A Lower Molecular Weight γ-Chain Variant in a Congenital Abnormal Fibrinogen (Kyoto)

By Nobuhiko Yoshida, Minoru Okuma, Masaaki Moroi, and Michio Matsuda

A γ-chain variant with a lower molecular weight than the normal γ chain was detected in a new congenital abnormal fibrinogen with impaired polymerization of the fibrin monomer and with normal release of fibrinopeptides A and B in a 45-year-old male. Purified fibrinogen analyzed on SDS-polyacrylamide gel electrophoresis under the reduced condition contained an abnormal protein band with an apparent molecular weight of 48,000 compared with the γ chain with a molecular weight of 50,000. This abnormal protein band was found to be a γ-chain variant from the molar ratio of Aa chain : Bβ chain : γ chain : abnormal protein (about 2 : 2 : 1 : 1), with positive staining for carbohydrate and crosslinking ability. Crosslinked fibrin contained three types of γ-γ dimers with apparent molecular weights of 94,000 (the same as normal major γ-γ dimer), 92,000 and 90,000, and the plasmic digests of crosslinked fibrin in the presence of calcium retained three types of γ-γ dimer remnants. This suggests that the abnormal γ-chain variant has a shorter polypeptide chain not in the NH₂-terminal but in the COOH-terminal portion, probably at or near the polymerization site. This patient's two daughters had the same abnormal fibrinogen. This unique inherited abnormal fibrinogen was designated as fibrinogen Kyoto, and the γ-chain variant as γKyoto.

Studies on purified fibrinogen. Fibrin monomer aggregation was studied according to the method of Belits et al14 as described by Gralnick et al.15 Release of fibrinopeptides A and B was examined by high-performance liquid chromatography.16 Release of fibrinopeptide A was also measured by radioimmunoassay.17 Immunoelectrophoresis was done as previously described.13 DEAE-Sephacel chromatography of fibrinogen was carried out by applying 10 mg of fibrinogen to a 40 μL column.18 Fibrinmonomer-Sepharose chromatography of fibrinogen was carried out as described previously.13 Crosslinked fibrin was prepared by adding 5 U/mL bovine thrombin and 1 U/mL purified human factor XIII to fibrinogen (2 mg/mL) in 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, 25 KIU/mL aprotinin, 5 mmol/L CaCl₂, pH 7.4. Reaction mixtures were incubated at 37°C for 80 minutes, and the reaction was terminated by the addition of 2% SDS and 10% β-mercaptoethanol and incubation at 100°C for 30 minutes. Plasmic digests of crosslinked fibrin in the presence of calcium were prepared essentially as described previously.13 Fibrinogen (1 mg) was incubated with 2 U of bovine thrombin and 0.1 U of factor XIII in 1 mL of 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, 10 mmol/L CaCl₂, pH 7.4, for two hours at 37°C and for another 12 hours at 4°C. Fibrin clots were squeezed out with a bamboo stick, washed twice with the above buffer, and digested by the addition of 0.25 mL of 0.013 mg/mL (0.4 CTAU/mL) human plasminogen19 and 172 U/mL streptokinase in the above buffer (plasmin solution) for six hours at 37°C. After six hours an additional 0.25 mL of plasmin solution was added, and the incubation was carried out for an additional 18 hours at 37°C. The reaction was carried out for an additional 18 hours at 37°C.

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Blood collections and coagulation studies. Venous blood was collected in one-tenth volume of 3.8% sodium citrate or in acid citrate dextrose (ACD). Platelet-rich plasma and platelet-poor plasma were prepared for the platelet aggregation and blood coagulation studies, respectively, as described previously.4 Coagulation studies were performed according to procedures described elsewhere.6,11 The reptilase time was done in a test system containing 0.1 mL plasma, 0.1 mL Tris-saline, pH 7.4 with or without 25 mmol/L CaCl₂, and 0.1 mL of 50 μg/mL enzyme (venom of Bothrops atrox, Pentapharm, Switzerland).

