Thrombosis Research 133 (2014) 1115–1123

Contents lists available at ScienceDirect

Thrombosis Research

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

Regular Article

Thromboelastographic phenotypes of fibrinogen and its variants: Clinical and non-clinical implications

Dennis K. Galanakis [a,*], Marguerite Neerman-Arbez [b], Stephen Brennan [c], Miriam Rafailovich [a], Luke Hyder [a], Oreanthi Travlou [d], Emmanuel Papadakis [e], Marilyn J. Manco-Johnson [f], Agnes Henschen [g,†], Inge Scharrer [h]

[a] Stony Brook University, Stony Brook, USA
[b] Division of Med. Genetics, University Med. Center, Geneva, Switzerland
[c] Canterbury Health Laboratories, Christchurch, NZ
[d] University of Athens, Greece
[e] Papaioanou Hospital, Salonika, Greece
[f] University of Colorado School of Medicine, Aurora, CO
[g] University of CA, Irvine
[h] Universtatskliniken, Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 14 January 2014
Received in revised form 20 February 2014
Accepted 4 March 2014
Available online 11 March 2014

Keywords:
Thromboelastography
Fibrinogen
Fibrin
Dysfibrinogenemia
Clot stiffness
Hydrophobic fibrinogen/fibrin adsorption

ABSTRACT

Introduction: Thromboelastography (TEG), a widely used clinical point of care coagulation test, is poorly understood. To investigate its fibrin determinants we used normal and variant fibrinogen isolates.
Materials and Methods: We focused mainly on the TEG maximum signal amplitude (MA), a shear modulus and clot stiffness indicator. Isolates included normal des-αC, cord, and abnormal congenital variants with amino acid substitutions or deletions that impaired fibrin polymerization. Heterophenotypic congenital isolates were from cryoprecipitate-depleted plasma owing to their more diminished clot MA than their cryoprecipitate counterparts. By colorimetric assay, the amount of fibrinogen adsorbed by untreated TEG cups was 83.5 ± 12.4 pM/cm^2, n = 18. Thrombin-induced clots were obtained at pH 6.4 or 7.4, the latter containing 8 mM $CaCl_2$, and 14% afibrinogenemic plasma with and without gel-sieved platelets.
Results and Conclusions: Measured by the water droplet contact angle, >90% reduction of surface hydrophobicity by exposure of TEG cup and pin to ozone plasma decreased MA by 74%. Increasing normal fibrinogen or thrombin concentrations progressively increased MA. Platelets increased MA further ~2 fold, except for ≥10 fold for des-αC clots. Examined in the absence of platelets, MA of heterophenotypic fibrin variants averaged 21%, n = 15. The results imply that essential MA determinants include hydrophobic fibrinogen/fibrin adsorption and each polymerization contact site, with substantial enhancement by platelets. Also, cryoprecipitate-harvested soluble fibrinogen/fibrin complexes contained mostly normal molecules, while cryoprecipitate-depleted plasma contained mostly variant molecules. Moreover, significantly decreased MA by fibrinogen anomalies and/or low level thrombin generation can potentially impact clinical interpretation of MA.

Introduction

Thromboelastography, first described by Hartert in 1948 [1], is frequently used in clinical and research settings for assessment of whole blood or plasma coagulation status. For example, the number of publications in PubMed during 2010-2013 that mentioned TEG use averaged 136/year. Common TEG uses include assessment and decision making for fibrinolytic and anticoagulant therapy and for transfusion after acute blood loss [2]. The procedure involves an oscillating cup in which a suspended immobile pin transmits the oscillation effect electromechanically to produce a tuning fork-like signal. The TEG signal is fibrin dependent, its amplitude is enhanced by platelets, and uniquely reflects the stretch and recovery (shear modulus) of the clot during its formation. More specific fibrin determinants remain unknown and formed the subject of the present study. The final step of blood clot formation involves insoluble fibrin network formed by conversion of fibrinogen to fibrin [3]. Fibrinogen is a 340 kDa hexameric glycoprotein composed of 2 sets of 3 different chains Aα, Bβ, γ linked by disulphide bonds. Following limited proteolysis by thrombin, the resulting fibrin monomers self-assemble and are covalently cross-linked to strengthen the clot. Thrombin cleaves 2 pairs of fibrinopeptides, FpA and FpB,

Abbreviations: TEG, thromboelastography; MA, maximum amplitude; HPLC, high performance liquid chromatography; FpA, fibrinopeptide A; FpB, fibrinopeptide B; SDS-PAGE, sodium dodecyl sulphate polyactrylamide gel electrophoresis; SD, standard deviation.

