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Fibrinogen Maracaibo: Hypo-Dysfibrinogenemia Caused by a Heterozygous Mutation in the Gen that Encodes for the Fibrinogen A α Chain (G.1194G>A: P.Gly13>Glu) with Diminished Thrombin Generation

Marchi R[1]*, Rojas H[2], Echenagucia M[3], Meyer M[4], Acosta M[5], Apitz R[3] and Ruiz-Sáez A[3]

[1]Centro de Medicina Experimental, IVIC, Caracas, República Bolivariana de Venezuela
[2]Instituto de Inmunología, Universidad Central de Venezuela. República Bolivariana de Venezuela
[3]CNH-Banco Municipal de Sangre, DC, República Bolivariana de Venezuela
[4]University of Applied Sciences, Department of Medical Engineering and Biotechnology, Jena, Germany
[5]Banco de Sangre del Estado Zulia. República Bolivariana de Venezuela

Abstract

Introduction: Hereditary fibrinogen abnormalities can be quantitative and/or qualitative. In hypofibrinogenemia and hypodysfibrinogenemia fibrinogen levels are below 150 mg/dL.

Objectives: The aim of the present work was to characterize the fibrinogen abnormalities in a family where the propositus (an asymptomatic four-year-old male) and his mother had prolonged thrombin time and low fibrinogen levels.

Methods: Fibrinogen genes were sequenced. Preliminary studies were performed on fibrin (ogen) function and fibrin network characteristics. Fibrin formation kinetic was done in plasma and purified fibrinogen. Fibrin network porosity was measured and fibrin structure visualized by laser scanning confocal microscopy (LSCM). In addition, global haemostatic tests such as thrombin generation and thromboelastography were performed.

Results: DNA analysis revealed a heterozygous mutation in the fibrinogen gen that encoded for the Aα chain (FGA g.1194G>A: p.Gly13>Glu) in the propositus and his mother. In plasma and purified fibrinogen, the rate of patients´ fibrin formation was approximately two times slower compared to control. Propositus´ fibrin porosity was similar to control, but diminished in his mother ($p<0.05$). By LSCM patients' clots morphology were similar to control. Thromboelastographic study was normal in both patients, and thrombin generation diminished in the propositus.

Conclusions: The mutation of fibrinogen at Aα Gly13>Glu impairs fibrin polymerization. The differences found in thrombin generation between the propositus and his mother highlights the utility of global assays for therapy individualization.

Keywords: Hypo-dysfibrinogenemia; Fibrin kinetic; Fibrin structure; Thrombin generation; Thromboelastography

Introduction

Fibrinogen or coagulation factor I (FI) is a glycoprotein of 340 k Da present in plasma at 2– 4 mg/mL, is synthesized predominantly in the liver and its levels increase under inflammatory stimuli [1,2]. Fibrinogen is secreted into the bloodstream as a hexamer composed by three pairs of identical chains (Aα, Bβ and γ)2, joined together by 29 disulphide bonds that form a dimer. Each fibrinogen chain is encoded by paralogous genes (FGA, FGB, and FGG for Aα, Bβ and γ chains, respectively) [3]. In the last step of the coagulation cascade, thrombin cleaves the bonds at Aα-Arg16 and Bβ-Arg14 removing short electronegative peptides (fibrinopeptides A and B, respectively). These modified fibrinogen molecules (fibrin monomers) polymerize spontaneously and form the 3-dimentional clot network that is further stabilized by activated factor XIII (FXIIIa) [4].

Inherited fibrinogen disorders affect either the quantity (hypofibrinogenemia, fibrinogen levels <150 mg/dL) and afibrinogenemia, characterized by the complete deficiency of fibrinogen or the quality of the circulating fibrinogen (dysfibrinogenemia) or both (hypo-dysfibrinogenemia) [5]. Up to date, approximately 115 mutations have been reported that cause dysfibrinogenemia, 67 hypofibrinogenemia, 75 afibrinogenemia, and 13 hypo-dysfibrinogenemia; 101 in the Aα, 63 in the Bβ and 93 in the γ chain. About 50% of approximately more than 600 cases reported in the literature are silent [6,7]. Thrombin binds to its substrate, fibrinogen, and remains bound to the product, fibrin, after fibrinopeptides are removed [8,9]. Different studies have established that Asp7 to Val20, particularly residues on the N-terminal side P1 to P10 (nomenclature is that suggested by Abramovitz [10]) are required for the binding of fibrinogen´s fibrinopeptides to thrombin [11]. Within the sequence of fibrinopeptide A there are both critical (nonvariable) residues and those that can be modified without impairs thrombin catalytic activity [12]. The amino acid sequence of FpA between Asp7 and Arg16 is highly conserved among mammalian species, suggesting that this region is critical for thrombin binding [13,14]. Several abnormal fibrinogens

