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The mutation in fibrinogen Bicêtre II (γ Asn_{308}→Lys) does not affect the binding of t-PA and plasminogen to fibrin

P. Grailhe, C. Boyer-Neumann, F. Haverkate, J. Grimbergen, M. J. Larrieu and E. Anglés-Cano

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The aim of this study was to investigate the interactions of t-PA and plasminogen with fibrin derived from an abnormal fibrinogen detected in a 40-year-old male patient who had had an episode of thrombophlebitis with pulmonary embolism. An abnormal fibrinogen was diagnosed on the basis of prolonged thrombin and reptilase times also detected in two other family members. Fibrinogen purified from plasma, in the presence of protease inhibitors, by glycine precipitations, gel filtration and affinity chromatography, was devoid of plasminogen, fibronectin, and vWf. SDS–PAGE analysis according to Laemmli under reducing conditions, showed an abnormal γ chain (~50% of the total) migrating in a more anodic position (M_r 48 kDa). By PCR amplification and DNA sequencing, the abnormality was identified as an Asn_{308}→Lys mutation of the γ chain. Since such a mutation constitutes a new plasmin cleavage site as first reported for fibrinogen Kyoto I, it may modify interactions of plasminogen and t-PA with carboxy-terminal lysine residues. Ligand-binding studies were therefore performed using intact and plasmin-degraded fibrin surfaces obtained from the abnormal fibrinogen. The plasminogen and t-PA binding isotherms obtained with the abnormal fibrinogen were similar to the control. Moreover, the stimulation by fibrin of plasminogen activation by t-PA was not different from the control. These results suggest (i) that the lysine 308 residue may not be exposed to plasmin cleavage in fibrin, and (ii) that the thrombotic accident of the propositus cannot be explained by an abnormality of the plasminogen/t-PA binding to fibrin.

Key words: Dysfibrinogenaemia, fibrinolysis, solid-phase fibrin, tissue plasminogen activator, plasminogen.

Introduction

More than 200 abnormal fibrinogens have been described (reviewed in Ref. 1). Most of the dysfibrinogenaemias showed a prolonged clotting time due to a decreased release of fibrinopeptides and/or a defect of polymerization. Indeed, a limited number of cases of dysfibrinogenaemia associated with thrombotic accidents and anomalies of the fibrinolytic system have been reported: fibrinogens Chapel Hill III,[2] Nijmegen,[3] Date[4] and Dusard.[5,6] Abnormal fibrinogens may result in abnormalities of the interaction of fibrin with proteins of the fibrinolytic system. Therefore, analytical studies on abnormal fibrinogens may be useful in investigating the structure/function relationships of fibrinogen with such molecules. In particular, interactions of plasminogen and t-PA with lysine residues of fibrin may be modified by lysine mutations. The present report studies a dysfibrinogenaemia detected in a 40-year-old male patient who developed a thrombotic accident. Since the mutation was identified as Asn_{308}→Lys of the γ chain, the interactions of fibrin

P. Grailhe, M. J. Larrieu and E. Anglés-Cano are with the Unité 143 of the Institut National de la Santé et de la Recherche Médicale at the Centre Hospitalo-Universitaire de Bicêtre, F-94275-Cedex, Bicêtre, France. Tel: (+33) 1–46–581544; Fax: (+33) 1–46–706446 and (+33) 1–46–719472. C. Boyer-Neumann is with the Service d'Hématologie Biologique, Hôpital A. Béclère, 92141-Cedex, Clamart, France. F. Haverkate and J. Grimbergen are with the Gaubius Laboratory, IVVO-TNO Leiden, The Netherlands. Address correspondence to Dr E. Anglés-Cano.

derived from this fibrinogen with tissue-type plasminogen activator (t-PA) and plasminogen were investigated.

