemented with 1.11 MBq (30μCi) L-[^{35}S]-methionine (Amersham Pharmacia Biotech Inc). The cells were incubated for 60 min at 37°C. After the pulse, the cells were rinsed twice with PBS, then 1 mL of fresh DMEM containing 2 mM unlabeled L-methionine (Wako, Osaka, Japan) was added, and the cells were incubated for various chase periods (0, 1, 3, 6, 8, and 24 hours). The media were harvested, and cell lysates were prepared in 120 μL of lysis buffer containing 1% IGEPAL CA-630, 150 mM NaCl, 5 mM EDTA, and 10 mM PMSF in 50 mM Tris-HCl buffer, pH 8.0. Each 100 μL of medium or cell lysate was added to an equivalent amount of 1:1000 diluted rabbit anti-fibrinogen polyclonal antibody (DAKO) and incubated overnight at 4°C, and immunocomplexes were precipitated using Protein A-Sepharose. The precipitates were dissolved in 40 μL Laemmli sample buffer, boiled for 5 minutes, and resolved on SDS-polyacrylamide gradient gels. Radioactive bands were detected using a Fuji Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film Co, Tokyo, Japan)

3. Results

3.1. Synthesis and secretion of recombinant variant fibrinogens in CHO cells

Four variant and normal fibrinogens were expressed in CHO cells, and the cell lysates and culture media of 8 to 14 fibrinogen-synthesizing cell lines were selected as described in the Materials and Methods. The fibrinogen concentrations in the cell lysates and culture media were measured by ELISA, and the results are shown in Figure 1. Normal fibrinogen concentrations (mean ± SD, n=8) were 1569 ± 297 ng/mL for cell lysates and 3400 ± 1006 ng/mL for the culture media. Surprisingly, the concentrations in the cell lysates for each of the variant fibrinogen-expressing cell lines were significantly elevated compared with that in normal cells; γ326A, 2.3-fold; γ326Y, 1.8-fold; γ326S, 2.8-fold; and γ339A, 3.2-fold, respectively (Fig. 1A). Whereas, the concentrations in the culture media for the variant fibrinogen-expressing cell lines were significantly lower than that in the normal cells; γ326A, 0.07-fold; γ326Y, 0.02-fold; γ326S, 0.04-fold; and γ339A, 0.05-fold, respectively (Fig. 1B). The fibrinogen ratios of the medium/cell lysate of the variant cell lines were markedly lower than that of the normal cells (Fig. 1C).

For selected cell lines for each variant fibrinogen-synthesizing cell line, SDS-PAGE and immunoblotting analysis were performed under non-reducing or reducing conditions for observation of the assembly and secretion of fibrinogen and/or three polypeptide chains, and the results are shown in Figure 2. In the normal-CHO cell lysate under non-reducing conditions intact fibrinogen, low levels of AαBβγ complex, intermediate complex, Aα-chain, and faint Bβ-, and γ-chains were observed (Fig. 2A, Lane N). In the γ326A-, γ326Y-, γ326S-, and γ339A-CHO cell lysates, variant fibrinogens, weak AαBβγ and intermediate complex, several unknown higher molecular mass bands, and a smear above the 100 kDa area were observed (Fig. 2 A). Furthermore, the three individual chains were also observed at higher level than in normal-CHO cells and an additional band was observed above the Aα-chain (Fig. 2 A). Under reducing conditions, in the normal-CHO cells, Aα-, Bβ-, and γ-chains were observed. For the γ326A-, γ326Y-, γ326S-, and γ339A-cells, Aα-, Bβ-, and γ-chains and at least four low-molecular-mass species below the γ-chain, which were presumed to be proteolytic degradation products (Fig. 2B). In addition, for the variant cell lysates several high-molecular-mass species above the Aα-chain were observed. Two bands were clear, and the upper one was a γ-γ dimer (present weakly in normal CHO cells) but the other was unidentified (Fig. 2B)

3.2. Pulse-chase analysis

Pulse-chase analysis was performed to examine the rate of fibrinogen synthesis, assembly, and secretion for normal and variant fibrinogen cell lines as described in the Materials and Methods. Analysis of the lysates from normal-CHO cells showed a 340 kDa band of intact fibrinogen (Fig. 3A). The band was present at a high level prior to the chase, increased in intensity by 1 h after the chase, and decreased thereafter. An additional lower-molecular-weight band was present in the samples, which was presuming to be a half molecule of fibrinogen, AαBβγ-complex. The normal fibrinogen band was seen in immunoprecipitates of the culture medium (Fig. 3D). This band became evident after a 1 h chase and increased with elevating incubation times. None of the fibrinogen bands were

5

seen in the γ153A cell lysates or culture media (Fig. 3B, E) during the 24 h chase period; whereas, a lower-molecular-weight band than the AαBβγ-complex was seen in the cell lysates without a chase and in a 1–6 h chase. (Fig. 3B*).