***Corresponding author:** Rita Marchi Cappelletti. Centro de Medicina Experimental, Laboratorio de Fisiopatología, Sección Biología del Desarrollo de la Hemostasia. Instituto Venezolano de Investigaciones Científicas, IVIC. Apartado 20632, Caracas 1020-A, República Bolivariana de Venezuela. Tel. +58-212-5041526 Fax: +58-212-5041086. E-mail: rmarchi@ivic.gob.ve

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have been reported with mutations in this region: Asp7>Asn [15], Phe8>Cys [6], Leu9>Pro [16], Glu11>Gly [17], Gly12>Val, and Gly13>Glu in fibrinogen Olovnice [18] and Krakow II [19].

Fibrinogen Maracaibo is a new venezuelan abnormal fibrinogen with an asymptomatic phenotype discovered during preoperative examination in a four-year-old boy. The Aα Gly13>Glu delayed fibrin formation; however, normal clot morphology was observed.

Methods

Blood collection and routine coagulation tests

Blood was collected in citrate (1 volume of 0.13 mol/l trisodium citrate and 9 volumes of blood) and immediately centrifuged at 2500 × g and 4°C, during 20 min. Plasma was aliquoted and kept frozen until use. Routine coagulation tests were performed with citrated plasma on coagulation analyzer STA Compact®, Stago, France. Fibrinogen level was determined by Clauss (Laboratoire Stago, Asnière, France) and clot weight method [20].

Mutation analysis

Genomic DNA was isolated using the Invisorb Spin Blood Mini Kit (Invitek GmbH, Berlin, and Germany) according to the manufacturer´s protocol. Sequences comprising all exons and exon-intron boundaries from the three fibrinogen genes: FGA, FGB, and FGG were amplified by the polymerase chain reaction (PCR) according to standard protocols. After purification of the PCR products using the Invisorb Spin PCRapid Kit® (Invitek, Berlin, FRG), direct DNA cycle sequencing was performed, applying the Big Dye kit from Applied Biosystems (Foster City, CA, USA), according to the manufacturer´s recommendations.

Fibrin network characterization

Fibrin polymerization: Fibrin polymerization was examined in plasma and purified fibrinogen. One hundred µL of plasma was mixed with 10 µL of bovine thrombin - $CaCl_2$ solution (0.6 units/mL and 20 mM, final, respectively); samples were run by triplicate. Purified fibrinogen (obtained by β-alanine precipitation [21]) at 1 mg/mL in Tris – buffered saline (50 mM Tris, 0.15 M NaCl), pH 7.4 was incubated for 1 min with 5 mM of $CaCl_2$ (final concentration), then clotted with 1 units/mL of thrombin (final concentration); samples were run by triplicate in three independent experiments. Changes in absorbance were followed during 1 h every 15 sec at 37°C in a Tecan Infinite M200 microplate reader (Vienna, Austria). The lag time, slope and maximum absorbance (MaxAbs) were calculated for each curve and averaged.

Permeation: Permeation through plasma clots was performed essentially as described elsewhere [22]. The clotting conditions used were 0.6 unit/mL of thrombin and 20 mM $CaCl_2$ (final concentrations). The buffer percolated through the column was Tris-buffered saline (50 mM Tris, 0.15 M NaCl, pH 7.4). In general, six clots were used and one measurement of each was taken. Experiments were done by triplicate except for the propositus where only 7 clots were run due to the scarcity of his plasma.

The permeation coefficient or Darcy constant (Ks) was calculated using the following equation [23]:

$$Ks = QL\eta/tAP$$

Where Q= volume of the buffer (cm^3), having a viscosity η (poise), flowing through a column of height L (cm) and area A (cm^2) in a given time (sec), under a hydrostatic pressure P (dyne/cm^2).

Laser scanning confocal microscopy of fibrin clot: Fibrin clots were formed inside the eight wells LabTek chambers (Invitrogen, Nalge Nunc International, Rochester, NY, USA). The plasma sample was mixed with Alexa Fluor 488-labeled fibrinogen (4 µg/215 µl final sample volume), then clotted with a thrombin - $CaCl_2$ solution (0.14 U/mL and 19 mM, respectively, final concentration). The chambers were placed in a moist environment for 2 h at 37°C for complete fibrin polymerization. The fibrin clots were observed in an Olympus laser scanning confocal microscopy (LSCM) system, Model FV1000, with an argon ion laser (473 nm excitation and 520/540 nm for emission). The objective used was UPLSAPO 60X W NA: 1.20 water immersions with a work distance of 0.28. The acquisition pinhole was set to 100 µm. The images were acquired with a field of view of 212 × 212 µm (0.331 µm/pixel). One z-stack of 30 µm thick (1 µm/slice) and one volume render was made for each field. Image analysis was performed as described elsewhere [24].

