A gamma methionine-310 to threonine substitution and consequent N-glycosylation at gamma asparagine-308 identified in a congenital dysfibrinogenemia associated with posttraumatic bleeding, fibrinogen Asahi.

K Yamazumi, … , N Takahashi, M Matsuda

J Clin Invest. 1989;83(5):1590-1597. https://doi.org/10.1172/JCI114056.

Research Article

In an abnormal fibrinogen with severely impaired polymerization of fibrin monomers, we identified a methionine-to-threonine substitution at position 310 of the gamma chain. Furthermore, asparagine at position 308 was found to be N-glycosylated due to a newly formed consensus sequence, asparagine(308)-glycine(309)-threonine(310). The two structural defects in the mutant gamma chain may well perturb the conformation required for fibrin monomer polymerization that is specifically assigned to the D domain of fibrinogen. This alteration also seems to affect the intermolecular gamma chain cross-linking of fibrin and fibrinogen, although the amine acceptor gamma glutamine-398 was found to function normally. These functional abnormalities may well be related to posttraumatic hemorrhage as observed in a 33-yr-old man with moderate hemorrhagic diathesis related to injuries since his early adolescence. The structure of the extra carbohydrate moiety attached to asparagine-308 was found to be identical with those derived from the normal B beta and gamma chains as evidenced by HPLC.

A γ Methionine-310 to Threonine Substitution and Consequent *N*-Glycosylation at γ Asparagine-308 Identified in a Congenital Dysfibrinogenemia Associated with Posttraumatic Bleeding, Fibrinogen Asahi

Kensuke Yamazumi, Kenji Shimura,* Shigeharu Terukina, Noriko Takahashi,‡ and Michio Matsuda
*Division of Hemostasis and Thrombosis Research, Institute of Hematology, Jichi Medical School, Tochigi 329-04, Japan; *Department of Internal Medicine, Asahi General Hospital, Asahi, Chiba 289-25, Japan; and ‡School of Nursing, Nagoya City University, Nagoya, Aichi 467, Japan*

Abstract

In an abnormal fibrinogen with severely impaired polymerization of fibrin monomers, we identified a methionine-to-threonine substitution at position 310 of the γ chain. Furthermore, asparagine at position 308 was found to be *N*-glycosylated due to a newly formed consensus sequence, asparagine(308)-glycine(309)-threonine(310). The two structural defects in the mutant γ chain may well perturb the conformation required for fibrin monomer polymerization that is specifically assigned to the D domain of fibrinogen. This alteration also seems to affect the intermolecular γ chain cross-linking of fibrin and fibrinogen, although the amine acceptor γ glutamine-398 was found to function normally. These functional abnormalities may well be related to posttraumatic hemorrhage as observed in a 33-yr-old man with moderate hemorrhagic diathesis related to injuries since his early adolescence.

The structure of the extra carbohydrate moiety attached to asparagine-308 was found to be identical with those derived from the normal Bβ and γ chains as evidenced by HPLC.

Introduction

The carboxy-terminal region of the fibrinogen γ chain has been shown to participate in various functions of fibrinogen including fibrin polymerization (1–7).

To form stable fibrin clots, several reactions take place sequentially or simultaneously. They include proteolytic cleavage of fibrinopeptides A and B by thrombin, polymerization of fibrin monomers to form fibrin gels, and intermolecular cross-linking mediated by activated blood coagulation factor XIII (Factor XIIIa) (4, 5). Although polymerization and gelation of fibrin have been extensively studied (1–3), little is known about the mechanisms involved in the reactions. For this purpose, congenital dysfibrinogens with well-elucidated structures may deserve careful study.

In this paper, we report a dysfibrinogen with two structural defects near the carboxy-terminal region of the γ chain. The structure of the D domain may thus be critically perturbed so that polymerization of fibrin monomers and intermolecular cross-linking of the γ chain can not proceed to form stable fibrin clots. The functional abnormalities are apparently associated with the clinical symptom of moderate bleeding as observed in the patient.

Methods

Hemostasis and blood coagulation studies. Blood was collected by venipuncture and immediately anticoagulated with 1/9 vol of 3.8% trisodium citrate. Plasma was harvested by centrifugation at 3,000 *g* for 30 min at 22°C.

Routine hemostasis and blood coagulation were studied by standard methods (8, 9) or as described elsewhere (10, 11). Plasma fibrinogen was determined by various methods including the thrombin time method of Clauss (12–14).

Plasma proteins and enzymes. 200 ml of blood was also collected into a plastic bag containing 30 ml of acid-citrate-dextrose (ACD)[1] (Formula A; U. S. Pharmacopeia, Terumo Corporation, Tokyo, Japan) and plasma was separated by centrifugation at 3,000 *g* for 30 min at 4°C.

Fibrinogen was purified from the ACD-plasma essentially as described (14–16). Portions of fibrinogen thus prepared were further treated with an anti-factor XIII (subunit A) MAb bound to protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove Factor XIII, a trace contaminant, and used for structure analysis. Plasminogen and Factor XIII were prepared from human plasma according to Deutsch and Mertz (17) and Lorand et al. (18), respectively. Absorbance coefficients were 15.1 for fibrinogen (19), 17.0 for plasminogen (20), and 13.8 for Factor XIII (21). Bovine thrombin was purified according to Lundblad (22). Ancrod, a snake venom thrombin-like enzyme, was a kind gift from Dr. Minoru Fukuda, Mochida Pharmaceutical Co., Tokyo, Japan. Streptokinase was from Kabi Vitrum, Stockholm, Sweden, from which the albumin component had been removed with blue-Sepharose CL-6B. Glycopeptidase A and neuraminidase were from Seikagaku Kogyo, Tokyo, Japan.

Studies on purified fibrinogen. The determinations of thrombin and Ancrod times and the polymerization of fibrin monomers were carried out as previously described (14–16, 23). Release of fibrinopeptides A and B was analyzed by reverse-phase HPLC (24). The gross structure of fibrinogen, its plasmic degradation products and cross-linking profiles mediated by Factor XIIIa were analyzed by SDS-PAGE according to Laemmli (25) or Weber and Osborn (26). The

Portions of this work were presented at the 1988 International Fibrinogen Workshop, Milwaukee, WI, 15 June 1988.

Dr. Yamazumi is on leave from the First Department of Surgery, Kagoshima University School of Medicine, Kagoshima, Kagoshima 890, Japan. Address reprint requests to Dr. Michio Matsuda, Division of Hemostasis and Thrombosis Research, Institute of Hematology, Jichi Medical School 3311-1 Yakushiji, Minamikawachi-Machi, Tochigi-Ken 329-04, Japan.

