Deletion of five residues from the coiled coil of fibrinogen (Bβ Asn167_Glu171del) associated with bleeding and hypodysfibrinogenemia

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ABSTRACT

Routine pre-surgical coagulation investigations led to the detection of a novel type of hypodysfibrinogenemia whose functional defect appears to result from an alteration in the spacing between the functional domains of the fibrinogen molecule. The detection, by reverse phase HPLC, of a minor isoform of Bβ chain with a 554 Da decrease in mass led to the identification of a deletion of five amino acids (NVVNE) from the center of the coiled coil. The variant chain contributed only 10% of the total Bβ material and the mutation (BβAsn167_Glu171del) was associated with both increased clotting times and low functional and physical fibrinogen concentrations in 3 family members. There was a significant history of pregnancy-associated bleeding and miscarriage within the first trimester. Mechanistically the 15-nucleotide deletion appears to arise from replication advancement during DNA synthesis caused by a flanking pentanucleotide repeat of AATGA.

Key words: fibrinogen, amino acid deletion, hypodysfibrinogenemia, protein expression, replication slippage.

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Introduction

The fibrinogen molecule is a symmetrical dimer with each half composed of three polypeptide chains (Aα, Bβ and γ) linked by a network of disulfide bonds to form the circulating 340 kDa protein.[1,2] The molecule has a linear trinodal D-E-D structure with a central E domain joined to outer D domains by a coiled coil of all three chains. Upon thrombin activation, the central E domain of one fibrin monomer binds to the outer D domains of two adjacent molecules forming a protofibril of half staggered subunits.

As well as being the focal point of the coagulation cascade, fibrinogen participates in other important processes such as wound healing and the maintenance of pregnancy. Individuals with very low fibrinogen levels encounter umbilical cord bleeding, spontaneous abortions, central nervous system bleeding and soft tissue hemorrhage.[3] Low levels of fibrinogen are associated with recurrent abortions, usually in the 6-8th week of pregnancy. For example, six consecutive early abortions were associated with fibrinogen Keokuk (Aα truncation at residue 328), and the woman with fibrinogen Otago (Aα truncation at residue 271) suffered four first trimester miscarriages.[4,5] As in these 2 cases, the presence of some dysfunctional fibrinogen molecules can further exacerbate bleeding[5] and contribute to thrombotic complications,[4] or lead to additional pathologies such as renal amyloidosis.[6] Mutations causing dysfibrinogenemia are usually clustered to functionally important regions such as the thrombin cleavage site in the E domain or the GPR-binding site in the γD domain. However remote mutations that cause structural deformations could alter function by disrupting the precise domain alignments needed for effective intermolecular interaction. Here we identify a novel deletion in the fibrinogen β gene that is associated with miscarriage and post-partum hemorrhage. The deletion of five amino acids from the center of the coiled coil results in impaired fibrin polymerization and enhanced clot lysis.

Design and Methods

Protein purification and analysis

Fibrinogen was purified by ammonium sulphate precipitation[7] and individual chains were isolated on a (25x0.46 cm) C-4 column using a 0.05% TFA/acetonitrile gradient system.[8]

Electrospray ionization mass spectrometry was performed as previously outlined.[9] Peak crests (20 μL) from reverse phase purified chains were directly injected into the ion

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source at a flow rate of 10 μL/min. The probe was charged at + 3500 V and the source maintained at 60°C. The mass range 800-1600 m/z was scanned every 2s with a cone voltage ramp of 30-60 V and the raw data was transformed using Max-Ent software.[9] Individual fibrinogen chains were digested with trypsin and m/z mapped as previously described.[8]

DNA analysis

The entire coding and flanking intronic sequences of all three fibrinogen genes were amplified[10,11] and sequencing was carried out on an ABI 3130xl genetic analyzer using BigDye Terminator v. 3.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA). Data was analyzed using SeqScape v. 2.5 software.

