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MATERIALS AND METHODS

Factor XIII A-subunit V34L variant affects thrombus cross-linking rather than size in a murine model of thrombosis

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Materials

Human α-thrombin (Calbiochem; Nottingham, UK) was reconstituted to 250U/mL, tissue plasminogen activator (tPA; Pathway Diagnostics; Dorking, UK) and Glu-plasminogen (ERL; Swansea, UK) were diluted in 0.05M Tris-HCl, 0.1M NaCl, pH7.4 (Tris-buffered saline; TBS) to 14nM and 11μM respectively, and stored at -80°C. EZ-link pentylamine-biotin (Thermo Scientific; Loughborough, UK) was diluted in water to 30μM and stored at -20°C. Recombinant murine alpha$_2$-antiplasmin (rmα$_2$-AP; R&D systems; Abingdon, UK) was reconstituted to 100μg/ml in water, and stored at -20°C. Recombinant murine thrombin (rmthrombin; Haematologic Technologies Inc; Vermont, USA) was reconstituted to 250U/ml in water, and stored at -80°C. AlexaFluor488 fibrinogen (Invitrogen; Paisley, UK) was diluted in TBS to 5mg/mL and stored at -80°C. AlexaFluor680 A15 peptide (Cambridge Research Biochemicals; Billingham, UK) was diluted in TBS to 2.78mM. All other chemicals were obtained from Sigma (Gillingham, UK) unless stated otherwise.

Fibrinogen preparation

Human plasma fibrinogen, plasminogen depleted (Calbiochem; Nottingham, UK), was further purified by immunoaffinity chromatography (IF-1 mAb, 10mg; Kamiya Biomedical; Seattle, USA) as previously described[1]. This preparation has previously been shown to be free of FXIII[2].

Factor XIII variants generation

Variants were generated from human wild-type (WT) FXIII-A (pGEX-FXIII-A, expression vector that codes for a N-terminal Glutathione S-Transferase (GST)-FXIII-A fusion protein) as previously described[3]. Site-directed mutagenesis was performed using primers (Table I in the online-only Data Supplement) and the QuickChange II Kit (Agilent Technologies; Stockport, UK), to generate FXIII-A variants (Fig. 1D) located in the activation peptide (T28A, V29A, E30A, L31A, Q32A, G33A, V34A, V34L, V34M, V35A, P36A, R37A) and immediately upstream of the thrombin cleavage site (G38A, V39A, N40A, L41A). Successful mutagenesis was checked by DNA sequencing.

Factor XIII variants expression and purification

FXIII-A variants and WT constructs were transformed into BL21-Gold DE3 *E. coli* (Agilent Technologies; Stockport, UK). Expression, cell lysis and purification were performed as previously described[3]. Expression of human FXIII-A variants by *E. coli* was induced by addition of 1mM isopropyl-β-D-thigalactopyranoside to 2L cultures (terrific broth, phosphate buffer, and ampicillin) and incubation for 16hrs at 30°C in an orbital shaker. Cells were pelleted, washed, and stored as pellets at -20°C.