Received for publication 3 August 1988 and in revised form 3 January 1989.

J. Clin. Invest.

0021-9738/89/05/1590/08 $2.00
Volume 83, May 1989, 1590–1597

1. *Abbreviations used in this paper:* ACD, acid-citrate-dextrose; CBB, Coomassie brilliant blue; CTA, Committee on Thrombolytic Agents; DFP, diisopropylfluorophosphate; FDP, fibrin fibrinogen degradation products; PTH, phenylthiohydantoin; TABS, 100 μl of 10 mM Tris-acetate, pH 7.6, containing 0.15 M NaCl, 10 mM EDTA, and 1.25 KIU/ml aprotinin; TBSE, 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 10 mM EDTA, and 1.25 KIU/ml aprotinin; TBSCa, 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 5 mM $CaCl_2$, and 1.25 KIU/ml aprotinin.

separated polypeptides were stained with Coomassie brilliant blue (CBB) for protein and with dansylhydrazine for carbohydrate (27). The γ chain and its derivatives were specifically studied by Western blot analysis (28) using a MAb that recognizes the plasmic core fragment of the γ chain (γ 85-302 residue segment) (15, 16).

Cross-linking of fibrinogen was specifically studied as follows: 10 U/ml Factor XIII was activated with 5 U/ml thrombin in the presence of 5 mM $CaCl_2$ for 60 min at 37°C. 2 mM diisopropylfluorophosphate (DFP) was then added to inactivate thrombin. 300 μg of Factor XIII-depleted fibrinogen was incubated with 0.5 U of Factor XIIIa at 37°C in the presence of 1.25 kallikrein inhibitor units (KIU) of aprotinin in 100 μl of TBS (0.05 M Tris–HCl, pH 7.4 containing 0.1 M NaCl) containing 5 mM $CaCl_2$. At various incubation times, aliquots of 1.5 μg protein were removed and analyzed by SDS-PAGE. We also studied incorporation of monodansylcadaverine into fibrinogen by Factor XIIIa essentially as described (5, 29).

Isolation of normal and abnormal fragment D_1 species from the plasmic digests of patient-derived fibrinogen. 40 μg of the patient-derived fibrinogen was digested with plasmin (a mixture of 5.67 Committee on Thrombolytic Agents (CTA) units of plasminogen and 6,000 U of streptokinase) and the digests were subjected to chromatofocusing chromatography to isolate fragment D_1 essentially as described (15). The isolated fragment D_1 species were tested for inhibition of thrombin clotting of normal fibrinogen and normal fibrin monomer polymerization, and also for direct binding to insolubilized and thrombin-activated fibrinogen.

Binding of isolated fragment D_1 species to insolubilized and thrombin-activated fibrinogen. Microtiter plates (Immuno Plate, Maxisorp F96; Nunc, Raskilde, Denmark) were coated with 1.5 μg per well of Factor XIII-depleted fibrinogen in 100 μl of 10 mM Tris-acetate, pH 7.6, containing 0.15 M NaCl, 10 mM EDTA, and 1.25 KIU/ml aprotinin (TABS) and then blocked with 1% (wt/vol) BSA in the same buffer for 1 h at 37°C (30–32). After a wash with TABS, the plates were treated with 0.5 NIH units of thrombin in 100 μl of TABS for 2 h at 37°C. The plates were rinsed with 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 10 mM EDTA, and 1.25 KIU/ml aprotinin (TBSE). Some plates were further rinsed with the same buffer in which EDTA was replaced with 5 mM $CaCl_2$ (TBSCa). 100-μl aliquots of various concentrations of fragment D_1 in either TBSE or TBSCa were pipetted into each well, and allowed to react with the insolubilized and thrombin-activated fibrinogen for 2 h of 37°C. The bound fragment D_1 was measured by an ELISA (28) using a horseradish peroxidase–conjugated MAb that recognizes fragment D species (D_1, D_2, and D_3) but not fibrinogen, fibrin, or their early plasmic fragments X and Y (manuscript in preparation), and 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as a chromogen.

Analysis of the carboxy-terminal peptides of fragment D_1. 500-μg samples of the two patient-derived fragment D_1 species were digested with plasmin and the released relevant peptides were separated and *S*-carboxymethylated after reduction essentially as described (15, 33). Each peptide was analyzed for amino acid sequence (34).

A 10-μmol portion of an aberrant peptide consisting of the γ 303–338 residues (See Results, PI'-2 in Fig. 7) was treated with 20 μl of neuraminidase (0.45 U/ml), digested with glycopeptidase A (0.02 U/ml) essentially as described (35), and finally separated by the same column for sequence analysis.

Analysis of N-linked oligosaccharides by HPLC. 30 nmol each of the aberrant γ 303-356 peptide, the Bβ chain isolated from the patient's fibrinogen, and the Bβ and γ chains from normal fibrinogen were desialylated by mild acid hydrolysis, digested with pepsin, and treated with glycopeptidase A at pH 5.0 as described (36). The oligosaccharide fractions were collected by gel filtration and reductively aminated with 2-aminopyridine (37). The pyridylamino derivatives of oligosaccharides thus prepared were fractionated and identified by reverse-phase HPLC on a conventional liquid chromatography-octadecyl silica column (Shim-pack, 6 × 150 cm; Shimadzu Corporation, Kyoto, Japan) according to Tomiya et al. (38).

Results

Description of the patient. A 33-yr-old man was hospitalized for bleeding from a 2-cm-long cut wound on his face and multiple excoriation wounds on his right arm and leg caused by a traffic accident. Bleeding was, however, satisfactorily controlled by surgical sutures and compression hemostasis. He had been suffering from moderate bleeding associated with injuries since his early adolescence.

Routine hemostasis and coagulation studies revealed that the patient had a markedly prolonged thrombin time (> 300 s, control 15.8 s) and Ancrod time (> 300 s, control 9.8 s), and an apparently reduced plasma fibrinogen level (< 50 mg/dl, normal 150–350 mg/dl) as determined by the thrombin time method (12). When plasma fibrinogen was measured by other methods, rather high values were obtained: 525 mg/dl by Laurell's method (13) and 565 mg/dl by the turbidimetric method (14). The bleeding time, platelet counts and platelet aggregation were normal, but the one-stage prothrombin time (14.9 s; control, 10.9 s) and activated partial thromboplastin time (41.7 s; control, 33.5 s) were moderately prolonged. The level of fibrin/fibrinogen degradation products (FDP) was 160 μg/ml. Blood coagulation Factors II, V, VII, VIII, IX, X, XI, XII, and XIII, plasminogen, antithrombin III, and α_2-plasmin inhibitor were all within normal ranges. These data appeared to be compatible with congenital dysfibrinogenemia.

