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Full Length Article

Fibrin clot structure in patients with congenital dysfibrinogenaemia

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ABSTRACT

The clinical phenotype of patients with congenital dysfibrinogenaemia is highly heterogeneous, from absence of symptoms to mild bleeding, or thrombosis. A few mutations are associated with a specific phenotype, but generally the clinical course is not predictable. We investigated whether fibrin clot properties are correlated with the patient's phenotype and/or genotype. *Ex vivo* plasma fibrin clot characteristics, including turbidity, fibrinolysis, clot permeability and fibrin fibre density assessed by laser scanner confocal microscopy were investigated in 24 genotyped patients with congenital dysfibrinogenaemia compared to normal pool plasma. Compared to normal pool plasma, the patients were characterised by slower fibrin polymerisation (lag time, 345.10 ± 22.98 vs. 166.00 s), thinner fibrin fibres (maximum absorbance, 0.15 ± 0.01 vs. 0.31), prolonged clot lysis time (23.72 ± 0.97 vs. 20.32 min) and larger clot pore size ($21.5 \times 10^{-9} \pm 4.48 \times 10^{-9}$ vs. 7.96×10^{-9} cm^2). Laser scanning confocal microscopy images confirmed disorganised fibrin networks in all patients. Patients with tendency to bleed showed an increased permeability compared to asymptomatic patients ($p = 0.01$) and to patients with a thrombotic history ($p = 0.02$) while patients with thrombotic history had a tendency to have a prolonged clot lysis time. Fibrin clot properties were similar among hotspot mutations. Further studies including a larger number of patients are needed to evaluate whether analysis of permeability and clot lysis time may help to distinguish the clinical phenotype in these patients and to assess differences according to the genotype.

1. Introduction

Congenital fibrinogen disorders include quantitative (afibrinogenaemia and hypofibrinogenaemia) and qualitative (dysfibrinogenaemia and hypodysfibrinogenaemia) diseases [1]. Congenital dysfibrinogenaemia is characterised by a discrepancy between the functional and the antigen levels of circulating fibrinogen, reflecting the altered functional properties of the molecule due to structural defects [2]. More than 100 causative mutations have been identified in congenital dysfibrinogenaemia, mainly heterozygous missense mutations in the amino-terminal portion of the Aα chain or in the carboxyl-terminal region of the γ chain [3]. Almost all these molecular anomalies lead to ineffective polymerisation of the fibrin clot and may affect several other functions of the fibrinogen/fibrin [4].

The clinical phenotype of dysfibrinogenaemic patients is highly heterogeneous, from absence of symptoms to major bleeding or thrombotic events, including pulmonary hypertension and renal amyloidosis [3]. During the natural course of the disease, even asymptomatic patients at the time of the diagnosis are at risk of developing adverse outcomes [5]. However, neither standard haemostasis assays nor specific genotypes, with exception of some thrombotic-related mutations [6], are able to predict the clinical phenotype.

Recently, abnormal fibrin structure has been correlated with several thrombotic and cardiovascular diseases [7,8]. Fibrin clots with high fibre density and increased resistance to fibrinolysis have been consistently associated with an increased risk of thrombosis [9] whereas abnormal clots with lower fibrin network density and increased porosity have been associated with a bleeding phenotype [10]. Many studies on dysfibrinogenaemic families have described how fibrinogen variants can affect the fibrin clot in both purified fibrinogen and plasma conditions [11–14]. Sugo et al. provided a classification of the fibrin network structures formed from congenital dysfibrinogenaemia, suggesting that specific clot architecture could be correlated with a given phenotype [15]. In the present study, we aimed to determine the structural properties of fibrin clots generated from dysfibrinogenaemic plasma and to assess whether they are predictive of clinical outcomes. In addition, we assessed whether the genotype is associated with distinct clot properties.

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2. Materials and methods

2.1. Patients

This study was performed with institutional review board approval and with written informed consent from all patients, in accordance with the Declaration of Helsinki. Known dysfibrinogenaemic patients were recruited in French (Lille, Besançon, Montpellier) and Swiss (Geneva, Bern, Sion) Hospitals. Patient plasma was obtained after collection of venous blood in 0.1 mol/L sodium citrate (9 parts blood per 1 part citrate). Within 1 h after the collection, the samples were centrifuged at 3000 *g* for 12 min at room temperature and stored at −80 °C until analysis. Normal pool plasma was obtained after collection of venous blood from the antecubital vein of 14 healthy volunteers aged between 20 and 35 in 0.1 mol/L sodium citrate (9 parts blood per 1 part citrate). Within 1 h after collection, the samples were centrifuged at 2400 *g* for 20 min at room temperature to obtain platelet-poor plasma. Plasma samples were pooled and frozen in aliquots in liquid nitrogen, and stored at −40 °C until analysis. Ethical approval was obtained from the local Leeds NHS Trust research ethics committee (Ref: 03/142). Plasma levels of functional fibrinogen were measured by the Clauss method (Multifibren* U, Siemens, Germany). The PT-derived fibrinogen assay is not recommended in the case of dysfibrinogenaemia as it can overestimate the plasmatic level of fibrinogen [16]. Levels of total fibrinogen antigen were measured by a latex immunoassay (Liaphen Fibrinogen, Hyphen BioMed, France) on a BCS® XP coagulometer (Siemens, Germany). The patient genotype was determined as previously reported [5]. Causative fibrinogen mutations are described with amino acid residues and substitutions numbered from the initiator methionine. Patients with a bleeding phenotype, defined by an ISTH/SCC bleeding assessment tool [17] greater than 2, were included in the bleeders group. The thrombotic group comprised patients with objectively documented venous or arterial thrombotic events.

