Fibrinogen Kyoto II, a New Congenitally Abnormal Molecule, Characterized by the Replacement of Aα Proline-18 by Leucine

By Nobuhiko Yoshida, Minoru Okuma, Hajime Hirata, Michio Matsuda, Kense kue Yamazumi, and Shinji Asakura

A new case of heterozygous dysfibrinogenemia characterized by an amino acid replacement in the NH₂-terminal region of the fibrin α-chain was found in a 27-year-old woman with a bleeding problem. Her one-stage prothrombin time and decrease in one peptide compared with the normal amount of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains. Reverse-phase high performance liquid chromatography (HPLC) of the lysylendopeptidase-cleaved purified Aα-chains showed a decrease in one peptide compared with the normal amount of fibrinogen under the reduced condition showed no abnormalities in the apparent molecular weights of its three chains.

and the appearance of an abnormal peptide peak. These peptides were treated with thrombin and further separated on HPLC. Amino acid sequence analysis of the abnormal peptide indicated that Aα proline-18, the second residue from the NH₂-terminus of the fibrin α-chain, was replaced by leucine. The synthetic peptide Gly-Pro-Arg-Pro inhibited both thrombin- and reptilase-induced fibrin aggregation, but Gly-Leu-Arg-Pro showed little or no inhibition under the same conditions. The discovery of this abnormal fibrinogen supports the findings that Aα proline-18 is important as part of the polymerization site in the NH₂-terminus of the fibrin α-chain. The propositus’ mother had the same abnormal fibrinogen. This unique inherited abnormal fibrinogen was designated as fibrinogen Kyoto II.

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Materials and Methods

Coagulation studies and fibrinogen purification. Coagulation studies were performed according to standard procedures. Immuno-logic fibrinogen concentration was determined by radial immunodiffusion on Nor-Partigen plates (Behringwerke, Marburg, Germany). Fibrinogen was purified from citrate- or citrate dextrose-plasma by centrifugation, the optical density of the supernatant solution at 280 nm was compared with that of the fibrinogen solution before thrombin- and reptilase-induced fibrin aggregation, but Gly-Pro-Arg-Pro inhibited both thrombin- and reptilase-induced fibrin aggregation, but Gly-Leu-Arg-Pro showed little or no inhibition under the same conditions. The discovery of this abnormal fibrinogen supports the findings that Aα proline-18 is important as part of the polymerization site in the NH₂-terminus of the fibrin α-chain. The propositus’ mother had the same abnormal fibrinogen. This unique inherited abnormal fibrinogen was designated as fibrinogen Kyoto II.

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used as the eluant; a linear gradient from 30% to 60% solvent system B in 2 hours with a flow rate of 0.5 mL/min was used, and the column effluent was monitored at 280 nm. Purified Aα-chain dissolved in 50 mmol/L Tris-HCl, 4 mol/L urea, pH 9.0, was digested with lysylendopeptidase (Wako Chemical) (3 μg/μmol Aα-chain) at 37°C for 18 hours. The lysylendopeptidase digests were fractionated on a Biofine RPC-SC18 reversed-phase HPLC column (Nihonbunko, Tokyo, Japan); a linear gradient from 0% to 45% solvent system B in 3 hours with a flow rate of 0.5 mL/min was used, and the column effluent was monitored at 214 nm. Relevant peaks obtained from 0.6 mg of Aα-chain digest were lyophilized, dissolved in 2 mL of 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, pH 7.4, and digested with 0.3 U/mL bovine thrombin at 37°C for 4 hours. The reaction was terminated by heating the mixture at 100°C for 15 minutes, and the cleaved peptides obtained by centrifugation of the heat-denatured samples were fractionated by HPLC with a linear gradient from 0% to 40% solvent system B in 80 minutes.

