Fibrinogen Kyoto III: A Congenital Dysfibrinogen With a γ Aspartic Acid-330 to Tyrosine Substitution Manifesting Impaired Fibrin Monomer Polymerization

By Sigeharu Terukina, Kensuke Yamazumi, Kiyoshi Okamoto, Hajime Yamashita, Yoko Ito, and Michio Matsuda

A congenital dysfibrinogen characterized by impaired fibrin monomer polymerization was found in an asymptomatic 50-year-old woman and her two sons. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run according to the method of Laemmli,'3 the purity was greater than 95%. The purified fraction was dissolved in 0.3 mol/L NaCl at >20°C; plasma was separated. Fibrinogen was purified from the plasma of a 50-year-old woman and her two sons. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run according to the method of Laemmli, we noticed two γ chain species in fibrinogen and its plasmic fragments D1 and D2, consisting of a normal species and an apparently lower molecular weight (mol wt) variant in respective fractions. However, in fragment D2 only a single γ chain remnant was observed. By chromatofocusing of the plasmic-CaCl2 digests of the abnormal fibrinogen, we separately isolated the normal and abnormal D1 species, the latter having been eluted in a slightly higher pH range. As expected, the abnormal D1 species failed to interfere with thrombin clotting of normal fibrinogen and normal fibrin monomer polymerization, whereas the normal D1 species inhibited them markedly. By analyzing the lysyl endopeptidase digests of the isolated γ chain, we identified a replacement of aspartic acid by tyrosine at position 330 of the mutant γ chain. The point mutation from an aspartic acid (pK for the β-carboxyl = 3.86) to a tyrosine (pK for the aromatic hydroxy = 10.07) may have perturbed the folding γ chain structure in the D domain of fibrinogen specifically required for polymerization.

MATERIALS AND METHODS

Coagulation studies. Coagulation studies were performed according to standard procedures20 or as described previously.11

Studies on purified fibrinogen. Because the patient, a 50-year-old woman, was scheduled to undergo gastrectomy for a gastric polyp, we collected 200 mL blood from one of her sons affected with the same coagulation abnormality after we obtained written consent (Table I). The blood was anticoagulated with 30 mL ACD (Formula A, U.S. Pharmacopeia) and centrifuged at 3,000 g for 60 minutes at 4°C; plasma was separated. Fibrinogen was purified from the plasma- and fibronectin-depleted plasma by repeated 25% ammonium sulfate saturation, essentially as described previously.13 The purified fraction was dissolved in 0.3 mol/L NaCl at >20 mg/mL and stored at -80°C until use. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run according to the method of Laemmli,13 the purity was greater than 95%. The thrombin and anadromous times with or without calcium ions and polymerization of fibrin monomers as monitored by absorbance at 350 nm (A350) were performed as described previously.14 Release of fibrinopeptides A and B was analyzed by reverse-phase high-performance liquid chromatography (HPLC) essentially as described previously.15

Gross structure of fibrinogen and its plasmic digests. Gross structure of fibrinogen and its plasmic digests were analyzed by SDS-PAGE according to the method of Laemmli with 7.5 or 10% separating gels and 4% stacking gels, or according to the method of Weber and Osborn16 with 5% gels.

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Purification of normal and abnormal fragment D1 species from the heterozygous dysfibrinogen. Fibrinogen (30 mg, 2 mg/mL in TBS containing 5 mmol/L CaCl2) was digested with 0.2 U/mL plasminogen and 1,500 U/mL streptokinase for approximately 7 hours at 37°C, and the reaction was terminated by addition of 200 KIU/mL aprotinin. The plasmic-CaCl2 digests were then dialyzed against 25 mm/L Tris-HCl, pH 7.8, and subjected to chromatofocusing, using a column of polybuffer exchanger gel (1.2 x 18 cm) at 4°C as described previously.17 Fragment D1, derived from normal fibrinogen, was eluted in a single peak, whereas that from the dysfibrinogen was eluted in two peaks, one in the same pH range as normal D1, and the other in a slightly higher pH range (Fig 1). The fractions were separately collected, concentrated, and dialyzed against TBS with CX-30 millipore membranes.