Functional studies

Thrombin catalyzed polymerization kinetics were determined in triplicate as previously described.[12] Fibrinolysis curves were generated by overlaying the resulting clots with 10 nM tissue plasminogen activator and 0.05 mg/mL Glu-plasminogen.[12]

Also, purified fibrinogen (100 μg) was incubated with 1 mU of porcine plasmin and the digestion profile analyzed on 7.5% reducing SDS PAGE. Bands corresponding to the Y and D1 fragments were quantified by densitometry.

Results and Discussion

The proband, a 20-year-old male, had a coagulation screen prior to wisdom teeth extraction. This showed normal liver function, platelet levels and bleeding time (6.5 min). However, APTT and INR were slightly raised at 41s and 1.3 respectively. Functional fibrinogen (Clauss) was low at 0.6 mg/mL (normal 1.5-4.0) and further testing showed prolonged thrombin and reptilase times of 24 sec each (normal 10-18 and 16-23 respectively). He reported good health with no bleeding or clotting problems, apart from a period of recurrent epistaxis which ceased following cauterization. He had a number of surgical interventions, including tonsillectomy, appendectomy and an eye operation without bleeding complications. He was given cryoprecipitate prior to the tooth extraction, which proceeded uneventfully. His parents are originally from the South of England and there is no history of bleeding problems in his siblings or his father.

His mother however, has had bleeding problems with all her pregnancies, both during pregnancy and following delivery, but never required blood transfusions. All the children were born in the UK and she does not recall any investigation into a *bleeding problem*. She has had two miscarriages, both in the first trimester. In addition she has had a history of menorrhagia and has always bled for a prolonged time from venipuncture sites. Surgeries however (including tonsillectomy and teeth extractions) were not associated with excess bleeding. The 17-year old sister has had a similar history of menorrhagia and the maternal grandmother (who was unavailable for investigation) has a history of miscarriage and problems with post-partum hemorrhage, which necessitated blood transfusion on at least one occasion. There was no family history of thrombosis and current family data is shown in Table 1.

Purified fibrinogen from all 5 family members that were available showed a normal pattern of Aα, Bβ and γ chains on reducing SDS PAGE, while non-reducing gels gave the expected pattern of 340 and 305 kDa bands. Reverse phase assays indicated normal fibrinopeptide release.

Reverse phase separation of reduced fibrinogen chains from the propositus, however, consistently showed an additional minor peak eluting just ahead of the normal $B\beta^{A}$ chain (Figure 1). The slight reduction in area of the $B\beta^{A}$ peak ($B\beta^{A}$ to γ ratio 1.03:1) compared to the control ($B\beta^{A}$ to g ratio 1.12:1) suggested the new peak ($B\beta^{X}$) might be an aberrant form of Bβ chain expressed at a lower than normal level. This was supported by electrospray mass analysis of the peaks. The monosialylated isoform of the γ chain appeared normal, with a mass of 48,358 Da compared to 48,359 Da for control γ chains. However, while the $B\beta^{A}$ peak from the proband (54,182 Da) had a comparable mass to the control (54,198 Da), the early eluting $B\beta^{X}$ peak was only 53,629 Da. This mass decrease of 553 Da was confirmed in a second HPLC separation, where a decrease of 554 Da was recorded. Tryptic peptide maps comparing the $B\beta^{X}$ and $B\beta^{A}$ peaks were uninformative, but did confirm that the new peak was derived from Bβ chain material.

Subsequent DNA sequencing of coding regions and the intron/exon boundaries of the fibrinogen β gene (*FGB*) indicated heterozygosity for a novel 15 nucleotide deletion (NM_005141.2:c.498_512del) in exon four (Figure 2A). The deletion segregated with low fibrinogen in 3 family members and was absent from the 2 normal individuals (Table 1).

No other mutations were detected in the coding regions of either *FGA* or *FGG*. The 15 nucleotide deletion (Chr4(NCBI36):g.155708202_155708216del) results in the loss of the five amino acids, NVVNE, that span positions 137-141 of the mature Bβ chain (Figure 2). The predicted mass decrease of 555 Da associated with this mutation was in excellent agreement with the measured decrease and this new variant has been named fibrinogen Epsom. The prolonged clotting times (43-47s) of all 3 carriers, together with the discrepancy between their functional and physical fibrinogen levels (Table 1), suggested the deletion might impact on func-

Table 1. Family fibrinogen levels and mutation status.