**Amino acid sequence analysis.** Amino acid sequence analysis of peptides was performed by automated Edman degradation (Model 470A Protein Sequencer to which a Model 120A PTH Analyzer was connected; Applied Biosystems, Foster City, CA) as described.20

**Effect of synthetic peptide on fibrin aggregation.** Gly-Pro-Arg-Pro (molecular weight, 425) and Gly-Leu-Arg-Pro (molecular weight, 441), kindly synthesized and supplied by Applied Biosystems Japan (Tokyo, Japan), were purified by HPLC. The concentrations of Gly-Pro-Arg-Pro and Gly-Leu-Arg-Pro were calculated using the extinction coefficient, absorbance of a 1% solution at 214 nm = 47.6 and 41.7, respectively, which were based on the dry weight of the lyophilized material. Fibrin aggregation was studied as described by Beck et al.14 Synthetic peptide was incubated with 0.1 mg/mL (0.294 μmol/L) of normal fibrinogen in 50 mmol/L Tris-HCl, 75 mmol/L NaCl, 1 mmol/L CaCl₂, pH 7.4, for 15 minutes, followed by the addition of thrombin or reptilase. The absorbance at 350 nm was continuously monitored at room temperature.

**PAGE.** SDS-PAGE was performed according to the method of Laemmli.25 Non–SDS-PAGE at alkaline pH was performed essentially as described by Soria et al.20

**Case report.** The propositus (T.H.), a 27-year-old woman with no past history of serious bleeding, had massive genital bleeding 2 weeks after her first delivery without any apparent precipitating cause. She received blood transfusions at a local hospital and was then referred to Kyoto University Hospital. The genital bleeding subsided spontaneously over the several days. Her one-stage prothrombin time was slightly prolonged (12.4 to 14.3 seconds v 11.3 to 12.1 seconds normal) and the value of serum fibrin degradation products (FDP) was slightly elevated, but platelet count was normal. The thrombin time method indicated that she had hypofibrinogenemia (73 to 90 mg/dL), but the immunologic method showed a normal concentration of plasma fibrinogen (216 mg/dL). Endometrial curettage was performed without any complications. The propositus' mother was also found to have hypofibrinogenemia by the thrombin time method and normal fibrinogen levels by the immunologic method.

**RESULTS**

The patient had normal levels of coagulation factors including factor XIII, antithrombin III, plasminogen, and α2-plasmin inhibitor, and her platelet count was within the normal range. Her one-stage prothrombin time and activated partial thromboplastin time were slightly prolonged. As shown in Table 1, the thrombin time and reptilase time of the patient’s plasma were prolonged, and the plasma fibrinogen concentration evaluated by the thrombin time method was about 25% of the normal value, although the turbidimetric and immunologic methods showed almost normal levels of plasma fibrinogen. The purified fibrinogen from the propositus had a markedly prolonged thrombin or reptilase time. Clotting times in the presence of calcium ions were also prolonged, although they became much shorter than those in the absence of calcium ions.

No abnormalities were shown by SDS-PAGE of purified propositus’ fibrinogen under the nonreduced or reduced condition, reduced and carboxymethylated fibrinogen, crosslinked fibrin under the reduced condition, and of the plasmic digests of fibrinogen (D₁, D₃, D₄, and E) under the nonreduced or reduced condition, or by non–SDS-PAGE at alkaline pH of reduced and carboxymethylated fibrinogen (data not shown). Release of fibrinopeptide A with thrombin as examined by radioimmunoassay was normal both with respect to the initial rate and the total amount released. Release of fibrinopeptides A and B as examined by HPLC also showed no substantial differences between the propositus’ fibrinogen and normal fibrinogen, which was also verified by the conversion velocity of Aα- and Bβ-chains to α- and β-chains, respectively, as monitored by SDS-PAGE (not shown). A fibrin monomer polymerization curve of the propositus’ fibrinogen (Fig 1) showed a long lag time (4 minutes v 2 minutes, normal), a decreased slope (ΔOD 350/min = 0.029 v 0.057, normal), and a decreased final amplitude (OD 350 at 30 minutes = 0.18 v 0.23, normal). These routine analyses failed to point out which chain is defective.

