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Research Article

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Molecular Basis for Fibrinogen Dusart (Aα 554 Arg → Cys) and Its Association with Abnormal Fibrin Polymerization and Thrombophilia

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Abstract

The molecular defect in the abnormal fibrinogen Dusart (Paris V) that is associated with thrombophilia was determined by sequence analysis of genomic DNA that had been amplified using the polymerase chain reaction. The propositus was heterozygous for a single base change (C → T) in the Aα-chain gene, resulting in the amino acid substitution Aα 554 Arg → Cys. Restriction analysis of the amplified DNA derived from the family members showed that his father and his two sons were also heterozygous. Electron microscopic studies on fibrin formed from purified fibrinogen Dusart demonstrated fibers that were much thinner than in normal fibrin. In contrast to the previously observed defective binding of plasminogen, the binding of thrombospondin to immobilized fibrinogen Dusart was similar to that of normal fibrinogen. Immunoblot analysis of plasma fibrinogen demonstrated that a substantial part of the fibrinogen Dusart molecules were disulfide-linked to albumin. The plasma of the affected family members also contained fibrinogen–albumin complexes. Furthermore, small amounts of high molecular weight complexes containing fibrinogen were detected in all the heterozygous individuals. These data indicate that the molecular abnormality in fibrinogen Dusart (Aα 554 Arg → Cys) results in defective lateral association of the fibrin fibers and disulfide-linked complex formation with albumin, and is associated with a family history of recurrent thrombosis in the affected individuals. (*J. Clin. Invest.* 1993. 91:1637–1643.) Key words: Aα chain • albumin, disulfide-linked • dysfibrogenemia • gene analysis

Introduction

Release of the fibrinopeptides FpA and FpB from fibrinogen exposes two types of polymerization sites, designated "A" and "B," in the amino-terminal portion of the fibrin molecule (1) that appear to function cooperatively in the fibrin self-assembly process (2, 3). Complementary polymerization sites, designated "a" and "b," respectively, are located in the carboxyl-terminal regions of the molecule (1). Fibrin assembly commences with formation of double-stranded fibrils (2–6), which then branch to form a three-dimensional matrix, concomitant with lateral association of fibrils, that results in increased fiber thickness (7–9). Fibril formation is predominantly due to interaction between the A and a sites (4); interaction between B and b sites contributes to lateral fibril association and augment thick fiber formation (4, 9). It has recently been suggested that the carboxyl-terminal region of the Aα chain constitutes an important component of the b polymerization site in fibrinogen (10).

Congenital abnormal fibrinogens not only provide tools for studying the structure–function relationship in fibrinogen, but they also offer us an opportunity to determine the relationship between the molecular defect and the clinical symptoms of the affected individuals. More than 240 cases of inherited dysfibrinogenemia have been reported (11), of which 48 are associated with clinically significant thromboembolic disease. In one such case, fibrinogen Dusart, the functional defect is related to reduced plasminogen binding (12), impaired plasminogen activation by the tissue-type plasminogen activator (t-PA[1] [13]), and abnormal fibrin polymerization (12).

In this paper we report the structural defect of fibrinogen Dusart (Paris V), inferred from genetic analysis using PCR (14). The presence of the mutation in the family members and the influence on fibrin polymerization was determined. The additional cysteine created by the mutation was involved in the formation of fibrinogen–albumin complexes in plasma. Furthermore, the family history of recurrent thrombosis and the analysis of the fibrinogen gene in the family members demonstrate a convincing association between the molecular defect and the thrombophilia.

Methods

Coagulation studies on plasma. Blood was collected by venepuncture and anticoagulated with 0.1 vol of 0.13 M trisodium citrate. Platelet poor plasma was prepared by centrifuging citrated blood at 2,000 *g* for 15 min at 15°C. Thrombin and Reptilase clotting times were performed as described before (15). Fibrinogen concentration was determined functionally according to Clauss (16) and immunologically according to Mancini et al. (17). Antithrombin III activity was measured by a chromogenic assay (18), protein C activity by using synthetic substrate (Behringwerke, Marburg, FRG), and protein S by immunoassay (STAGO, Asnières, France).

Protein purification. Fibrinogen was purified from plasma of the propositus and a normal individual (19) and further analyzed as described (12). Fibrinogen that lacked the carboxyl-terminal region was prepared as described (20). Thrombospondin was purified from the

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1. *Abbreviations used in this paper:* HRP, horseradish peroxidase; t-PA, tissue-type plasminogen activator.

supernatant of thrombin-activated platelets by heparin-Sepharose affinity chromatography followed by Sepharose-4B gel filtration, essentially as described by Margossian et al. (21) and modified as described (22). The calcium concentration was maintained at 2 mM throughout the purification procedure to avoid structural modification of the thrombospondin molecule. Purified thrombospondin was radiolabeled with carrier-free ^{125}I using the chloramine-T procedure to a specific activity of ~ 0.2 μCi/μg protein. The radiolabeled thrombospondin had the same electrophoretic mobility as the unlabeled counterpart, and exhibited specific and saturable binding to thrombin-activated platelets (22). Protein concentrations were determined spectrophotometrically at 280 nm, the $A^{1\%}_{1cm}$ used for fibrinogen and thrombospondin were 15.0 and 10.4, respectively.