	Age	Fibrinogen (mg/mL) Functional	Fibrinogen (mg/mL) Physical	TCT (sec)	Δ NVVNE
Father	47	3.3	2.9	21	–
Mother	44	0.6	1.4	43	+
Proband	20	0.7	1.5	43	+
Brother	19	2.8	3.2	21	–
Sister	17	0.7	2.0	47	+
Normal		1.5-4.0	1.5-4.0	18-24	

Functional fibrinogen was measured using a modified Clauss method and physical levels by quantitation of fibrinopeptide B.[7]

tion. This was supported by studies on purified fibrinogen, which showed abnormalities in all kinetic parameters (*data not shown*). The patient curves displayed a lag time to protofibril formation of 132s (control 69s), a V_{max} of 0.0105 OD/s (control 0.0132 OD/s) and a final OD of 0.2 (control 0.35). After the clot had formed fibrinolysis was examined by overlaying the clot with plasminogen/tPA and measuring the decrease in optical density. Comparison of patient clot lysis to 2 normal controls revealed an enhanced lysis rate 1.3 times faster. Similarly, plasmin catalyzed lysis of native fibrinogen was also found to have a significant 1.25 fold increase in susceptibility to proteolysis.

Mechanistically the Epsom mutation most likely arises from replication slippage during DNA synthesis, where the priming strand partly detaches from the template strand, loops out and reanneals to a common sequence later in the template.[14] In this case the deletion is flanked by direct AATGA repeats which would facilitate the reannealing process (Figure 2A).

At a protein level the five residue deletion would be expected to shorten the Bβ coil of the coiled coil by ~1.5 turns of helix, or 7.5 Å. This would tend to tension and deform the super coil shortening the distance between the E and D domains. On coagulation this would perturb the close packing of fibrin monomers and be reflected in aberrant polymerization and a phenotype of dysfibrinogenemia. The coagulation data (Table 1) confirm this and indicate both a functional and quantitative defect. While the low plasma expression of the variant chain ($\beta^X/\beta^A + \beta^X$; 0.1:1.0) was confirmed by reverse phase HPLC it remains unresolved as to whether the hypofibrinogenemia results from accelerated intracellular degradation of misfolded protein or increased plasma turnover, or a combination of both. The deletion does occur close to the plasmin sensitive site in the coiled coil and we have shown that molecules containing variant chains have an increased susceptibility to plasmin cleavage. This may be reflective of a general increase in susceptibility to proteolytic activity and increased circulatory turnover. As 75% of newly assembled molecules would contain at least

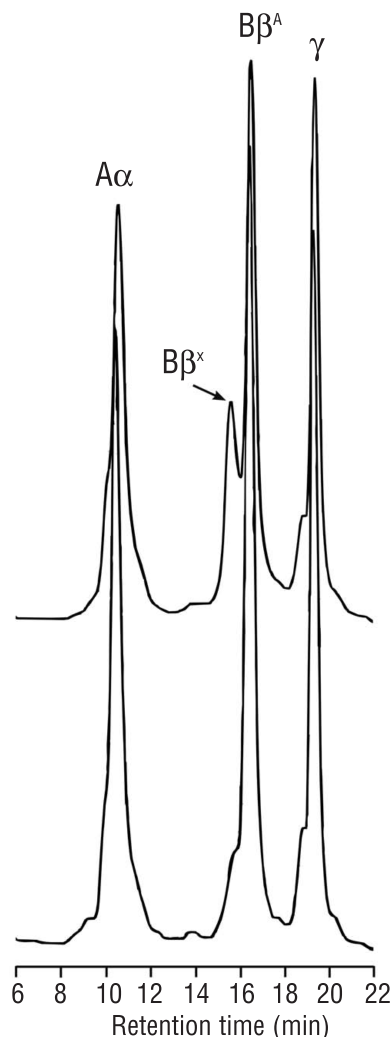

Figure 1. Reverse phase HPLC profile (215 nm) of purified fibrinogen from control (lower trace) and from individual with fibrinogen Epsom (upper trace). In addition to the normal Bβ chain, a minor more hydrophilic chain, $B\beta^X$, was detected in carriers of the Epsom mutation.

