A New Congenital Abnormal Fibrinogen Ise Characterized by the Replacement of Bβ Glycine-15 by Cysteine

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A new case of heterozygous dysfibrinogenemia characterized by the replacement of NH₂-terminal amino acid of fibrin β-chain was found in a 50-year-old man. Despite a prolonged thrombin time, the propositus' fibrinogen had a normal reptilase time with the normal release of fibrinopeptide A. Release of fibrinopeptide B by thrombin was strongly affected, but a very high concentration of thrombin almost completely released fibrinopeptide B with a normal elution pattern on reversed-phase high performance liquid chromatography (HPLC). Lysylendopeptidase-cleavage of purified Bβ-chains analyzed on HPLC showed the decrease of one peptide compared with the normal 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 demonstrated that Bβ glycine-15, NH₂-terminal of the fibrin β-chain, was replaced by cysteine. These findings will be of particular importance because they strongly support the hypothesis that the NH₂-terminal portion of the fibrin β-chain is involved in the polymerization reaction by thrombin. The propositus' daughter and two sisters had the same abnormal fibrinogen. This unique inherited abnormal fibrinogen was designated as fibrinogen Ise. During these studies, we found that a very high concentration of thrombin cleaves not only the Arg19-Val20 bond but also the COOH-terminal region of α-chains, which results in the generation of further degraded α-chains with apparent molecular weights of 44,000 or less.

Materials and Methods

Coagulation studies and purification of fibrinogen. Coagulation studies were performed as described previously.13 Fibrinogen was purified using lysine-Sepharose 4B chromatography, gelatin-Sepharose 4B chromatography, and fractionation by ammonium sulfates,13 and further purified on diethyl aminomethyl (DEAE)-Sephadex.14 Clottability of fibrinogen was assayed according to the method of Laki17 with minor modifications and that of propositus' fibrinogen had the normal value of 94%.

Studies on purified fibrinogen. Release of fibrinopeptides A and B was examined by high performance liquid chromatography (HPLC) and by the conversion velocity of α- and Bβ-chains to α- and β-chains, respectively, on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described.13 Fibrin monomer polymerization was studied as described by Grañick et al14 using their second method. Fibrin aggregation was studied as described by Beck et al.16

Bβ-chain digestion with lysylendopeptidase and thrombin. Preparation of reduced and carboxymethylated fibrinogen and its separation on HPLC column was performed as described.13 Purified Bβ-chain dissolved in 50 mmol/L Tris-HCl, 4 mol/L urea, pH 9.0, was digested with lysylendopeptidase (Wako Chemical, Tokyo, Japan) (0.2 μg/nmol Bβ-chain) at 37°C for 18 hours. Lysylendopeptidase digests were fractionated on a Biofine RPC-SC18 reversed-phase HPLC column (Nihonbunko, Tokyo, Japan). A 0.09% trifluoroacetic acid (solvent system A) and 0.09% trifluoroacetic acid in acetonitrile (solvent system B) gradient system was used as the eluant; a linear gradient from 5% to 45% solvent system B in 2 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 Bβ-chain digest were lyophilized, dissolved in 1.5 mL of 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, pH 7.4, digested with 20 U/mL bovine thrombin at 37°C for 4 hours, and fractionated by HPLC with a linear gradient from 0% to 40% solvent system B in 80 minutes. These analyses were also performed using reduced and pyridylethylated19 fibrinogen as the starting material.

PAGE and electroblotting. SDS-PAGE was performed according to the method of Laemmli20 as described previously.20 Electroblotting of proteins for sequence analysis was performed according to the method of Matsudaira.20 Polyvinylidine difluoride membranes (Clear Blot Membrane-P) were obtained from ATTO (Tokyo, Japan). In this study about 1 nmole of protein was used for the first step SDS-PAGE.

Amino acid sequence analysis. Amino acid sequence analysis of...
peptides was performed by automated Edman degradation as described.25

Case report. The propositus (H.M.), 50-year-old man, was admitted to a local hospital to undergo a cholecystectomy for gallstone. During the routine hematologic study, it was discovered that he had hypofibrinogenemia (66 mg/dL) by the thrombin time method but the turbidimetric method showed the normal concentration of plasma fibrinogen (300 mg/dL). The propositus’ two sisters and a daughter were also found to have hypofibrinogenemia by the thrombin time method. These four individuals 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, platelet count, bleeding time, and ADP-, collagen-, epinephrine-, or ristocetin-induced platelet aggregation were all within the normal range. As shown in Table 1, thrombin time of the plasma was prolonged; and the plasma fibrinogen concentration evaluated by the thrombin time method was much lower than that by the turbidimetric or immunologic method. The plasmas of two sisters and a daughter also showed a discrepancy between the fibrinogen concentration assayed by the thrombin time method (127, 109, and 151 mg/dL, respectively) and by the turbidimetric method (227, 174, and 281 mg/dL, respectively). The propositus’ purified fibrinogen also showed a prolonged thrombin time, but the reptilase time was entirely normal (Table 1). Fibrin aggregation curve by the addition of thrombin was impaired, but that by the addition of reptilase was normal (figures not shown), corresponding to the normal reptilase time.