* Corresponding author at: Blood Bank, University Hospital L5, Stony Brook, NY 11790. Tel.: +1 631 444 2625; fax: +1 631 444 3137.
E-mail address: dennis.galanakis@stonybrookmedicine.edu (D.K. Galanakis).
† Deceased.

http://dx.doi.org/10.1016/j.thromres.2014.03.026
0049-3848/

from the N-termini of fibrinogen polypeptide chains Aα and Bβ, respectively. This exposes pairs of "A" and "B" binding sites or knobs [4] in the central (or E) part of the molecule. These knobs interact with constitutive sites a and b, known as holes or pockets located in the D regions of each fibrinogen molecule. Knob A consists of two portions, one located at the amino terminal region of the Aα chain composing the amino-terminal α17-20 sequence GPRV [5,6], and the other within Bβ15-42 [7]. Pocket a encompasses γ337-379 of the D region γ module [8,9]. Knob B is also located in β15-42, and pocket b in β397-432 of the D region β module [10,11]. During fibrin self-assembly, a two molecule thick fibril (protofibril) forms by binding of two molecules to a third via D:E contacts mediated by knob:pocket interactions. The resulting D:E:D contact set forms a staggered alignment of monomers that continues by D:E:D repeats enabling elongation of the protofibril. Another part of this is the D:D interaction which additionally promotes elongation, and is mediated by the D interface that resides between γ275R and γ300S [9]. Following sufficient elongation soluble fibrils associate laterally and branch leading to fibers and three-dimensional polymers that coalesce to form the insoluble clot. The αC region originates as the Aα chain emerges from the D region at Aα220 and terminates at Aα610 [12]. This region enhances lateral polymerization. In fibrinogen two αCs interact with each other and with the central (E) region while in fibrin they switch to intermolecular interactions to form αC-linked polymers [13]. The elastic capacity of the clot resides in its fibers, which can stretch to 2.8x before sustaining permanent lengthening and 3.3x before breaking [14]. The clot's mechanical unraveling reflects the combined effects of unfolding of the two (C-terminal) γ modules, the reversible extension-contraction of the coiled coil connector [15], and the αC region [16]. This property, known as clot stiffness, and the clot's constitutive capacity to adhere to extravascular surfaces are among its pivotal hemostasis functions. Studies of naturally occurring fibrinogen variants corroborated other data that identified functional sites [17]. Impaired function of variant structures near or far from such sites (vide supra) intimated direct or allosteric effects [18].

Our investigations focused on three thromboelastography areas. One was the cup and pin (hydrophobic) surface, prompted by evidence that fibrinogen binds rapidly and tightly to hydrophobic and hydrophilic surfaces [19]. The second area was thrombin and fibrinogen dose response, in view of evidence the concentration of either can significantly influence the network structure and stiffness. For example, relatively low thrombin concentrations induce predominantly coarse and relatively high concentrations predominantly fine and more stiff networks [20]. The third focus area comprised the fibrin networks of available fibrinogen variants. The results demonstrate pronounced diminution of MA by each variant clot, by low thrombin and by low fibrinogen concentrations, and by >90% reduction in cup and pin surface hydrophobicity.

Materials and Methods

General

Reagents and supplies were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Water was de-ionized (DI) by ELIX 10 equipped with continuous UV irradiator (Millipore, Danvers, MA). Buffers used were either 10 mM Tris-HCL,pH 7.4, 150 mM NaCl (TBS), or 10 mM PO_4, pH 6.4, 150 mM NaCl (PBS). Human factor XIII was a gift from Dr. R. Chung. Afibrinogenemic and normal human plasma were obtained by plasmapheresis, stored at -80°, and thawed at 37° prior to use. The afibrinogenemic proband had a life-long hemorrhagic diathesis with fibrinogen undetectable by functional and by immunoassay (i.e. <10 mg/dl). Plasma from the other probands was from blood collected into standard citrate anticoagulant. Monoclonal IgG antibodies (mAbs) against Aα241-476, Aα518-584 were produced and stored as described [21]. Recombinant fibrinogen fragments, Aα221-391 (αC-connector) and Aα392-610 (αC-domain), were prepared, measured, and stored as described[22]. Plasma-free platelets were obtained by Sepharose 2B chromatography [23].

