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Molecular characterization of 7 patients affected by dys- or hypo-dysfibrinogenemia: Identification of a novel mutation in the fibrinogen Bbeta chain causing a gain of glycosylation

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ABSTRACT

Fibrinogen is a hexameric glycoprotein consisting of two sets of three polypeptides (the Aα, Bβ, and γ chains, encoded by the three genes *FGA*, *FGB,* and *FGG*). It is involved in the final phase of the coagulation process, being the precursor of the fibrin monomers necessary for the formation of the hemostatic plug. Rare inherited fibrinogen disorders can manifest as quantitative deficiencies, qualitative defects, or both. In particular, dysfibrinogenemia and hypo-dysfibrinogenemia are characterized by reduced functional activity associated with normal or reduced antigen levels, and are usually determined by heterozygous mutations affecting any of the three fibrinogen genes.

In this study, we investigated the genetic basis of dys- and hypo-dysfibrinogenemia in seven unrelated patients. Mutational screening disclosed six different variants, two of which novel (*FGB*-p.Asp185Asn and *FGG*-p.Asn230Lys). The molecular characterization of the *FGG*-p.Asn230Lys mutation, performed by transient expression experiments of the recombinant mutant protein, demonstrated that it induces an almost complete impairment in fibrinogen secretion, according to a molecular mechanism often associated with quantitative fibrinogen disorders. Conversely, the *FGB*-p.Asp185Asn variant was demonstrated to be a gain-of-glycosylation mutation leading to a hyperglycosylation of the Bβ chain, not affecting fibrinogen assembly and secretion. To our knowledge, this is the second gain-of-glycosylation mutation involving the *FGB* gene.

Introduction

Fibrinogen is a ~340-kDa plasma glycoprotein with a pivotal role in the haemostatic system both as precursor of the clot-forming protein fibrin and as adhesion molecule essential for platelet aggregation [1–3].

The fibrinogen molecule is a hexamer composed of two sets of three homologous polypeptide chains (Aα, Bβ, and γ), encoded by the *FGA*, *FGB,* and *FGG* gene [2]. Its assembly is a stepwise process taking place in the endoplasmic reticulum of hepatocytes, starting with the formation of Aα-γ and Bβ-γ dimers, and continuing with the addition of the third chain that gives rise to trimeric half molecules. These eventually dimerize originating a trinodular structure in which the N-termini of all chains contribute to the formation of the central E domain, whereas the C-termini of the Bβ and γ chains constitute the two lateral globular D-domains; the E and the D domains are linked by two coiled-coil triple helices [4]. Biosynthesis of fibrinogen also contemplates the occurrence of multiple post-translational modifications, including N-glycosylation (at Bβ-364 and γ-52 positions), O-glycosylation, sulfation, sialylation, phosphorylation, hydroxylation, oxidation, and deamidation [5,6]. The assembled and modified molecule is finally secreted into bloodstream to yield a normal plasma concentration of 160 to 400 mg/dL; the circulating molecule has a half-life of approximately four days [7].

Abbreviations: FGA, fibrinogen alpha chain gene; FGB, fibrinogen beta chain gene; FGG, fibrinogen gamma chain gene; Aα, fibrinogen alpha chain polypeptide; Bβ, fibrinogen beta chain polypeptide; γ, fibrinogen gamma chain polypeptide; ELISA, enzyme-linked immunosorbent assay; PT, prothrombin time; aPTT, activated partial thromboplastin time; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's Medium; PNGase F, peptide-N-glycosidase F; Fg:C, fibrinogen coagulant activity; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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Congenital fibrinogen disorders include quantitative defects (afibrinogenemia and hypofibrinogenemia) and qualitative defects (dysfibrinogenemia and hypo-dysfibrinogenemia). Afibrinogenemia is an autosomal recessive disorder characterized by the absence of circulating fibrinogen and caused by homozygosity or compound heterozygosity for mutations in one of the three fibrinogen genes [8–10]. In the past considered as a different clinical entity, "true" hypofibrinogenemia is characterized by low fibrinogen levels accompanied by bleeding/thromboembolic disease, although generally to a milder extent than in afibrinogenemia [9,11]. In most cases, it results from heterozygosity for null or missense mutations, each causing a complete lack or a major decrease of the corresponding chain in circulating hexameric fibrinogen molecules [8–10]. As for qualitative defects, congenital dysfibrinogenemia is characterized by normal fibrinogen antigen levels associated with low functional activity [12]. However, in some cases, antigen levels below the normal range are also observed, leading to the presence of low levels of a dysfunctional protein in the circulation, a condition more properly defined as hypo-dysfibrinogenemia. These conditions are generally caused by heterozygosity for missense mutations, even though homozygous or compound heterozygous patients have been reported [8]. While dysfibrinogenemia shows mutational hotspots at residues *FGA*-Arg35 (exon 2) and *FGG*-Arg301 (exon 8), hypo-dysfibrinogenemia results from substitutions/truncations in any of the three fibrinogen genes [8,13], often involving the globular domains of the molecule, which encompass several functionally important sites (e.g. the calcium binding and polymerization sites) [14].

