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Severe Bleeding In a Woman Heterozygous for the Fibrinogen γ R275C Mutation

Chantelle M. Rein^a, Brian L. Anderson^d, Morgan M. Ballard^d, Christopher M. Domes^d, Joshua M. Johnston^d, R. Jared Madsen Jr.^d, Kathryn K. M. Wolper^d, Andrew S. Terker^d, John M. Strother^e, Thomas G. Deloughery^b, and David H. Farrell^c

^a Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA

^b Department of Medicine, Oregon Health & Science University, Portland, OR, USA

^c Department of Pathology, Oregon Health & Science University, Portland, OR, USA

^d School of Medicine, Oregon Health & Science University, Portland, OR, USA

^e Hematology Oncology of Salem, Salem, OR, USA

Abstract

The dysfibrinogen γ R275C can be a clinically silent mutation, with only two out of seventeen cases in the literature reporting a hemorrhagic presentation, and four cases reporting a thrombotic presentation. We describe here a particularly severe presentation in 54-year-old female patient who required a hysterectomy at 47 years of age due to heavy menstrual bleeding. Coagulation studies revealed a prolonged prothrombin time and thrombin time, a normal fibrinogen antigen level, and a low fibrinogen activity level. Molecular analysis of the patient's DNA revealed a γ chain gene mutation resulting in an amino acid substitution at residue 275 (γ R275C). Protein sequencing of the fibrinogen γ chain confirmed this mutation, which was named Fibrinogen Portland I. This case demonstrates that the γ R275C mutation can lead to a severe hemorrhagic phenotype.

Keywords

fibrinogen; hemorrhage; mutation

Introduction

Fibrinogen is a 340,000 kDa protein synthesized in the liver that plays an essential role in clot formation. Fibrinogen is composed of two sets of three polypeptide chains: $\text{A}\alpha$, $\text{B}\beta$ and γ , which are assembled with the stoichiometry $(\text{A}\alpha\text{B}\beta\gamma)_2$ [1]. Thrombin, a serine protease that is activated during coagulation, cleaves fibrinopeptides A and B from the $\text{A}\alpha$ and $\text{B}\beta$ chains, respectively, exposing cryptic polymerization sites within the α and β chains of fibrinogen [2]. This allows for interaction of neighboring fibrin monomers to form the matrix that is the foundation of a fibrin clot. The transglutaminase factor XIIIa stabilizes the newly formed clot by forming bonds between neighboring monomers, creating an insoluble fibrin mesh [3].

Defects in fibrinogen can be either quantitative or qualitative. Quantitative defects leading to the absence of (afibrinogenemia), or a significant decrease in the amount of

(hypofibrinogenemia) circulating protein, are typically associated with bleeding [4]. Qualitative defects, or dysfibrinogenemia, lead to a defect in the structure of fibrinogen which may in turn lead to bleeding, thrombosis or no symptoms at all [5].

The γ chain mutation γ R275C has been identified in 17 named dysfibrinogens [6]. All of these reported dysfibrinogens have the classic phenotype of a mutant fibrinogen, with a prolonged clotting time and a high antigen to activity ratio [7]. Yet the phenotype of these dysfibrinogens varies considerably, with the majority of dysfibrinogens containing the γ R275C mutation being phenotypically silent. However, Fibrinogen Hannover IV and Hershey IV are associated with hemorrhage, while Fibrinogens Bellingham, Bologna, Cedar Rapids, and Villajoyosa are associated with thrombosis, although all these dysfibrinogens contain a γ R275C mutation. A mechanistic explanation for the hemorrhagic phenotype associated with Fibrinogen Hershey IV is also complicated by the fact that Hershey IV proband is a compound heterozygote with a novel γ V411I mutation in the platelet integrin α IIb β 3 binding site [8]. The present report provides evidence that the γ R275C mutation alone in Fibrinogen Portland I can result in a hemorrhagic phenotype.

Methods

Clinical testing

All experiments were conducted with the understanding and the consent of the proband, and the experiments were approved by the OHSU Institutional Review Board, IRB #2792. Standard clinical coagulation tests were performed, including the activated partial thromboplastin time, prothrombin time, thrombin time, and fibrinogen level, following informed consent. Fibrinogen antigen testing was performed using radial immunodiffusion by ARUP Laboratories (Salt Lake City, UT, USA).