SDS-PAGE of purified propositus’ fibrinogen under the reduced condition (Fig 1, left panel, lane 2) showed no abnormalities in apparent molecular weights of three chains. The left panel of Fig 1 shows the release of fibrinopeptides A and B from fibrinogen (0.2 mg/mL) with 0.2 U/mL thrombin as monitored by SDS-PAGE. Conversion velocity of the propositus’ αα-chains to α-chains was the same as normal, but that of Bβ-chains to β-chains was much slower than the normal control and about 50% of Bβ-chains remained as Bβ-chains after 80 minutes of incubation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thrombin time (sec)</th>
<th>Studies</th>
<th>Propositus</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Without calcium ions</td>
<td>22.4</td>
<td>12.0-13.8</td>
<td>9.2-9.3</td>
</tr>
<tr>
<td></td>
<td>With calcium ions</td>
<td>14.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Thrombin time method</td>
<td>129</td>
<td>150-350</td>
<td>277</td>
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<tr>
<td></td>
<td>Turbidimetric method</td>
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<td>150-350</td>
<td>277</td>
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<td></td>
<td>Immuno logic method</td>
<td>256</td>
<td>150-350</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>Factor XIII (%)</td>
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<td>Fibrinogen</td>
<td>Reptilase time (sec)</td>
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<td>5.2-7.2</td>
<td>7.8</td>
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<tr>
<td></td>
<td>Without calcium ions</td>
<td>16.3</td>
<td>14.8-19.9</td>
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<tr>
<td></td>
<td>With calcium ions</td>
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</table>

Release of fibrinopeptide A as examined by HPLC showed no substantial difference between the propositus’ fibrinogen and normal fibrinogen, but the amount of released fibrinopeptide B that had the same retention time as normal on HPLC was about 50% of normal after 80 minutes of incubation with thrombin (data not shown). However, more than 50% of propositus’ Bβ-chains were converted to β-chains with an increase of thrombin concentration (Fig 1, right panel). Incubation of propositus’ fibrinogen (1 mg/mL) with a high concentration of thrombin (50 U/mL) for 6 hours at 37°C and for another 12 hours at 4°C resulted in almost complete conversion of Bβ-chains to β-chains (Fig 2) and the amount of the released fibrinopeptide B was almost the same as normal (Fig 3). Such a high concentration of thrombin digested α-chains of both normal and abnormal fibrinogens further into smaller degraded α-chains with apparent molecular weights of about 44,000 or less (Fig 2), and the fibrin clots once formed became abnormally weak and translucent and sometimes portions of clots became soluble, which supports the observation made by Triantaphyllopoulos and Chandra that thrombin could cleave fibrin in many places and could even dissolve a fibrin clot.24 Amino acid sequences of the first three residues of the degraded α-chains were analyzed after electrophoblotting procedure. Two sequences of Gly-Pro-Arg (about 57%) and Val-Val-Glu (about 43%) corresponding to residues 17-19 and 20-22, respectively, were obtained, which suggests that not only the NH2- but the COOH-terminal region of α-chains were digested by a high concentration of thrombin. Release of fibrinopeptide A with reptilase showed no difference between the propositus’ fibrinogen and normal fibrinogen (not shown).

As mentioned above, it was impossible to obtain the propositus’ fibrin monomers by treatment with thrombin without further degradation of α-, β-, or γ-chains. Accordingly, polymerization studies of des-AB fibrin monomers, which are devoid of fibrinopeptides A and B, could not be
Fig 2. Effect of a high concentration of thrombin on fibrinogen chains. Fibrinogen (1 mg/mL) in buffer A was incubated with 50 U/mL thrombin as described in the text. 1 and 4, fibrinogen; 2 and 3, fibrin. deg. α, degraded α-chains with apparent molecular weights of 44,000 or less.

Assessed precisely, but the polymerization of propositus’ des-AB fibrin monomers obtained was defective compared with the normal control (Fig 4, upper panel). Polymerization of fibrin monomers obtained by the treatment of reptilase showed no substantial difference between the propositus’ fibrinogen and normal fibrinogen (Fig 4, lower panel).