Materials and methods

Chemicals and reagents

All chemicals obtained were of the best reagent grade commercially available. Specific products were purchased from the following sources: ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), and Tween 20 (Serva, Heidelberg, Germany); Ultrogel AcA 44, gelatin-Ultrogel and DEAE-Trisacryl (IBF, Villeneuve-la-Garenne, France); chromogenic substrates: D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) and L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2366) (Chromogenix, Montpellier, France); D-dimer test, hirudin and chromogenic substrate methylmalonylhydroxyprolylarginine *p*-nitroanilide (CBS 1065; Diagnostica Stago, Asnières, France); Sepharose-6B, lysine-Sepharose 4B, PD-10 Sephadex G-25 M columns and low molecular mass reference proteins (Pharmacia, Uppsala, Sweden); poly (vinyl) chloride (PVC) U-shaped microtitration plates and plate sealers (Dynatech, Guyancourt, France); glutaraldehyde 25% aqueous solution (TAAB Laboratories, Reading, UK); sodium dodecyl sulphate (SDS), acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulphate and Temed (Bio-Rad, Richmond, CA, USA); 1,3,4,6-tetrachloro-3a,6a-diphenilglycoluril (Iodo-Gen™, Pierce Chemical Co., Rockford, IL, USA); human plasmin (Boehringer, Mannheim, Germany); $Na^{125}I$ from Amersham (Bucks., UK). L-glutamyl-glycyl-L-arginine chloromethylketone (GGACK) and D-valyl-L-phenylalanyl-L-lysine chloromethylketone (VPLCK) from France Biochem (Meudon, France); *trans*-4-(aminomethyl)-cyclohexane carboxylic acid (AMCHA) from Sigma (St Louis, MO, USA); diisopropylfluorophosphate (DFP) and 6-aminohexanoic acid (6-AHA) from Aldrich (Strasbourg, France). Unfractionated heparin (Calciparine) was from Sanofi-Choay (Paris, France).

Buffers

Buffer A: 0.05 M sodium phosphate, pH 7.4, containing 0.08 M NaCl. Assay buffer: buffer A containing 2 mg/ml BSA and 0.01% Tween 20. Binding buffer: 0.05 M sodium phosphate, pH 6.8, containing 4 mg/ml BSA, 0.01% Tween 20 and 2 mM EDTA. Mass buffer was binding buffer containing 40 mg/ml (w/v) bovine serum albumin.

Blood collection

Blood was drawn from the propositus, family members or healthy subjects and immediately mixed with one-ninth volume of 3.8% (w/v) trisodium citrate. Following two centrifugations at 2 400 × *g* for 20 min at 4°C, plasma was separated, frozen in dry ice in small aliquots and stored at −80°C. A reference pool plasma consisting of 20 plasmas from healthy subjects between the ages of 20 and 40 was prepared and stored under similar conditions. For DNA analysis, 15 ml of anticoagulated blood was immediately frozen at −80°C.

Haemostatic tests

Platelet count, bleeding time, euglobulin lysis time, thrombin time, reptilase time, assays of fibrinogen and factors II, V, VII and X were performed as previously described.[7] D-dimers were measured by an enzyme immunoassay (Diagnostica Stago).

Coagulation and fibrinolytic assays

Plasma levels of plasminogen antigen (M-Partigen-plasminogen, Behringwerke, Marburg, Germany), t-PA antigen (Asserachrom t-PA, Diagnostica Stago), PAI-1 antigen (Asserachrom PAI-1, Diagnostica Stago) and α_2-antiplasmin activity[8] were measured as indicated.