For the cell lysates of γ326A cells, a low level of fibrinogen was present prior to the chase, and its intensity increased with chase periods between 1 and 24 h (Fig. 3C). In addition, an AαBβγ-complex band and an unknown lower-molecular-weight band (present in γ153A cell lysates) were seen prior to the chase and in all chase times. For the media of γ326A cells, a weak variant fibrinogen band was observed at 6 h, but it became fainter at 8 h (Fig. 3F). These results indicated that the rates of both assembly and secretion for the γ326A variant fibrinogen were slower than those of normal fibrinogen.

4. Discussion

To examine the assembly and/or secretion of variant fibrinogens substituted at residues 326 or 339 in the γ-chain, four variant fibrinogens, γ326Y, γ326S, γ326A, and γ339A, were expressed in CHO cells as stable transfectants. All these cell lines synthesized mutant γ-chains, assembled these into fibrinogen in the cells, and secreted small amounts of variant fibrinogens into the culture media. Of interest, the fibrinogen concentrations in the cell lines were much higher than those in the normal cell line, whereas the levels of secreted fibrinogen from these variant cells were much lower than those in the normal cell line. It was first speculated that the variant fibrinogens were assembled at a similar rate to normal fibrinogen in the cells but had a reduced rate of secretion into the culture media. Contrary to this expectation, pulse-chase analysis demonstrated that the rates of not only secretion but also assembly of γ326A fibrinogens was slower compared with that of normal fibrinogen.

Chain-assembly and/or secretion of variant fibrinogens substituted at residues 326 or 339 (γ326S, γ339S, γ339A, and γ326S+γ339S) have already been studied in transiently transfected COS-1 cells by Zhang *et al* [8]. They demonstrated that variant γ-chains assembled into fibrinogen in the cells but its secretion was not observed using a highly sensitive radiolabeling method [8]. Although there is a possibility that characteristics of secretion of fibrinogen might be somewhat different between COS-1 and CHO cells, variant fibrinogens substituted at residues γ326 or γ339 were essentially not secreted or only secreted at very low levels into the culture media. In the same fashion, Vu *et al.* demonstrated that truncated γ-chain variant, γ387X, was assembled into fibrinogen inside the COS-7 cells, yet this was not secreted into medium [17]. This result appears to contradict data from our study showing no fibrinogen is detected when this truncated γ-chain is transfected in CHO cells [15]. Profoundly different from the present analysis, Zhang *et al* did not measure the fibrinogen concentration in cells, so it is not clear whether the accumulation of variant fibrinogen in COS-1 cells was higher or not than that of normal fibrinogen [8].

Three heterozygous cases of two types of variant fibrinogen at γ326 substituted by Y or S have been reported [9-11]. Plasma fibrinogen concentrations of the γ326C>Y (Suhl) [9], γ326C>Y (Frostburg) [10], and γ326C>S (Córdoba) [11] were <0.6/1.5, 0.5/0.4, and 0.12/1.42 g/L (functionally determined value/ immunologically determined value),

6

respectively (Table 1). Namely, in addition to functional levels all plasma protein concentrations were lower or minimum levels as normal. The expression level of the Frostburg variant fibrinogen was indicated as very low by mass analysis [10] and the plasma fibrinogen from the Suhl patient indicated the presence of a small amount of albumin binding fibrinogen [9]. In addition, all of the purified fibrinogens showed impaired fibrin polymerization [9-11]. These results indicated that variant fibrinogens were present in the patient's plasma and showed severely impaired function or inhibited the function of the normal molecules in fibrin fiber formation. Experiments using CHO cells do not reflect the function of human hepatocytes properly *in vivo*; however, the present results concerning fibrinogen assembly and secretion are coincident with the observation of naturally occurring variant fibrinogens.