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Table 1. Thrombin and Ancrod Times and Fibrinogen Concentrations in the Propositus and Two Sons

<table>
<thead>
<tr>
<th>Variable</th>
<th>Propositus</th>
<th>Son 1</th>
<th>Son 2</th>
<th>Control or Normal</th>
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</thead>
<tbody>
<tr>
<td>Thrombin time (s)</td>
<td></td>
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<tr>
<td>Without Ca²⁺</td>
<td>20.4</td>
<td>18.2</td>
<td>20.8</td>
<td>10.1</td>
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<tr>
<td>With Ca²⁺</td>
<td>9.1</td>
<td>9.5</td>
<td>9.8</td>
<td>6.7</td>
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<tr>
<td>Ancrod time (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Ca²⁺</td>
<td>34.1</td>
<td>24.7</td>
<td>24.7</td>
<td>17.0</td>
</tr>
<tr>
<td>With Ca²⁺</td>
<td>27.2</td>
<td>22.6</td>
<td>20.9</td>
<td>10.1</td>
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<td>Plasma fibrinogen (mg/dL)</td>
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<td>Thrombin time method</td>
<td>68</td>
<td>65</td>
<td>64</td>
<td>200-400</td>
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<tr>
<td>SRID</td>
<td>290</td>
<td>299</td>
<td>331</td>
<td>200-400</td>
</tr>
</tbody>
</table>

Abbreviation: SRID, single radial immunodiffusion.

Isolelectric focusing of fibrinogen subunits and their plasmic digests. Normal and abnormal fibrinogens and their plasmic fragments D, were analyzed by isoelectric focusing (IEF) in slabs after reduction essentially as described elsewhere or according to the manual supplied by the manufacturer (Pharmacia, Uppsala, Sweden). The gels contained a nonionic detergent, polyoxyethylene alcohol to remove SDS from the protein at a ratio to protein of 8 to 1.

Separation of the fibrinogen γ chain and its lysyl endopeptidase digestion. Five milligrams (14.5 nmol) each of the normal and abnormal fibrinogens were reduced and S-pyridylethylated, and their three subunit polypeptides were separated by reverse-phase HPLC with a TMS-250 column (4.5 × 75 mm) as described previously. They were eluted in the order of Aα, Bβ, and γ chains with a linear gradient from 20% to 50% acetonitrile in 60 minutes. The pyridylethylated γ chain (Pe-γ) was collected and lyophilized. The Pe-γ (2 mg/mL, 450 μg) was digested with lysyl endopeptidase (enzyme/substrate ratio = 1/100, wt/wt) for 8 hours at 37°C in 50 mmol/L Tris-HCl, pH 9.0, containing 3 mol/L urea. The digests were analyzed by reverse-phase HPLC with a Cosmosil 5C18P column (4.6 × 150 mm), applying a gradient from 0.1% trifluoroacetic acid (TFA)/water (solvent A) to 60% of 0.1% TFA/acetonitrile (solvent B) in 120 minutes. The flow rate was 0.5 mL/min, and the column effluent was monitored by A210. The relevant peaks were further purified by rechromatography on the same column as described.

Immunoblotting analysis of the γ chain. The fibrinogen γ chain and its remnants in the plasmic digests were specifically examined by immunoblotting using an anti-human fibrinogen γ chain monoclonal antibody (MoAb) that recognizes the γ (86 to 302) residue peptide segment (7/D3) as described previously. This antibody was tentatively designated as “anti-γ core antibody.”

Amino acid composition and sequence determination. Purified peptides were analyzed for the total amino acid composition and primary sequence as described previously.

Case report. The patient was a 50-year-old woman undergoing gastrectomy for a gastric polyp. From routine coagulation studies...
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before surgery, she was suspected to have a dysfibrinogenemia on the basis of a markedly reduced level of plasma fibrinogen determined by the thrombin time method but a normal level by single radial immunodiffusion. The thrombin time and the ancrod time were both prolonged, although they were partially corrected by addition of CaCl₂. The results are summarized in Table 1. The one-stage prothrombin time was also slightly prolonged (13.9 seconds, control 10.9 seconds) but activated partial thromboplastin time, thrombo-test, and bleeding time were within normal limits. Blood coagulation factors V, VII, VIII, IX, X, and XIII, antithrombin III, plasminogen, α₂-plasmin inhibitor, and protein C were all normal.