Electron microscopic studies on fibrin. Fibrin for critical-point drying was prepared by addition of human α-thrombin (0.1 U/ml final concentration) to a solution of fibrinogen (50 μg/ml in 50 mM Tris/HCl, 100 mM NaCl, pH 7.4, buffer; ionic strength 0.14) followed by incubation for 60 min at room temperature. A specimen of the fibrin clot was picked up on a carbon-coated 200-mesh grid, fixed with glutaraldehyde/tannic acid, stained with uranyl acetate, dehydrated, and then critical-point dried. Electron microscopy (EM) was carried out in a model 400 electron microscope (Philips Electronic Instruments, Mahwah, NJ) at 120 kV.

Binding of thrombospondin to fibrinogen. Microtiter wells were coated in duplicate with 0.1 ml of a 10 μg/ml freshly prepared fibrinogen solution in 10 mM Tris/HCl, 150 mM NaCl, pH 7.4 (Tris buffer) containing 2 mM $CaCl_2$, overnight at 22°C in a humid chamber. Wells were rinsed twice with Tris buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.05% (vol/vol) Tween 20 (Tris/Tween buffer), and subsequently incubated for 1 h with Tris/Tween buffer containing 1.5% (wt/vol) BSA. The wells were rinsed four times and incubated with increasing concentrations of ^{125}I-thrombospondin in Tris/Tween-buffer for 3 h. The wells were washed four times with Tris/Tween buffer and cut out, and the radioactivity associated with each well was counted. Nonspecific binding was determined by measuring the binding of ^{125}I-thrombospondin to wells coated only with BSA.

Immunoblot analysis of plasma. SDS-PAGE was performed on 5–25% gradient gels according to Laemmli (23) or on 2–16% precast gradient gels (Pharmacia, Uppsala, Sweden) using an electrophoresis buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM disodium EDTA, 0.2% (wt/vol) SDS, pH 7.4, and a sample buffer containing 10 mM Tris/HCl, 1 mM disodium EDTA, 1% (wt/vol) SDS, pH 8.0. Molecular mass was estimated using a low M_r calibration kit (Pharmacia) containing proteins with an M_r ranging from 14.4 to 94 kD or using a mixture of purified fibrinogen (340 kD), fibronectin (450 kD) (purified from fibrinogen by affinity chromatography on gelatin-Sepharose), and purified mouse monoclonal IgM (900 kD) (a generous gift from Dr. R. Bos, Gaubius Laboratory).

Proteins from the SDS-PAGE gels were electroblotted onto nitrocellulose (24) for 16 h at 400 mA and 10°C. The nitrocellulose sheets were blocked by incubating them in Tris/Tween buffer, pH 7.4, containing 0.15 M NaCl, 0.5% (wt/vol) gelatin for 2 h. After blocking, the blots were washed with Tris/Tween buffer and incubated for 2 h at room temperature with mouse monoclonal antifibrinogen Y18 (25) conjugated to horseradish peroxidase (HRP) diluted in Tris/Tween buffer (kindly provided by Dr. W. Nieuwenhuizen, Gaubius Laboratory). Identical blots were incubated for 2 h at room temperature with goat anti-human albumin conjugated to HRP (Nordic, Tilburg, The Netherlands) diluted in Tris/Tween buffer. The protein bands reacting with the different immunoconjugates were visualized by incubation with a substrate solution containing 4-chloro-1-naphthol (26).

DNA amplification and sequencing. Genomic DNA was isolated from blood cells as described (27). Oligonucleotides were synthesized on a model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Oligonucleotides β2a(5′GCCTCTAAGGTTGTAGGAATTCTTCAG3′) and β2b(5′ ATCAGTGCACCCACCAAGTCTGGG3′) were used to amplify the Bβ gene segment coding for amino acids 9–72. Oligonucleotides α6a (5′GGCACGCTGGATGGGTTC3′) and α6b (5′GGACTTACAGTCGACCACAAAAACAGACC3′) were used to amplify the part of the Aα-chain gene coding for amino acids 492–625 and a 121-bp nontranslated 3′ sequence. Oligonucleotides α1a (5′TGTCTCCTCTTCTGGCTA3′) and α2b (5′GTTATTGGCTGAGGAAAAATCGCC3′) were used to amplify the Aα gene segment coding for amino acids 1–95. Oligonucleotides γ4a (5′GAAGCATCCTACGAAAGAGGG3′) and γ4b (5′AACTTGGAATCTAAGAAAGGAAAACTAACC3′) and γ5a (5′CTTCATAGACTTGCAGAG3′) and γ5b (5′AAGCAAGTCGACTGTCCAATAGGAAAAATA3′) were used to amplify the γ gene segment coding for amino acids 259–411. Amplification by PCR (14) was performed in a 100-μl reaction volume containing 1 μg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia), 0.2 μM each of primer a and b in 1× reaction buffer (10 mM Tris/HCl, pH 8.3, at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$ and 0.001% [wt/vol] gelatin). The DNA was denatured at 94°C for 4 min and 2.0 U Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT) was added. Cycles consisted of a 1-min at 94°C, 0.5-min at 58°C, and 1.5-min at 72°C incubation. After 30 cycles, the amplified DNA was precipitated with ethanol, dried, dissolved in 20 μl of distilled water, and run on a 1.0% (wt/vol) ultra low gelling agarose gel (A-5030, Sigma Chemical Co., St. Louis, MO). The bands with the appropriate size, as predicted from the DNA sequence of the Aα (11), Bβ (28), and γ (29) chain genes were cut out of the gel in a volume of ~ 20 μl and heated to 55°C. 1 μl of the melted agarose, containing ~ 10 ng of DNA, was mixed with 1 μl of the appropriate amplification primer (60 ng), and 2 μl of 5× annealing buffer (T7 sequence kit, Pharmacia), the volume was brought up to 14 μl with distilled water. The mixture was heated to 95°C for 3 min and immediately after this was put on ice, and labeling (5 min) and termination (10 min) reactions were performed using the T7 DNA sequence kit according to the manufacturer's instructions.