Bβ-chains purified from reduced and carboxymethylated fibrinogen were digested with lysylendopeptidase, and the digests were analyzed by HPLC. The HPLC elution pattern of propositus’ sample (Fig 5, left panel, I) showed a decrease of about 50% in one peak (designated IN) compared with the corresponding normal peak (designated NN), and the appearance of an abnormal peak designated as IA. As these peaks were found to be NH₂-terminal portion of Bβ-chains from pyroglutamate aminopeptidase digestion (data not shown), three peaks (NN, IN, and IA) were digested with thrombin and the digests were analyzed by HPLC. The NN digest profile (data not shown) was the same as that of the IN digest (Fig 5, right panel, IN). The IN digest contained two peaks, INT with a retention time of 35.6 minutes and FPB. The IA digest (Fig 5, right panel, IA) contained three peaks, IAT with a retention time of 37.5 minutes, FPB, and an undigested one. Peak FPB proved to be fibrinopeptide B (data not shown). As shown in Table 2, the amino acid sequence of INT corresponded to residues 15-22 of the normal Bβ-chain and that of IAT was the same as INT except for the substitution of cysteine.
for glycine at residue 15, which is the NH₂-terminus of fibrin β-chain. When we used reduced and pyridylethylated fibrinogen as the starting material, pyridylethylated-Cys was detected as the NH₂-terminal amino acid of IAT, confirming this substitution (data not shown). The HPLC elution pattern of the lysylendopeptidase digest of propositus’ Aα- or γ-chains was the same as the normal control (data not shown).

The role of Bβ-chains in fibrin clot formation has been the subject of numerous investigations. Laudano et al showed that Gly-His-Arg-Pro, the NH₂-terminal tetrapeptide of the fibrin α-chain, binds to the site for Gly-His-Arg-Pro as well as to its major binding site, which is present not in fragment D, but in fragment Dγ. Enhancement of fibrin polymerization by Gly-His-Arg-Pro was explained by Laudano et al as follows: synthetic peptide Gly-His-Arg-Pro binds to its binding site, thereby inhibiting the binding of NH₂-terminus of α-chain to the Gly-His-Arg-Pro binding site.

Fibrinogen Ise demonstrated that the delayed release of fibrinopeptide B and the resultant delayed exposure of the NH₂-terminus of β-chain prolongs thrombin time. In other words, these findings strongly suggest the positive role of Bβ-chains in fibrin clot formation by thrombin; NH₂-terminus of β-chain binds to its binding site (GHR site), thereby inhibiting the binding of the NH₂-terminus of α-chain to GHR site and promoting the binding of the NH₂-terminus of α-chain to its correct binding site (GPR site).

Indeed, Furlan et al showed that the onset of visible clot formation occurred following release of about 60% fibrinopeptide A by reptilase, whereas it was noted already after removal of 30% fibrinopeptide A, in addition to about 5% fibrinopeptide B, by thrombin, which is in good agreement with our explanation on the role of NH₂-terminus of β-chain. In addition, Shainoff and Dardik showed that des-B fibrin prepared by addition of copperhead venom procoagulant enzyme can exist as soluble trimers at 37°C, which cannot be explained without assuming the binding of the NH₂-terminus of β-chain to its binding site in another molecule at 37°C. We do not know the reason why fibrinogen Detroit with nonfunctional NH₂-terminal polymerization domain could polymerize to insoluble fibrin clots at 37°C after prolonged incubation with thrombin and the concomitant release of considerable amount of fibrinopeptide B.

Delayed exposure of the NH₂-terminus of β-chain in
fibrinogen Ise will result in the consumption of the NH₂-terminus of α-chain for the binding to GHR site and will be the main reason for the prolonged thrombin time. We can not tell precisely if the NH₂-terminus of β-chain of fibrinogen Ise could bind to GHR site, because des-AB fibrin monomers contained a considerable amount of NH₂-terminus-degraded α-chains, which resulted in the defective fibrin monomer polymerization even in the normal control (Fig 4). Cys-His-Arg-Pro was not synthesized because the state of the sulphydryl group of the substituted cysteine is not involved in disulfide bond formation with another fibrinogen molecule or with other plasma proteins bearing free sulphydryl groups. Lysylendopeptidase digests of native fibrinogen or with other plasma proteins bearing free sulphydryl groups, of fibrin monomers, of or NH₂-terminal disulfide knot or plasmic digests of fibrinogen were analyzed on HPLC to find the abnormal peptide with the native structure, but we failed to find it (data not shown) and we do not know whether the substituted cysteine is involved in disulfide bond formation with other cysteines in the same fibrinogen molecule. At most we can say that the replacement of Bβ glycine-15 by cysteine did not affect the structure and function of Aα-chains.

Finally, we obtained important information from fibrinogen Ise. A high concentration of thrombin cleaves not only the Aα Arg19-Val20 bond but also the COOH-terminal region of α-chains, which results in the further degradation of α-chains. In addition, a high concentration of thrombin should be used for the analysis of abnormal fibrinogens if the release of fibrinopeptide A and/or B is defective.

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A new congenital abnormal fibrinogen Ise characterized by the replacement of B beta glycine-15 by cysteine

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