Although only his brother was available and found to be unaffected, we tentatively designated this abnormal fibrinogen as "fibrinogen Asahi."

Studies on purified fibrinogen. As summarized in Table I, both the thrombin and Ancrod times were markedly prolonged regardless of the presence or absence of calcium ions. The lack of correction of thrombin clotting of fibrinogen by calcium ions seems to be rather unique as compared with most of the other abnormal fibrinogens reported heretofore (39).

By SDS-PAGE after reduction, we noticed two bands for the γ chain of the patient-derived fibrinogen, one with a normal molecular weight and the other with a higher molecular weight by ~ 3,000, as indicated by "γ-Asahi" in Fig. 1 (lanes *2, 4,* and *6*). Although the release of fibrinopeptides A and B was normal as evidenced by HPLC (figures not shown), polymerization of fibrin monomers was severely impaired in both the absence and presence of calcium ions as shown in Fig. 2.

We then analyzed cross-linking profiles of fibrin mediated by Factor XIIIa. As shown in *A* and *B* in Fig. 3, the abnormal

Table I. Thrombin and Ancrod Times and Thrombin Clottability of Purified Fibrinogen

Studies	Normal	Patient's
Thrombin time (*s*)		
Without calcium ions	16.7	>180
With calcium ions	12.8	>180
Ancrod time (*s*)		
Without calcium ions	9.6	>180
With calcium ions	5.5	>180
Thrombin clottability (%)		
Without calcium ions	88	32
With calcium ions	95	30

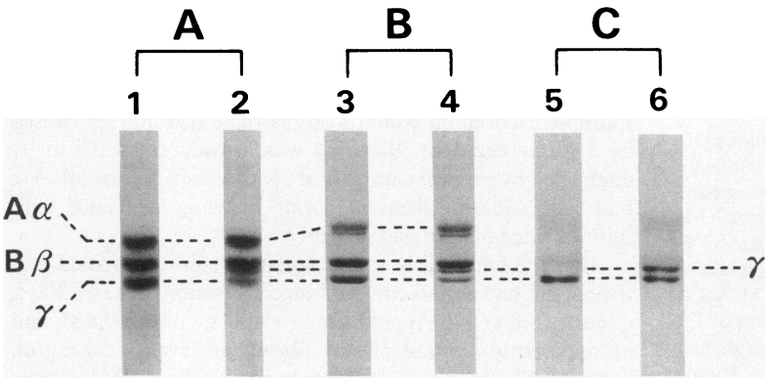

Figure 1. Subunit polypeptides of fibrinogen examined by SDS-PAGE and Western blotting. (*A*) Weber and Osborn gel, 5%; (*B*) Laemmli gel, 10%; (*C*) Western blot analysis with an anti-γ core (the γ 85–302 residue segment) antibody. Lanes *1*, *3*, and *5* are normal fibrinogen and lanes *2*, *4*, and *6* are the patient's fibrinogen. To each lane, 1.5 μg of protein was loaded.

γ chain species was converted to the γ-γ dimer very slowly as compared with the normal one. As the incubation proceeded, three γ-γ dimers appeared, probably in such combinations as: (*a*) two normal γ chains, (*b*) one each of the normal and γ-Asahi chains and (*c*) two γ-Asahi chains, as indicated by N·γ-N·γ, N·γ-A·γ and A·γ-A·γ, respectively. Judging from the intensity of the bands for the γ-γ dimers, formation of the N·γ-N·γ dimer proceeded predominantly at the initial stage of reaction. However, N·γ-A·γ dimer formation, first noted at 5 min of incubation (lane *3*), did not progress any further as the incubation proceeded, most probably because of the unavailability of normal γ chains for the cross-linking. On the contrary, formation of the A·γ-A·γ dimer progressed slowly in accordance with the disappearance of the γ-Asahi chain over 24 h. The extremely retarded cross-linking between the two abnormal γ chains may be accounted for by severely impaired polymerization of fibrin monomers. Therefore, we examined the cross-linking profile of fibrinogen to minimize the effect of polymerization. Here again, the mutant γ chain species was not cross-linked even after 24 h of incubation (profiles not shown).

To see whether the cross-linking sites of the γ-Asahi chain function normally, we examined the amine acceptor γ glutamine (Gln)-398 by monitoring the incorporation of monodansylcadaverine. Fluorescence was equally incorporated into both the normal and γ-Asahi chains (profiles not shown), indicating that the amine acceptor of the mutant γ chain functions normally.

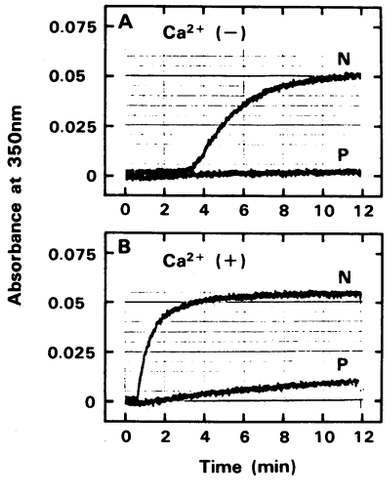

Figure 2. Aggregation profiles of fibrin monomers. 20 μl of fibrin monomers (1.94 mg/ml) derived from normal (N) fibrinogen or that from the patient (P) was diluted with 500 μl of 0.02 M imidazole-buffered saline, pH 7.4, containing either (*A*) 0.15 M NaCl or (*B*) 0.14 M NaCl and 5 mM $CaCl_2$.

Isolation of normal and abnormal fragment D_1 species. As depicted in Fig. 4, fragment D_1 derived from the patient's fibrinogen was eluted in two peaks: one eluted at the same pH range between 5.8 and 5.5 as normal fragment D_1, and the other eluted at a slightly more acidic pH range between 5.5 and 4.8. The peak fractions were collected and combined as indicated by horizontal bars I and II. By SDS-PAGE, peak I was identical with normal fragment D_1, but peak II was found to possess a 3-kD higher molecular weight γ chain (inset in Fig. 4 and lanes *3* and *4* in *A*, Fig. 6). The peak I fraction prolonged the clotting time of normal fibrinogen dose dependently, namely, 11.6, 14.2, 16.8, 21.1, and 27.5 s at the fragment D_1/fibrinogen molar ratios (D_1/Fbg) of 0, 1, 2, 3, and 4, respectively. On the contrary, the peak II fraction did not significantly affect it (11.6, 11.8, 12.4, 12.6, and 12.5 s at the corresponding D_1/Fbg molar ratios). Likewise, the peak I fraction inhibited polymerization of normal fibrin monomers significantly, but the peak II fraction did not do so (profiles not shown). These data indicate that the polymerization site(s) specifically assigned to the D domain is functional in the peak I but dysfunctional in the peak II fractions.

