Severe hypodysfibrinogenemia in compound heterozygotes of the fibrinogen AαIVS4+1 G>T mutation and an AαGln328 truncation (Fibrinogen Keokuk)

Short Title: Fibrinogen Keokuk

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Abstract

Two hypofibrinogenemic siblings have lifelong trauma-related bleeding. Recently, the brother experienced recurrent thrombosis after cryoprecipitate infusions post-surgery; the sister has suffered 6 miscarriages. Their plasma clots were resistant to compression and fibrinolysis, and were soluble in 5 M urea. Examination by SDS-PAGE revealed only the presence of crosslinked $\gamma-\gamma$ fibrin chain dimers without high polymers of $\alpha_n$. Fibrin clots contained an abnormal 35 kDa constituent recognized by an antibody to the mature fibrinogen $A\alpha$-chain residues 241-476 but not by antibodies to $A\alpha291-348$ or $A\alpha349-406$. DNA analysis revealed a heterozygous CAA$\rightarrow$TAA mutation at the codon for amino acid 328 of the $A\alpha$ gene in these siblings and 2 asymptomatic family members. The Gln328stop mutation (Fibrinogen Keokuk) predicted a 46% truncation and the production of a 35 kDa $A\alpha$ chain. Analysis of purified fibrinogen revealed expression of the abnormal $A\alpha$ chain in 4 family members, but found no normal fibrinogen in the 2 hypofibrinogenemic patients. This paradox was resolved when they, and their asymptomatic mother, were found to be heterozygous for a second $A\alpha$ mutation, a GT$\rightarrow$TT splice site mutation in intron 4 (IVS4+1 G$\rightarrow$T). However, compound heterozygosity for both mutations was required for the expression of severe hypodysfibrinogenemia and clinical symptoms.
Introduction

Fibrinogen is an approximately 340 kDa disulfide-bonded dimer of 3 different protein chains. The Aα, Bβ, and γ chains are encoded by 3 separate genes within a 50-kb region of chromosome 4q28-q31 (reviewed in Nieuwenhuizen et al.\(^1\)). The glycoprotein is synthesized in hepatocytes and secreted to yield a normal steady-state plasma concentration of 1.5 to 3.5 mg/mL with a half-life of about 4 days.\(^2\) As the precursor of the clot forming protein fibrin and as a mediator of platelet aggregation, fibrinogen is a key component of the hemostatic system.\(^1\) As evidenced by the high rate of spontaneous abortions in hypofibrinogenemic women,\(^3\) it is also important for the successful conclusion of pregnancy.

Fibrinogen abnormalities can be classified according to whether there is low or no circulating levels of normal protein (hypo- or afibrinogenemia), a mutated species (dysfibrinogenemia) or a combination (hypodysfibrinogenemia). Reports\(^1,4\) on approximately 350 families with dysfibrinogenemia reveal that about half of the cases are clinically silent, a quarter of them have a tendency towards bleeding and another quarter show predisposition for thrombosis with or without bleeding. Mutations have been found on all the fibrinogen chain genes, but those of the Aα chain have been most prevalent,\(^5,6\) particularly a mutation of the invariant 5’ splice site of intron 4 from GT to TT (IVS4+1 G>T), which results in afibrinogenemia in the homozygous state.\(^7\) Several nonsense mutations leading to a truncated fibrinogen Aα-chain have been reported.\(^7-14\) Clinical complications can vary widely, from asymptomatic to afibrinogenemic, depending on whether the patient is heterozygous or homozygous, as well as both the extent of the truncation and any confounding mutations.

In this study we describe a novel fibrinogen mutation – designated Fibrinogen Keokuk – that truncates the Aα-chain at Gln328 of the mature protein. Four family members were shown to
be heterozygous for this mutation, but only 2 of them presented with hemostatic complications. These individuals were found to be compound heterozygotes for the truncation as well as the IVS4+1 G>T mutation, resulting in hypodysfibrinogenemia.