Haemostasis global tests

Thromboelastography: The extrinsic (PT-Fibrinogen Recombinant, HemosIL, Instrumentation Laboratory) and the intrinsic (APTT-SP, HemosIL, Instrumentation Laboratory) blood coagulation pathway were evaluated by thromboelastography in a ROTEM® instrument (Pentapharm, Germany). The parameters of clot time (CT), rate of clot formation (CFT), maximum clot firmness (MCF), alpha angle (α), maximum lysis (ML), and amplitude at 10, 15 and 20 min were calculated. Patients´ samples were run by duplicate and values averaged.

Calibrated Automated Thrombin Generation (CAT): Thrombin generation in plasma was measured by calibrated automated thrombography (CAT). Plasma was prepared by centrifuging twice at 2900 × g for 10 min at room temperature, essentially as described elsewhere [25]. Reactions were triggered with 1 pM TF/4 µM lipid in a Fluoroskan Ascent fluorometer (TermoLabsystem, Helsinki, Finland). Thrombin generation parameters were calculated using Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands).

Statistical Analysis

The data obtained from the different assays are represented as the mean ± standard deviation (SD). Statistical analysis was done using Origin Pro version 8.1. Purified fibrinogen polymerization, Ks, and LSCM results were compared using the Student's t-test and a $p<0.05$ was considered statistically significant.

Results

Case report

A four-year-old boy was referred to the Banco Municipal de Sangre of Caracas due to low functional fibrinogen levels, found during preoperative examination for hernia and hydrocele repair. There was not personal or family history of haemorrhagic diathesis. The mother of the propositus was a 31 year-old woman with normal menstrual flow, and no haemorrhagic complications during teeth extraction, orthopedic surgery, and caesarian. She told that only an aunt of her mother had a venous thromboembolism episode at the age of 40. Coagulation screening tests revealed a prolonged thrombin time +10.9 and +10.1 sec for the propositus and his mother, respectively, and low functional fibrinogen concentration determined by Clauss [26]. Antigenic factor von Willebrand, factor VII, protein C and S, and antithrombin levels were normal. In Table 1 the coagulation screening

	Control	Propositus	Mother
Coagulation test Prothrombin time (sec)	12 - 14	16.4	16.1
Thrombin time (sec)	16 - 19	29.9	29.1
aPTT (sec)	27 - 35	29.1	29.5
Fibrinogen (mg/dL): Clauss	200 - 400	80 - 100	95 - 111
Polimerization			
Plasma:	300	ND	280
Fibrinogen (mg/mL)[1]	15	15	15
Lag time (sec)	3.3 ± 0.7	1.6 ± 0.7	1.4 ± 0.4
Slope (mOD/sec)	708 ± 30	743 ± 11	785 ± 17
MaxAbs (mOD)	9.1 ± 7.5	ND	15*
Purified Fibrinogen:	0.386 ± 0.129		0.183 ± 0.038*
Lag time (sec)	90 ± 23		74 ± 22*
Slope (mOD/sec)			
MaxAbs (mOD)			
Permeation			
Fibrinogen (mg/mL)[1]	280	125	300
Ks (×10^{-9} cm^2)	8.2 ± 0.3 (18)	9.0 ± 1.4 (7)	5.0 ± 0.5* (17)
LSCM			
FWHM (μm)	0.58 ± 0.32 (1220)	0.56 ± 0.31 (1055)	0.58 ± 0.32 (1573)
Density (peaks/μm)	0.457 ± 0.1	0.367 ± 0.08*+	0.436 ± 0.08

[1]Fibrinogen quantified according reference 20. aPTT: partial activated thromboplastin time; MaxAbs: maximum absorbance. Ks: permeation coefficient or Darcy constant. LSCM: laser scanning microscopy. ND: not done. FWHM: full width at half maximum

*p<0.05 compared to control; + p<0.05 compared to the propositus´ mother

Table 1: Summary of the coagulation screening tests, fibrin polymerization, permeation and laser scanning confocal microscopy studies. Results are reported as mean (± SD), and the number of measurements are in brackets.

tests are summarized. DNA analysis revealed a heterozygous missense mutation in the fibrinogen gen that encoded for the Aα chain (FGA g.1194G>A: p.Gly13>Glu) in the propositus and his mother, close to the thrombin cleavage site at Aα Arg16/Gly17. Informed consent was obtained from the propositus´ mother. We have named this new hypodysfibrinogenemia as fibrinogen Maracaibo.