Detection of mutation in family members by restriction analysis. PCR samples containing the Aα fragment from the family members were incubated with the restriction endonuclease BSAJ I (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. After incubation the restriction digests were analyzed on a 2% (wt/vol) agarose gel (A-6013, Sigma Chemical Co.) and stained with ethidium bromide. DNA molecular size markers were prepared by Rsa I (New England Biolabs) digestion of M13mp18 DNA.

Results

Case report and laboratory data. The propositus (II.2 in Fig. 1) is a male Caucasian who developed spontaneous phlebitis at the age of 37 yr, which was treated by heparin followed by oral anticoagulant. At the age of 41 yr, he suffered from pulmonary embolism and deep vein thrombosis of the left leg. The throm-

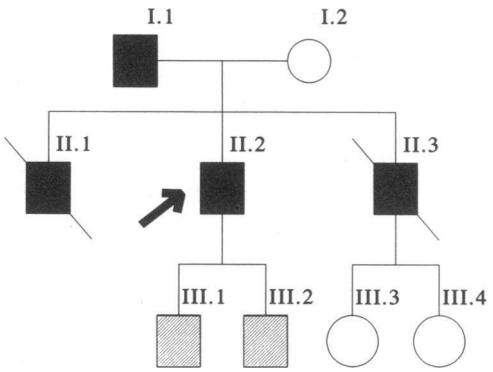

Figure 1. Pedigree of the Dusart family. The arrow indicates the propositus. Symbols used: (□) male, (○) female, (■) thrombotic disorder, (■) fatal thrombotic disorder, (□) treated prophylactically with oral anticoagulant.

Table I. Summary of Laboratory Data of the Propositus with Fibrinogen Dusart (II.2) and His Family Members

	I.1	I.2	II.1	II.2	II.3	III.1	III.2	III.3	III.4
Heterozygous CGT (Aα 554 Arg) → TGT (Cys) mutation in genomic DNA	Yes	ND	ND	Yes	ND	Yes	Yes	No	No
Prolonged thrombin clotting time in plasma	Yes	No	ND	Yes	Yes	Yes	Yes	No	No
Disulfide-linked fibrinogen–albumin complexes in plasma	Yes	ND	ND	Yes	ND	Yes	Yes	No	No

ND, not determined.

bin clotting time of the propositus was prolonged (35 s, control 20 s) as well as the Reptilase clotting time (29.5 s, control 20 s). His father (I.1) developed three episodes of spontaneous deep vein thrombosis at the age of 46 yr. At the age of 52 yr he had an episode of pulmonary embolism, and between the age of 54 and 70 yr he developed three episodes of thrombotic disorder (deep vein thrombosis and superficial thrombosis). At the age of 76 yr he suffered a pulmonary embolus confirmed by angiography without accompanying evidence of deep venous thrombosis. The thrombin and Reptilase clotting times of the father were also prolonged. Two brothers (II.1 and II.3) of the propositus died at the age of 20 and 30 yr, respectively, from pulmonary embolism after several episodes of thrombophlebitis. Brother II.3 showed a prolonged thrombin clotting time; brother II.1 was not investigated. The two sons (III.1 and III.2) of the propositus were born respectively in 1962 and 1964; both had prolonged thrombin and Reptilase clotting times. They were treated prophylactically with oral anticoagulants from 1981 and no history of thrombosis was reported. Two nieces (III.3 and III.4) had normal thrombin and Reptilase clotting times and no history of thrombotic disorders (see also Table I). Antithrombin III, protein C and protein S levels, determined in the plasma of the propositus (II.2) and his father (I.1) during an interruption of oral anticoagulant therapy, were all within the normal range.

Amplification and direct sequencing of genomic DNA fragments. Based on the evidence suggesting that lateral association of fibrin fibrils was impaired (see below), we amplified the genomic DNA coding for the amino terminus of the Aα chain (amino acids 1–95) and Bβ chain (amino acids 9–72), and the carboxy terminus of the Aα chain (amino acids 492–625) and γ chain (amino acids 259–411) of the fibrinogen molecule. After amplification, fragments with the size predicted from the genomic DNA sequence for the Aα, Bβ, and γ chain genes were sequenced. The fragments containing the DNA sequence coding for the amino-terminal segments of the Aα chain and Bβ chain and the carboxyl-terminal segment of the γ chain were completely normal. The fragment containing the DNA encoding the carboxyl-terminal segment of the Aα chain of fibrinogen Dusart II.2 had a single base substitution (Fig. 2) in the codon normally coding for arginine at position 554. This mutation changed the codon CGT (arginine) to TGT which codes for cysteine. The normal sequence was also present in Dusart II.2, indicating that the propositus was heterozygous for this mutation.

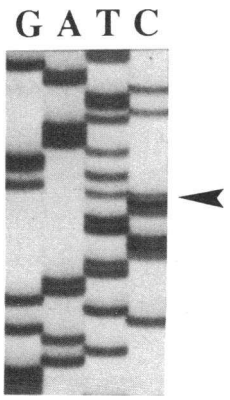

Figure 2. Part of the DNA sequence of the amplified Aα-chain gene fragment coding for amino acids 492–611. Direct sequence analysis of the amplified fragment demonstrated a heterozygous mutation at the position indicated by the arrow.