Aα-chains purified from reduced and carboxymethylated fibrinogen were digested with lysylendopeptidase, and the digests were analyzed by HPLC (Fig 2). The HPLC elution pattern of the propositus’ sample (Fig 2, KII) showed that one peak was decreased about 50% (designated KII) compared with the corresponding normal peak (designated NN) and the appearance of an abnormal peak designated as KIIA. Because these peaks were supposed to be derived from the NH₂-terminal portion of the Aα-chains, the three peaks (NN, KII, and KIIA) were digested with thrombin

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**Table 1. Studies for Fibrinogen Function**

<table>
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<tr>
<th>Sample</th>
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<th>Propositus</th>
<th>Control</th>
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<td>Thrombin time (sec)</td>
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<td>22.3</td>
<td>11.7-12.7</td>
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<td></td>
<td>With calcium ions</td>
<td>14.3</td>
<td>9.2-9.3</td>
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<tr>
<td></td>
<td>Reptilase time (sec)</td>
<td>&gt; 100</td>
<td>11.2-14.3</td>
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<tr>
<td></td>
<td>Without calcium ions</td>
<td>36.8</td>
<td>7.7-8.7</td>
</tr>
<tr>
<td></td>
<td>With calcium ions</td>
<td>6.7</td>
<td></td>
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<tr>
<td><strong>Fibrinogen</strong></td>
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<tr>
<td></td>
<td>With calcium ions</td>
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and the digests were then analyzed by HPLC (Fig 3). The NN digest profile (data not shown) was the same as that of the KIIN digest. The KIIN digest contained two main peaks, KIINT, with a retention time of 34.4 minutes, and FPA. The KIIA digest also contained two main peaks, KIIAT, with a retention time of 40.6 minutes, and FPA. Peak FPA proved to be fibrinopeptide A (data not shown).

As shown in Table 2, the amino acid sequence of KIINT corresponded to residues 17-29 of the normal Aα-chain and that of KIIAT was the same as KIINT except for the substitution of leucine for proline at residue 18, which is the second residue from the N-terminus of the fibrin α-chain. The amino acid sequence of the very small peak, V-K, with a retention time of 27.4 minutes in the KIIA digest, corresponded to residues 20-29 of the normal Aα-chain. The size of this peak increased with prolonged thrombin digestion concomitant with the decrease of KIIAT or KIINT (figure not shown), which indicates the additional cleavage of the Aα Arg19-Val20 bond by thrombin. Gly-Leu-Arg or Gly-Pro-Arg, residues 17-19 of the Aα-chain, was

<table>
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<th>pmol</th>
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<td>Lys</td>
<td>33</td>
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*Corresponding residues of the Aα-chain.
Amino acid sequence analysis (Table 2) demonstrated the replacement of Αα proline-18 by leucine, which can arise from a point mutation involving a single nucleotide change in the codon (CCA) responsible for position Αα 18. The codon is most likely altered from CCA to CTA.

Variant fibrinogens with an abnormal NH₂-terminal region in the fibrin α-chains have been limited to four cases: fibrinogens Detroit (Αα 19Arg → Ser substitution), Munich I (Αα 19Arg → Asn substitution), Aarhus (Αα 19Arg → Gly substitution), and Mannheim I (Αα 19Arg → Gly substitution). These four cases and the patient with fibrinogen Kyoto II have a common characteristic in that their thrombin- and/or reptilase-induced fibrinogen clotting (polymerization) is markedly delayed but the individuals do not exhibit common clinical symptoms. Four of five affected members of the Detroit family had mild to severe bleeding problems. All the affected family members with fibrinogens Munich I and Mannheim I had mild bleeding tendencies. In contrast, the homozygous proposita of fibrinogen Aarhus had no abnormal bleeding tendency.

The patient with fibrinogen Kyoto II experienced a bleeding problem, although her mother, with the same fibrinogen, had no bleeding tendency. The etiology of the heterogeneity of clinical symptoms remains to be established.