Fibrin network characterization

The patients´ fibrin kinetic formation was slower than control. The parameter more affected was the slope (the stage of fibrin fibers formation and association), both in plasma and purified fibrinogen, approximately two times less than control (Figure 1 and Table 1). The permeation coefficient (Ks) of the propositus was similar to control, while that of his mother was approximately 1.6 times less than control (p<0.05). By laser scanning confocal microscopy the patients´ fibrin meshwork had normal fibrin morphology (Figure 2).

Haemostasis global tests

Thromboelastography showed only prolonged INTEM - CT both in the propositus and his mother (Table 2). Interestingly, the propositus had impaired thrombin generation. Peak thrombin and ETP were approximately 3 and 2.5 times less than control (Table 3).

Discussion

In the clinical practice dysfibrinogenemia is suspected when the thrombin time is prolonged and a low ratio between clottable fibrinogen to its antigen is found. However, the ultimate diagnosis is established based on molecular fibrinogen defect tests [27]. A new case of dysfibrinogenemia was found in a venezuelan family with an Aα Gly13>Glu mutation, molecular defect already described in fibrinogen Olovnice [18] and Krakow II [19], reported as mild bleeders. However, the carriers of fibrinogen Maracaibo were asymptomatic. This mutation was first reported by Gaja et al. [28] in a Czech Republic´ family, one carrier had thrombosis and three were asymptomatic.

The N-terminal part of the Aα and Bβ fibrinogen chains are cleaved by thrombin at Arg 16/Gly17 and Arg14/Gly15, respectively, initiating clot formation. The thrombin - fibrinogen interaction is very specific. The change of glycine, a neutral and small amino acid, by glutamic acid, an acidic and larger one at AαGly13 impairs the substrate binding in the thrombin active site [29], decreasing the efficiency of fibrinopeptides A and B release [18], lengthening clot formation but without affecting clot´s morphology, as was observed in fibrinogen Olovnice and Krakow II.

The permeation coefficient or Darcy constant is a measure of the surface available for flow [30]. Under a pressure-driven system the quantity of buffer that percolates through the fibrin clot is quantified as fibrin meshwork porosity. In our experiments, the Ks was not related to fibrinogen molecular defect, since it was normal in the propositus and decreased in his mother. Clots made with plasma are rather a complex system, and it is well known the effects of other plasma proteins in fibrin structure [31,32]. In contrast, in fibrinogen Krakow II the Ks of three family members were consistently increased.

It is important to remark that the fibrin molecules that make up the clots are normal, since FpAs are released. This could explain the almost normal clot morphology. By computer modeling, a lengthening in FpAs release predicts an increase in MaxAbs [33].

By thromboelastography the patients´ maximum clot firmness (MCF) was normal. Since clot elastic properties are related to the clot structure, these results confirmed the normality of clot morphology observed by LSCM. Interestingly, the propositus´ thrombin generation was decreased. Probably this fact was due to the subject´s young age, since it has been reported diminished thrombin generation in childhood [34].

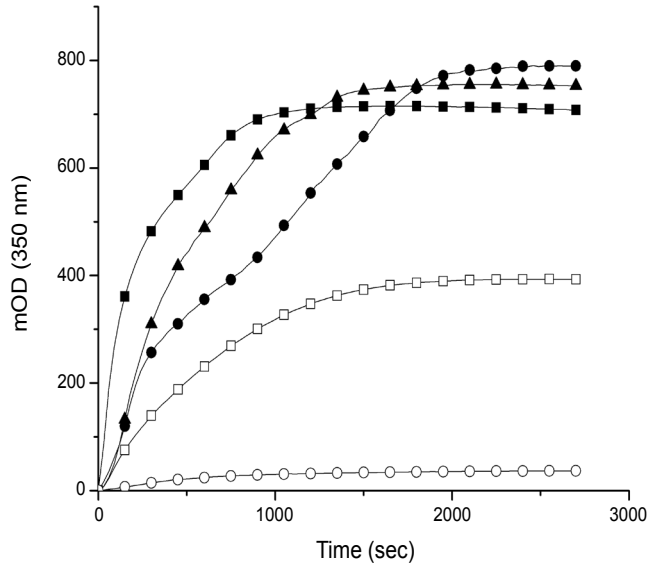

Figure 1: Fibrin polymerization kinetic followed by turbidity at 350 nm. Curves performed with plasma (filled symbol): Control (■), Propositus (▲), and Propositus´ mother (●); and with purified fibrinogen (empty symbol): Control (□) and Propositus´ mother (○).