Harvested cells were resuspended in 88ml phosphate buffer and lysed for 30min at 25°C with 1mg/ml lysozyme and 1mM dithiothreitol (DTT), followed by a further incubation with 4mM benzamidine, 2μg/ml aprotinin, 1μM pepstatin-A and 10μM leupeptin for 30min at 4°C. The lysate was then incubated with 0.5mM phenylmethanesulphonylfluoride and 0.05% [v/v] sodium deoxycholate for 30min at 25°C, before being vigorously mixed with 0.02% [v/v] triton X-100. Following DNase treatment, the lysate was centrifuged at 22,000G for 20min at 4°C, and the supernatant was incubated with 10mg/ml streptomycin sulphate for a few seconds. The suspension was centrifuged at 22,000G for 20min at 4°C, and the supernatant was filtered using 0.22μm PES membrane filters. Purification of rhFXIII-A_2 was performed by GST-affinity chromatography using an AKTAprime purification system and a GSTrap FF column (GE Healthcare; Little Chalfont, UK). Purified rhFXIII-A variants and WT were eluted in phosphate-buffered saline containing 15% glycerol [w/v] (PBS-glycerol) following in-column cleavage of the GST tag using PreScission Protease at 2U/100μg protein. Successful cleavage of the GST tag was verified on NuPAGE Novex 4-12% Bis-Tris Protein Gel (Life Technologies; Paisley, UK). For measurement of the rate of FXIII activation by thrombin, no in-column cleavage of the GST tag was performed, GST-rhFXIII-A was eluted as a fusion protein by addition of 20mM glutathione in PBS-glycerol. Endotoxins were removed using Pierce High-Capacity Endotoxin Removal Resin (Thermo Scientific; Loughborough, UK), and the level of endotoxin was tested to be under 0.05EU/ml using Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific; Loughborough, UK)..

Rate of FXIII activation by thrombin

GST-rhFXIII-A_2 variants and WT (15μg) were incubated with thrombin (0.15U) in 30μl TBS with 2.5mM $CaCl_2$, at 37°C for 0, 1, 5, 10, 20, 40, and 60 minutes. Negative controls consisted of samples without thrombin. The reactions were stopped with 10μl of 4X NuPAGE LDS Sample Loading Buffer (Life Technologies; Paisley, UK), and 10μg of protein samples were immediately run on a NuPAGE Novex 4-12% Bis-Tris Protein Gel (Life Technologies; Paisley, UK) in reducing conditions. Band densitometry was performed for each time point using a Kodak Camera-Scanner Image Station 2000R with ID Image Analysis Software v3.6 (Kodak; Watford, UK), and rhFXIII-A_2 cleavage rate by thrombin was determined by measuring the intensity of the FXIIIa band for each time-point (Fig. 1C). Experiments were performed in triplicate.

Factor XIII activity assay

Measurement of Factor XIII activity was performed using a modified 5-(biotinamido)pentylamine incorporation assay[4]. Nunc-Immuno 96 MicroWell plates were coated with 100μl of 10μg/ml N,N-dimethylated casein overnight at 4°C, then blocked with 300μl 1% bovine serum albumin (BSA) in TBS for 90min at 37°C. Plates were then washed with 4x 300μl TBS and 10μl of samples (rhFXIII-A_2 or murine plasma) were added to the wells in triplicate. 90μl of activation mix (111μM DTT, 0.3μM biotinylated pentylamine, 11mM $CaCl_2$, 2.2U/ml thrombin) was added and the reactions were stopped at 0, 20, 40, 60, 80, 100, 120 min by adding 200μl of 200mM ethylenediaminetetraacetic acid (EDTA). Plates were washed with 4x 300μl 0.1% [v/v] Tween20 in TBS, and 100μl of 2μg/ml of streptavidin in 1% [w/v] BSA (in TBS-Tween) were added for 60min at 37°C. Following washes with 4x 300μl TBS-Tween, 100μl of 1mg/ml phosphatase substrate (in 1M diethanolamine) were added, and the

reaction was stopped by adding 100µl of 4M NaOH. Absorbency was measured at 405nm, using a SpectraMax 190 absorbance microtiterplate reader (Molecular Devices; Wokingham, UK). The rate of pentylamine incorporation over time was then used as an indicator of FXIII activity. Experiments were performed in triplicate.

Turbidity analysis of fibrin polymerization

Polymerization of fibrin was studied by turbidity analysis as previously described[1,5,6]. Fibrinogen (0.5mg/mL), $CaCl_2$ (5mM) and rhFXIII-A_2 (3.7µg/mL) were diluted in TBS and premixed in 96-well plates in triplicate. Thrombin (0.1U/mL final concentration) was added to initiate clotting, and absorbency was measured at 340nm, every 12s for 2hrs at room temperature, using a SpectraMax 190 absorbance microtiterplate reader (Molecular Devices; Wokingham, UK). Experiments were performed in triplicate.