Patients and methods

Patients

Three generations of a non-consanguineous American family of European descent were studied: the mother (referred to as I.2), her 3 children (II.1, II.2, II.3), and 2 grandchildren (III.1 and III.2) from her third child. II.1 and II.2 have no offspring, though II.1 had 6 miscarriages. II.1’s husband, and II.3’s wife, the mother of III.1 and III.2, were not studied. The father (I.1), deceased of lung cancer, was not known to have any hemostatic disorders. Only II.1 and II.2 had hypofibrinogenemia and problems with bleeding and thrombosis. Informed consent was obtained from all participants.

Sample collection and preparation

Blood for analysis of hemostasis was collected into tubes containing 1/10 volume of 0.105 M trisodium citrate anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Platelet-rich plasma was obtained by centrifugation (100g x 10 minutes, room temperature (RT)). The remaining blood was centrifuged (1000g x 10 minutes, 4°C) to obtain platelet-poor plasma (PPP), aliquoted, and stored at -80°C. Blood for DNA analysis was collected into tubes containing 1/90 volume 15% K$_2$EDTA. Unfractionated samples were transported at RT for up to 3 days prior to analysis. Genomic DNA was isolated from whole blood as previously described.$^{15}$
**Hemostasis functional analysis**

Total fibrinogen concentration was measured using the Laurell immunoelectrophoresis method.\(^{16}\) As the Laurell method had a sensitivity level of only 0.45 mg/mL, fibrinogen levels for II.2, II.3, and III.2 were also measured by fibrinopeptide A release as previously described.\(^{17}\)

Platelet count was performed with a Thrombocounter C (Beckman Coulter, Brea, CA). Platelet aggregation was studied using a PAP-4 (Bio-Data Corp., Horsham, PA) by adding 5 µL of 2 µM calcium ionophore U-46619 (Sigma, St. Louis, MO) to 250 µL of platelet-rich plasma and stirring (1000 rpm, 4 minutes, 37°C).

ELISA kits were used for measuring t-PA and PAI-1 levels (BioPool, Burlington, ON, Canada), and anticardiolipin antibodies (TheraTest Laboratories, Chicago, IL). Analysis for anti-fibrinogen and anti-factor XIII antibodies was carried out by ELISA as previously described,\(^{18}\) using purified fibrinogen or FXIII as antigens.

Factor XIII activity was assessed by examining clot solubility in 5 M urea.\(^{19,20}\) Activated partial thromboplastin time (aPTT), prothrombin time (PT), mixing tests, and assays for factors VIII, IX, and XI, and clottable fibrinogen (using a modified Clauss method\(^{21,22}\)) were performed using an MLA Electra 800 instrument and antithrombin levels were measured using an MLA Electra 900 instrument (both from Medical Laboratory Automation, Inc., Pleasantville, NY). Thrombin time (TT) and plasminogen activity were determined with the respective STA kits, while Lupus anticoagulant and functional Protein C and Protein S levels were measured by the respective STACLOT procedures, all performed on an ST-4 instrument (Diagnostica Stago, Asnieres-Sûr-Seine, France). Whole blood clotting time was determined by observing 1.0 mL of whole blood in a glass clotting tube, incubated at 37°C for 2 minutes, then tilted every 15 seconds until the tube could be inverted without significant movement of the blood in the tube.
Fibrinogen purification

Three mL of PPP was dialyzed against 10 mM sodium phosphate, pH 6.5, 1 mM EDTA and subjected to anion exchange chromatography on a DEAE-Sepharose column equilibrated with the same buffer. After loading, the column was washed with 10 mM sodium phosphate, pH 6.0, 1 mM EDTA until the absorbance at 280 nm returned to baseline. Remaining plasma proteins, including fibrinogen, were eluted with 10 mM sodium phosphate, pH 6.0, 1 mM EDTA, 200 mM NaCl, and fractions corresponding to these proteins were pooled. Fibrinogen was purified from this pool by precipitation with 35% saturated ammonium sulfate. After washing the pellet twice with 40% saturated ammonium sulfate it was resuspended in 20 mM HEPES, pH 7.4, 150 mM NaCl and dialyzed against the same buffer. Normal fibrinogen was purified from 3 mL plasma using the same method or by precipitation with 22% saturated ammonium sulfate. The pellet was washed twice with 25% saturated ammonium sulfate, resuspended in the HEPES buffer and dialyzed.