DNA sequencing

Genomic DNA was purified from whole blood using the QIAamp DNA blood kit (Qiagen, Valencia, CA, USA). DNA sequencing of the coding regions of each of the three fibrinogen genes as well as the intron-exon boundaries was performed as described previously [9]. The DNA sequence for the coding regions was obtained for the $A\alpha$, $B\beta$ and γ genes (*FGA*, *FGB*, and *FGG*, respectively) from both strands.

Fibrinogen purification

Citrate-anticoagulated plasma was collected from the proband and from an anonymous control donor. Fibrinogen was purified from each sample using glycine precipitation [10]. Purified fibrinogens were analyzed on a 10% sodium dodecyl sulfate polyacrylamide gel under reducing conditions followed by Coomassie blue staining.

Western blotting

Fibrinogen was immunoprecipitated following purification using a Co-Immunoprecipitation Kit (Pierce, Rockford, IL, USA) according to the manufacturers instructions. Briefly, AXL203 polyclonal rabbit anti-human fibrinogen antibody (Zymed, Inc., South San Francisco, CA, USA) was covalently linked to agarose resin. Antibody bound-beads were washed and incubated with 250 μ g of purified control and proband fibrinogen overnight at 4 °C. Bound proteins were eluted in a low pH buffer and were separated on a 10% sodium dodecyl sulfate polyacrylamide gel under reducing conditions. Protein was transferred to a nitrocellulose membrane and the membrane was blocked in 1% gelatin for two hours. The membrane was then probed using a rabbit anti-human albumin antibody at 4 °C overnight. The membrane was then incubated with a goat anti-rabbit Alexafluor 680 secondary antibody for one hour at room

temperature and the membrane was imaged using a LiCor Odyssey Imaging System (Lincoln, NE, USA).

Protein sequencing

Five to 10 µg portions of purified control and mutant fibrinogen were dried by vacuum centrifugation and dissolved in 10 µl of 8M urea/1.0M Tris/0.2M methylamine, pH 8.5. One µl of 0.2M dithioerythritol was then added, and the samples were incubated at 50 °C for 15 minutes, followed by the addition of 2 µl of 0.5M iodoacetamide and incubation at room temperature for 15 minutes. An additional 2 µl of 0.2M dithioerythritol was then added and samples were diluted to a final 40 µl volume with 5% formic acid. One µg of each reduced and alkylated sample was injected onto a micro protein trap cartridge (Michrom Bioresources, Inc., Auburn, CA, USA) and fibrinogen subunits were separated by reverse phase chromatography using a 1.0 × 250 mm C4 column (214 MS C4, Vydac, Hesperia, CA, USA). The column used a 2–60% acetonitrile gradient over 70 minutes in a mobile phase containing 0.1% formic acid and a 19 µl/minute flow rate. Spectra were collected in profile mode over a 600–2000 m/z range while averaging 20 µscans. Data collected during the elution of the fibrinogen γ chain were averaged and deconvoluted using BioMass Calculation and Deconvolution Software (Bioworks 3.2, ThermoFinnigan, San Jose, CA, USA). Mass measurements with an error of less than 0.01% were confirmed using horse myoglobin as a standard.

Results

Case Report

The proband is a 54 year old female with hepatitis C who presented in 2001 with heavy menstrual bleeding that became progressively worse, required a hysterectomy in 2002, and was diagnosed at that time with dysfibrinogenemia. She has a history of excessive menstrual blood flow since menarche at age nine, but delivered three babies with no noted bleeding abnormalities. Minor cuts and abrasions generally stopped bleeding spontaneously in 1–2 minutes. She has had rare epistaxis, the last one in her late teens, and has had dental extractions without excessive bleeding. Prior to hysterectomy, she had not been challenged with major surgical procedures, but did have a D&C with polyp removal. She was bleeding at the time of the D&C and continued to have some vaginal bleeding following the procedure. She had not required transfusion therapy prior to hysterectomy for her bleeding episodes. Her hysterectomy was managed with the use of cryoprecipitate prior to surgery, and she did well without excessive bleeding. No fibrinolytic inhibitors were used.

The family history was unremarkable; the proband's mother (age 76) had a history of breast cancer, and her father had died at age 72 of what was believed to be a ruptured abdominal aortic aneurysm. The most recent laboratory coagulation tests on the proband revealed a prolonged thrombin time of 47.9 seconds (Table 1). Fibrinogen antigen levels were normal at 308 mg/dL, as determined by radial immunodiffusion, but the fibrinogen activity level was relatively low at 132 mg/dL (Table 1). Liver function test results showed only very mild abnormalities. Alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase were slightly elevated, but total bilirubin and albumin were within the normal range. These laboratory results suggested little impairment of liver function that might have accounted for a bleeding diathesis.