Fibrinolysis analysis by turbidity

Fibrin clot lysis was studied using an adapted turbidity assay (above) in which tPA and plasminogen were included in the reaction mixture. Fibrinogen (0.5mg/mL), $CaCl_2$ (5mM), tPA (100pM), Glu-plasminogen (0.24µM) and rhFXIII-A_2 (3.7µg/mL) were diluted in TBS and premixed in 96-well plates in triplicate. Thrombin (0.1U/mL final concentration) was added to initiate clotting, and changes in absorbency were monitored at 340nm, every 12s for 2hrs at room temperature, using a SpectraMax 190 absorbance microplate reader (Molecular Devices; Wokingham, UK). Experiments were performed in triplicate.

Incorporation of α_2-AP into fibrin

Nunc-Immuno 96 MicroWell plates were coated with 110µl of 80µg/ml purified fibrinogen for 40min at room temperature, then blocked with 200µl 3% bovine serum albumin (BSA) in TBS for 60min at 37°C. Plates were then washed with 3x 300µl TBS and 10µl of samples (22µg/ml rhFXIII-A_2) or TBS were added to the wells in triplicate. 90µl of activation mix (111µM DTT, 11µg/ml rmα_2-AP, 5.5mM $CaCl_2$, 1.1U/ml rmthrombin) was added and the reactions were stopped at 0, 5, 10, 30, 60, 120min by adding 200µl of 200mM EDTA. Plates were washed with 3x 300µl 0.05% [v/v] Tween20 in high salt TBS (1M NaCl), 3x 300µl high salt TBS, 3x 300µl TBS, and 100µl of rabbit anti-mAP antibody (1/1000; Molecular Innovations; Newmarket, UK) were added and incubated for 60min at room temperature. Following washes with 3x 300µl TBS, 100µl of HRP-conjugated goat anti-rIgG (1/2000; Dako; Ely, UK) were added and incubated for 60min at room temperature. Wells were washed with 3x 300µl TBS before adding 100µl of 0.7mg/ml *o*-phenylenediamine dihydrochloride (Sigma) containing 0.01% H_2O_2. The reactions were stopped by adding 50µl of 0.5M H_2SO_4. Absorbency was measured at 490nm, using a SpectraMax 190 absorbance microtiterplate reader (Molecular Devices; Wokingham, UK). Experiments were performed in triplicate.

Analysis of rates of α- and γ-chain cross-link formation

Rates of α- and γ-chain cross-link formation were studied by SDS-PAGE as previously described[7]. Fibrinogen (2mg/mL), $CaCl_2$ (10mM), rhFXIII-A_2 (15µ/mL) and thrombin (0.5U/mL) were mixed into a final volume of 20µl and incubated at 37°c. The reaction was stopped by adding equal volume of a mixture of 10X Sample Reducing Buffer and 4X NuPAGE LDS Sample Loading Buffer (Life Technologies),

and boiling at 95℃ for 10 minutes. Samples were immediately run on a NuPAGE Novex 4-12% Bis-Tris Protein Gel (Life Technologies) in reducing conditions. Band densitometry was performed for each time point (α-, β-chains, and γ-γ crosslinked chains) using a Kodak Camera-Scanner Image Station 2000R with ID Image Analysis Software v3.6 (Kodak; Watford, UK). Relative band intensity was determined for each variant at each timepoint, by dividing the α-chains or γ-γ cross-linked chains intensities by the β-chains intensity. The relative amount α-α cross-linked chains was determined for each timepoint by substracting the relative α-chains intensity from the α-chains intensity at 2min.

In-vivo thrombosis formation and visualisation by intra-vital microscopy

In-vivo visualisation of clot formation is described separately (Ali *et al*, submitted). All procedures were approved by the University of Sheffield Ethics Committee, and performed under the Home Office Animals (Scientific Procedures) Act 1986.