Fibrin structure determined by electron microscopy. The fibrin fibers formed from fibrinogen Dusart at physiological pH and ionic strength were much thinner than the fibers present in normal fibrin (Fig. 3), indicating impaired lateral association of the fibrin fibrils formed from fibrinogen Dusart. These results are consistent with previous observations that showed low turbidity of polymerized fibrin Dusart compared with normal fibrin (12).

Binding of thrombospondin to fibrinogen. Thrombospondin binds to the carboxyl-terminal part of the fibrinogen Aα- and Bβ-chain (30, 31), and is thought to be an inhibitor of complex formation among fibrin, plasminogen, and t-PA (32). As the mutation in fibrinogen Dusart is located in the carboxyl terminus of the Aα-chain, the binding of thrombospondin to immobilized fibrinogen Dusart was compared with binding to normal fibrinogen. ^{125}I-thrombospondin bound to normal fibrinogen and fibrinogen Dusart in a concentration-dependent manner (Fig. 4). Thrombospondin binding to fibrinogen lacking the carboxyl-terminal segment containing amino acids 491–611 was reduced ~ 74% as compared to intact fibrinogen (data not shown). The specificity of the binding to fibrinogen was demonstrated by the fact that wells coated with only BSA retained negligible amounts of ^{125}I-thrombospondin. The binding of ^{125}I-thrombospondin to fibrinogen Dusart was similar to that of normal fibrinogen in terms of maximal amounts of thrombospondin bound and apparent dissociation constants ($K_d = 1.3 \times 10^{-8}$ M, Fig. 3), indicating that the mutation in the Aα-chain did not influence thrombospondin binding.

Detection of the mutation in family members. The mutation found in the Aα gene fragment of fibrinogen Dusart abolishes the recognition sequence (CCNNGG) for the restriction

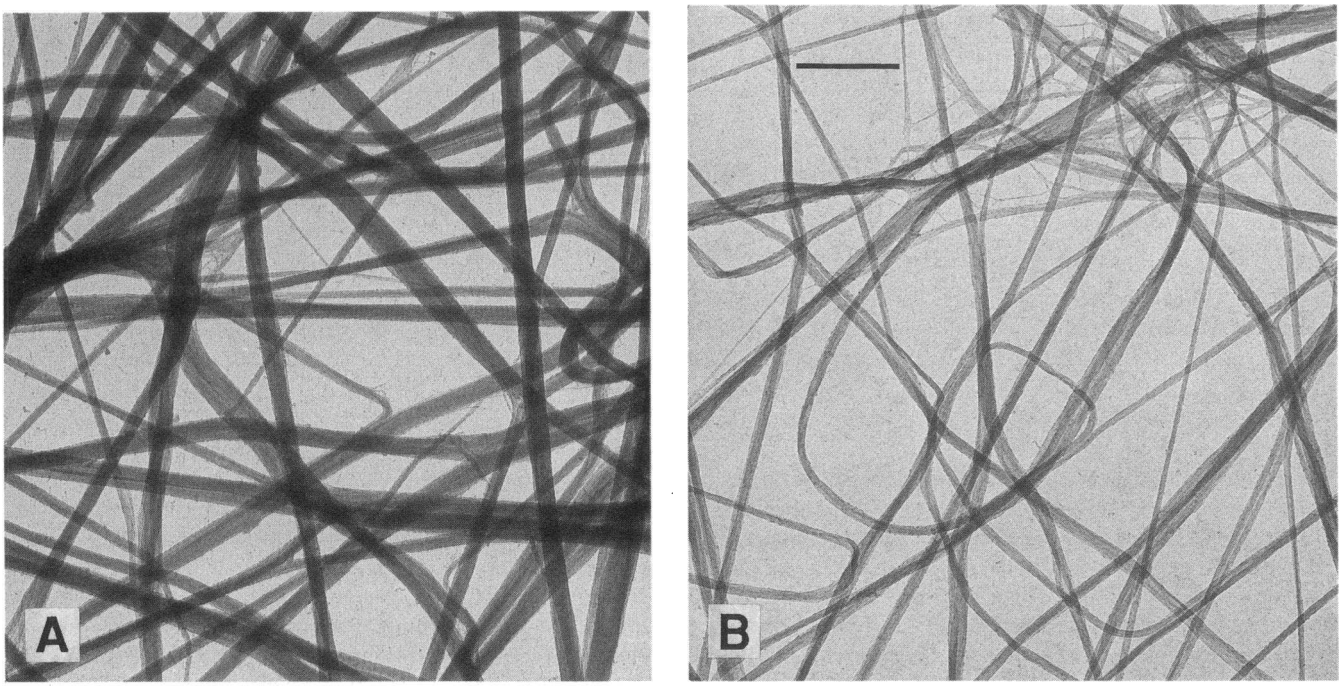

Figure 3. Electron microscopic images of fibrin formed by thrombin from (*A*) normal fibrinogen and (*B*) fibrinogen Dusart (bar, 500 nm; ×27, 245).

endonuclease BSAJ I, which is found in the normal Aα gene fragment. The loss of the restriction site at this position results in the formation of a 275-bp band for the Aα fragment of Dusart in place of the 251-bp band for normal Aα fragment. Restriction analysis of the amplified Aα gene fragment from a normal individual and the family members of the propositus (Fig. 5) showed that the propositus (II.2), his father (I.1), and his two sons (III.1 and III.2) contained both the normal fragment (251 bp) and the abnormal fragment (275 bp). These results indicate that the father and the two sons of the propositus are also heterozygous for the mutation found in the propositus. The normal individual and the two nieces (III.3 and III.4) contained only the normal fragment (251 bp), indicating that they do not contain the mutation in the Aα gene fragment. The DNA of the two brothers (II.1 and II.3) of the propositus was not studied. However, one of them (II.3) showed a prolonged thrombin clotting time, indicating that he probably contained the molecular defect (see also Table I).