Purification of fibrinogen

Venous blood from the propositus (II–3, Figure 1), his mother (I–2, Figure 1) and from normal donors (C) was collected on ice from the forearm on one-tenth volume of 0.11 M sodium citrate containing 100 KIU/ml aprotinin, 1 μM GGACK, 1 μM VPLCK, 1 U/ml hirudin, 4 mM benzamidine and 20 IU/ml heparin (final concentrations). Plasma was separated by two centrifugations (20 min at 2 400 × *g*, 4°C) and then supplemented with 1 mM DFP. Fibrinogen was purified from plasma according to Kazal *et al.*[9] with the following modifications. After two barium sulphate precipitations (90 g/l, 1 h, 22°C), the pellet was discarded and the supernatant was precipitated twice with glycine (0.15 g/l, 30 min, 22°C). After centrifugation (20 min, 11 950 × *g*, 22°C) the pellet was dissolved in 25% volume of the barium sulphate supernatant with 0.05 M sodium citrate adjusted to pH 7.4 with citric acid and containing 0.5 M NaCl, 2 mM EDTA, 5 KIU/ml aprotinin, 150 mM 6-AHA and 1 μM GGACK. Then, fibrinogen was purified by gel filtration on Sepharose-6B to eliminate vWF. Fractions eluted corresponding to fibrinogen were pooled and passed through lysine–Sepharose 4B and gelatin–Ultrogel equilibrated with 0.05 M phosphate buffer, pH 7.4, containing 0.5 M NaCl, 1 mM benzamidine, 5 KIU/ml aprotinin, 2 mM EDTA and 0.01% NaN_3 to eliminate plasminogen and fibronectin, respectively.

Fibrinogen was then precipitated with 0.16 g/l glycine and stirred for 1 h at 22°C. After centrifugation for 15 min at 11 950 × *g*, the pellet was dissolved in a minimal volume of 0.05 M sodium citrate buffer, pH 7.4, containing 0.3 M NaCl and 5 KIU/ml aprotinin. After exhaustive dialysis against 0.05 M phosphate buffer, pH 7.4, containing 0.3 M NaCl using a dialysis bag with 12–14 kDa cut-off pores, fibrinogen concentrations calculated from the absorbance at 280 nm using an extinction coefficient $E^{1\%}_{1\ cm} = 15.1$[10] were: 4.6 (II–3), 3.37 (I–2) and 1.51 mg/ml (C). The purified fibrinogen was free of von Willebrand factor, plasminogen and fibronectin as determined by an enzyme-linked immunosorbent assay specific for these proteins; the absence of plasminogen or plasmin was confirmed by incubating for 72 h at 37°C on fibrin-agar plates prepared with the purified products supplemented with t-PA when indicated. Fibrinogen preparations were more than 98% clottable.

SDS–Polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions was performed using 0.75 mm thick, 4% stacking and 10% separating slab gels according to Laemmli.[11] Each sample was diluted (1:2, v/v) with sample buffer containing 0.125 M Tris–HCl buffer, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue. After 5 min at 90°C the samples were electrophoresed at a constant current of 5 mA at 4°C. The gels were stained with Coomassie blue (0.025%) in isopropanol (2.5%) and acetic acid (10%) for 30 min at 22°C, and then destained using isopropanol (25%) and acetic acid (10%).

Glu-plasminogen was purified from DFP-treated fresh-frozen human plasma by affinity chromatography on lysine–Sepharose 4B,[12] gel filtration on Ultrogel AcA 44 and ion-exchange chromatography on DEAE–Trisacryl. All procedures were performed at 4°C in the presence of aprotinin. No contaminant plasmin activity was detected by incubating the plasminogen with the chromogenic substrate CBS 1065 (1.5 mM final concentration) for 48 h at 37°C. The concentration of plasminogen was measured at 280 nm in buffer A using an $E^{1\%}_{1\ cm} = 16.8$.[13]

Human t-PA (>95% single-chain) purified from Bowes melanoma cell-conditioned medium on immobilized monoclonal antibody anti-t-PA was obtained from Biopool (Umeå, Sweden). It had a specific activity of 680 000 IU/mg as determined with the solid-phase fibrin-t-PA spectrophotometric assay[14] by reference to the First International Standard for t-PA, preparation coded 83/517, provided by the National Institute for Biological Standards and Control (London, UK). Goat anti-human uterine t-PA IgG was obtained from Biopool.

Radioiodination

Glu-plasminogen and IgG against t-PA were radioiodinated with $Na^{125}I$ using the Iodo-Gen™ method of Fraker and Speck[15] with the following modifications. Protein (10 μg in 0.2 M phosphate buffer, pH 7.4) and 1 mCi of the radioisotope were added (20 μl final volume) to a conical plastic centrifuge vial (Eppendorf) precoated with Iodogen (10 μg) according to the manufacturer's instructions. After an iodination time of 4 min at 4°C, the labelled protein was separated from free $Na^{125}I$ by gel filtration on a PD-10 Sephadex G-25 column (medium grade). The specific radioactivities obtained were 20 nCi/ng of protein for the Glu-plasminogen and 5 nCi/ng for the IgG.