Male WT (CBA/129) mice and CBA/129 lacking FXIII-A ($FXIII^{-/-}$, obtained from G. Dickneite[8]) were anaesthetised by intraperitoneal injection of ketamine/atropine/xylazine. A carotid artery was cannulated to allow for administration of anaesthetics and injection of 100μg AlexaFluor488 fibrinogen, 20nmol AlexaFluor680 A15 peptide (based on the N-terminal sequence of α_2-antiplasmin; GNQEQVSPLTLLKWC[9,10]), and 54μg rhFXIII-A_2 or saline, for 5min prior to exposing the femoral vein and applying a 10% [v/v] $FeCl_3$ saturated filter paper for 3min. Real-time observation of clot formation was started 2min after removal of the $FeCl_3$ filter paper, using an upright Nikon Eclipse E600-FN microscope (Nikon; Kingston-upon-Thames, UK), equipped for fluorescence microscopy with a water-immersion 40/0.80W objective. Both 488nm (green) and 680nm (red) channels were simultaneously recorded using Slidebook Imaging Software v5.0 (Intelligent Imaging Innovation; Denver, USA). Clot size was determined as a combination of area and intensity of green pixels; and A15 incorporation within the clot, was measured by calculating Pearson's correlation coefficient for co-localisation of red and green pixels, for each time-point. Experiments were repeated 5 times.

Statistical analysis

All data are presented as mean±SEM, and all statistical analyses (t-test for *in-vitro* analysis, Kruskall-Wallis and Mann-Whitney test for *in-vivo* analysis) were performed using GraphPad. P-values less than 0.05 were considered to indicate statistical significance.

REFERENCES

1. Duval C, Allan P, Connell SD, Ridger VC, Philippou H, Ariens RA. Roles of fibrin alpha- and gamma-chain specific cross-linking by FXIIIa in fibrin structure and function. *Thromb Haemost*. 2014;111:842-50.
2. Hethershaw EL, Cilia La Corte AL, Duval C, Ali M, Grant PJ, Ariens RA, Philippou H. The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis. J Thromb Haemost 2014;12:197-205.
3. Smith KA, Adamson PJ, Pease RJ, Brown JM, Balmforth AJ, Cordell PA, Ariens RA, Philippou H, Grant PJ. Interactions between factor XIII and the alphaC region of fibrinogen. *Blood*. 2011;117:3460-8.

4. Kohler HP, Ariens RA, Whitaker P, Grant PJ. A common coding polymorphism in the FXIII A-subunit gene (FXIIIVal34Leu) affects cross-linking activity. *Thromb Haemost*. 1998;80:704.
5. Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys J*. 1992;63:111-28.
6. Allan P, Uitte de WS, Abou-Saleh RH, Connell SD, Ariens RA. Evidence that fibrinogen gamma' directly interferes with protofibril growth: implications for fibrin structure and clot stiffness. *J Thromb Haemost*. 2012;10:1072-80.
7. Ariens RA, Philippou H, Nagaswami C, Weisel JW, Lane DA, Grant PJ. The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. *Blood*. 2000;96:988-95.
8. Lauer P, Metzner HJ, Zettlmeissl G, Li M, Smith AG, Lathe R, Dickneite G.Targeted inactivation of the mouse locus encoding coagulation factor XIII-A: hemostatic abnormalities in mutant mice and characterization of the coagulation deficit. *Thromb Haemost*. 2002;88:967-74.
9. Ichinose A, Tamaki T, Aoki N. Factor XIII-mediated cross-linking of NH2-terminal peptide of alpha 2-plasmin inhibitor to fibrin. *FEBS Lett*. 1983;153:369-71.
10. Jaffer FA, Tung CH, Wykrzykowska JJ, Ho NH, Houng AK, Reed GL, Weissleder R. Molecular imaging of factor XIIIa activity in thrombosis using a novel, near-infrared fluorescent contrast agent that covalently links to thrombi. *Circulation*. 2004;110:170-6.