Binding of the isolated fragment D_1 species to insolubilized and thrombin-activated fibrinogen. We then examined the binding ability of the normal and abnormal fragment D_1 species to thrombin-activated fibrinogen insolubilized onto microtiter plates in the presence or absence of calcium ions. As depicted in Fig. 5, the binding of abnormal fragment D_1 was significantly lower than that of normal fragment D_1 under comparable conditions. Although the binding of abnormal fragment D_1 was apparently increased in the presence of calcium ions, it was still evidently lower than that of normal fragment D_1 in the absence of calcium ions.

Carbohydrate staining of the mutant γ chain. The finding that the γ-Asahi chain and the γ remnant of abnormal fragment D_1 were apparently larger than the normal counterparts prompted us to investigate whether the γ chain itself was elongated in the D domain, or whether some extra substances were attached to a normal length polypeptide. We first examined possible extra glycosylation to the mutant γ chain by staining the polypeptide remnants of fragment D_1 with dansylhydrazine. As shown in Fig. 6, the γ-Asahi remnant of fragment D_1 was clearly stained for carbohydrate (lane *4* in *B*), whereas the normal γ chain remnant that lacks carbohydrate was not stained as expected (lane *3* in *B*). Furthermore, the γ-Asahi chain showed strongly positive staining for carbohydrate (lane *2* in *B*) as compared with the normal Bβ and γ chains (lanes *1* and *2* in *B*).

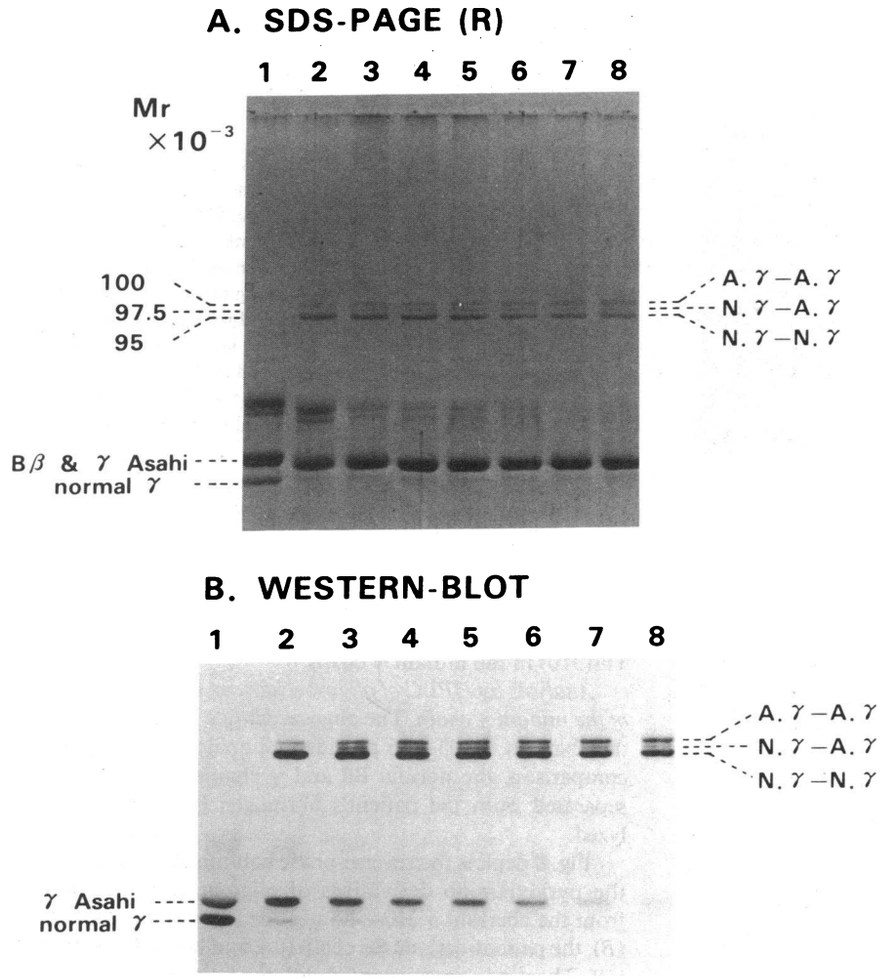

Figure 3. Factor XIIIa-mediated cross-linking of fibrin analyzed by SDS-PAGE and Western blotting after reduction. 50-μg aliquots of factor XIII-depleted fibrinogen were clotted with 0.2 NIH units of thrombin and 0.1 U of factor XIII in the presence of 1.0 KIU of aprotinin in 80 μl of 0.05 M Tris-HCl, pH 7.4, and 0.1 M NaCl, containing 5 mM $CaCl_2$. At various time intervals at 37°C, the reaction mixtures were treated with 1% SDS-1% DTT, and 1.5 μg protein samples were subjected to SDS-PAGE in the Laemmli system in duplicate, using 7.5% acrylamide slab gels. One gel was stained with CBB for proteins (*A*), and the other gel was subjected to Western blot analysis using the anti-γ core MAb (*B*). Lane *1*, 0 min; lane *2*, 2 min; lane *3*, 5 min; lane *4*, 10 min; lane *5*, 15 min; lane *6*, 30 min; lane *7*, 2 h and lane *8*, 24 h.

Identification of the extra glycosylation in the mutant γ chain. To locate the extra carbohydrate moieties in the mutant γ chain, we digested the abnormal fragment D_1 with plasmin in the presence of EGTA, and analyzed the digests by SDS-PAGE and Western blotting. We found that the molecular weight for the mutant γ remnant was higher in fragments D_1 and D_2, but not so in fragment D_3 as compared with the corresponding normal γ remnants (profiles not shown). This indicated that the carboxy-terminal peptide removed upon conversion of fragment D_2 to fragment D_3; i.e., the aberrant γ 303–356 residue peptide, is most probably glycosylated. We thus attempted to isolate the putative aberrant γ 303–356 peptide by reverse-phase HPLC. As shown in Fig. 7 *A*, we

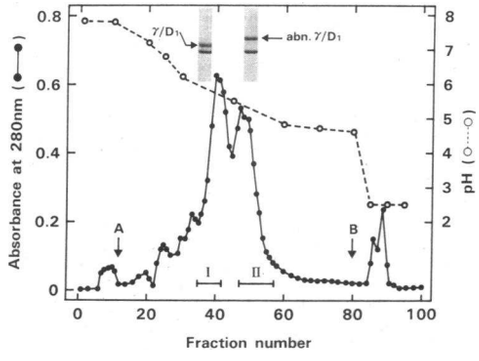

Figure 4. Separation of normal and abnormal fragment D_1 species by chromatofocusing. 40 μg of plasmic digests of the patient's fibrinogen was applied onto a polybuffer exchanger gel column (1.2 × 18 cm) and eluted with 350 ml of polybuffer, pH 4.7, followed by 100 ml of 0.025 M glycine-HCl, pH 2.5, containing 0.1 M NaCl, as indicated by vertical arrows A and B, respectively. The flow rate was 20 ml/h and 5-ml fractions were collected. (*Inset*) SDS-PAGE of peak I and peak II fractions after reduction.