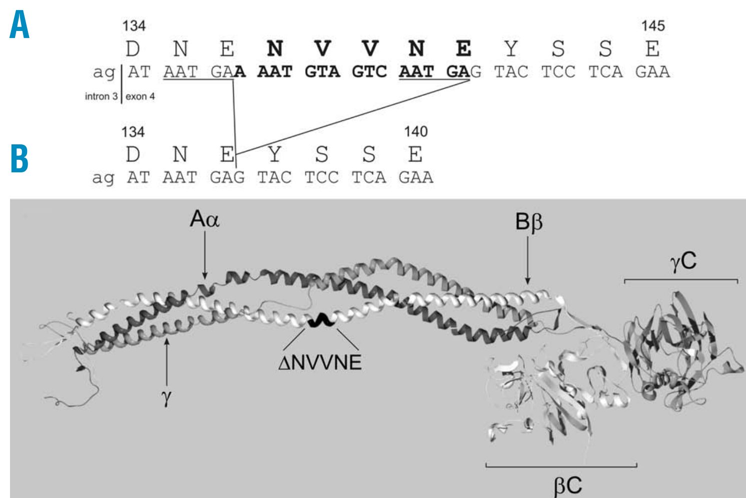

Figure 2. (A) DNA and protein sequence of relevant region of the fibrinogen Bβ chain showing the normal (upper) and new sequence in fibrinogen Epsom (lower). The deletion is flanked by an AATGA repeat (underlined), which could facilitate replication slippage and deletion of the intervening 15 nucleotides. (B) Molecular model showing the coiled coil and outer D domain, with the unresolved E domain on left. Black shading indicates the location of the NVVNE deletion that removes 1.5 turns of helix resulting in instability and altered fibrin polymerization. Created from PDB co-ordinate file 1M1J (13) using spdb viewer v.3.7 and POV-Ray v.3.6 software.

one variant Bβ chain, a destabilizing event that decreases the expression level of $B\beta^{X}$ to 10% of the total would have a significant impact on plasma fibrinogen concentration and result in an overall phenotype of hypodysfibrinogenemia.

Low fibrinogen is a recognised cause of spontaneous abortion within the first trimester and in knock-out mice fibrinogen has been shown to facilitate placental-maternal attachment during embryonic development.[15] Also, during pregnancy fibrinogen levels become elevated and there is a shift to less highly sialylated oligosaccharide side chains; both of these adaptations protect against bleeding, because the lower sialic acid content decreases the clotting time.[16] If the Epsom deletion favored increased sialylation then its potential effect on bleeding might be more apparent during pregnancy. As one of the oligosaccharide side chains is attached to the coiled coil (γ52 Asn) and one to the βD domain it is possible the mutation could have an increased effect on bleeding during pregnancy. The bleeding diathesis associated with fibrinogen Epsom can be explained by the cumulative effects of decreased fibrinogen concentration and altered function. These together with the increased rate of fibrinolysis would all be expected to contribute to a bleeding tendency. However, they are probably not the sole cause of bleeding; mutation or genetic variation in other (unidentified) coagulation genes will undoubtedly contribute to the overall phenotype.

Authorship and Disclosures

SOB performed the protein structural analysis, wrote the draft manuscript and approved the final version. RLD performed the protein functional analysis, prepared the figures, and helped write the manuscript. RL performed the DNA sequencing and assisted in other experiments. AR identified the initial hematologic problem, obtained the family histories and blood samples, and helped write the manuscript.

The authors reported no potential conflicts of interest.

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