Purification of fibrinogen. Fifty mL of ACD plasma was mixed with 5 mmol/L benzamidine, 10 KIU/mL aprotinin, and 2 mmol/L EDTA, and passed through a column of lysine-Sepharose 4B (25 mL) and a column of gelatin-Sepharose 4B (25 mL) that were connected in tandem and equilibrated with 50 mmol/L Tris-HCl, 0.1 mol/L NaCl, 5 mmol/L benzamidine, 10 KIU/mL aprotinin, 2 mmol/L EDTA, pH 7.4, at room temperature, and columns were washed with the same buffer. Two hundred mL of unbound pass through fractions was pooled, brought to 25% saturation with ammonium sulfate solution, stirred for 30 minutes, and centrifuged at 13,000 x g for 30 minutes. The pellet was washed twice with 25% ammonium sulfate solution, suspended in an appropriate amount of 0.3 mol/L NaCl, dialyzed extensively against the same solution at 4°C, and finally the insoluble portion was removed by centrifugation at 13,000 x g for 30 minutes. The recovery of purification was 75% and thrombin clotability was 92%. Purified fibrinogen was stored at -80°C until use.


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was terminated by the addition of 500 KIU/mL aprotinin and 0.1 mmol/L p-amidinophenyl-methylsulfonyl fluoride (Calbiochem-Behring, La Jolla, Calif). To obtain plasmic digests of crosslinked fibrin in the presence of EGTA, crosslinked fibrin was washed with the buffer with 20 mmol/L EGTA and digested with plasmin solution with 20 mmol/L EGTA.

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5% or 7.5% separation gels with 5% stacking gels. Gels were stained for proteins with Coomassie Brilliant Blue R-250 and for carbohydrate by the method of Eckhardt et al.

**Case report.** The propositus (YN) was a 45-year-old male with mild hypertension. He experienced a brief episode of dizziness and difficulty in walking at the age of 18, sometimes suffered from numbness of extremities since the age of 32, and had angina pectoris since the age of 40.

His paternal grandmother and a paternal aunt died suddenly in their forties without any sign of disease. His father died of cerebral thrombosis and ruptured common iliac artery at the age of 63. Another paternal aunt had hypermenorrhea for a long time and died of acute pneumonia in her forties. The propositus' youngest brother died of acute pneumonia with nasal bleeding and purpura around the mouth at the age of 8 months. The propositus was advised by his home doctor to undergo a blood examination at a local hospital, and the propositus was referred to our hospital. His two daughters were also found to have hypofibrinogenemia, but had no history of thrombosis or hemorrhage.

**RESULTS**

Coagulation studies, including one-stage prothrombin time, activated partial thromboplastin time, factor XIII, antithrombin III, plasminogen, α2-plasmin inhibitor, and platelet aggregation induced by ADP or collagen, were all within the normal range. As shown in Table 1, thrombin time and reptilase time of the propositus' plasma were markedly prolonged and the plasma fibrinogen concentration evaluated by the thrombin time method was about half the normal value, although the turbidimetric method and immunologic method showed normal levels of plasma fibrinogen. Purified fibrinogen also showed a prolonged thrombin time. The plasma of the two daughters also gave prolonged thrombin and reptilase times and a discrepancy between the plasma fibrinogen concentration assayed by the thrombin time method and that by the turbidimetric or immunologic method (Fig 1).

Release of fibrinopeptide A with thrombin as examined by radioimmunosay was normal, both with respect to initial rate and total amount. Maximum fibrinopeptide A cleavage from the propositus and normal fibrinogen was 2.09 and 2.03 mol/mol fibrinogen, respectively. Release of fibrinopeptide B as well as of fibrinopeptide A as examined by high-performance liquid chromatography at time intervals after the addition of thrombin also showed no substantial differences between the propositus' fibrinogen and normal fibrinogen. This was also verified by the conversion velocity of Bβ chain to β chain on SDS-PAGE (pictures not shown). A fibrin monomer polymerization curve of the propositus' fibrinogen showed a long lag time (seven minutes v two minutes normal) and a decreased slope (ΔOD 350/min = 0.04 v 0.138 normal), but the final amplitude was similar to normal (not shown).

Purified fibrinogen was analyzed on SDS-PAGE. Under the reduced condition, the propositus' fibrinogen had an extra protein band with an apparent molecular weight of about 48,000 in addition to the normal γ chain with a molecular weight of 50,000 (γ50) (Fig 2, lane 2). This abnormal protein band positively stained for carbohydrate to the same extent as the normal γ chain (Fig 2, lane 4). Densitometric scan of the gel electrophoreogram of the propositus' fibrinogen was performed, and the molar ratio of the Aα chain, Bβ chain, γ chain, and abnormal protein band was calculated as 2 : 1.84 : 1.02 : 1.14 (about 2 : 2 : 1 : 1). No difference in mobility between the propositus' fibrinogen and normal fibrinogen could be detected under the nonreduced condition. These results suggested that this abnormal protein band is a lower molecular weight γ-chain variant. Fibrogenins of the two daughters also showed the presence of a lower molecular weight γ-chain variant to the same extent as propositus' fibrinogen on SDS-PAGE (not shown).