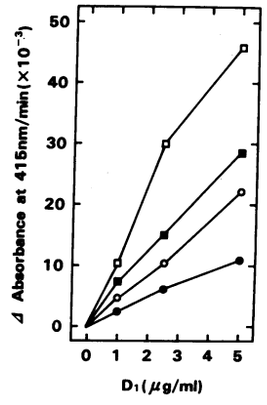

Figure 5. Binding of the two fragment D_1 species derived from the patient's fibrinogen to insolubilized and thrombin-activated normal fibrinogen as determined by an ELISA. (●) Abnormal fragment D_1 in the absence of calcium ions, (○) abnormal fragment D_1 in the presence of 5 mM $CaCl_2$; (■) normal fragment D_1 in the absence of calcium ions; and (□) normal fragment D_1 in the presence of 5 mM $CaCl_2$.

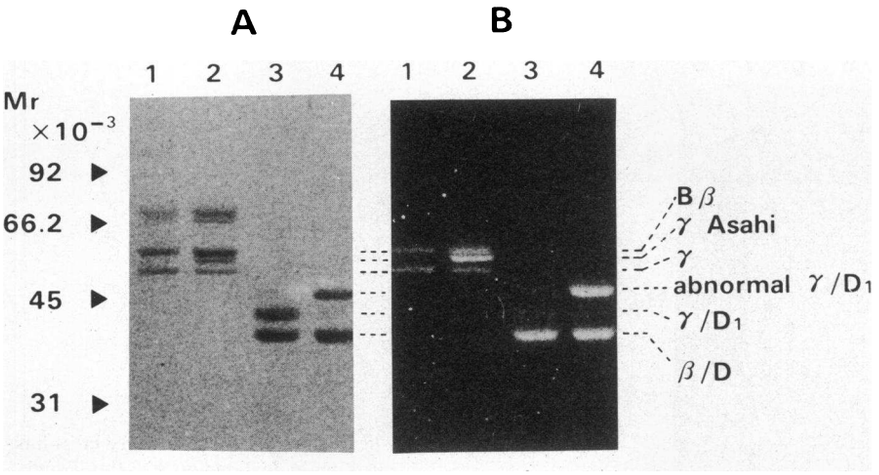

Figure 6. Analysis of the patient's fibrinogen and its two fragment D_1 species by carbohydrate staining. SDS-PAGE was performed by Laemmli's method using 10% gels. (*A*) CBB staining for proteins; (*B*) carbohydrate staining by dansylhydrazine. Lane *1*, normal fibrinogen; lane *2*, patient's fibrinogen; lane *3*, normal fragment D_1 species derived from the patient's fibrinogen; and lane *4*, abnormal fragment D_1 species derived from the patient's fibrinogen.

identified an aberrant peak PI′ that was eluted earlier than its normal counterpart PI. These peptide peaks were reduced, *S*-carboxymethylated, and subjected to rechromatography, and an aberrant peptide PI′-2 was isolated (lower figure in *B*). A portion of this peptide was desialylated and then treated with glycopeptidase A. This peptide was designated as PI′-2G. As shown in Table II, PI′-2 was identified as the γ 303-338 residue peptide on the basis of the known sequence for the γ chain (2, 3, 40), but a methionine (Met) at cycle 8 of this peptide was found to be replaced by a threonine (Thr). Furthermore, an asparagine (Asn) at cycle 6 identified in the normal peptide was not detectable in this aberrant peptide, suggesting that Asn-308 in the mutant γ chain is linked to an oligosaccharide. Indeed, in PI′-2G, we identified an aspartic acid (Asp) at cycle 6, which was certainly derived from *N*-glycosylated Asn upon treatment with glycopeptidase A. We thus concluded that Met-310 is replaced by a Thr and consequently, Asn-308 is *N*-glycosylated because of a newly constructed consensus sequence of Asn(308)-glycine(Gly)(309)-Thr(310) in the mutant γ chain.

Analysis by HPLC of oligosaccharides linked to γ Asn-308 of the mutant γ chain. The oligosaccharide was separated from the aberrant γ 303–356 peptide and analyzed by HPLC. For comparison, the normal Bβ and γ chains and the Bβ chain separated from the patient's fibrinogen (41) were also analyzed.

Fig. 8 depicts the reverse-phase column elution profiles for the pyridylamino derivatives of oligosaccharides separated from the aberrant γ 303–356 peptide (*A*), the normal γ chain (*B*), the patient-derived Bβ chain (*C*), and the normal Bβ chain (*D*). There are no substantial differences among elution profiles for all the oligosaccharides. Among them, peak 5 was found to represent a biantennary oligosaccharide proposed for

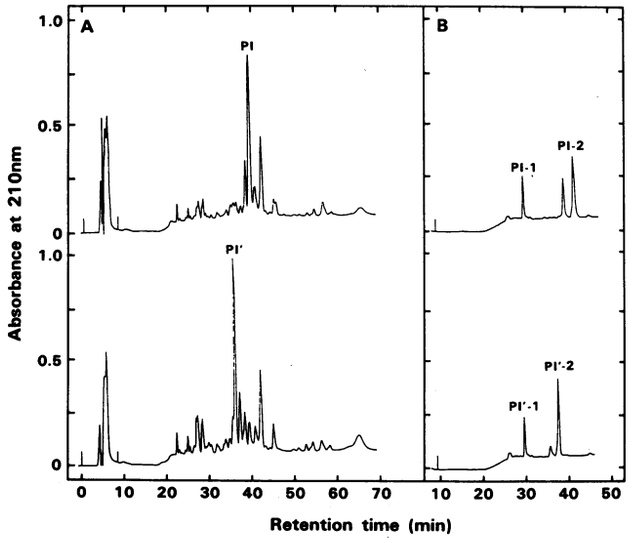

Figure 7. HPLC elution profiles of the peptides released from the two fragment D_1 species upon their conversion to fragment D_3. (*A*) The peptides derived from the normal (*top*) and abnormal (*bottom*) fragment D_1 species. (*B*) Rechromatography of the reduced and *S*-carboxymethylated aberrant peptide PI′ and its normal counterpart PI using the same column. The aberrant peptide PI′-2 was separated and subjected to amino acid analysis. See text for details.