In the same way, release of fibrinopeptides A and B is also at variance. Blomback and Blomback showed that release of fibrinopeptide A from fibrinogen Detroit was unaffected, but that of fibrinopeptide B was slower than normal. However, they described later that the experiments were performed at a very low concentration of thrombin and that it is possible that considerable amounts of fibrinopeptide B are released from fibrinogen Detroit. Release of fibrinopeptide A from fibrinogen Munich I is reported to be reduced to one-third of the normal amount, but we could not obtain any information about the release of fibrinopeptide B. Releases of fibrinopeptides A and B from fibrinogen Aarhus have been shown to occur to the same extents as those from normal fibrinogen, but the rates of release were slower. For fibrinogen Mannheim I, the release of fibrinopeptide A was shown to be delayed.

Release of fibrinopeptides A and B by thrombin was normal in fibrinogen Kyoto II. Regardless of these heterogeneities, markedly delayed fibrinogen clotting in these cases is attributable to the abnormal NH₂-terminal portion of the fibrin α-chain except for the case of fibrinogen Munich I.

It is now well accepted that the NH₂-terminal portion of the fibrin α-chain plays a central role in fibrin clot formation as the binding site for the polymerization site that is believed to reside in the COOH-terminal portion of the γ remnant of fragment D₄. Laudano and Doolittle showed that Gly-Pro-Arg-Pro, a synthetic tetrapeptide including the NH₂-terminal tripeptide of the normal fibrin α-chain, is a potent inhibitor of fibrin polymerization, but that Gly-Pro-Ser-Pro, a synthetic tetrapeptide including the NH₂-terminal tripeptide of the fibrin α-chain of fibrinogen Detroit, neither binds to fibrinogen nor inhibits fibrin polymerization, confirming the importance of Αα Arg-19 in

FIG. 4. Effect of synthetic peptide on thrombin- or reptilase-induced fibrinogen aggregation. The upper and lower panels show thrombin- and reptilase-induced fibrinogen aggregation, respectively. 1C, control for 2L and 3P; 4C, control for 5L and 6P; 2L, 5L. In the presence of 0.2 and 0.4 mmol/L Gly-Leu-Arg-Pro, respectively; 3P, 6P, in the presence of 0.2 and 0.4 mmol/L Gly-Pro-Arg-Pro, respectively.

not retained by the column or was too small to be recognized as a peak. The HPLC elution pattern of the lysylendopeptidase digest of the propositus’ Bβ- or γ-chains was the same as that of the normal control (data not shown).

The replacement of Αα proline-18 by leucine led us to investigate the effect of synthetic peptide Gly-Leu-Arg-Pro on fibrin aggregation (Fig 4). Synthetic peptide Gly-Pro-Arg-Pro inhibited both thrombin- and reptilase-induced fibrin aggregation in a dose-dependent manner, but Gly-Leu-Arg-Pro caused little or no inhibition under the same conditions. Effects of Gly-Pro-Arg-Pro or Gly-Leu-Arg-Pro on clotting times were not investigated because of the limited amounts of synthetic peptides.

**DISCUSSION**

Heterozygous, congenitally abnormal fibrinogen designated as fibrinogen Kyoto II is characterized by a bleeding history, normal release of fibrinopeptides A and B, and defective polymerization of the fibrin monomer (Fig 1).
fibrin formation. They also showed that replacement of proline by valine or histidine eliminated the inhibition of thrombin-induced clotting of human fibrinogen. 24

We compared the effect of Gly-Pro-Arg-Pro and Gly-Leu-Arg-Pro on fibrin aggregation, and confirmed that Gly-Pro-Arg-Pro had an inhibitory effect but Gly-Leu-Arg-Pro had very little effect under our experimental conditions (Fig 4). We believe that fibrinogen Kyoto II is important because its molecular abnormality and associated coagulation defect is the first unequivocal demonstration of the requisite role of Aa Pro-18 in fibrin formation. For fibrinogen Detroit, 21 fibrin monomer-Sepharose was used to investigate the ability of the NH₂-terminus of fibrin α-chains to bind to normal fibrinogen. However, this investigation was not performed for fibrinogen Kyoto II, because fibrinogen Kyoto II is heterozygous and we could not separate the abnormal homodimer molecules from the normal ones.

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