To determine whether this abnormal protein band cross-links with activated factor XIII, fibrinogen was treated with

### Table 1. Studies for Fibrinogen Function

<table>
<thead>
<tr>
<th>Sample</th>
<th>Study</th>
<th>Propositus</th>
<th>Normal or Control</th>
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<td>Plasma</td>
<td>Thrombin time (sec)</td>
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<tr>
<td></td>
<td>without calcium ions</td>
<td>47.6</td>
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<td>with calcium ions</td>
<td>11.4</td>
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<td>Reptilase time (sec)</td>
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<td>without calcium ions</td>
<td>&gt;200</td>
<td>13.3 to 18.4</td>
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<tr>
<td></td>
<td>with calcium ions</td>
<td>24.7</td>
<td>8.4 to 11.7</td>
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<td>Turbidimetric method</td>
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<td>Immunologic method</td>
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<td>Factor XIII (%)</td>
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<tr>
<td>Purified Fibrinogen</td>
<td>Thrombin time (sec)</td>
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<td>without calcium ions</td>
<td>179.0</td>
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<td>Fibrin monomer polymerization</td>
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FIBRINOGEN KYOTO WITH A SHORTER γ-CHAIN

Fig 1. Pedigree of fibrinogen Kyoto. Figures represent plasma fibrinogen concentrations (mg/dL) measured by the thrombin time method and in parentheses, those determined by turbidimetric (upper) and immunologic methods (lower).

Thrombin and factor XIII in the presence of calcium. The propositus' fibrinogen crosslinked to the same extent as normal; the α chain, γ chain, and the abnormal protein band disappeared, resulting in the appearance of α polymers and γ-γ dimers which appeared to exist as three bands but to have smaller molecular weights than γ-γ dimers (Fig 2, lane 6). To make it clear, fibrinogen was purified on DEAE-Sephacel chromatography. A chromatographic profile for propositus' fibrinogen was the same as normal with the appearance of a single type of major peak (peak A, peak 18), and the ascending limb, peak, and descending limb of a major peak showed the presence of low molecular weight γ-chain variant (γKyoto) to the same extent on SDS-PAGE (not shown). Application of limited amounts on DEAE-Sephacel made it difficult to obtain enough minor peak (peak C, peak 24) for further analysis.

Crosslinked fibrin was prepared from a major peak and electrophoresed on 5% polyacrylamide gel (Fig 2, lanes 7–9). Normal crosslinked fibrin contained only one γ-γ dimer (Fig 2, lane 7) as shown by Francis et al.18 Crosslinked fibrin of propositus' fibrinogen (Fig 2, lane 9) clearly showed the presence of three γ-γ dimers which had apparent molecular weights of 94,000 (the same as major γ-γ dimer of normal fibrin, Fig 2, lane 8), 92,000, and 90,000, and their molar ratio appeared to be about 1:2:1, although the three bands which migrated closely did not allow us to measure accurately the intensity of each band densitometrically. This suggested that the three γ-γ dimers are composed of γ50' Yp50', γ50' γKyoto', and γKyoto' γKyoto'. All three bands were positively stained for carbohydrate (data not shown).

The fragment D dimer obtained from plasmic digestion of crosslinked fibrin in the presence of calcium migrated as a single but somewhat broader band than normal under the nonreduced condition (Fig 3, lane 2), but retained the three γ-γ dimer remnants with apparent molecular weights of 80,000, 77,000, and 74,000 under the reduced condition (Fig 3, lane 4). In contrast, plasmic digestion of crosslinked fibrin in the presence of EGTA resulted in the formation of an identical γ remnant with apparent molecular weight of 25,000 as normal (not shown).

In the following analysis the propositus' fibrinogen could not be separated into two or three populations under the present experimental conditions: The propositus' fibrinogen showed a single precipitation arc with the same migration as normal fibrinogen on immunoelctrophoresis. Fibrinogen bound to fibrinmonomer-Sepharose and was eluted in a single symmetrical peak with the elution buffer. There was no thrombin- or reptilase-nonclottable fibrinogen.

The γKyoto cannot be derived from the purification step of
fibrinogen; the plasma of the propositus and his two daughters was treated with anti-fibrinogen IgG, or incubated for five minutes at 56 °C. All three immunoprecipitates and heat-precipitates proved to contain γKyo as noted on SDS-PAGE (not shown).