Her two sons had the same type of abnormality (Table 1), indicating that the abnormality was hereditary. Although none of them had bleeding or thrombotic tendencies clinically, we tentatively designated this abnormal fibrinogen as fibrinogen Kyoto III.

RESULTS

Fibrinogen was purified from the plasma derived from the second son (son 2 in Table 1) of the propositus. The clotting by thrombin or ancrod of purified fibrinogen was also significantly impaired; The clotting times were 134.7 seconds for thrombin (control 11.1 seconds) and more than 300 seconds for ancrod (control 15.3 seconds). Additions of calcium ions at 8 mmol/L corrected them partially (ie, 17.4 seconds for thrombin [control 7.1 seconds] and 30.1 seconds for ancrod [control 9.9 seconds]).

The HPLC elution profiles for fibrinopeptides A and B released on thrombin or ancrod treatment were indistinguishable from those for normal profiles (profiles not shown). Polymerization of fibrin monomers was impaired, as evidenced by a prolonged lag phase and a reduced amplitude of polymerization curve as monitored by A₃₅₀ (Fig 2).

By SDS-PAGE of purified fibrinogen run according to the method of Laemmli, we observed two γ chain species as indicated by γ and γ Kyoto III (lane 2, panel A, Fig 3). The γ Kyoto III variant possessed an apparently lower mol wt (LMW) than the normal γ chain. The dual mol wt of the γ chain was confirmed by immunoblotting using the anti-γ core antibody that specifically stained the two γ chain species derived from the heterozygous dysfibrinogen (lane 2, panel B, Fig 3). In the system of Weber and Osborn, however, the γ chain doublet was not noted (lane 2 in panel C, Fig 3).

When cross-linked fibrin was analyzed after reduction by SDS-PAGE according to Laemmli, the γ-γ dimer formation proceeded almost normally (profiles not shown). The LMW γ chain remnants were also noticeable in the plasmic fragments D₁ and D₂, as indicated by γ/D₁, Kyoto III and γ/D₂ Kyoto III, respectively (lanes 8 through 10, panel A, Fig 4). The LMW γ chain remnants were more clearly identified by immunoblotting with the anti-γ core antibody (lanes 8 through 10, panel B, Fig 4). However, in fragment D₁, the LMW γ chain remnant was no longer discernible (lane 10 in panels A and B), suggesting that the abnormality resided most probably in the carboxy-terminal peptide of the γ chain remnant of fragment D₁ (γ 303 to 356) that is removed on conversion to fragment D₂.

Figure 1 shows a chromatofocusing profile of the plasmic-CaCl₂ digests of fibrinogen where fragment D₁ was eluted in two peaks, peaks I and II. Peak II was eluted at the same position as the normal fragment D₁, but peak I was eluted slightly earlier than the normal one. By SDS-PAGE according to the method of Laemmli, the latter had an apparently LMW γ chain remnant (Fig 1, inset).

On the basis of the elution positions and SDS-PAGE according to the method of Laemmli (Fig 1, inset), we assigned peak I to the abnormal fragment D₁ species and peak II to the normal fragment D₁ species. These two fragment D₁ species were tested for inhibition of thrombin clotting of normal fibrinogen and normal fibrin monomer polymerization. As expected, the abnormal D₁ species did not inhibit thrombin clotting of normal fibrinogen substantially, whereas the normal D₁ species inhibited it dose dependently (namely, 13.5, 14.3, 15.8, 16.7, and 17.7 seconds for abnormal fragment D₁ and 13.5, 16.1, 21.7, 27.7, and 34.1 seconds for normal fragment D₁). The polymerization of normal fibrin monomers was unaffected by the abnormal D₁ species (profile not shown).