Immunoblot analysis of plasma. To determine whether the additional cysteine created by the mutation in fibrinogen Dusart (Aα 554 Arg → Cys) is involved in disulfide-linked complex formation with other proteins (33–35), plasma of the propositus, his siblings, and normal plasma was analyzed by immunoblotting after separation of plasma proteins on 2–16% gradient SDS-PAGE gels. The blots were incubated with a mouse monoclonal antifibrinogen antibody (Y18) and a goat

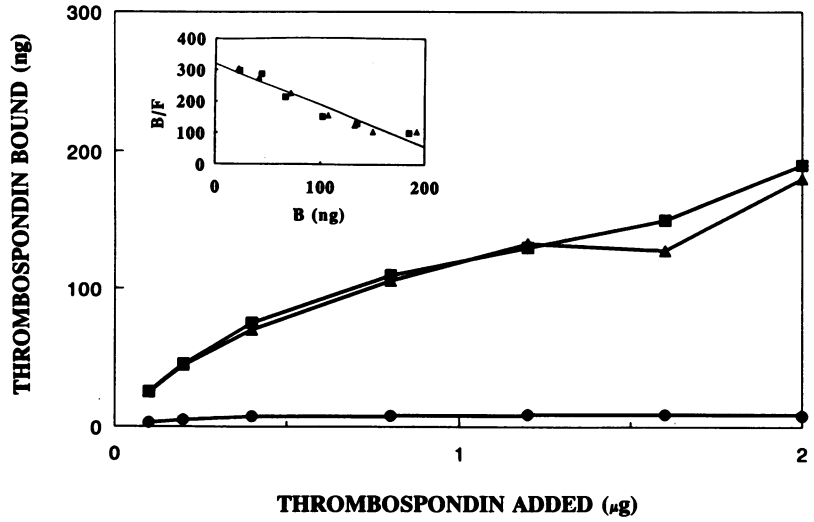

Figure 4. Binding of ^{125}I-thrombospondin to immobilized normal fibrinogen (■), fibrinogen Dusart (▲), and BSA (●). The data are expressed as the total amount of the relevant protein, and the total amount bound to the well. *Inset*: Scatchard plot of binding data.

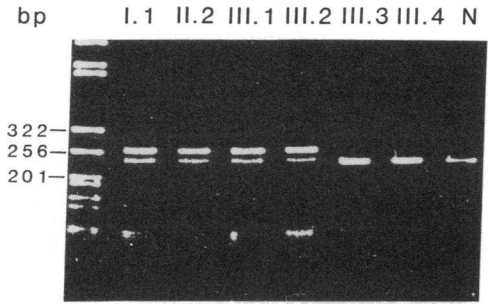

Figure 5. EtBr stained gel of restriction digests by BSAJ I of amplified Aα-chain gene fragment from the propositus (II.2), his siblings (I.1, III.1, III.2, III.3, and III.4) and a normal individual (N). DNA molecular size marker: M13mp18 Rsa I digest.

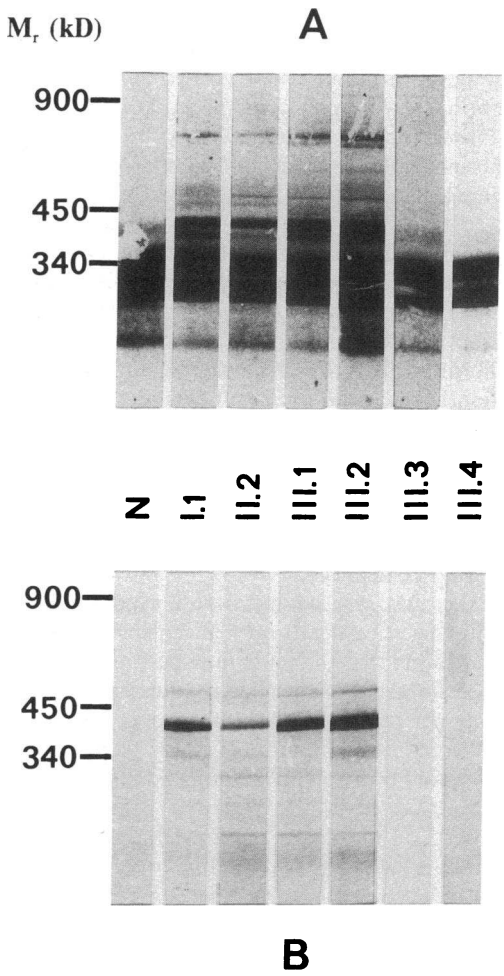

Figure 6. Immunoblot of plasma of the propositus (II.2), his siblings (I.1, III.1, III.2, III.3, and III.4) and a normal individual (N) after separation of the plasma proteins on a 2–16% gradient SDS-PAGE gel, using (*A*) Y18/HRP, a monoclonal antibody specific for fibrinogen and (*B*) goat anti-human albumin/HRP.