Fibrin clot function and structure

TPA-induced fibrinolysis for II.1 and II.2 was determined in duplicate by release of $^{125}$I-fibrinogen (196 μCi/mg, Amersham Pharmacia Biotech, Arlington Heights, IL) from different thrombin (kindly provided by J.W. Fenton, New York State Dept. of Health, Albany, NY)-induced PPP clots over several time points as previously described. Percent-lysed fibrinogen was calculated as $\left[\frac{\text{supernatant cpm} - \text{background cpm}}{\text{total cpm} - \text{background cpm}}\right] \times 100$ and plotted on a histogram versus time incubated at 37°C. The time to achieve 50% lysis was derived from the curves fitted to those points.
Plasma clot compaction was determined in triplicate by mixing 60 µL of PPP with 2.5 mM CaCl₂ and 5 U/mL bovine thrombin (Instrumentation Laboratory, Lexinton, MA) and immediately injecting the solution into heparinized micro-hematocrit capillary tubes (Curtin Matheson Scientific, Inc., Houston, TX) which were then sealed with Seal-ease® tube sealing compound (Clay Adams, Parsippany, NJ). A tiny air bubble was left between the plug and plasma to assist the clot in disengaging from the glass to ensure more uniform compaction. The plasma was allowed to clot for 2 to 3 minutes at RT, then centrifuged (12,600g x 3 minutes, RT) in a TRIAC Centrifuge (Clay Adams). The length of the compacted clot was measured on a Leitz DM IL inverted microscope (Leica, Wetzlar, Germany) using an ocular ruler. To test normal fibrinogen clot compaction at a concentration similar to the proband’s, clots were made from 6 µL of normal PPP ([fibrinogen] = 3.0 mg/mL) mixed with 54 µL of fibrinogen-depleted plasma, obtained from the supernatant of PPP heated to 56°C for 5 minutes, then centrifuged at 1,500g for 15 minutes.

Thrombin-catalyzed fibrin polymerization was initiated by addition of 20 µL of 1 U/mL thrombin (Thrombostat, Parke-Davis, Morris Plains, NJ) to purified fibrinogen (0.15 mg/mL in 180 µL 20 mM HEPES, pH 7.4, 150 mM NaCl) and absorbance at 350 nm was monitored for 50 minutes at RT. Triplicate measurements were made on fibrinogen purified from control and patient II.2 plasma.

Fibrin crosslinking was carried out with 100 µL or 200 µL (for hypofibrinogenemic samples) PPP brought to a total volume of 500 µL with buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10,000 IU/mL Trasylol, 12.5 U/mL bovine thrombin, and either 1 mM EDTA or 10 mM CaCl₂). Following incubation (60 minutes, 37°C), 0.5 mL of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA was added and each clot centrifuged (20,000g x 10 minutes, 4°C). The
pellets were washed twice with 1 mL of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, centrifuged and solubilized in 150 µL or 75 µL (for hypofibrinogenemic samples) of 50 mM Tris-HCl, pH 6.8, 9 M urea, 2% SDS, 40 mM dithiothreitol for 30 minutes at 37°C prior to SDS-PAGE.\textsuperscript{25,26} 6 µL or 8 µL (hypofibrinogenemic samples) of the solubilized clots were applied for electrophoresis on 10% polyacrylamide gels. Gels were stained with Coomassie brilliant blue R, destained, dried and scanned.

Electrophoretic and immunological analysis of clots and purified fibrinogen preparations

Plasma was diluted with MilliQ water and matched for similar fibrinogen loading on gels (~0.02 µg). Samples were electrophoresed on a 7.5% reducing SDS-PAGE gel, electroblotted onto nitrocellulose, blocked with casein blocking solution (5% (w/v) low-fat milk powder in Tris-buffered saline w/ Tween-20), and incubated for 1 hour at RT with rabbit polyclonal antibody to human fibrinogen (immunoglobulin fraction, 1:1,000 dilution in casein blocking solution; Dako, Glostrup, Denmark). Antibody staining was visualized with an ECL Western blotting analysis system (Amersham Pharmacia Biotech) using a secondary antibody specific to rabbit IgG (1:10,000 dilution) and X-Omat AR film (Kodak, Rochester, NY).