Preparation of the fibrin surfaces

Solid-phase fibrin was prepared as previously described.[14,16] Briefly, PVC-bound stable poly(glutaraldehyde) derivates were first produced by treating U-microtitration PVC plates with 2.5% glutaraldehyde in 0.1 M sodium bicarbonate buffer, pH 9.5, for 2 h at 22°C. Then, fibrinogen (0.3 μM in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM $CaCl_2$) was covalently fixed for 18 h at 4°C. After washing, a fibrin network was generated by treatment (2 h at 37°C) with thrombin (20 N. I. H. units/ml in assay buffer containing 1 mM $CaCl_2$). The excess thrombin was eluted by three washes with a high ionic strength solution (0.5 M NaCl, 8 mM $CaCl_2$, 0.05% Tween 20). A final wash with 5 mM sodium phosphate buffer (pH 6.8 containing 0.05% Tween 20) was done and 100 μl per well of binding-buffer containing 0.02 M L-lysine and 0.01% NaN_3 was added. The plate was sealed and stored at 4°C until further use. The ability of thrombin to cleave fibrinogen in solid-phase was monitored using a horseradish peroxidase-labelled mouse monoclonal antibody (Y18-HRP) kindly provided by Organon Teknika (Fresnes, France). The immunoreactivity of this antibody with the Aα stretch 1–51 of human fibrinogen disappears upon treatment with thrombin thus indicating release of fibrinopeptide A.[17] When indicated the fibrin surfaces were degraded with plasmin as previously described.[16] Briefly, after washing the plate three times with assay buffer, solutions of 0–25 nM plasmin in mass buffer were incubated with the fibrin surface for 30 min at 37°C. In the next step plasmin in the fluid phase was discarded, the surface washed three times with assay buffer and finally incubated for 24 h at 22°C with assay buffer containing 0.2 M AMCHA, 1 mM benzamidine and 10 mM VPLCK. This procedure eliminates bound plasmin; indeed, no significant amidolytic activity was detected after 48 h incubation with 1.5 mM CBS 1065 at 37°C as indicated from measurements of the absorbance at 405 nm. The

degradation of the fibrin surface by plasmin was verified by the direct binding of a monoclonal antibody (FDP-14) directed against fibrin degradation products,[18] kindly provided by Dr Willem Nieuwenhuizen.

Binding of t-PA to solid-phase fibrin

Various concentrations of t-PA in mass buffer were incubated for 1 h at 37°C on the fibrin plates, after three washes with binding buffer fibrin bound t-PA was measured with a radiolabelled IgG directed against t-PA (500 000 counts/min/well in binding buffer) incubated with the surface for 1 h at 37°C. After washing, the plate was cut and the radioactivity counted in a γ-radiation counter.

Plasminogen activation by fibrin-bound t-PA was studied in a photometric assay as previously described.[14] In brief, t-PA was bound to fibrin as described above, then 50 μl of an activation mixture containing Glu-plasminogen (200 nM) and 1 mM of the chromogenic substrate S-2251 in assay buffer were added per well, and the plasmin generated was detected by measuring the release of pNA from the chromogenic substrate. The effect of varying concentrations of normal fibrin on the binding and activation of plasminogen by fibrin-bound t-PA was tested with fibrin surfaces prepared with different concentrations of normal fibrinogen. Absorbance readings were performed at double wavelength absorbance ratio (405/490 nm) at regular intervals with a microtitre plate counter (MR 5000, Dynatech Laboratories) equipped with a thermostatic device to maintain a constant 37°C. Reaction rates were determined from the time course of the overall reaction as indicated elsewhere.[14] The statistical analysis, curve fitting and graphical procedures were worked out on an IBM 8555-X31 microcomputer using a program developed in our laboratory.