Fibrinogen and its plasmic digests were analyzed by IEF after reduction, and the separated bands were compared with those for normal profiles. For normal fibrinogen, two bands were assigned to the γ chain (lane 1, panel A, Fig 5), whereas for the abnormal fibrinogen, one additional band is obvious and the second band probably overlaps one of the normal γ chain components, as indicated by γ Kyoto III (lane 2, panel A, Fig 5). The heterogeneity could be attributed to the oligosaccharide moiety attached to the γ chain (γ Asn-52), since the γ remnant of normal fragment D₁ lacking the oligosaccharide (the γ 86-411 residue peptide) migrated as a single band (lane 1, panel B, Fig 5). The γ remnant of the normal fragment D₁ species derived from the heterozygous abnormal fibrinogen also migrated as a single component to the normal position (lane 2, panel B, Fig 5), whereas that of the abnormal fragment D₁ species shifted obviously to a more...
Fig 3. The subunit polypeptides of fibrinogen analyzed by SDS-PAGE. (A) SDS-PAGE according to the method of Laemmli with 10% gels stained with CBB. (B) Immunoblotting using the anti-γ core antibody after SDS-PAGE according to the method of Laemmli. (C) SDS-PAGE according to the method of Weber and Osborn with 5% gels stained with CBB.

Fig 4. Plasmic degradation of the fibrinogen γ chain analyzed by SDS-PAGE according to the method of Laemmli and immunoblotting with the anti-γ core antibody. At various incubation times, 20-µL aliquots of the plasmic digests were removed, diluted fivefold with TBS, and treated with 1% SDS-1% DTT. Two micrograms of protein was subjected to SDS-PAGE. (A) SDS-PAGE using 10% gels stained with CBB. (B) Immunoblotting using the anti-γ core antibody. After SDS-PAGE, resolved proteins were transferred to a nitrocellulose membrane and allowed to react with the anti-γ core antibody. The fibrinogen γ chain and its remnants in fragments D1α, D1, D2, and D3 were indicated by γ, γ/D1a, γ/D1, γ/D2, and γ/D3, respectively. Corresponding fibrinogen γ chain and its derivatives derived from fibrinogen Kyoto III (arrows). The γ/D1a has an extension of the γ 83-85 residues to the γ/D1.
Fig 5. IEF of reduced fibrinogen and fragment D1. (A) Fibrinogen: Lane 1, normal fibrinogen; lane 2, heterozygous dysfibrinogen. (B) Fragment D1: Lane 1, derived from normal fibrinogen; lanes 2 and 3, normal and abnormal fragment D1 species, respectively, derived from the heterozygous dysfibrinogen.

cathodal region (γ/D1 Kyoto III, lane 3, panel B, Fig 5). This finding suggested that the variant γ chain polypeptide itself had a higher pI. Therefore, four bands in all could be assigned to the γ chain derived from the heterozygous dysfibrinogen.

When the lysyl endopeptidase digests of the Pe-γ derived from the heterozygous dysfibrinogen were subjected to reverse-phase HPLC with a Cosmosil 5C18P column, we noticed a decrease in the height of K28 as compared with that of K28 in the normal Pe-γ and the presence of an extra peak K28' (panel B, Fig 6). This extra peak was isolated, further purified by rechromatography on the same column, and analyzed for the primary sequence and amino acid composition.

By amino acid sequence analysis, we assigned peptides K28 and K28' to the normal and aberrant γ 322-338 residue peptides, respectively. In the latter, Asp at cycle 9 (ie, at position 330 of the γ chain) was replaced by Tyr. Table 2 summarizes the data on the first 12 cycles of the aberrant peptide in comparison with the normal sequence. This finding was supported by the total amino acid composition study, in which a Tyr residue absent in the normal peptide K28 was present (1.0 residue/molecule); instead, one residue less Asp was identified in the aberrant peptide (1.9 residues per molecule) than in the normal one (2.4 residues per molecule; the theoretical value is three residues per molecule).