Solubilized clots (EDTA samples only) were matched for similar protein loading (3-8 µL/well) electrophoresed and electroblotted onto nitrocellulose,\textsuperscript{27} blocked with Blotto (2% non-fat dry milk in PBS) and incubated overnight at RT with antibodies to either whole human fibrinogen (rabbit immunoglobulin fraction, 1:20,000 dilution in Blotto; Dako, Carpenteria, CA) or its Aα241-476 (rabbit antiserum HS2-6, 1:100,000 dilution in Blotto; kindly provided by J. Sobel, Columbia University, New York, NY), Aα291-348 or Aα349-406 segments (mouse monoclonal hybridoma
supernatants from Dr. G. Samokhin, Northwestern University, Chicago, IL). Antibody staining was visualized with an ECL Western blotting system (Amersham Pharmacia Biotech) using secondary antibodies specific to rabbit or mouse IgG (1:10,000 dilution) and Biomax ML film (Kodak, Rochester, NY).

**DNA sequence analysis**

Exon 5 of the fibrinogen Aα-chain gene was amplified by 37 cycles of PCR using the following primers: AαVF corresponding to nucleotides 3715-3732, (5’CAGGAACTCAATAGACGT 3’) and AαVR primer (5’GACCAGTTTTTCTGTGTGGTACTC 3’) corresponding to the complementary nucleotides 4577-4554. 28 The PCR product was run on a 1% agarose gel and the band with the expected size was purified with the Gel Extraction Kit (QIAGEN, Santa Clarita, CA). The purified products were sequenced using both AαVF and AαVR primers (GlaxoWellcome DNA sequence facility, UNC at Chapel Hill, NC).

All exons and intron-exon boundaries of the fibrinogen genes were amplified from genomic DNA using standard PCR protocols. PCR products were purified in MultiScreen96 PCR plates (Millipore, Billerica, MA), and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and a 3100-Avant capillary DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primers were designed from the published FGA (Genbank M64982), FGB (Genbank M64983) and FGG (Genbank M10014) sequences.

**Results**

**Case Histories**
Two Caucasian siblings, a 53-year-old female (II.1) and a 49-year-old male (II.2) (arrow in Figure 1) have a life-long history of prolonged bleeding following trauma. In addition, II.1 had six miscarriages. Both were previously diagnosed with hypofibrinogenemia and were reported to be negative for Factor V Leiden. Their parents (I.1 and I.2), a 44-year-old male sibling (II.3) and his 2 children (III.1 and III.2) have no history of bleeding or thrombosis. As an adult, II.1 reported having oral surgery for impacted molars with cryoprecipitate infused prior to the procedure and “a few units” infused every three days post-surgery until bleeding stopped, all without complication.

At age 43, II.2 underwent total hip arthroplasty due to aseptic necrosis of the left hip. He was prepared for surgery with cryoprecipitate to raise his circulating fibrinogen to ~1.5 mg/mL. The procedure was uneventful, and postoperatively he was given 10-15 units of cryoprecipitate daily to maintain the fibrinogen concentration between 1.0 and 1.5 mg/mL. Five days after surgery, he began experiencing weakness and malaise, and on day eight cryoprecipitate was discontinued. On day 10, after complaining of chest pain, imaging studies revealed multiple pulmonary emboli and thrombi in both femoral veins. An inferior vena cava filter was inserted, and he was treated with anticoagulants for 3 months.
Eighteen months after surgery II.2 presented with leg ischemia secondary to emboli originating from a thrombus in the thoracic descending aorta. Removal of the aortic thrombus was performed and cryoprecipitate was given post-operatively for several days. Once again, he developed pulmonary emboli and intravenous catheters became occluded by thrombi. He was treated with subcutaneous low molecular weight heparin and the thrombi slowly resolved. Three years after the original hip surgery, thrombectomy was performed to remove femoral arterial clots. No cryoprecipitate was administered, and no excessive bleeding was observed. He is currently receiving long-term anticoagulation with low molecular weight heparin and aspirin, and has had no recent thrombotic episodes.