TEG

The thromboelastograph 5000 was employed according to manufacturer procedure (Haemonetics Corp., Braintree, MA). Its disposable cup and pin were manufactured from acrylic polymer (i.e. hydrophobic surface). The pin is a fixed probe linked to a torsion wire and suspended in the cup that oscillates 4.5° every 5 s. By adhering to both, the forming clot links cup and pin generating a tuning fork-like time course graph. The MA (maximum signal amplitude) reflects the capacity of forming clot to recover from the stretching and disruptions (shear modulus) caused by the oscillating cup, and it is regarded as a measure of clot stiffness. MA is fibrinogen concentration dependent [24], is increased ~2x by factor XIIIa-catalyzed fibrin crosslinking [25], and is also increased ~2x by platelets [24], the latter increase mediated by fibrin(ogen) binding to $\alpha_{IIb}\beta_3$ platelet integrin [26].

The cup and pin were exposed to ozone plasma to decrease their surface hydrophobicity, illustrated in Fig. 1panel I, a decrease that results from altered surface end groups [27]. The decrease was demonstrated by measuring the contact angle of a 5 μl water droplet at room temperature using the CAM200 Optical Contact Angle meter (KSV instruments LTD., Helsinki, Finland). For this purpose, the cup wall was cut and the interior surface of the cup bottom was used. The water droplet images were recorded and stored at a rate of 1 frame per 10 s, and 7 or more droplets were tested for each experiment. Values of contact angle were acquired by analyzing the images with the CAM200 software.

For some experiments clots were obtained in PBS, but for most they were obtained in TBS containing 8 mM $CaCl_2$ with or without 14% afibrinogenemic plasma. Fibrinogen and thrombin concentrations were varied as specified in the Results. For adsorption experiments, 360 μl of 0.5 μM fibrinogen in TBS containing 0.5 mM ethylenediaminetetraacetate was incubated in the cup and pin for 3 hours. After removing the solution and washing with excess buffer x3, adsorbed fibrinogen was measured by the color developed by adding the bicinchoninic acid reagent (Protein Assay kit, Pierce, Rockford, ILL) according to manufacturer instructions. Among normal and abnormal fibrinogen variants the amounts adsorbed were 83.5 ± 12.4(SD) pM/cm^2.

Fibrinogen Isolation

Immunoaffinity chromatography was employed using the monoclonal IF1 anti-fibrinogen IgG (Kamiya Biomedical Company, Seattle, WA). This was covalently linked to CNBr activated Sepharose (Sigma-Aldrich, St. Louis, MO) according to the latter manufacturer instructions. The gravity-packed 7×0.5 cm column displayed a maximum fibrinogen binding of 6.3 mg. After equilibration with 2 mM $CaCl_2$ in TBS, an aliquot of 3 ml citrated plasma was mixed with an equal volume of TBS containing 20 mM $CaCl_2$, to neutralize citrate, and applied. Unbound proteins were removed by gravity flow with ≥5 column volumes of buffer. Fibrinogen was eluted with 2 mM EDTA in TBS, and concentrated by precipitation with 25% saturated $(NH)_2SO_4$. The precipitate was dissolved in 0.3 M NaCl containing 20 nM phenylmethylsulfonylfluoride to neutralize possible protease contaminants, and after 30 or more minutes it was dialyzed in 0.3 M NaCl, and stored at -70°. Fibrinogen concentration was measured [28] using the extinction coefficient (280 nm, 1 cm, 1%) of 15.5. Purity and intactness of its subunit chains were ascertained after dialysis by electrophoresis in dodecyl sulfate polyacrylamide (SDS-PAGE) gels [32]. Some heterophenotypic proband plasmas formed minor precipitates on dilution with the TBS/$CaCl_2$ buffer, and these tended to markedly slow column flow. This was attributed to precipitation of soluble fibrin/fibrinogen complexes, was prevented by using cryoprecipitate-depleted plasma, and led to comparisons of fibrinogen isolated [30] from plasma cryoprecipitate [31] and from its plasma supernatant of 15 different probands. Maximum clot opacities