In conclusion, the molecular fibrinogen defect Aα Gly13>Glu lengthened fibrin formation but did not alter clot structure. The differences found in thrombin generation between the propositus and his mother highlights the utility of global assays for therapy individualization.

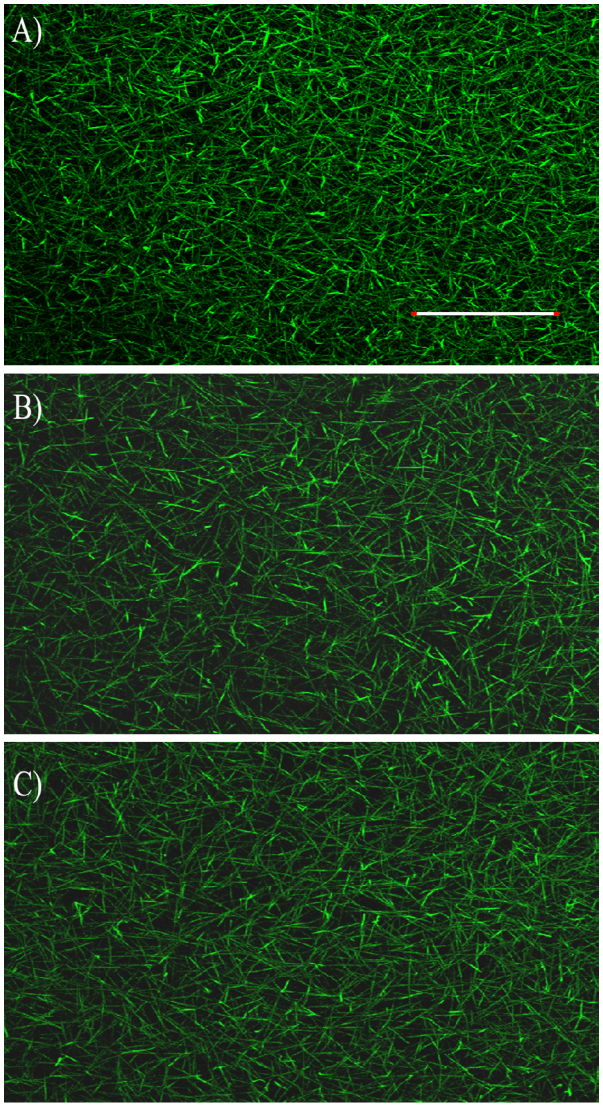

Figure 2: Laser scanning confocal microscopy images of plasma clots. Fibrin is labeled with Alexa 488. Each image was formed from a Z-stack of 30 µm. a) Control, b) Propositus, and c) Propositus´ mother. The magnification bar represents 50 µm.

	Control	Propositus	Mother
Lag time (min)	3.4 ± 0.7	6.6	3.4
Peak thrombin (nM)	168 ± 34	53	148
ETP (nM.min)	786 ± 110	318	974

ETP: Endogenous thrombin potential

Table 3: Calibrated Automated Thrombin Generation (CAT) results. Thrombin formation was triggered by adding 1 pM tissue factor.

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	CT (sec)	CFT (sec)	MCF (mm)	α angle	ML (%)	A10 (mm)	A15 (mm)	A20 (mm)
EXTEM								
Reference Values	38 - 79	34 - 159	50 -72	63 -83	< 15	43 - 65	48 - 69	50 - 71
Propositus	66	54	70	79	8	55	64	67
Propositus´ mother	52	59	75	78	-	57	67	71
INTEM								
Reference Values	100 – 173	34 – 108	50 – 72	70- 83	<15	44 – 66	48 – 69	50 – 71
Propositus	275	120	69	68	2	44	57	63
Propositus´ mother	223	76	77	75	-	51	63	68

EXTEM: extrinsic activator thromboelastometry; INTEM: intrinsic activator thromboelastometry; CT: clotting time; CFT: Clot formation time; MCF: maximum clot firmness; A10, A15, A20: clot firmness (amplitude) after 10, 15 and 20 min

Table 2: Thromboelastographic results performed using whole blood, the extrinsic and intrinsic coagulation pathway were analyzed.

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