<table>
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<th>Table 1. Hemostatic indices for patients II.1 and II.2</th>
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<td>Test</td>
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<td>Fibrinogen (clottable; mg/mL)</td>
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<td>Factor VIII (%)</td>
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<td>Factor XI (%)</td>
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n.d. = not determined.
* = Normal plasma; [fibrinogen] = 2-3 mg/mL. Clots from normal plasma diluted to 0.3 mg/mL with defibrinogenated plasma compressed to 0.007 mm.
Hemostasis factor studies

Results of coagulation studies for the 2 hypofibrinogenemic patients (II.1 and II.2) are presented in Table 1. The PT and TT times were greatly prolonged, but admixture of normal plasma normalized both. Factor VIII was found to be somewhat elevated in II.1 (Table 1), and in family members I.2 and II.3 (data not shown). Plasminogen levels for both II.1 and II.2 were normal; t-PA and PAI-1 were normal in II.1, but were elevated in II.2 as might be expected in patients experiencing thrombosis. Both had a normal platelet count and normal platelet aggregation was obtained with the calcium ionophore U-46619. Protein C, protein S, and antithrombin levels for II.2 were normal (I.1’s levels were not determined), and tests for lupus anticoagulant, antifibrinogen, anti-FXIII and anticardiolipin antibodies were negative for II.1 and II.2.

Fibrinogen and fibrin clot studies

No fibrinogen could be detected in the platelet-poor plasma of patients II.1 and II.2 by immunoelectrophoresis but, as indicated in Table 1, low concentrations of fibrinogen were found with the modified Clauss procedure and fibrinopeptide quantitation. Fibrinogen concentrations were normal in the other family members (Figure 1).

Plasma clot lysis induced by t-PA was greatly retarded, and plasma clots from II.2 were resistant to compression by centrifugation, especially when compared to normal plasma clots made with the same fibrinogen levels as II.2 (Table 1). In addition, II.2’s clots were soluble in 5 M urea.

Examination of fibrin chain profiles by SDS-PAGE (Figure 2) revealed the presence of an abnormal fibrin(ogen). Thrombin-induced clots of PPP from patients II.1 and II.2 had essentially
no normal α chain (compare lanes 3 and 5 to lane 1 in Figure 2). But, perhaps the most remarkable feature of these clots was the presence of a new abnormal band at ~35kDa (labeled $\alpha_{Keokuk}^\text{Keokuk}$, in Figure 2, lanes 3-8, 13, and 14). The weak Coomassie blue staining remaining at the position of normal α chains (66 kDa) (Figure 2, lanes 3 and 5) is probably due to trapped albumin which would be expected to migrate in this position in both the presence and absence of Ca$^{2+}$. Further evidence for the absence of normal α chains in II.1 and II.2 was a failure to form crosslinked polymeric $\alpha_n$ structures when clotting was carried out in the presence of Ca$^{2+}$ (compare lanes 4 and 6 with lane 2 in Figure 2). However, the β and γ chain patterns (Figure 2, lanes 3 and 5), including the formation of γ-γ chain dimers (Figure 2, lanes 4 and 6), were normal for II.1 and II.2.

Figure 2. Electrophoretic profile of fibrin clots. SDS-PAGE showing fibrin chain profiles of clots generated in the absence (odd numbered lanes) and presence (even numbered lanes) of Ca$^{2+}$ from the citrated plasma of patient II.1, II.2, their mother (I.2), brother (II.3) and his children (III.1 and III.2). II.1 and II.2 lack normal α chain, and a much shorter constituent chain ($\alpha_{Keokuk}^\text{Keokuk}$), that cannot produce crosslinked $\alpha_n$ polymers, is seen in II.1, II.2, II.3 and III.1 samples.