Table II. First 10 Cycles of Amino Acids for Peptides PI-2, PI′-2, and PI′-2G

		PI-2		PI′-2		PI′-2G	
Cycle	Position	Amino acid		Amino acid		Amino acid	
			pmol		*pmol*		*pmol*
1	303	Phe	340	Phe	493	Phe	495
2	304	Phe	283	Phe	424	Phe	334
3	305	Thr	139	Thr	251	Thr	140
4	306	Ser	NQ*	Ser	NQ*	Ser	NQ*
5	307	His	9	His	21	His	6
6	308	Asn	15	Nil‡		Asp	10
7	309	Gly	11	Gly	29	Gly	7
8	310	Met	15	Thr	17	ND§	
9	311	Gln	8	Gln	21	ND§	
10	312	Phe	8	Phe	24	ND§	

Phe, phenylalanine; Thr, threonine; Ser, serine; His, histidine; Asn, asparagine; Asp, aspartic acid; Gly, glycine; Met, methionine; Gln, glutamine.

* Not quantitated.

‡ No PTH amino acid was identified.

§ Not determined.

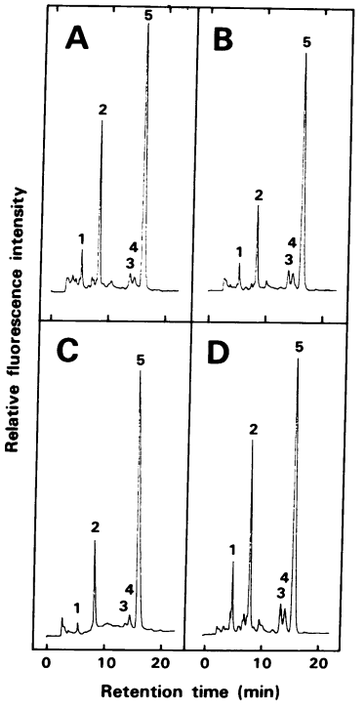

Figure 8. Analysis of the extra oligosaccharide attached to the mutant γ chain by reverse-phase HPLC. HPLC elution profiles of oligosaccharides derived from *A*, the aberrant γ 303–356 residue peptide; *B*, the normal γ chain, *C*, the Bβ chain of the patient's fibrinogen; and *D*, the normal Bβ chain. The main peak, peak 5, is an oligosaccharide with a biantennary structure shown below.

Galβ(1,4)GlcNAcβ(1,2)Manα(1,3)↘
Galβ(1,4)GlcNAcβ(1,2)Manα(1,6)↗ Manβ(1,4)GlcNAcβ(1,4)GlcNAcβ-Asn

Gal, galactose; GlcNAc, *N*-acetylglucosamine; and Man, mannose. Peaks 1 and 2 are its pyridylamino derivatives, and peaks 3 and 4, oligosaccharides chains lacking the Galβ(1,4) attached to the Manα(1,3) (peak 3) or that attached to the Manα(1,6) (peak 4).

the carbohydrate attached to the normal Bβ and γ chains of human fibrinogen (42). There were also two small peaks, 3 and 4, to be assigned to oligosaccharides that lack the β(1,4) galactose attached to the α(1,3)-linked mannose (peak 3) and to the α(1,6)-linked mannose (peak 4), as compared with the elution positions for the authentic oligosaccharides with known structures (38).

Discussion

We identified a new type of mutation, a γ Met-310 to Thr substitution, and consequent *N*-glycosylation at γ Asn-308 in an abnormal fibrinogen designated as fibrinogen Asahi.

The abnormality seems to be associated with posttraumatic bleeding as observed in the patient, a 33-yr-old man who has been manifesting clinically mild-to-moderate hemorrhagic diathesis related to injuries since his early adolescence.

The functional abnormality could be categorized as severely impaired polymerization that is not readily correctable by calcium ions and significantly delayed cross-linking of the mutant γ chain. Indeed, a point mutation together with a bulky extra oligosaccharide structure attached near the carboxy-terminal region of the γ chain may well perturb the local conformation of the D domain that is critical for fibrin polymerization (1–3). As proposed by several investigators (43–46), the contact between the thrombin-activated E domain of one molecule and the D domain of another with an overlap of one-half molecule is believed to play a primary role at the initial stage of fibrin oligomer formation. In fibrinogen Asahi, this type of contact is significantly impaired as evidenced by the decreased binding of isolated abnormal fragment D_1 to insolubilized and thrombin-activated normal fibrinogen even in the presence of calcium ions (Fig. 5). Furthermore, the close contact between the two D domains of different molecules may also participate in the polymerization mechanisms by eliciting a stable D · D structure that can fit in its complementary structure exposed on the central E domain of fibrinogen exposed upon thrombin-treatment (1, 14, 43–45). This type of contact seems to proceed very slowly in fibrinogen Asahi as deduced from the protracted γ chain cross-linking of fibrin and fibrinogen.

Because the intermolecular γ chain cross-linking of fibrinogen takes place at the same amine acceptor (Gln-398) and donor (Lys-406) as fibrin in an antiparallel fashion (43, 44), our data seem to imply that the two abnormal D domains are unable to contact each other closely enough to achieve an antiparallel alignment of the carboxy-terminal part of their γ chains. Nevertheless, the carboxy-terminal plasmic peptide (γ 374–411) containing a normally functioning amine acceptor, γ Gln-398, was identified at exactly the same position as its normal counterpart on HPLC (profiles not shown). This indicates that both amine donor and acceptor sites are located normally in the mutant γ chain.

The impaired intermolecular γ chain cross-linking mediated by activated Factor XIIIa seems to be unique, as another abnormal fibrinogen with impaired γ chain cross-linking, fibrinogen Paris I (47, 48), has been characterized to have a 3.1-kD peptide extension at the carboxy terminus of its γ chain that renders the amine acceptor site unavailable for the cross-linking reaction (48).

Despite the two structural defects in or near the putative calcium binding site, the γ 303–356 (3) or the γ 311–336 residue segment (49), the calcium binding ability seems to be well preserved based on the observed normal calcium effect on the protection against plasmic cleavage of the γ remnant of abnormal fragment D_1 (Figs. 4 and 6).