Binding of plasminogen to carboxy-terminal lysines of degraded fibrin surfaces

A plate containing surfaces with an increasing number of carboxy-terminal lysines was washed with binding buffer and incubated for 18 h at 4°C with 50 μl per well of mass buffer containing 500 nM Glu-plasminogen supplemented with a trace amount of ^{125}I-Glu-plasminogen (50 μl per well = 500 000 counts/min, specific radioactivity: 80 pCi/ng). The plate was then washed three times with binding buffer and the radioactivity in the wells counted in a γ-radiation counter.

DNA amplification and sequence analysis

Genomic DNA was isolated from blood cells.[19] Two primer pairs (5′-GAAGCATCCTACGAAAGAGGG-3′-5′-AACTTGGAATCTAAGAAAGGAAACTACC-3′ and 5′-CTTCATAGACTTGCAGAG-3′-5′-AAGCAAGTCGACTGTCCAATAGGAAAAATA-3′) were used to amplify a 386 and a 883 base pair fragment containing exon VIII, IX, and X coding for the carboxyl terminus of the γ chain. Amplification by PCR[20] was performed in 100 μl containing 1 μg genomic DNA, 0.2 mM (each) dATP, dCTP, dGTP and dTTP, 0.3 μg (each) primer in 10 mM Tris–HCl (pH 8.3 at 25°C), 50 mM KCl, 3.0 mM $MgCl_2$, 0.001% gelatin, and 1 U of *Taq* DNA polymerase. The samples were heated at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 59°C for 0.5 min, and 72°C for 1.5 min. Amplified DNA was precipitated with ethanol, dried, and dissolved in 20 μl of distilled water, and run on a 1.0% (w/v) ultra-low-gelling agarose gel. The 386 and the 883 base pair bands were cut out of the gel in a gel volume of approximately 20 μl, heated to 55°C and 1 μl was combined with 60 ng of either of the amplification oligos, heat denatured, annealed on ice and directly sequenced using a T7 DNA sequencing kit.

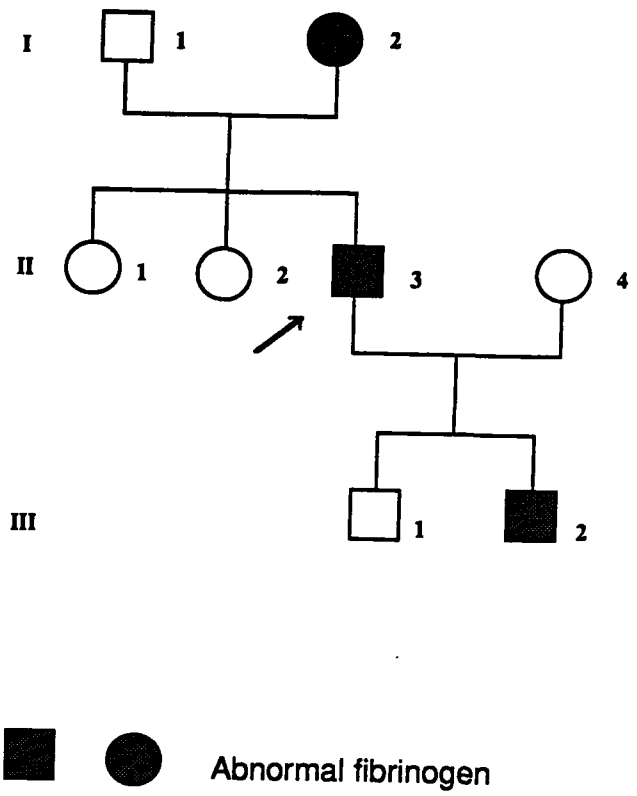

Figure 1. Pedigree of fibrinogen Bicêtre II. The propositus is indicated by an arrow.