In general, misfolded and/or secretion-impaired proteins are subject to quality control and degraded by the ubiquitin-proteasome system [18, 19]. When protein breakdown occurs by this system, an endoplasmic reticulum storage disease (ERSD) might be induced. ERSD caused by the storage of variant fibrinogen has been reported in four families of heterozygous variant fibrinogens, γ 284G >R [20], γ375R>W [21, 22]), and deletion of γ346-350 [23]). All of these patients showed no significant levels of the variant fibrinogen in plasma, namely, hypofibrinogenemia. Contrary to these variants, all of the three heterozygous families with γ326Y and γ326S have not been described as showing liver dysfunction as suspected for the presence of ESDR. Finally, the present results lead to the prediction that variant fibrinogens substituted at residues γ326 or γ339 might escape from the quality control system and/or degradation by the ubiquitin-proteasome system, resulting in the accumulation of variant fibrinogens in CHO cells for a long period but without the aberrant storage of fibrinogen in the endoplasmic reticulum.

In conclusion, the current results indicate that the γ326-339 intrachain disulfide bond has a crucial role in maintaining the tertiary structure of the C-terminal domain of the γ-module and is necessary for fibrinogen assembly and specifically for fibrinogen secretion. Namely, the γ326-339 disulfide bond connects between a α-helix composed of residues γ326-331 and a random coil region (γ337-340). The combination of the present results and the observation of naturally occurring heterozygous cases of γ326Y and γ326S suggest that small amounts of variants might be secreted into plasma and show impaired fibrin polymerization, resulting in hypodysfibrinogenemia.

References

[1] Doolittle RF, Bouma IH, Cottrell BA, Strong D, Watt KWK. The covalent structure of human fibrinogen. In: Bing DH, Ed. The Chemistry and Physiology of the Human Plasma Proteins. New York, NY: Pergamon Press 1979;77-95.

[2] Doolittle RF. Fibrinogen and fibrin. Sci Amer 1981;245:92-101.

[3] Weisel JW, Stauffacher CV, Bullitt E, Cohen C. A model for fibrinogen: domains and sequence. Science 1985;230:1388-91.

[4] Côté HC, Lord ST, Pratt KP. γ-Chain dysfibrinogenemias: molecular structure-function relationships of naturally occurring mutations in the gamma chain of human fibrinogen. Blood 1998;92:2195-212.

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

[6] Terasawa F, Okumura N, Kitano K, Hayashida N, Shimosaki M, Okazaki M, Lord ST. Hypofibrinogenemia associated with a heterozygous missense mutation γ153Cys to Arg (Matsumoto IV): In vitro expression demonstrates defective secretion of the variant fibrinogen. Blood 1999;94:4122-31.

[7] Terasawa F, Fujita K, Okumura N. Residue γ153Cys is essential for the formation of the complexes Aαγ and Bβγ, assembly intermediates for the AαBβγ complex and intact fibrinogen. Clin Chim Acta 2004;353:157-64.

[8] Zhang JZ, Redman C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. J Biol Chem 1996;71:30083-8.

[9] Meyer M, Franke K, Richter W, Steiniger F, Seyfert UT, Schenk J, Treuner J, Haberbosch W, Eisert R, Barthels M. New molecular defects in the γ subdomain of fibrinogen D-domain in four cases of (hypo)dysfibrinogenemia: fibrinogen variants Hannover VI, Homburg VII, Stuttgart and Suhl. Thromb Haemost 2003;89:637-46.

[10] Dear A, Brennan SO, George PM. Familial hypodysfibrinogenaemia associated with second occurrence of γ326 Cys ->Tyr mutation. Thromb Haemost 2005;93:612-3.

[11] Guglielmone HA, Sanchez MC, Abate Daga D, Bocco JL. A new heterozygous mutation in gamma fibrinogen gene leading to 326 Cys ->Ser substitution in fibrinogen Córdoba is associated with defective polymerization and familial hypodysfibrinogenemia. J Thromb Haemost 2004;2:352-4.

[12] Rooney MM, Parise LV, Lord ST. Dissecting clot retraction and platelet aggregation. Clot retraction does not require an intact fibrinogen γ chain C terminus. J Biol Chem 1996;271:8553-5.