DISCUSSION

We identified an amino acid replacement of Asp by Tyr at position 330 of the mutant γ chain derived from a heterozygous congenital dysfibrinogen. This dysfibrinogen designated as fibrinogen Kyoto III is characterized by impaired polymerization of fibrin monomers. On SDS-PAGE according to the method of Laemmli, the mutant γ chain and its plasmic remnants in fragments D1, D2 (γ/D1 and γ/D2) migrated faster than the normal counterparts, but the mutant γ chain remnant in fragment D3 (γ/D3) migrated normally. The result suggested that the structural abnormality resided most probably in the carboxy-terminal peptide of the γ/D2 (ie, the γ 303-356 residue peptide), which was removed by plasmin from γ/D2 on its conversion to γ/D1. Indeed, we identified a point mutation at position 330 of the mutant γ chain, which is certainly included in this peptide.

Fig 6. HPLC elution profile of the lysyl endopeptidase digests of reduced and pyridylethylated γ chain (Pe-γ). (A) Normal Pe-γ. (B) Pe-γ derived from the heterozygous dysfibrinogen.
No other abnormalities were found to account for the apparently lower mol wt for this particular peptide segment, however, such as a peptide deletion or premature termination of the mutant $\gamma$ chain. Such an increase in mobility was not observed in the system of Weber and Osborn (Fig 3); thus, the apparently lower mol wt for the mutant $\gamma$ chain in the system of Laemmli could be an artifact. Indeed, we and other investigators also observed similarly increased mobilities of the mutant $\gamma$ chains with a single amino acid substitution (ie, investigators the apparently lower mol wt for the mutant $\gamma$ chain in the fibrinogens Tochigi 1,24 Osaka ii,25 respectively). On the contrary, the mutant $\gamma$ chain with an arginine to cysteine substitution at position 275 migrated more slowly and failed to interfere with thrombin clotting of normal fibrinogen and polymerization of normal fibrin monomers. Nevertheless, the site(s) on the D domain should not necessarily be restricted to this particular region of the $\gamma$ chain. Recent investigations from this laboratory and others showed that mutations at position 275,14,17,24,25,26 308,22 or 31018 of the $\gamma$ chain and position 335 of the $\beta$ chain33 critically affected the polymerization site(s) assigned to the D domain. Therefore, the polymerization site(s) specifically assigned to the D domain of fibrinogen appears to include a more extended structure than had been previously postulated23 and to depend on the tertiary structure involving the carboxy-terminal $\gamma$ chain regions containing $\gamma$ Asp-330. Although the structure in the D domain required for fibrin polymerization has not yet been defined, this conserved region is very likely involved in the putative polymerization site(s), as deduced from this and other studies5,23 of abnormal fibrinogens characterized by impaired fibrin monomer polymerization. No other abnormalities were found to account for the apparently lower mol wt for this particular peptide segment, however, such as a peptide deletion or premature termination of the mutant $\gamma$ chain. Such an increase in mobility was not observed in the system of Weber and Osborn (Fig 3); thus, the apparently lower mol wt for the mutant $\gamma$ chain in the system of Laemmli could be an artifact. Indeed, we and other investigators also observed similarly increased mobilities of the mutant $\gamma$ chains with a single amino acid substitution (ie, investigators the apparently lower mol wt for the mutant $\gamma$ chain in the fibrinogens Tochigi 1,24 Osaka ii,25 respectively). On the contrary, the mutant $\gamma$ chain with an arginine to cysteine substitution at position 275 migrated more slowly and failed to interfere with thrombin clotting of normal fibrinogen and polymerization of normal fibrin monomers. Nevertheless, the site(s) on the D domain should not necessarily be restricted to this particular region of the $\gamma$ chain. Recent investigations from this laboratory and others showed that mutations at position 275,14,17,24,25,26 308,22 or 31018 of the $\gamma$ chain and position 335 of the $\beta$ chain33 critically affected the polymerization site(s) assigned to the D domain. Therefore, the polymerization site(s) specifically assigned to the D domain of fibrinogen appears to include a more extended structure than had been previously postulated23 and to depend on the tertiary structure involving the carboxy-terminal $\gamma$ chain regions containing $\gamma$ Asp-330.

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