anti-human albumin antibody, both conjugated with HRP. After reaction with Y18/HRP (Fig. 6 *A*), normal plasma and the plasma of Dusart III.3 and III.4 showed two intense bands with M_r's of ~ 340,000 and 300,000 D, corresponding to the high- and low-M_r form of fibrinogen (36, 37). Plasma of the four other family members of Dusart (I.1, II.2, III.1, and III.2) contained the same two bands as normal plasma, and an additional intense band with a M_r of ~ 400,000 D. Several other faint bands were also present in the plasma of these individuals (I.2, II.2, III.1, and III.2). After reaction with goat anti-human albumin/HRP (Fig. 6 *B*) normal plasma and plasma from Dusart III.3 and III.4 showed no bands which reacted with this antibody. Plasma samples from Dusart I.1, II.2, III.1, and III.2 showed one intense band with an M_r of 400,000 D after reaction with the goat anti-human albumin/HRP conjugate. As indicated by the intense band with M_r 400,000 D, which reacted with antifibrinogen and antialbumin antibodies, the mutation in fibrinogen Dusart results in the formation of covalent complexes between the mutated fibrinogen and plasma albumin. Immunoblot analysis of reduced plasma samples with Y18/HRP (data not shown), did not show any high-M_r complex, indicating that the complex formation involves disulfide bridges between the different proteins.

In summary, the presence of the mutation in the Aα-chain of fibrinogen Dusart is accompanied by a prolonged thrombin clotting time and the presence of disulfide-linked fibrinogen-albumin complexes in plasma of the affected family members (Table I).

Sulfhydryl content. To determine whether the cysteine residue at position Aα 554 in fibrinogen Dusart had been oxidized to disulfides, we evaluated the titratable sulfhydryl content using Ellman's reagent (38). The analysis was performed in the presence of 8 M urea to denature the protein, because sulfhydryl groups can be present in native proteins in a nontitratable state (39). Calculation of the number of free sulfhydryl groups showed that both normal fibrinogen and fibrinogen Dusart did not contain any detectable free sulfhydryl groups (< 0.05 mol/mol).

Discussion

Using the polymerase chain reaction, we identified a single-base substitution in the gene coding for the Aα chain of the abnormal fibrinogen Dusart. The mutation in fibrinogen Dusart is associated with abnormal fibrin polymerization (12), reduced binding of Lys-plasminogen (12), and defective t-PA induced plasminogen activation (13). Because the defect was found to affect fibrin polymerization, genomic DNA segments coding for the amino terminus of the Aα chain and the Bβ chain, and the carboxy terminus of the Aα chain and γ chain were amplified. Sequence analysis of the amplified products, demonstrated that the propositus (II.2) was heterozygous for a single-base substitution in the codon for Aα Arg 554 (CGT) resulting in a Cys (TGT) at this position. The absence of titratable sulfhydryl groups in fibrinogen Dusart indicated that the additional cysteine residue at position Aα 554 had been oxidized. Immunoblot analysis of plasma from the propositus and his family members demonstrated that, in all four heterozygous family members, fibrinogen was linked to other proteins by disulfide bonds. The predominant complex was identified as a fibrinogen–albumin complex. The occurrence of a similar complex was first described for the variant antithrombin III molecule Northwick Park (33, 34) and recently for two abnormal fibrinogens with an Arg → Cys substitution in the amino terminus of the fibrinogen Bβ-chain (35). The other faint

bands present on the blot, could mean that a small amount of fibrinogen Dusart is linked to other proteins as well. Intramolecular disulfide bond formation between two abnormal Aα chains in one fibrinogen molecule could also occur.

It has been known for many years (40) that fibrin polymers formed from fibrinogen lacking carboxyl-terminal regions of Aα chains develop less turbid clots, suggesting that fibers in the clot matrix are reduced in thickness. Recent studies by Hasegawa and Sasaki (10) provided direct evidence that this region of the Aα chain plays a critical role in the process of lateral fibril association, and constitutes all or part of the so-called b polymerization site in the fibrin molecule. Electron-microscopic images of the fibrin formed from fibrinogen Dusart showed a marked reduction in the width of fibrin fibers in the clot matrix. These observations support the conclusion that the carboxyl-terminal region of the Aα chain plays an important role in lateral fibril association. Whether the substitution of cysteine at Aα 554, per se, or whether the albumin molecules bound to the fibrinogen at this position cause the defective function, cannot yet be deduced.

Removal of the carboxyl-terminus from fibrinogen Dusart by limited plasmin digestion nearly normalized fibrin polymerization (20), indicating that the remaining part of fibrinogen Dusart contained no mutation which could explain the impaired fibrin polymerization. Moreover, sequence analysis of amplified DNA coding for the terminal parts of fibrinogen, which are involved in fibrin polymerization, did not reveal any structural defect.

Previous reports on fibrinogen Dusart demonstrated that fibrin formed from it had a reduced binding of Lys-plasminogen (12) and a reduced accelerating effect on t-PA–induced plasminogen activation (13). An explanation for these effects could be that Aα Arg 554 is part of a plasminogen binding site in normal fibrin, or that the presence of albumin, linked to Aα Cys 554, masks this binding site in fibrin Dusart. However, thrombospondin, thought to bind to the carboxyl-terminal end of the fibrinogen Aα chain (31), bound normally to fibrinogen Dusart. Another explanation is that the functional defects related to fibrinolysis are the result of the impaired lateral association of fibrin fibrils in fibrin Dusart. The latter explanation is in agreement with the observation that inhibition of fibrin polymerization reduces the acceleratory effect of fibrin on t-PA–induced plasminogen activation (40–43). Further evidence for this hypothesis is presented by Mirshahi et al. (44) who showed that fibrin formed from low-M_r fibrinogen, which results in reduced lateral association (10), is more resistant to fibrinolysis induced by t-PA than fibrin prepared from high-M_r fibrinogen.