[13] Kingston RE, Chen CA, Okayama H. Transfection of DNA into eukaryotic cells. Calcium phosphate transfection, in Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds): Current Protocol in Molecular Biology. New York, NY, Wiley 1989.

[14] Binnie CG, Hettasch JM, Strickland E, Lord ST. Characterization of purified recombinant fibrinogen: Partial phosphorylation of fibrinopeptide A. Biochemistry 1993;32:107-13.

[15] Okumura N, Terasawa F, Tanaka H, Hirota M, Ota H, Kitano K, Kiyosawa K, Lord ST. Analysis of fibrinogen γ-chain truncations shows the C-terminus, particularly gammaIle387, is essential for assembly and secretion of this multichain protein. Blood 2002;99:3654-60.

[16] Kani S, Terasawa F, Yamauchi K, Tozuka M, Okumura N. Analysis of fibrinogen variants at γ387Ile shows that the side chain of γ387 and the tertiary structure of the γC-terminal tail are important not only for assembly and secretion of fibrinogen but also for lateral aggregation of protofibrils and XIIIa-catalyzed γ-γ dimer formation. Blood 2006;108:1887-94.

[17] Vu D, de Moerloose P, Batorova A, Lazur J, Palumbo L, Neerman-Arbez M. Hypofibrinogenaemia caused by a novel *FGG* missense mutation (W253C) in the γ chain globular domain impairing fibrinogen secretion. J Med Genet 2005;42e:1-5.

[18] Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67: 425-79.

[19] Redman CM, Xia H. Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion. Ann N Y Acad Sci 2001;936:480-95.

[20] Brennan SO, Wyatt J, Medicina D, Callea F, George PM. Hepatic endoplasmic reticulum storage and hypofibrinogenemia because of a γ284 Gly->Arg mutation. Amer J Pathology 2000;157:189-96.

[21] Brennan SO, Maghzal G, Shneider BL, Gordon R, Magid M, George PM. Novel Fibrinogen γ375 Arg->Try mutation (Fibrinogen Aguadilla) causes hepatic endoplasmic reticulum storage and hypofibrinogenemia. Hepatology 2002;36:652-8.

[22] Francalanci P, Santorelli FM, Talini I, Boldrini R, Devito R, Camassei FD, Maggiore G, Callea F. Severe liver disease in early childhood due to fibrinogen storage and de novo gamma375Arg->Try gene mutation. J Pediatr 2006;148:396-8.

[23] Dib N, Quelin F, Ternisien C, Hanss M, Michalak S, De Mazancourt P, Rousselet MC, Calès P. Fibrinogen angers with a new deletion (γ GVYYQ 346-350) causes hypofibrinogenemia with hepatic storage. J Thromb Haemost 2007;5:1999-2005.

Table 1. Clinical data for three variant fibrinogens substituted at γ326C

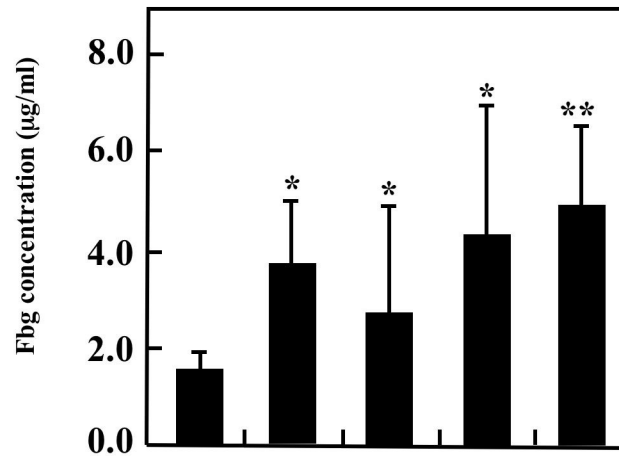| Mutation	Variant name	Patient	Type	Functional fibrinogen level (g/L)	Antigenic fibrinogen level (g/L)	Variant fibrinogen in plasma	Thrombin catalyzed fibrin polymerization	Haemorrhage	Thrombosis	Reference
γ326 Cys/Tyr	Suhl	propositus	dys	<0.6	1.5	albumin binding(+)	impaired	No	Yes	9
γ326 Cys/Tyr	Frostburg	propositus	hypo/dys	0.5	0.4	very low	impaired	No	Yes	10
		sister 1		0.7	0.5	ND		No	No	
		sister 2		0.6	0.6	ND		No	No	
γ326 Cys/Ser	Córdoba	propositus	dys	0.12	1.42	ND	impaired	Yes	No	11
		son		0.25	0.81	ND		No	No	

