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Regular Article

Recombinant γT305A fibrinogen indicates severely impaired fibrin polymerization due to the aberrant function of hole 'a' and calcium binding sites

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ABSTRACT

Introduction: We examined a 6-month-old girl with inherited fibrinogen abnormality and no history of bleeding or thrombosis. Routine coagulation screening tests showed a markedly low level of plasma fibrinogen determined by functional measurement and also a low level by antigenic measurement (functional/antigenic ratio = 0.295), suggesting hypodysfibrinogenemia.
Materials and methods: DNA sequence analysis was performed, and γT305A fibrinogen was synthesized in Chinese hamster ovary cells based on the results. We then functionally analyzed and compared with that of nearby recombinant γN308K fibrinogen.
Results: DNA sequence analysis revealed a heterozygous γT305A substitution (mature protein residue number). The γT305A fibrinogen indicated markedly impaired thrombin-catalyzed fibrin polymerization both in the presence or absence of 1 mM calcium ion compared with that of γN308K fibrinogen. Protection of plasmin degradation in the presence of calcium ion or Gly-Pro-Arg-Pro peptide (analogue for so-called knob 'A') and factor XIIIa-catalyzed fibrinogen crosslinking demonstrated that the calcium binding sites, hole 'a' and D:D interaction sites were all markedly impaired, whereas γN308Kwas impaired at the latter two sites. Molecular modeling demonstrated that γT305 is localized at a shorter distance than γN308 from the high affinity calcium binding site and hole 'a'.
Conclusion: Our findings suggest that γT305 might be important for construction of the overall structure of the γ module of fibrinogen. Substitution of γT305A leads to both dysfibrinogenemic and hypofibrinogenemic characterization, namely hypodysfibrinogenemia. We have already reported that recombinant γT305A fibrinogen was synthesized normally and secreted slightly, but was significantly reduced.

Introduction

Fibrinogen is a 340 kDa plasma hexameric glycoprotein composed of two sets of three different polypeptide chains (Aα: 610, Bβ: 461, and γ: 411 residues) [1], and encoded by three genes, *FGA*, *FGB*, and *FGG*, respectively. Each chain is synthesized, assembled into a three-chain monomer, (Aα-Bβ-γ), by disulfide bonds, further held together into a six-chain dimer, $(Aα\text{-}Bβ\text{-}γ)_2$, by disulfide bonds in the hepatocytes [2], finally secreted into the blood and is present at 1.8-3.5 g/L in plasma. The six chains are arranged into three globular nodules. The central E region contains the N-termini of all chains and the distal D regions contain the C-termini of the Bβ, γ and a short segment of the Aα chains. The C-termini of the Aα chains (αC domains) extend briefly through the D regions and fold back into coiled-coil connectors. Coiled-coil connectors composed of all three chains link the E and D nodules [3].

During thrombin-catalyzed fibrin polymerization, thrombin cleaves fibrinogen, releasing fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from the N-termini of the Aα and Bβ chains, respectively, and converting fibrinogen to fibrin monomers [4]. Fibrin monomers

Abbreviations: APTT, activated partial thromboplastin time; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; FpA, fibrinopeptide A; FpB, fibrinopeptide B; GPRP, Gly-Pro-Arg-Pro; HBS, N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], pH 7.4, 0.12 M NaCl; HEPES, N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]; PAGE, polyacrylamide gel electrophoresis; PT, prothrombin time; SDS, sodium dodecyl sulfate; SEM, scanning electron microscopy.

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polymerize spontaneously through a two-step process. In the first step, the release of FpA exposes a new N-terminal segment, knob 'A', which starts with sequence Gly-Pro-Arg-, and binds to hole 'a' in the γ module of another fibrin molecule. These A:a interactions mediate the formation of double-stranded protofibrils with half-staggered overlap between molecules in different strands [4]. The end-to-end alignment of monomers in each protofibril strand requires so-called D:D interaction, which abuts the γ-chain of two adjacent molecules [5]. In the second step, these protofibrils grow in length and thrombin cleaves FpB, which exposes a new N-terminal segment, knob 'B', which likely interacts with hole 'b' in the β module of the D region of another molecule to promote lateral aggregation of the protofibrils [6], resulting in the formation of thicker fibers and finally an insoluble fibrin clot consisting of a multi-stranded and branched fiber network [7].

Inherited fibrinogen disorders causing quantitative or qualitative alterations of this molecule have been phenotypically as hypofibrinogenemia, afibrinogenemia, dysfibrinogenemia. As many as 400 families with inherited fibrinogen disorders have been analyzed genetically and/or structurally. These are listed on the GEHT homepage [8] (updated on 26/01/2012) (http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html). Crystallographic studies have provided high-resolution structures of the γ module, which has several important sites relating to fibrin polymerization, including the high affinity calcium binding site, hole 'a' and the D:D interaction site [9,10]. Many amino acid substitutions in the fibrinogen γ module lead to reduced fibrin polymerization, namely dysfibrinogenemia [8] and some substitutions lead to impaired synthesis and/or secretion of fibrinogen in hepatocytes, namely hypofibrinogenemia caused by heterozygotes [8,11], but no substitutions lead to afibrinogenemia caused by homozygotes [8]. Amino acid substitutions in the γ module rarely show both characteristics of dysfibrinogenemia and hypofibrinogenemia, so-called hypodysfibrinogenemia [12,13]. The analysis of bases of dysfibrinogenemia and hypofibrinogenemia has provided useful information for understanding the molecular details of the mechanisms of thrombin-catalyzed fibrin polymerization [14,15] and fibrinogen synthesis and/or secretion of hepatocytes [11,16,17], respectively.

