Thrombosis Research 135 (2015) 710–717

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Novel heterozygous dysfibrinogenemia, Sumida (AαC472S), showed markedly impaired lateral aggregation of protofibrils and mildly lower functional fibrinogen levels

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ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form 23 December 2014
Accepted 11 January 2015
Available online 15 January 2015

Keywords:
Fibrinogen
Dysfibrinogenemia
Lateral aggregation of protofibrils
Fibrin fiber
Clot lysis

ABSTRACT

Introduction: We encountered a 6-year-old girl with systemic lupus erythematosus. Although no bleeding or thrombotic tendency was detected, routine coagulation screening tests revealed slightly lower plasma fibrinogen levels, as determined by functional and antigenic measurements (functional/antigenic ratio = 0.857), suggesting hypodysfibrinogenemia.
Materials and methods: DNA sequence and functional analyses were performed on purified plasma fibrinogen, and recombinant variant fibrinogen was synthesized in Chinese hamster ovary cells based on the results obtained.
Results: DNA sequencing revealed a heterozygous AαC472S substitution (mature protein residue number) in the αC-domain. AαC472S fibrinogen indicated the presence of additional disulfide-bonded molecules, and markedly impaired lateral aggregation of protofibrils in spite of slightly lower functional plasma fibrinogen levels. Scanning electron microscopic observations showed a thin fiber fibrin clot, and t-PA and plasminogen-mediated clot lysis was similar to that of a normal control. Recombinant variant fibrinogen-producing cells demonstrated that destruction of the Aα442C-472C disulfide bond did not prevent the synthesis or secretion of fibrinogen, whereas the variant Aα chain of the secreted protein was degraded faster than that of the normal control.
Conclusion: Our results suggest that AαC472S fibrinogen may cause dysfibrinogenemia, but not hypofibrinogenemia. The destruction and steric hindrance of the αC-domain of variant fibrinogen led to the impaired lateral aggregation of protofibrils and t-PA and plasminogen-mediated fibrinolysis, as well as several previously reported variants located in the αC-domain, and demonstrated the presence of disulfide-bonded molecules.

Introduction

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ) [1,2], and physiologically functions in blood coagulation, fibrinolysis, wound healing, and angiogenesis [3]. Each chain is coded by the *FGA*, *FGB*, *FGG* genes, respectively, and then synthesized, assembled into a six-chain molecule in hepatocytes, which is expressed as $(Aα, Bβ, γ)_2$, and secreted into the blood. The six chains are arranged into three globular regions (a central E region and two distal D regions) connected by linear segments called coiled-coil connectors. The E region contains the N-termini of all chains [3].

During blood coagulation, thrombin cleaves four short peptides from fibrinogen to form fibrin monomers. In brief, two sets of fibrinopeptide A and fibrinopeptide B are released from the N-termini of the Aα and Bβ chains, respectively, which exposes the knobs 'A' and knobs 'B'. The fibrin monomers spontaneously polymerize in a two-step fashion [3]. In the first step, the so-called knob 'A', hole 'a', and D-D interactions form half-staggered, double-stranded protofibrils and the second step of polymerization, termed lateral aggregation, occurs. The release of fibrinopeptide B promotes lateral aggregation [4]. The final product is an insoluble fibrin network consisting of multi-stranded, branched fibers [3].

Abbreviations: APTT, activated partial thromboplastin time; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; 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]; HMW, high molecular weight; LMW, low molecular weight; LMW', low molecular weight prime; PAGE, polyacrylamide gel electrophoresis; PCR, Polymerase chain reaction; PT, Prothrombin time; SDS, sodium dodecyl sulfates; SEM, Scanning electron microscopy; t-PA, tissue type-plasminogen activator.

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http://dx.doi.org/10.1016/j.thromres.2015.01.013
0049-3848/

Genetic mutations in fibrinogen chain genes have been implicated in afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia, as listed on the GEHT homepage [5] (updated on 26/01/2012). As many as 307 families with dysfunctional fibrinogens have been analyzed genetically and/or structurally. Most of these variants are present in either the Aα chain (46 species, 170 families) or γ chain (49 species, 97 families), while variants of the Bβ chain have been detected in 40 families (24 species). Three clinical manifestations; a tendency towards bleeding (26.1%), thrombosis (18.6%), and renal amyloidosis (7.2%), are also associated with dysfibrinogenemia, whereas 48.2% of carriers are asymptomatic. The αC-domains of the fibrinogen molecule (Aα chain amino acid sequence 392–610) are folded into a globular structure, interact with the central region of the molecule, and play an important role in enhancing lateral aggregation [6,7]. Furthermore, several variants have been identified in this domain, namely truncated or albumin-binding forms or pathogenic forms of familial renal amyloidosis [5].