ND: not described

Figure legends

Figure 1. 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): n=8; γ326A: n=14; γ326Y: n=8; γ326S: n=8; and γ339A: n=12. Significantly different from N: (*P<0.01, **P<0.001).
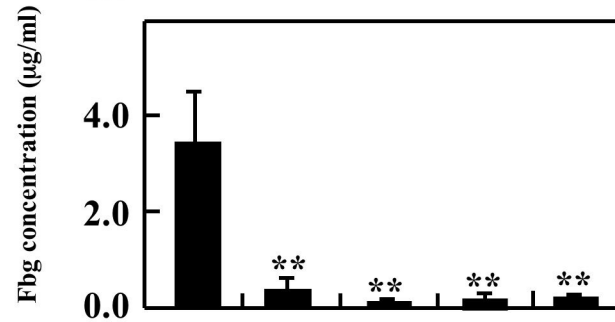
Figure 2. Immunoblotting analysis of the cell lysate. The same volumes of samples from cell lysates were subjected to 8% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under reducing conditions (B). The blots were probed using a polyclonal antibody to fibrinogen and reactive bands were detected by chemiluminescence, as described in Materials and Methods. Samples from individual CHO cell lines were electrophoresed in lanes 2: normal (N)-20; 3: γ326A-57; 4: γ326Y-23; 5: γ326S-31; 6: γ339A-28; Lane 1: purified fibrinogen from normal plasma; and 7: from parent AαBβ-CHO cells. Int: intermediates of fibrinogen, Right sides of panel A and B show molecular size markers.