The proportion of truncated α chains in the simple heterozygotes was calculated from the gel band intensities as: $\alpha_{Keokuk}^\% = \{(2 \times \alpha_{Keokuk}^\text{Keokuk}) / [(2 \times \alpha_{Keokuk}^\text{Keokuk}) + \alpha_{\text{normal}}]\} \times 100$. A correction factor of 2 was used to compensate for the reduced capacity of the approximately half-normal
\( \alpha^{\text{Keokuk}} \) chain to bind the Coomassie blue stain and allowance was also made for the presence of normal \( \alpha \) chain cleavage products that migrate close to the truncated \( \alpha \) chain position, in that \( \alpha(\text{total}) \) always includes any cleavage products; for normal samples it is all cleavage products; for Fibrinogen Keokuk samples it would be a combination, probably proportional to the amount of normal fibrinogen. From subsequent gene analysis, we concluded that patients II.1 and II.2 carried only the truncated \( \alpha \) chain and that family members I.2 and III.2 were normal. From densitometric scanning of the gel in Figure 2 we estimated that some 10\% of the circulating fibrinogens in II.3 and his son III.1 was derived from the truncated species.

Immunoblotting experiments (shown for patient II.2 in Figure 3) with an antiserum specific for the A\( \alpha \)241-476 segment of human fibrinogen confirmed that the \( \sim35 \) kDa constituent was a derivative of the A\( \alpha \) chain of the normal protein.

**Figure 3. Characterization of aberrant A\( \alpha \) chain.** A) Immunoblot of plasma, following SDS-PAGE, with antibody to A\( \alpha \)241-476 before and after addition of thrombin. Lanes 1 and 2 = normal control; lanes 3 and 4 = patient II.2. The patient lacks normal A\( \alpha \) chains, but fibrinopeptide A is successfully removed from the N-terminus of the defective chain by thrombin, suggesting C-terminal truncation. B) Immunoblot of SDS-PAGE using antiserum to whole fibrinogen. Lane 1, normal plasma; lane 2, patient II.2; lane 3 homozygote for A\( \alpha \)\( \text{Otago} \) truncation with a predicted mass of 30,862 Da.
A slightly larger form of this abnormal chain was present in citrated plasma than in the clot (compare the band marked $\alpha_{\text{Keokuk}}$ in lane 3 with that of $\alpha_{\text{Keokuk}}$ in lane 4 of Figure 3a for patient II.2), consistent with the notion that thrombin cleavage produced the $\alpha_{\text{Keokuk}}$ to $\alpha_{\text{Keokuk}}$ conversion by removal of the fibrinopeptide A moiety. It was further evident from the findings in lane 4 that the abnormal $\alpha_{\text{Keokuk}}$ chain could not form high molecular weight crosslinked homopolymers under Factor XIIIa-catalyzed conditions (compare lanes 2 and 4 of Figure 3a), beyond perhaps a certain extent of dimerization (that would yield a protein band close to the position of the normal $\alpha$ chain in lane 4).

Taken together, the results indicated that the C-terminal half of the $\alpha$ chain was missing in protein expressed from the Keokuk mutation (similar results were obtained for samples from patient II.1; data not shown). Additional immunoblots were negative with monoclonal antibodies to $\alpha_{291-348}$ and to $\alpha_{349-406}$ (data not shown), suggesting that truncation occurs upstream of residue 348, but downstream of the Otago truncation at 271. Indeed, as presented in Figure 3b for purified fibrinogen preparations, the $\alpha_{\text{Keokuk}}$ chain is somewhat larger ($\sim$35kDa, lane 2) than the previously described $\alpha_{\text{Otago}}$ ($\sim$30 kDa, lane 3).\textsuperscript{11}

**Figure 4. Fibrin polymerization curves.** The clotting defect in patient II.2 is characterized by a much slower than normal rate of fibrin assembly and by an approximately 5-fold reduction of clot turbidity (ordinate).
The nature of the defect implied by the prolonged thrombin time (Table 1) was explored further by examining the kinetics of thrombin-catalyzed polymerization. Polymerization curves on purified fibrinogen (Figure 4) showed significant defects in all stages of fibrin polymerization, with a lag phase of 430 seconds, a $V_{\text{max}}$ of $0.78 \times 10^{-4}$ units/s, and a final turbidity of 0.023 units compared to control values of 220 seconds, $2.22 \times 10^{-4}$ units/s, and 0.111 units respectively.