Here, we report the mutational screening of seven patients affected by qualitative fibrinogen disorders: analysis of the fibrinogen gene cluster revealed six different mutations, two of which hitherto unknown (*FGB*-p.Asp185Asn and *FGG*-p.Asn230Lys). The two newly-identified variants were characterized by expression experiments of mutant fibrinogens in COS-1 cells. These experiments allowed us to demonstrate, for the *FGB*-p.Asp185Asn mutation, a pathogenic mechanism not common for fibrinogen deficiencies, i.e. the hyperglycosylation of the Bβ chain due to the introduction of a novel N-glycosylation motif.

Materials and Methods

This study was approved by the local Ethics Committee and conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants and from the parents of the underage subject.

Blood Collection and Coagulation Tests

Venous blood was collected in 1:10 volume of 0.125 M trisodium citrate, pH 7.3. Plasma was obtained by blood centrifugation at 2,000 g for 10 minutes. Fibrinogen was measured in plasma by a functional assay based on fibrin polymerization time using a commercial kit (Laboratoire Stago, Asnieres, France); the sensitivity of the test was 10 mg/dL. An in-house developed enzyme-linked immunosorbent assay (ELISA) was used to measure the antigen fibrinogen level (sensitivity: 0.05 mg/dL) [15]. The immunoassay was performed for each patient at least in triplicate on the same plasma samples. The normal range for both assays was 160-400 mg/dL. All the other screening tests were performed according to standard laboratory procedures.

DNA Sequence Analysis

DNA extraction was performed from peripheral blood using an automated DNA extractor (Maxwell 16 System; Promega, Madison, WI, USA). DNAs were quantified by using a PicoGreen assay (Invitrogen, Carlsbad, CA, USA) and the microplate reader Wallac 1420 VICTOR3 V (Perkin Elmer, Waltham, MA, USA).

FGA, *FGB*, and *FGG* coding sequences, including about 60 intronic bp at each splice junction and 500 bp of the three promoter regions, were PCR amplified using primer couples designed on the basis of the known genomic sequence of the genes (GenBank, accession numbers: NG_008832.1, NG_008833.1, and NG_008834.1). PCRs were performed on 10 ng of genomic DNA in a 25-μL final volume, following standard protocols. Primer sequences and cycling conditions for each amplicon are available on request. Direct sequencing of purified PCR products was performed on both strands using the BigDye Terminator Cycle Sequencing Kit version 3.1 and an automated ABI-3130 XL DNA sequencer (Applied Biosystem, Foster City, CA, USA). Mutation detection was performed using the Variant Reporter software (Applied Biosystems).

Expression Vectors

Full-length Aα, Bβ, and γ cDNAs, each cloned in the expression vector pRSV-Neo, were kind gifts of Dr C.M. Redman (Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY). The two newly-identified missense variants (*FGB*-p.Asp185Asn and *FGG*-p.Asn230Lys) were introduced in the relevant vector using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The correct introduction of the mutations was checked by direct sequencing. Plasmids were purified using the PureYield™ Plasmid Miniprep System kit (Promega).

Cell Cultures, Transfections, and Metabolic Labeling

The African green monkey kidney COS-1 cell line was cultured in Dulbecco's modified Eagle's Medium (DMEM; EuroClone, Wetherby, UK), supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA), glutamine (1%; EuroClone), and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; EuroClone). Cells were grown according to standard procedures at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2.

Semi-confluent cells were co-transfected with equimolar quantities of the three plasmids coding for the fibrinogen chains, either wild-type or mutant, using the Fugene reagent (Roche, Indianapolis, IN, USA), according to the manufacturer's protocols. As a negative control, COS-1 cells were also mock transfected with the unrelated pUC18 plasmid. Transfections were carried out in 10-cm diameter dish plates.

Twenty-four hours after transfection, cells were washed twice with methionine- and cysteine-free DMEM (ICN Biomedicals, High Wycombe, Berks, UK) and incubated for 16 hours in 1.5 mL/well of methionine- and cysteine-free DMEM supplemented with 200 μCi [^{35}S]-labeled methionine and cysteine (Translable; ICN Biomedicals), 10% dialyzed fetal calf serum, 2 mM L-glutamine, 2.5 mM $CaCl_2$, 5 mg/mL bovine serum albumin, and 0.1 mg/mL heparin.

Protein Analysis

After metabolic labeling, preparation of cell lysates and conditioned media, as well as immunoprecipitation experiments were performed essentially as described [16]. Briefly, immunoprecipitations were performed by preadsorbing the polyclonal rabbit anti-human fibrinogen antibody (Dako, Zug, Switzerland) to magnetic polystyrene beads covalently bound to recombinant protein G (Dynabeads Protein G; Dynal Biotech, Oslo, Norway). Immunoprecipitated proteins were hence released from protein G, by boiling for 5 minutes, and resolved by non-reducing 4% SDS-PAGE. Gels were dried under vacuum at 80 °C for 20 minutes, and then exposed over night to a storage phosphor screen (Amersham Pharmacia Biotech, Uppsala, Sweden) to visualize labeled proteins. Scans of the gels were performed using a Typhoon 9200 phosphor imager and the ImageQuant software (Amersham Pharmacia Biotech).

To analyze the glycosylation pattern of recombinant wild-type/mutant fibrinogens, the modifying enzyme peptide-N-glycosidase F (PNGase F; Roche) was used. Aliquots of 5 μL of the fibrinogen immunoprecipitated from conditioned media were initially boiled at