Upon amino acid sequence analysis of the aberrant γ 303–338 residue peptide PI'-2 by Edman degradation, the phenylthiohydantoin (PTH) derivative of an Asn was not detectable at cycle 6 of the aberrant peptide, suggesting that the Asn was glycosylated. Indeed, a PTH derivative of an Asp was recovered at cycle 6 as a product from the *N*-glycosylated Asn, when the peptide had been digested with glycopeptidase A that specifically hydrolyzes β-aspartylglycosylamine linkages (36, 50, 51). This extra glycosylation is certainly attributed to a newly constructed tripeptide sequence, Asn(308)-Gly(309)-Thr(310) because of a Met-310-to-Thr substitution. Such a mutation was formerly proposed for the mutant Bβ chain of fibrinogen Pontoise (52), namely, a replacement of the Bβ Ala-335 by a Thr and possible glycosylation at the Bβ Asn-333 based on an amino acid analysis of an aberrant tryptic peptide. However, no detailed data have so far been provided on the possible extra carbohydrate moiety in this abnormal molecule.

In this study, we provided evidence that the extra carbohy-

drate moiety linked to Asn-308 of the mutant γ-Asahi chain is identical to those linked to the normal Bβ (Bβ Asn-364) and γ (γ Asn-52) chains (42) (Fig. 8). The carbohydrate moiety was found to be composed of the major oligosaccharide with a biantennary structure (42) and two minor components lacking the β(1,4) galactose attached to the α(1,3)-linked mannose or that attached to the α(1,6)-linked mannose (38). The biantennary structure has also been reported for the oligosaccharides of the mutant γ chain of fibrinogen Nagoya (53).

Acknowledgments

This work was supported in part by Scientific Research Grants-in-Aid 61480272 and 63480293 and Grant-in-Aid for Scientific Research on Priority Areas 63616512 from the Ministry of Education of the Government of Japan, Research Grants for Intractable Disease and for Cardiovascular Disease, 60-C, from the Ministry of Health and Welfare of the Government of Japan, and a grant from the Japan Private School Promotion Foundation (to M. Matsuda).

References

1. Olexa, S. A., and A. Z. Budzynski. 1980. Evidence for four different polymerization sites involved in human fibrin formation. *Proc. Natl. Acad. Sci. USA* 77:1374–1378.

2. Southan, C., E. Thompson, M. Panico, T. Etienne, H. R. Mouis, and D. A. Lane. 1985. Characterization of peptides cleaved by plasmin from the C-terminal polymerization domain of human fibrinogen. *J. Biol. Chem.* 260:13095–13101.

3. Varadi, A., and H. A. Scheraga. 1986. Localization of segments essential for polymerization and for calcium binding in the γ-chain of human fibrinogen. *Biochemistry.* 25:519–528.

4. Chen, R., and R. F. Doolittle. 1971. γ-γ cross-linking sites in human and bovine fibrin. *Biochemistry.* 10:4486–4491.

5. Lorand, L., D. Chenoweth, and A. Gray. 1972. Titration of the acceptor cross-linking sites in fibrin. *Ann. NY Acad. Sci.* 202:155–171.

6. Timmons, S., M. Kloczewiak, and J. Hawiger. 1984. ADP-dependent common receptor mechanism for binding of von Willebrand factor and fibrinogen to human platelets. *Proc. Natl. Acad. Sci. USA.* 81:4935–4939.

7. Nieuwenhuizen, W., M. Voskuilen, A. Vermond, F. Haverkate, and J. Hermans. 1982. A fibrinogen fragment D (D intermediate) with calcium binding but without anticlotting properties. *Biochim. Biophys. Acta.* 707:190–192.

8. Tocantins, L. A., and L. A. Kazal, editors. 1964. Blood Coagulation, Hemorrhage and Thrombosis. Methods of Study. Grune & Stratton, Inc., New York. 29–150.

9. Duke, W. W. 1910. The relation of blood platelets to hemorrhagic disease: description of a method for determining the bleeding time and coagulation time and report of three cases with hemorrhagic disease relieved by transfusion. *JAMA (J. Am. Med. Assoc.).* 55:1185–1188.

10. Aoki, N., M. Moroi, Y. Sakata, N. Yoshida, and M. Matsuda. 1978. Abnormal plasminogen. A hereditary molecular abnormality found in a patient with recurrent thrombosis. *J. Clin. Invest.* 61:1186–1195.

11. Sakata, Y., and N. Aoki. 1980. Cross-linking of α_2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J. Clin. Invest.* 65:290–297.

12. Clauss, A. 1957. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematol. (Basel).* 17:237–246.

13. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* 15:45–52.

14. Matsuda, M., M. Baba, K., Morimoto, and C. Nakamikawa. 1983. Fibrinogen Tokyo II. An abnormal fibrinogen with an impaired polymerization site on the aligned DD domain of fibrin molecules. *J. Clin. Invest.* 72:1034–1041.

15. Terukina, S., Matsuda, M., Hirata, H., Takeda, Y., Miyata, T., Takao, T. and Shimonishi, Y. 1988. Substitution of γ Arg-275 by Cys in an abnormal fibrinogen, "fibrinogen Osaka II". Evidence for a unique solitary cystine structure at the mutation site. *J. Biol. Chem.* 263:13579–13587.

16. Yoshida, N., Terukina, S., Okuma, M., Moroi, M., Aoki, N. and Matsuda, M. 1988. Characterization of an apparently lower molecular weight γ-chain variant in fibrinogen Kyoto I. The replacement of γ Asn-308 by Lys which caused an accelerated cleavage of fragment D_1 by plasmin and the generation of a new plasmin cleavage site. *J. Biol. Chem.* 263:13848–13856.

17. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen. Purification from human plasma by affinity chromatography. *Science (Wash. DC).* 170:1095–1096.

18. Lorand, D., R. B. Credo, and T. J. Janus. 1981. Factor XIII (fibrin-stabilizing factor). *Methods Enzymol.* 80:333–341.

19. Marder, V. J., N. R. Shulman, and W. F. Carroll. 1969. High molecular weight derivatives of human fibrinogen produced by plasmin. I. Physiochemical and immunological characterization. *J. Biol. Chem.* 244:2111–2119.

20. Barlow, G. H., L. Summaria, and K. C. Robbins. 1969. Molecular weight studies on human plasminogen and plasmin at the μg level. *J. Biol. Chem.* 244:1138–1141.

21. Shwartz, M. L., Pizzo, R. L. Hill, and P. A. Mckee. 1973. Human factor XIII from plasma and platelets. *J. Biol. Chem.* 248:1395–1407.