We examined a girl with a low plasma fibrinogen level determined by both functional and antigenic measurement but each level was incompatible (functional/antigenic ratio = 0.295), suggesting hypodysfibrinogenemia. DNA sequence analysis revealed the heterozygous substitution of Ala for Thr at γ305 residue (mature protein; γT305A). We designated this variant as fibrinogen Nagakute. Since we had already reported the synthesis and/or secretion of γT305A fibrinogen [18], in this report, we purified the recombinant γT305A fibrinogen and analyzed the markedly impaired function of fibrin polymerization, and compared it with a nearby recombinant variant fibrinogen, γN308K [19], which was synthesized based on dysfibrinogenemia [20].

Materials and Methods

This study was approved by the Ethics Review Board of Shinshu University School of Medicine. After informed consent had been obtained from the patients, blood samples were collected for biochemical and genetic analyses.

Patients and Coagulation Screening Tests

The proposita of Nagakute was a 6-month-old girl with suspected Kawasaki disease who had no history of bleeding or thrombosis. Nine volumes of blood were collected from intermediate cephalic vein into plastic tubes containing one volume of 3.2% trisodium citrate. Plasma was separated by centrifugation at 1500 x g for 10 minutes at 4 °C. Routine coagulation screening tests showed a markedly low concentration of plasma fibrinogen.

Prothrombin time (PT), activated partial thromboplastin time (APTT), and the fibrinogen concentration, which were determined by the thrombin time method, were measured with an automated analyzer, Coagrex-800 (Sekisui Medical Co., Tokyo, Japan). The immunological fibrinogen concentration was determined by a latex photometric immunoassay using anti-fibrinogen antibody-coated latex particles (Mitsubishi Chemical Medience Co., Tokyo, Japan) [21].

Polymerase Chain Reaction (PCR) and DNA Sequence Analysis

Genomic DNA was extracted from white blood cells using a DNA Extraction WB Kit (Wako Pure Chemical Ltd, Osaka, Japan), according to the manufacturer's instructions. To analyze all exons and exon–intron boundaries of the fibrinogen genes, long-range PCR for *FGA*, *FGB* and *FGG* was performed using the TaKaRa LA Taq (Takara Bio Inc., Otsu, Japan) and the three pair of primers, as described elsewhere [17]. The purified PCR products were directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 3100 Genetic analyzer (both from Applied Biosystems, Foster City, CA) and 28 pairs of primers.

Preparation of Recombinant Variant Fibrinogens

Recombinant variant fibrinogen was prepared as previously described. Briefly, the variant fibrinogen γ-chain expression vector, pMLP-γT305A, was altered from pMLP-γ [18] at codon 305 ACA (Thr) to GCA (Ala) by oligonucleotide-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The resultant expression vector γT305A was cotransfected with the histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Aα and Bβ chains (AαBβ CHO cells), using a standard calcium-phosphate co-precipitation method [11]. A selected and cloned cell line was cultured in serum-free medium using the roller bottle culture system. Recombinant fibrinogen, γT305A was purified from harvested culture medium by immunoaffinity chromatography, utilizing a calcium-dependent monoclonal antibody (IF-1; Iatron Laboratories, Tokyo, Japan) [14]. The fibrinogen concentration was determined from A280-320, assuming that a 1 mg/mL solution has an absorbance of 1.51. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reduced conditions (10% polyacrylamide gel). Purified γN308K fibrinogen, the neighboring residue at γT305, and wild-type (normal) fibrinogen were used as control fibrinogen [19].

Thrombin-Catalyzed Fibrin Polymerization and Clottability

Polymerization was followed by measuring the change in turbidity at 350nm at ambient temperature using a UV-140-02 spectrophotometer (Shimadzu, Tokyo, Japan). Reactions were performed in a final volume of 100 μl, as described elsewhere [15]. Briefly, fibrinogen (90 μl at 0.44 or 0.22 mg/mL) in 20 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (HBS buffer) was mixed with human α-thrombin (Enzyme Research Laboratories, South Bend, MA, USA, 10 μl at 0.5 U/mL). Three parameters, lag time, the maximum slope, and the absorbance change (ΔAbs) for 30 min, were obtained from the turbidity curves, as previously described [14]. The reactions were performed in triplicate for each sample.

The clottability of purified fibrinogens was determined essentially as described before [14], human α-thrombin (final concentration, 0.05 U/mL) being mixed with fibrinogen (final concentration, 0.4 mg/mL) in HBS buffer. Samples were incubated overnight at ambient temperature. After centrifugation at 15 000 rpm for 10 minutes, the fibrin(ogen) not incorporated into the fibrin gel was determined from the A_{280} value of the supernatant, and clottability was calculated as