We recently found a novel heterozygous dysfunctional fibrinogen, in which Ser was substituted for Aα472Cys in the αC-domain, analyzed its molecular characterization, fibrin polymerization, fibrin clot and fiber structure, and clot lysis by tissue-type plasminogen activator (t-PA)-mediated plasminogen activation, and compared these with those of other fibrinogen variants located in the αC-domain, which are present as albumin-binding variant fibrinogens.

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 parents, blood samples were collected for biochemical and genetical analyses.

Patient

The proposita was a 6-year-old girl with systemic lupus erythematosus. Since she was positive for serum anti-cardiolipin-$\beta_2$glycoprotein I antibodies, she had taken warfarin to prevent the thrombosis associated with anti-phospholipid syndrome. Before being diagnosed, her plasma fibrinogen concentration was continuously lower than the lower-level of reference intervals without bleeding or a thrombotic tendency. Her mother had a similar plasma fibrinogen concentration to the proposita, but had no history of bleeding or a thrombotic tendency, and her father's plasma fibrinogen concentration was normal.

Coagulation Screening

Nine volumes of blood were collected from the 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. The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration determined by the thrombin time method were measured with the automated analyzer, Coagrex-800 (Sekisui Medical Co., Tokyo, Japan). The immunological fibrinogen concentration was determined using anti-fibrinogen antibody-coated latex particles (LSI Medience Co., Tokyo, Japan) [8].

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 fibrinogen genes, long-range PCR for *FGA*, *FGB* and *FGG*, and direct DNA sequencing for their purified PCR products were performed as described elsewhere [9].

Purification of Plasma Fibrinogen

Fibrinogen was purified from citrated plasma obtained from the proposita, her mother, and a normal control subject by immunoaffinity chromatography using an IF-1monoclonal antibody (LSI Medience Co), and concentrations were determined as described elsewhere [9]. The purity of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reduced conditions (10% polyacrylamide gel). The characterization of the proteins was determined by SDS- PAGE under non-reduced conditions (5% polyacrylamide gel) followed by an immunoblot analysis with a rabbit anti human fibrinogen antibody (Dako, Carpinteria, CA, USA) and horse radish peroxidase-conjugated goat anti rabbit IgG antibody (Medical and Biological Laboratories Inc., Nagoya, Japan), or a rabbit anti-human albumin antibody (Dako) with the reacting species being visualized with the aid of an alkaline phosphatase-conjugated goat anti rabbit IgG antibody (EY Laboratories Inc., San Mateo, CA, USA) as described elsewhere [10].

Preparation of Mutant Expression Vectors and Recombinant Variant Fibrinogens

Recombinant variant fibrinogens were prepared as described previously [11]. Briefly, the variant fibrinogen Aα chain expression vector, pMLP-Aα472S or -Aα442S, was altered from pMLP-Aα at codon 472 TGT (Cys) to TCT (Ser) or 442 TGC (Cys) to TCC (Ser) by oligonucleotide-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following primer pairs (the altered base is underlined); 5'-GAT GGT TCT GAC TCT CCC GAG GCA ATG-3' (sense) and 5'-CAT TGC CTC GGG AGA GTC AGA ACC ATC-3' (antisense) for Aα472S, 5'-CCA CGC GTC GTT CAT CCT CTA AAA CCG TTA C-3' (sense) and 5'-GTA ACG GTT TTA GAG GAT GAA CGA CGC GTG G-3' (antisense) for Aα442S [11]. The resultant expression vector for Aα472S or Aα442S was co-transfected with the histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Bβ and γ chains (Bβγ-CHO cells) as described elsewhere [11]. Ten colonies from each variant of fibrinogen-synthesizing CHO cells (Aα472S- or Aα442S-CHO cells) were selected at random and expanded to examine the synthesis of fibrinogen. Fibrinogen concentrations in culture media or cell lysates were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [11]. Both recombinant fibrinogens, Aα472S and Aα442S, were purified from harvested serum-free medium by immunoaffinity chromatography and concentrations were determined as described elsewhere [9].

Thrombin-catalyzed Fibrin Polymerization and Thrombin Clotting Time

Polymerization was followed by measurements of changes in turbidity at 350 nm at ambient temperatures using the UVmini-1240 spectrophotometer (Shimadzu, Tokyo, Japan). Reactions were performed as described elsewhere [9] using 20 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (HBS buffer) and human α-thrombin (Enzyme Research Laboratories, South Bend, MA, USA). Three parameters: lag time, the maximum slope, and absorbance changes for 30 min (final OD), were obtained from the turbidity curves, as previously described [12]. Thrombin clotting time was also measured in duplicate as described elsewhere [8].

Scanning Electron Microscopy (SEM)

To make fibrin clot, thrombin (10 μL at 0.5 U/mL) was added to fibrinogen solution (40 μL at 0.4 mg /mL). The SEM preparation was performed as described previously [9]. Images were viewed on a JSM-6000 F (Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan) and taken at 3,000x or 20,000x with a 5-kV accelerating voltage. Fiber