**DNA analysis**

Because of the presumed site of the Keokuk putative mutation deduced from immunoblotting (Figure 3a), DNA sequence analysis focused first on examining exon 5 of the fibrinogen A$\alpha$-chain gene. A cytosine to thymine point mutation was found that changes the triplet CAA coding for A$\alpha$Gln328 to a TAA stop codon (Figure 5a). Family members II.1, II.2, II.3, and III.1 were heterozygous for this mutation (named Fibrinogen Keokuk after the family’s town of origin). However, in order to explain the fact that only 2 of the 4 individuals in the family had extreme hypofibrinogenemia and suffered coagulation problems, further genetic analysis was deemed necessary. These tests revealed another mutation at the 5’ end of intron 4 of the A$\alpha$ gene. The mutation converts the invariant GT splice site to TT (Figure 5b) and 3 family members (I.2, II.1, and II.2) were found to be heterozygous for this mutation. Interestingly, the heterozygous carriers possessing either the Fibrinogen Keokuk (II.3 and III.1) or the IVS4+1 G>T (I.2) mutation alone have normal concentrations of fibrinogen and seem to be free of disease, whereas the compound heterozygous combination of these mutations found in II.1 and II.2 causes extreme hypodysfibrinogenemia and coagulopathy. Complete sequencing of all exons and intron-exon boundaries of the 3 fibrinogen subunit genes from II.2 found no other mutations.
Figure 5. DNA sequence analysis. A) DNA sequencing of exon 5 of the fibrinogen Aα gene showed that, patients II.1 II.2, their brother (II.3) and his son (III.1) had a heterozygous C/T mutation that changed the CAA codon for residue Gln328 to a TAA stop codon and was the cause for the Keokuk truncation of the Aα constituent chain of the protein. B) Patients II.1, II.2 and their mother (I.2) had a heterozygous mutation (IVS4+1 G>T) in intron 4 at the invariant 5’ splice site of this gene. Representative examples of each mutation are shown.

Discussion

DNA sequencing established that the 2 symptomatic individuals (II.1 and II.2) with hypofibrinogenemia were compound heterozygotes for 2 different Aα gene mutations: the IVS4+1 G>T splice site mutation and a novel stop mutation at codon AαGln328 of the mature protein (the Keokuk mutation). Heterozygosity for either the splice site or the Keokuk mutation, by themselves, caused neither clinical symptoms nor hypofibrinogenemia, with fibrinogen concentrations of 3.1 mg/mL for the former (I.2) and 2.4 and 2.6 mg/mL for the latter individuals (II.3 and III.1). Homozygosity for the splice mutation is recognized as a frequent cause of afibrinogenemia14, indicating that no viable Aα chains should be produced from the aberrantly spliced allele. Thus, the compound heterozygotes should express only a truncated Aα chain of 35,977 Da. This was confirmed in SDS PAGE of their purified fibrinogen (Figure 2) that showed a single Aα band migrating above the truncated AαOtago chains of 30,862 Da in size.
Nonsense mutations have been reported along the whole length of the fibrinogen Aα-chain gene, and homozygosity can result in dysfibrinogenemia, hypodysfibrinogenemia or often afibrinogenemia depending on the extent of the truncation. As with Fibrinogen Keokuk, heterozygous family members usually had normal fibrinogen concentrations, though with diminished allelic expression and in other cases a significant polymerization defect. For instance, heterozygotes for Fibrinogen Perth and Fibrinogen Otago (with Aα truncations of 15 and 56% respectively) had normal fibrinogen concentrations but decreased expression of their truncated chain; the ratio of α<sub>Perth</sub> to Aα was 0.2:1.0 and there were no α<sub>Otago</sub> chains in the plasma fibrinogen of Fibrinogen Otago heterozygotes. The 46% truncation in fibrinogen Keokuk lead to an allelic expression ratio (α<sub>Keokuk</sub>/Aα) of only about 0.1:1.0 as seen in individuals II.3 and III.1. This suggests that the AαC domain is involved in assembly of the fibrinogen molecule in the hepatocyte, as the shorter the α chain, the less the truncated species is found in circulating fibrinogen.