Restriction analysis of the Aα gene fragments demonstrated that the three family members with a prolonged thrombin clotting time (I.1, III.1, and III.2) were also heterozygous for the defect, whereas the two family members with a normal thrombin clotting time (III.3 and III.4) did not contain the mutated DNA sequence. From the family history of recurrent and massive thrombosis and the genetic analysis of the family members, the association of the clinical symptoms with the defect in fibrinogen Dusart is convincing. The mechanism responsible for the recurrent thrombosis in this family is most likely related to the decreased plasminogen binding to fibrin and the reduced acceleratory effect of fibrin on t-PA–induced plasminogen activation. This would indicate that the cofactor function of fibrin in regulating fibrinolysis is an important in vivo mechanism for inducing efficient thrombolysis and for preventing the occurrence of thrombosis.

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References

1. Kudryk, B. J., D. Collen, K. R. Woods, and B. Blombäck. 1974. Evidence for localization of polymerization sites in fibrinogen. *J. Biol. Chem.* 249:3322–3325.

2. Shainoff, J. R., and B. N. Dardik. 1979. Fibrinopeptide B and aggregation of fibrinogen. *Science (Wash. DC).* 204:200–202.

3. Shainoff, J. R., and B. N. Dardik. 1983. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. *Ann. N.Y. Acad. Sci.* 408:254–268.

4. Olexa, S. A., and A. Z. Budzynski. 1980. Evidence for four different polymerization sites involved in human fibrin formation. *Proc. Natl. Acad. Sci. USA.* 77:1374–1378.

5. Olexa, S. A., A. Z. Budzynski, R. F. Doolittle, B. A. Cottrell, and T. C. Greene. 1981. Structure of fragment E species from human cross-linked fibrin. *Biochemistry.* 21:6139–6145.

6. Budzynski, A. Z., S. A. Olexa, and B. V. Pandya. 1983. Fibrin polymerization sites in fibrinogen and fibrin fragments. *Ann. N.Y. Acad. Sci.* 408:301–314.

7. Carr, M. E., Jr., L. L. Shen, and J. Hermans. 1977. Mass-length ratio of fibrin fibers from gel permeation and light scattering. *Biopolymers.* 16:1–15.

8. Hantgan, R. R., and J. Hermans. 1979. Assembly of fibrin: a light scattering study. *J. Biol. Chem.* 254:11272–11281.

9. Mosesson, M. W. 1990. Fibrin polymerization and its regulatory role in hemostasis. *J. Lab. Clin. Med.* 116:8–17.

10. Hasegawa, N., and S. Sasaki. 1990. Location of the binding site "b" for lateral polymerization of fibrin. *Thromb. Res.* 57:183–195.

11. Ebert, R. F., editor. 1991. Index of Variant Human Fibrinogens, 1991 Edition. CRC Press, Boca Raton, FL.

12. Soria, J., C. Soria, and J. P. Caen. 1983. A new type of congenital dysfibrinogenaemia with defective fibrin lysis-Dusard syndrome: possible relation to thrombosis. *Br. J. Haematol.* 53:575–586.

13. Lijnen, H. R., J. Soria, C. Soria, D. Collen, and J. P. Caen. 1984. Dysfibrinogenemia (fibrinogen Dusard) associated with impaired fibrin-enhanced plasminogen activation. *Thromb. Haemostasis.* 51:108–109.

14. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 39:487–491.

15. Caen, J., M. J. Larrieu, and M. Samama. 1975. L'Hémostase, Méthode d'Exploration et de Diagnostic Pratique. L'Expansion Scientifique Française, Paris.

16. Clauss, A. 1957. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta. Haematol.* 17:237–246.

17. Mancini, G., A. O. Carbonara, and J. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* 2:235–254.

18. Abilgaard, U., O. R. Odengard, and M. Lie. 1975. Heparin cofactor activity measured with an amidolytic method. *Thromb. Diath. Haemorrh.* 6:287–297.

19. Blombäck, B., and M. Blombäck. 1956. Purification of human and bovine fibrinogen. *Ark. Kem.* 10:415–443.

20. Siebenlist, K. R., M. W. Mosesson, J. P. DiOrio, J. Soria, C. Soria, and J. P. Caen. 1991. The polymerization of fibrinogen Dusart (Aα 554 Arg → Cys) after removal of carboxyl terminal regions of the Aα chains. *Blood Coagulation and Fibrinolysis.* In press.

21. Margossian, S. S., J. W. Lawler, and H. S. Slayter. 1981. Physical characterization of platelet thrombospondin. *J. Biol. Chem.* 256:7495–7500.

22. Dubernard, V., and C. Legrand. 1991. Characterization of the binding of thrombospondin to human platelets and its association with the platelet cytoskeleton. *J. Lab. Clin. Med.* 118:446–457.

23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.

24. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

25. Koppert, P. W., C. M. G. Huijsmans, and W. Nieuwenhuizen. 1985. A monoclonal antibody, specific for human fibrinogen, fibrinopeptide A-containing fragments and not reacting with free fibrinopeptide A. *Blood.* 66:503–507.