22. Lundblad, R. L. 1971. A rapid method for the purification of bovine thrombin and the inhibition of the purified enzyme with phenylmethylsulfonyl fluoride. *Biochemistry.* 10:2501–2506.

23. Gralnick, H. R., H. M. Givelber, J. R. Shainoff, and J. S. Finlayson. 1971. Fibrinogen Bethesda: a congenital dysfibrinogenemia with delayed fibrinopeptide release. *J. Clin. Invest.* 50:1819–1830.

24. Kehl, M., F. Lottspeich, and A. Henschen. 1981. Analysis of human fibrinopeptides by high-performance liquid chromatography. *Hoppe-Seyler's Z. Physiol. Chem.* 362:1661–1664.

25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

26. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.

27. Eckhardt, A. E., C. E. Hayes, and I. J. Goldstein. 1976. A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. *Anal. Biochem.* 73:192–197.

28. Wakabayashi, K., Y. Sakata, and N. Aoki. 1986. Conformation-specific monoclonal antibodies to the calcium-induced structure of protein C. *J. Biol. Chem.* 261:11097–11105.

29. Fretto, L. J., E. W. Ferguson, H. M. Steinman, and P. A. Mckee. 1978. Localization of the α-chain cross-link acceptor sites of human fibrin. *J. Biol. Chem.* 253:2184–2195.

30. Doellgast, G. J., and H. Rothberger. 1985. Enzyme-linked coagulation assay: a clot-based, solid-phase assay for thrombin. *Anal. Biochem.* 147:529–534.

31. Doellgast, G. J., and H. Rothberger. 1986. Enzyme-linked coagulation assay II. a sensitive assay for tissue factor and factors II, VII, and X. *Anal. Biochem.* 152:199–207.

32. Doellgast, G. J. 1987. Enzyme-linked coagulation assay III. sensitive immuno assays for clotting factors II, VII, and X. *Anal. Biochem.* 162:102–114.

33. Yoshida, N., K. Ota, M. Moroi, and M. Matsuda. 1988. An apparently higher molecular weight γ-chain variant in a new congenital abnormal fibrinogen Tochigi characterized by the replacement of γ arginine-275 by cysteine. *Blood.* 71:480–487.

34. Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J.

Dreyer. 1981. A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* 256:7990–7997.

35. Nishibe, H., and N. Takahashi. 1981. The release of carbohydrate moieties from human fibrinogen by almond glycopeptidase without alteration in fibrinogen clottability. *Biochim. Biophys. Acta.* 661:274–279.

36. Takahashi, N. 1977. Demonstration of a new amidase acting on glycopeptides. *Biochem. Biophys. Res. Commun.* 76:1194–1201.

37. Hase, S., T. Ibuki, T. Irimura, and T. Osawa. 1984. Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins. *J. Biochem. (Tokyo).* 95:197–203.

38. Tomiya, N., M. Kurono, H. Ishihara, S. Tejima, S. Endo, Y. Arata, and N. Takahashi. 1987. Structural analysis of N-linked oligosaccharides by a combination of glycopeptidase, exoglycosidases, and high-performance liquid chromatography. *Anal. Biochem.* 163:489–499.

39. Menache, D. 1973. Abnormal fibrinogens. A review. *Thromb. Diath. Haemorrh.* 29:525–535.

40. Chung, D. W., W.-Y. Chan, and E. W. Davie. 1983. Characterization of a complementary deoxyribonucleic acid coding for the γ chain of human fibrinogen. *Biochemistry.* 22:3250–3256.

41. Miyata, T., S. Terukina, M. Matsuda, A. Kasamatsu, Y. Takeda, T. Murakami, and S. Iwanaga. 1987. Fibrinogen Kawaguchi and Osaka: an amino acid substitution of Aα arginine to cysteine which forms an extra interchain disulfide bridge between the two Aα chains. *J. Biochem. (Tokyo).* 102:93–101.

42. Townsend, R. R., E. Hilliker, Y.-T. Li, R. A. Laine, W. R. Bell, and Y. C. Lee. 1982. Carbohydrate structure of human fibrinogen. *J. Biol. Chem.* 257:9704–9710.

43. Fowler, W. E., H. P. Erickson, R. R. Hantgan, J. McDonagh, and J. Hermans. 1981. Cross-linked fibrinogen dimers demonstrate a feature of the molecular packing in fibrin fibers. *Science (Wash. DC).* 211:287–289.

44. Fowler, W. E., R. R. Hantgan, J. Hermans, and H. P. Erickson. 1981. Structure of the fibrin protofibril. *Proc. Natl. Acad. Sci. USA.* 78:4872–4876.

45. Ferry, J. D. 1952. The mechanism of polymerization of fibrin. *Proc. Natl. Acad. Sci. USA.* 38:566–569.

46. Doolittle, R. F. 1973. Structural aspects of the fibrinogen-fibrin conversion. *Adv. Protein. Chem.* 27:1–109.

47. Budzynski, A. Z., V. J. Marder, D. Menache, and M.-C. Guillin. 1974. Defect in the gamma polypeptide chain of a congenital abnormal fibrinogen (Paris I). *Nature (Lond.).* 252:66–68.

48. Mosesson, M. W., D. L. Amrani, and D. Menache. 1976. Studies on the structural abnormality of fibrinogen Paris I. *J. Clin. Invest.* 57:782–790.

49. Dang, C. V., R. F. Ebert, and W. R. Bell. 1984. Localization of a fibrinogen calcium binding site between γ-subunit positions 311 and 336 by terbium fluorescence. *J. Biol. Chem.* 260:9713–9719.

50. Takahashi, N., and H. Nishibe. 1978. Some characteristics of a new glycopeptidase acting on aspartylglycosylamine linkages. *J. Biochem. (Tokyo).* 84:1467–1473.

51. Takahashi, N., and H. Nishibe. 1981. Almond glycopeptidase acting on aspartylglycosylamine linkages. *Biochim. Biophys. Acta.* 657:457–467.

52. Kaudewitz, H., A. Henschen, J. Soria, and C. Soria. 1986. Fibrinogen Pontoise: a congenital abnormal fibrinogen with defective fibrin polymerization but normal fibrinopeptide release. *In* Fibrinogen-Fibrin Formation and Fibrinolysis. Vol. 4. D. A. Lane, A. Henschen, and M. K. Jasani, editors. Walter de Gruyter, Berlin. 91–96.

53. Mizuochi, T., T. Taniguchi, Y. Asami, J. Takamatsu, M. Okude, S. Iwanaga, and A. Kobata. 1982. Comparative studies on the structures of the carbohydrate moieties of human fibrinogen and abnormal fibrinogen Nagoya. *J. Biochem. (Tokyo).* 92:283–293.