The AαC domain of fibrin is intimately involved in all stages of polymerization and, while cleavage of fibrinopeptide A is the primary driver of polymerization, cleavage of fibrinopeptide B not only exposes the GlyHisArg sequence, but facilitates the release of the αC domain from intramolecular association with the central E domain. These tethered αC appendages associate intermolecularly and are thought to promote lateral aggregation of the protofibrils. Polymerization curves for the clotting of purified fibrinogen Keokuk support this scenario with a doubling of the lag phase, indicating a delay in the onset protofibril formation, and a 3-fold decrease in V<sub>max</sub> that indicates impaired protofibril assembly into fibers. The most significant aberration, however, was that the final turbidity (measured by absorbance at 350 nm)
was only about one fifth of the normal control, implying that the Keokuk clot was composed of thinner fibers.

The deletion of Aα residues 328-610 results in the removal of key side chains directly involved the XIIIa mediated covalent cross-linking of the Aα chain, notably Gln328 and Gln366 and Lys508. While analysis of plasma clots by SDS-PAGE (Figure 1, lanes 4 and 6) indicated normal γ-γ dimer formation, covalent α, polymers were notably absent. Interestingly, plasma clots of fibrinogen Keokuk were readily soluble in 5 M urea indicating that, while the covalent fusion of γ chains along the linear array of fibrin molecules might be necessary for stabilizing the clot network, it is the XIIIa-catalyzed ’spotwelding’ of α chains that renders a clot insoluble in urea.

While bleeding episodes can be readily explained by a combination of low fibrinogen, severely impaired polymerization kinetics and defective α chain cross-linking, the principal pathology was thrombosis in II.2 and recurrent miscarriages in II.1. Thin fibers and decreased pore size lead to clots that are physically more resistant to degradation and this may have contributed to the thrombotic history of II.2. Other mutations within the AαC domain have been associated with rigid clots showing diminished final turbidities, thin fibers, and history of thrombosis. It is well established that fibrinogen contains 2 cryptic plasminogen and t-PA binding sites (Aα148-160 and γ312-324) that become available in fibrin. However more recent expression studies using purified full length AαC (residues 221-610) and its N and C-terminal fragments established that the AαC domain contains additional independent cryptic t-PA and plasminogen binding sites located in the region from 392-610. The loss of these sites in fibrinogen Keokuk through truncation at residue 328 may contribute to the thrombotic phenotype
observed in II.2. The pro-thrombotic signals commonly generated by major surgery presumably
acted upon the fibrinolysis-resistant Fibrinogen Keokuk to form small clots, which may have
accumulated over time and combined with the large quantity of added fibrinogen to eventually
result in the thrombotic complications that finally became clinically significant after more than a
week of cryoprecipitate infusions. Patients with similar hypodysfibrinogenemias, including II.1
of the Keokuk family, have been reported tolerant of moderate fibrinogen replacement therapy but the levels infused into II.2 may have been excessive, especially in the light of a major surgery successfully completed later without any cryoprecipitate infusions.

The miscarriages suffered by I.1 were all in the first trimester, primarily around 6 to 8
weeks. Similarly, the Fibrinogen Otago patient had 4 miscarriages in the 6-8th week of pregnancy. These miscarriages are at the time when trophoblasts infiltrate the myometrium. Experimentally, afibrinogenemic mice also miscarry at the time of trophoblast infiltration (day 9-10).

This family study provides a unique insight as to how 2 mutations, without untoward
consequences in carriers of either mutation, may produce severe manifestations in compound
heterozygotes, originally diagnosed with simple hypofibrinogenemia. Regardless of the precise molecular explanations for thrombogenicity, the near fatal post-surgical thrombosis following the administration of cryoprecipitate to our patient II.2 illustrates the risk associated with aggressive replacement therapy for raising the circulating concentration of fibrinogen without adequately characterizing the nature of the hypodysfibrinogenemia.

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References


Severe hypodysfibrinogenemia in compound heterozygotes of the fibrinogen AαIVS4+1 G>T mutation and an AαGln328 truncation (Fibrinogen Keokuk)

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