2.2. Turbidity and lysis

For the fibrin polymerisation assessment [18], plasma samples were diluted 1/6 with TBS (50 mM Tris, 100 mM NaCl, pH 7.4) and incubated with 0.1 U/ml human thrombin (Calbiochem; Nottingham, UK) and 5 mM $CaCl_2$ (final concentrations) in a final volume of 150 μl in polystyrene 96-well plates (Greiner Bio-one International, Stonehouse, UK). Absorbance was monitored at 340 nm, every 12 s for 60 min, using a BioTek PowerWave HT microplate reader (BioTek; Swindon, UK). For the fibrinolysis measurement, plasma samples were diluted 1/6 with TBS and incubated with 85 ng/ml tissue plasminogen activator (tPA; TechnoClone GmbH; Vienna, Austria), 0.5 U/ml human thrombin (Calbiochem; Nottingham, UK) and 22.5 mM $CaCl_2$ (final concentrations) in a final volume of 150 μl in polystyrene 96-well plates. Absorbance was monitored at 340 nm, every 12 s for 180 min, using the BioTek PowerWave HT microplate reader (BioTek; Swindon, UK). All measurements were made in triplicate and analysed with the Gen5™ software (BioTek; Swindon, UK). Lag time and maximum absorbance were calculated from the turbidity curves as previously described [19]. The clot lysis time was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity to the midpoint in the transition from maximum turbidity to the final baseline turbidity.

2.3. Permeation

The permeability assay was performed with slight modifications as previously described [20]. Briefly, plasma samples (100 μl) were incubated with 1 U/ml human thrombin (Calbiochem; Nottingham, UK) and 16 mM $CaCl_2$ (final concentrations), in a final volume of 110 μl, into a clotting tip and placed for 2 h in a humidity chamber at room temperature. The clotting tips were connected via plastic tubing to a reservoir containing TBS with a constant pressure drop of 4 cm. After washing the clots for 2 h, flow rates of buffer through the fibrin gels were measured every 30 min for 2 h for each tube. The permeation coefficient (Ks, Darcy constant) was calculated as described [20]. All samples were analysed in triplicate.

2.4. Laser scanner confocal microscopy

Laser scanning confocal microscopy was performed as previously described [21,22]. In brief, plasma samples were diluted 1/6 with TBS and incubated with 0.6 U/ml of human thrombin (Calbiochem; Nottingham, UK), 50 μg/ml AlexaFluor 488 fibrinogen (Invitrogen; Paisley, UK) and 5 mM $CaCl_2$ (final concentration), in a final volume of 60 μl. The reaction mixture was transferred into the channel of an uncoated Ibidi slide (Ibidi GmbH, München, Germany) and left in a humidity chamber for 4 h at room temperature. Imaging was performed using an upright Zeiss LSM700 microscope (Oberkochen, Germany) with a 63× oil immersion objective lens. The fibrin density was determined by counting the number of fibres crossing an arbitrary line of 100 μm drawn through a single optical section using an in-house macro for Image J (Fiji, National Institute of Health, Bethesda, Maryland, USA). Each fibrin clot was prepared in duplicate and 20 density measurements were performed on each sample.

2.5. Statistical analysis

Data are expressed as means (with standard deviation, SD) or medians (with Interquartile ranges, IQR) as appropriate. Means of normal pool plasma (normal distribution) were compared to patients by chi-square test. Means of groups were compared by an unpaired Student t-test with Welch's correction. Pearson correlation (r) was used to test for correlation between functional and antigen fibrinogen levels and fibrin clot properties. Two-sided p-values < 0.05 were considered statistically significant. Statistical analyses were performed using STATA® version 11.2 (StataCorp, College Station, TX, USA) and graphs were prepared using GraphPad Prism® version 6.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Demographic data

A total of 24 patients (12 probands and 12 relatives) with congenital dysfibrinogenaemia were included in this study. Demographic, clinical and biological data of proband and relatives are summarised in Table 1. A total of 5 (21%) patients reported a bleeding phenotype, including cutaneous bleeding, menorrhagia or post-partum haemorrhage (one requiring treatment with a fibrinogen concentrate). The median ISTH bleeding assessment tool was 4 (range 2–5). A total of 8 (33%) patients experienced 12 thrombotic events, 11 venous (6 provoked) and 1 arterial. Three patients suffered from recurrent venous thrombosis. Patients with history of thrombosis neither carried a Leiden mutation of factor V nor a G20210A mutation of factor II. All patients presented a discrepancy between normal antigenic and decreased functional fibrinogen levels, with a mean ratio activity/antigenic of 0.23 (SD 0.1) compatible with the dysfibrinogenaemia diagnosis. As expected, most patients carried a hotspot mutation (n = 20, 83.3%) either at residue Arg301 of exon 8 in *FGG* (n = 13), or at Arg35 of exon 2 of *FGA* (n = 7). All other patients harboured a heterozygous missense mutation in *FGA* (Arg38Gly, n = 2; Cys184Arg, n = 1; Arg573Cys, n = 1). The latter mutation is known to be the fibrinogen Paris V (also named Chapel Hill III), which is strongly associated with a thrombotic phenotype. Clinical phenotype according to genotype is resumed in Table 1. No patient was receiving antithrombotic treatment at the time of inclusion.