DISCUSSION

The hereditary congenital dysfibrinogenemia described here, designated as fibrinogen Kyoto, is characterized by normal release of fibrinopeptides A and B, defective polymerization of the fibrin monomer, the presence of a γ-chain variant with a lower molecular weight than normal (γKyo), and normal crosslinking ability of γ chains.

A fibrin γ-chain polymerization site has recently been shown to reside in segment Thr-374 to Glu-396, and abnormal fibrinogens with an abnormal γ chain have been shown to have defective fibrin polymerization.

Fibrinogens Nagoya, Bern I, and Haifa, however, have a normal molecular weight of the γ chain on SDS-PAGE, and fibrinogens Paris I and Grenoble have a larger molecular weight γ chain than normal. Although defective polymerization of fibrin monomer in these abnormal fibrinogens could arise from the abnormalities at or near the γ-chain polymerization site itself, no conclusive arrangement has ever been pinpointed in the γ chain of these abnormal molecules in relation to the impaired function. Only one exception is a recently reported abnormal fibrinogen, fibrinogen Milano I, in which γ-330 Asp has been shown to be substituted by Val.

The abnormal protein band found in fibrinogen Kyoto was considered to represent a lower molecular weight γ chain, because of (1) its molar ratio of Aα chain : Bβ chain : γ chain : abnormal protein, (2) positive staining for carbohydrate to the same extent as γ chain, and (3) the crosslinking with each other or with the γ chain resulting in the formation of dimers (Fig 2). Crosslinked fibrin contained three types of γ-γ dimers, which shows that crosslinking sites of γKyoto, Gln-398 and Lys-406 in normal γ chain, are intact and γKyoto has the same crosslinking ability as the normal γ chain. The appearance of three γ-γ dimer remnants in the fragment D dimer of plasmic digests of crosslinked fibrin in the presence of calcium (Fig 3, lane 4) and the disappearance of γ remnants with different molecular weights in plasmic digests of crosslinked fibrin in the presence of EGTA suggest that the COOH-terminal portion of γKyoto has shorter peptide chains than normal. If NH2-terminal portion which is cleaved by plasmin had shorter peptide chains, smaller molecular weight γ-γ dimer remnants could not be retained. These data suggest that a lower molecular weight γ chain, γKyoto, comes from a deletion of a certain short segment in γ303-411 of the normal γ chain which has been shown to be cleaved off by plasmin in the presence of EGTA. Thus, there is a possibility, though not confirmed, that a certain short segment which is critical for or may affect polymerization is missing in γKyoto. This deletion will result in a lower molecular weight γ chain with abnormal fibrin monomer polymerization. A good candidate for the missing segment may be the γ-chain polymerization site, Thr-374 to Glu-396. The possible deletion may correspond to the molecular weight difference of approximately 2,000 between normal γ chain and γKyoto. Another candidate for it may be a recently characterized calcium binding site, γ311-336, which is suggested to affect fibrin-monomer polymerization.

Three types of fibrinogen molecules, first discussed for fibrinogen Louisville, are possible in fibrinogen Kyoto as suggested in the case of fibrinogen New York with a deletion of Bβ (9-72): one with two normal γ chains, one with two γKyoto and one with a normal γ chain and a γKyoto chain. However, DEAE-Sephacel chromatography, fibrin monomer-Sepharose chromatography, immunoelectrophoresis, or fibrin clot formation failed to separate the propositus' fibrinogen into two or three main populations. These results leave open the possibility that fibrinogen Kyoto may contain hybrid fibrinogen molecule.

Henschen and Edman identified two minor γ-chain populations in fibrinogen obtained from the pooled plasma of many donors, and showed that the variant isolated in a larger amount is smaller than the main γ chain on gel-filtration chromatography. Although we do not know whether this variant is identical with γKyoto, we could not find a γ-chain population with a lower molecular weight in normal fibrinogen obtained from the pooled plasma of at least 20 donors. The cause of sudden death in two family members is not known and three members with γKyoto are now free of symptoms. There is a possibility, however, that abnormal fibrinogen Kyoto affects normal hemostasis and that family members who died suddenly without any sign of disease had fibrinogen Kyoto. The propositus' mother and a maternal aunt have normal fibrinogen, and no maternal family members have had a sudden death or hemorrhagic tendency. In summary, a new congenital abnormal fibrinogen Kyoto with impaired fibrin monomer polymerization had a γ-chain variant, γKyoto, with a lower molecular weight than normal. Investigation of the precise structural abnormality of γKyoto will provide us with additional information on the role of γ chain in fibrin polymerization.

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FIBRINOGEN KYOTO WITH A SHORTER γ-CHAIN

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