Characterization of the Mutation

All three fibrinogen genes were sequenced on both DNA strands. Sequencing revealed heterozygosity for a mutation in the fibrinogen γ chain within exon 8 (Fig. 1). This lead to an amino acid substitution of Cys for Arg at residue 275 (γR275C), as confirmed by protein sequencing of the γ chain. This mutation has been reported previously to cause dysfibrinogenemia [6]. The mutation is located at the interface between D-domains (Fig. 2) in

polymerized fibrin [11]. Purified Fibrinogen Portland I showed a very slight change in mobility in the γ chain, consistent with a point mutation (Fig. 3).

Albumin Binding Studies

Introduction of a new unpaired Cys residue has been reported to result in disulfide-linked albumin conjugates in Fibrinogen Milano VII containing a γ Ser358Cys mutation [12]. To determine whether the proband's fibrinogen contained bound albumin, both proband and normal fibrinogen were immunoprecipitated and western blotted with an antibody against human albumin. There was no detectable binding of albumin to either the proband's or the control fibrinogen (data not shown). In addition, mass spectrometry did not detect any peptide sequences in the purified fibrinogen that corresponded to albumin fragments.

Discussion

The γ R275C mutation most often results in a lack of clinical symptoms, as seen in Fibrinogens Tokyo II [13], Milano V [14], and Villajoyosa [15]. Extensive biochemical studies have revealed that this mutation does not lead to abnormal t-PA or plasminogen binding [15] and does not affect factor XIIIa mediated crosslinking [13]. However, this mutation does lead to formation of abnormal fibers within the clot network, suggesting that the γ R275C mutation leads to an abnormal D:D association, resulting in impaired fibrin formation [13]. These results are in agreement with the location of the mutation at the critical D:D interface within the fibrinogen molecule (Fig. 2). In contrast to the asymptomatic patients with the molecular defects described above, the clinical symptoms of this dysfibrinogen usually present as thrombosis [16,17] and not hemorrhage as seen in the patient described here.

Excessive bleeding was also reported in another patient with the γ R275C mutation whose dysfibrinogen, Fibrinogen Hershey IV, was characterized previously by our laboratory [8]. In this case, the proband was a 52-year-old woman who gave a history of frequent, sometimes prolonged epistaxis as a child. In addition, she had menorrhagia, most likely secondary to uterine fibroids. Like the proband for fibrinogen Portland I, this patient also underwent a hysterectomy, and a work-up prior to surgery showed a prolonged thrombin time and low functional plasma fibrinogen. However, this patient was a compound heterozygote for yet another mutation, γ V411I, which interrupted the platelet binding site for integrin α IIb β 3, resulting in lower affinity fibrinogen binding. The contribution of each mutation to the bleeding diathesis was therefore unclear. The present case report of fibrinogen Portland I suggests that the γ R275C mutation is sufficient to account for the bleeding diathesis, which may have been exacerbated by an underlying hepatitis C infection.

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Fig. 1. Sequencing of Fibrinogen Portland I γ gene. A heterozygous C to T transition is shown at nucleotide position 981 that changes a CGC codon to TGC, resulting in a γ Arg275 to γ Cys275 mutation.

Fig. 2.

Location of the γ Arg275Cys mutation at the interface of fibrin D-dimers. The γ Arg275 residue that is mutated to a Cys in Fibrinogen Portland I is shown in red. This figure shows the critical location of this amino acid at the interface between polymerized D-domains.

Fig. 3. Purified Fibrinogen Portland I and control fibrinogen. The fibrinogens were purified using glycine precipitation and analyzed by 10% SDS-PAGE under reducing conditions. Lane 1, Fibrinogen Portland I; lane 2, control fibrinogen. The molecular weights in kDa are shown at left.

Table I

Clinical laboratory results

Parameter (normal range)	Value
Fibrinogen antigen (149–353 mg/dl)	308 mg/dl
Fibrinogen activity (250–450 mg/dl)	132 mg/dl
Prothrombin time (11–13s)	19.6 s
Thrombin time (15–17s)	47.9 s
APTT (28–38s)	36.8s
Alkaline phosphatase (35–105 units/ml)	120 units/ml
Aspartate aminotransferase (5–32 units/ml)	44 units/ml
Alanine aminotransferase (5–35 units/ml)	43 units/ml
Total bilirubin (0.1–1.0 mg/dl)	0.4 mg/dl
Albumin (3.5–5.2 g/dl)	4.4 g/dl