26. Hong, C. S., B. M. Stadler, M. Wälti, and A. L. De Weck. 1986. Dot-immunobinding assay with monoclonal anti-IgE antibodies for the detection and quantitation of human IgE. *J. Immunol. Methods.* 95:195–202.

27. Gustafson, S., J. A. Proper, E. J. W. Bowie, and S. S. Sommer. 1987. Parameters affecting the yield of DNA from human blood. *Anal. Biochem.* 165:294–299.

28. Chung, D. W., B. G. Que, M. W. Rixon, M. Mace, Jr., and E. W. Davie. 1983. Characterization of complementary deoxyribonucleic acid and genomic deoxyribonucleic acid for the β chain of human fibrinogen. *Biochemistry.* 22:3244–3250.

29. Rixon, M. W., B. W. Chung, and E. W. Davie. 1985. Nucleotide sequence of the gene for the γ-chain of human fibrinogen. *Biochemistry.* 24:2077–2086.

30. Bacon-Baguley, T., B. J., Kudryk, and D. A. Walz. 1987. Thrombospondin interaction with fibrinogen: evidence for binding to the Aα and Bβ chains of fibrinogen. *J. Biol. Chem.* 262:1927–1930.

31. Tuszynski, G. R., S. Srivastava, H. I. Switalska, J. C. Holt, C. S. Cierniewski, and S. Niewiarowski. 1985. The interaction of human platelet thrombospondin with fibrinogen: thrombospondin purification and specificity of interaction. *J. Biol. Chem.* 260:12240–12245.

32. Silverstein, R. L., L. L. K. Leung, P. C. Harpel, and R. L. Nachman. 1984. Complex formation of platelet thrombospondin with plasminogen: modulation of activation by tissue activator. *J. Clin. Invest.* 74:1625–1633.

33. Erdjument, H., D. A. Lane, H. Ireland, M. Panico, V. Di Marzo, I. Blench, and H. R. Morris. 1987. Formation of a covalent disulfide-linked antithrombin-albumin complex by an antithrombin variant, antithrombin "Northwick Park." *J. Biol. Chem.* 262:13381–13384.

34. Erdjument, H., D. A. Lane, M. Panico, V. Di Marzo, and H. R. Morris. 1988. Single amino acid substitution in the reactive site of antithrombin leading to thrombosis. *J. Biol. Chem.* 263:5589–5593.

35. Koopman, J., F. Haverkate, J. Grimbergen, L. Engesser, I. Nováková, A. Kerst, and S. T. Lord. 1992. Abnormal fibrinogens Ijmuiden (Bβ Arg 14 → Cys) and Nijmegen (Bβ Arg 44 → Cys) form disulfide-linked fibrinogen–albumin complexes. *Proc. Natl. Acad. Sci.* USA. 89:3478–3482.

36. Mosesson, M. W., D. K. Galanakis, and J. S. Finlayson. 1974. Comparison of human plasma fibrinogen subfractions and early plasmic fibrinogen derivatives. *J. Biol. Chem.* 249:4656–4664.

37. Lipinska, I., B. Lipinski, and V. Gurewich. 1974. Fibrinogen hetrogeneity in human plasma: electrophoretic demonstration and characterization of two major fibrinogen components. *J. Lab. Clin. Med.* 84:509–516.

38. Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82:70–77.

39. Takahashi, N., and M. Hirose. 1990. Determination of sulfhydryl groups and disulfide bonds in a protein by polyacrylamide gel electrophoresis. *Anal. Biochem.* 188:359–365.

40. Mosesson, M. W., N. Alkjaersig, B. Sweet, and S. Sherry. 1967. Human fibrinogen of relatively high solubility: comparative biophysical, biochemical, and biological studies with fibrinogen of lower solubility. *Biochemistry.* 6:3279–3287.

41. Suenson, E., and L. C. Petersen. 1986. Fibrin and plasminogen structures essential to stimulation of plasmin formation by tissue-type plasminogen activator. *Biochim. Biophys. Acta.* 870:510–519.

42. Koopman, J., L. Engesser, M. Nieveen, F. Haverkate, and E. J. P. Brommer. 1986. Fibrin polymerization associated with tissue-type plasminogen activator (t-PA) induced Glu-plasminogen activation. *In* Fibrinogen and Its Derivatives: Biochemistry, Physiology and Pathophysiology. G. Muller-Berghaus, U. Scheefers-Borchel, E. Selmayr, and A. Henschen, editors. Elsevier, Amsterdam. 315–318.

43. Soria, J., C. Soria, O. Bertrand, M. Mirshahi, F. W. Dunn, E. Svenson, P. Desvignes, P. Bonnet, M. Samama, and J. P. Caen. 1986. Role of fibrin polymerization in fibrinolysis: comparison between polymerisable and unpolymerisable fibrins in the potentiating effect of plasminogen activation by tissue-type plasminogen activator. *In* Fibrinogen—Fibrin Formation and Fibrinolysis, Volume 4. D. Lane, A. Henschen, and M. K. Jasani, editors. Walter de Gruyter & Co., Berlin. 153–158.

44. Mirshahi, M., J. Soria, and C. Soria. 1990. Fibrinogen populations in plasma: differences in their degradability. *In* Fibrinogen 4: Current Basic and Clinical Aspects. M. Matsuda, S. Iwanaga, A. Takada, and A. Henschen, editors. Elsevier, Amsterdam. 123–130.