Figure 3. Analysis of pulse-labeled fibrinogen in transfected CHO cells. Cells were labeled for 1 h with [^{35}S]-methionine and chased for the indicated periods (0, 1, 3, 6, 8, or 24 h) with an excess of unlabeled methionine. Immunoprecipitates from the cell lysates or conditioned media were analyzed by 4% to 12% gradient SDS-PAGE under non-reducing conditions and labeled bands were detected by autoradiography. Lane Inh in the normal cell lysate or medium includes the addition of excess purified plasma fibrinogen to the reaction mixtures of the 6 h chase experiment to demonstrate the antibody specificity, and lane PC is the medium after a 6 h chase in normal-CHO cells as a positive control. Fbg: synthesized mature fibrinogens, *: unknown band.
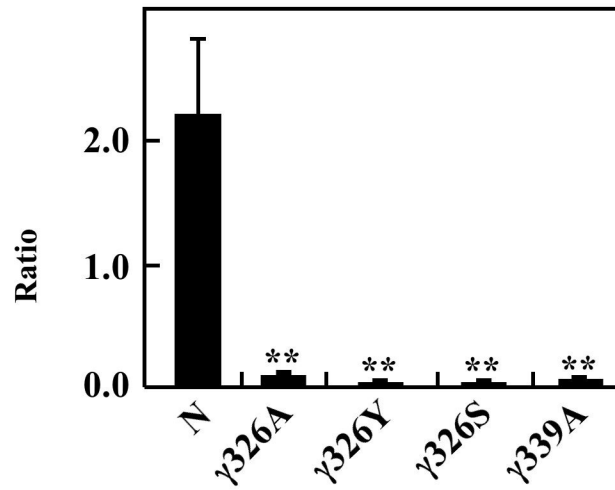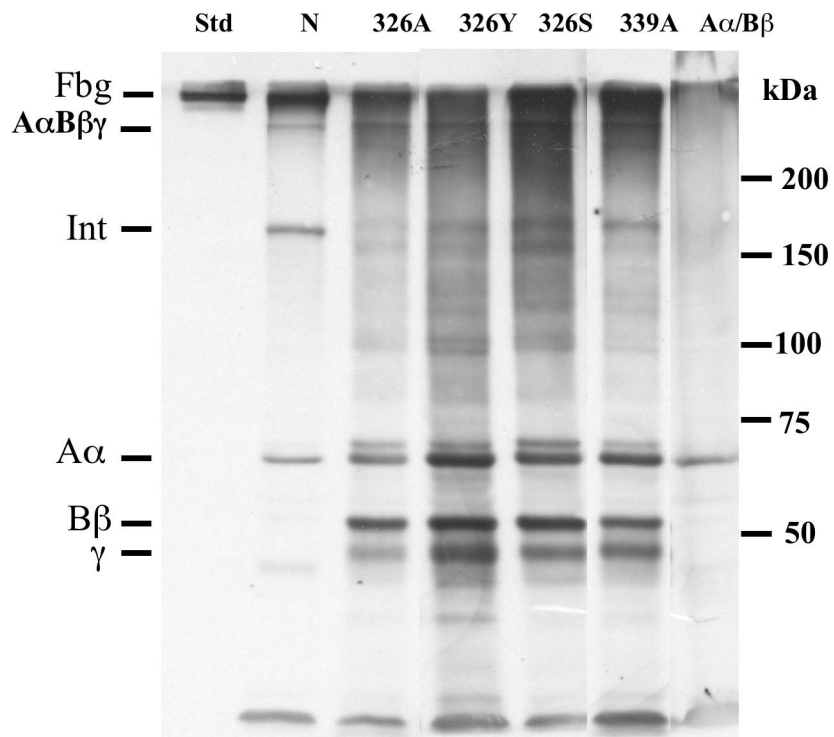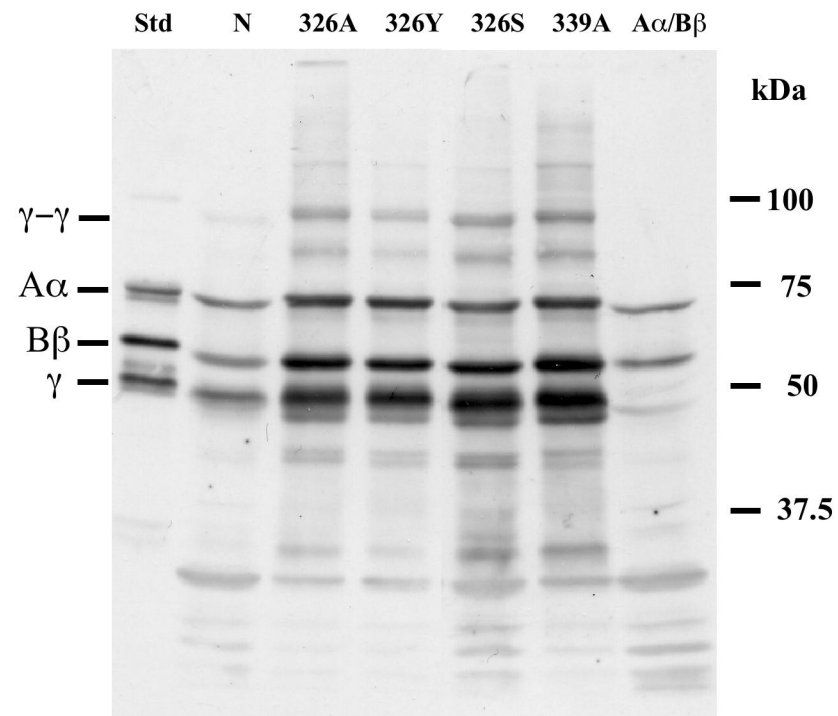
A

Std	N	326A	326Y	326S	339A	Aα/Bβ	

Fbg
AαBβγ
Int
Aα
Bβ
γ

kDa

200
150
100
75
50

B

Std	N	326A	326Y	326S	339A	Aα/Bβ	

γ–γ
Aα
Bβ
γ

kDa

100
75
50
37.5

Cell lysate

A: N

| 0 | 1 | 3 | 6 | 8 | 24 | Inh | PC |

Fbg –
AαBβγ –

B: γ153A

| 0 | 1 | 3 | 6 | 8 | 24 |

* –

C: γ326A

| 0 | 1 | 3 | 6 | 8 | 24 |

Fbg –
AαBβγ –
* –

Medium

D: N

| 0 | 1 | 3 | 6 | 8 | 24 | Inh | PC |

Fbg –

E: γ153A

| PC | 0 | 1 | 3 | 6 | 8 | 24 |

Fbg –

F: γ326A

| 0 | 1 | 3 | 6 | 8 | 24 |

Fbg –