Results

Case report

A previously healthy man developed a thrombotic event at age 40, consisting of spontaneous deep venous

Table 1. Thrombin and reptilase times, and fibrinogen levels in the three affected family members

Case No.	Thrombin time (s)	Reptilase time (s)	Fibrinogen clottable (g/l)	Fibrinogen antigen (g/l)
I–2 (BP)	39	37	1.6	4.7
II–3 (BM)	40	29	1.3	3.7
III–2	42	33	0.85	2.3
Normal range	< 20	< 23	2–4	2–4

Table 2. Plasma levels of t-PA, PAI-1, plasminogen and α_2-antiplasmin

Samples	t-PA antigen (ng/ml)	PAI-1 antigen (ng/ml)	Plasminogen antigen (μg/ml)	α_2-Antiplasmin activity (%)
Plasma pool	4.8	13.69	1.48	100
II–3 (BM)	5.4	13.07	1.4	115
I–2 (BP)	8.2	23.02	1.45	115

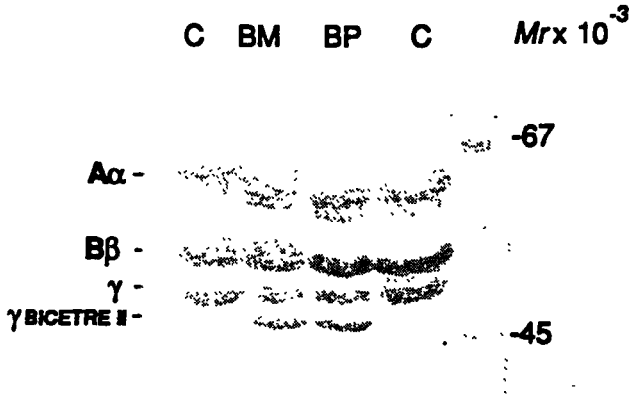

Figure 2. SDS–PAGE analysis of purified fibrinogens from BM (II–3), BP (I–2) and the control (C) after Coomassie blue staining. Electrophoresis was performed under reducing conditions using a 7.5% polyacrylamide slab gel. Molecular mass reference proteins (bovine serum albumin, 67 kDa; ovalbumin, 45 kDa) are indicated on the right. The slight difference in the migration of the Aα chains is artefactual.

thrombophlebitis of the right leg. Diagnosis was confirmed by phlebography. He was admitted to hospital and treated with unfractionated heparin. In the meantime pulmonary embolism was suspected and confirmed by scintigraphy. Oral anticoagulant therapy was then started. Smoking and obesity were the two risk factors observed. No familial history of thrombosis could be documented. The propositus (II–3), his mother (I–2) and his son (III–2) had prolonged thrombin and reptilase times and the haemostatic parameters of these three affected members were determined. The pedigree of the investigated family with dysfibrinogenaemia is shown in Figure 1.

Haemostatic studies

These results are summarized in Table 1. The prolongation of the thrombin and reptilase times were observed in the three affected members of this family. The fibrinogen level was decreased to 1.3 g/l when measured by the chronometric method, whereas this level was higher when measured by clot weight or immunological assays, with values of 3.7 and 3.5 g/l, respectively. Total protein S antigen, C4 binding protein, protein C, antithrombin III and fibrin(ogen) degradation products were in the normal range (data not shown). The plasma levels of t-PA, PAI-1, plasminogen and α_2-antiplasmin were normal (Table 2).

SDS–PAGE of purified fibrinogen

Purified fibrinogen of the propositus and his mother was analysed by SDS–PAGE under reducing conditions on 7.5% slab gel according to Laemmli.[11] The Aα and Bβ chains of the abnormal fibrinogens migrated at a position similar to that of the control. In contrast, two types of γ chains were observed, one migrating as the control γ chain with a relative M_r of 50 000 and the other (about 50% determined by scanning densitometry) migrating in a more anodic position with an M_r of 48 000 (Figure 2).

Binding of t-PA to fibrin, and plasminogen activation by fibrin bound t-PA

The binding of t-PA to the fibrin surface was detected with a polyclonal radiolabelled IgG directed against t-PA. The binding isotherms of t-PA to fibrin as a function of the concentration of t-PA is shown in Figure 3. No difference was observed between fibrin obtained from a normal control and fibrin obtained from the abnormal fibrinogen. Plasminogen activation

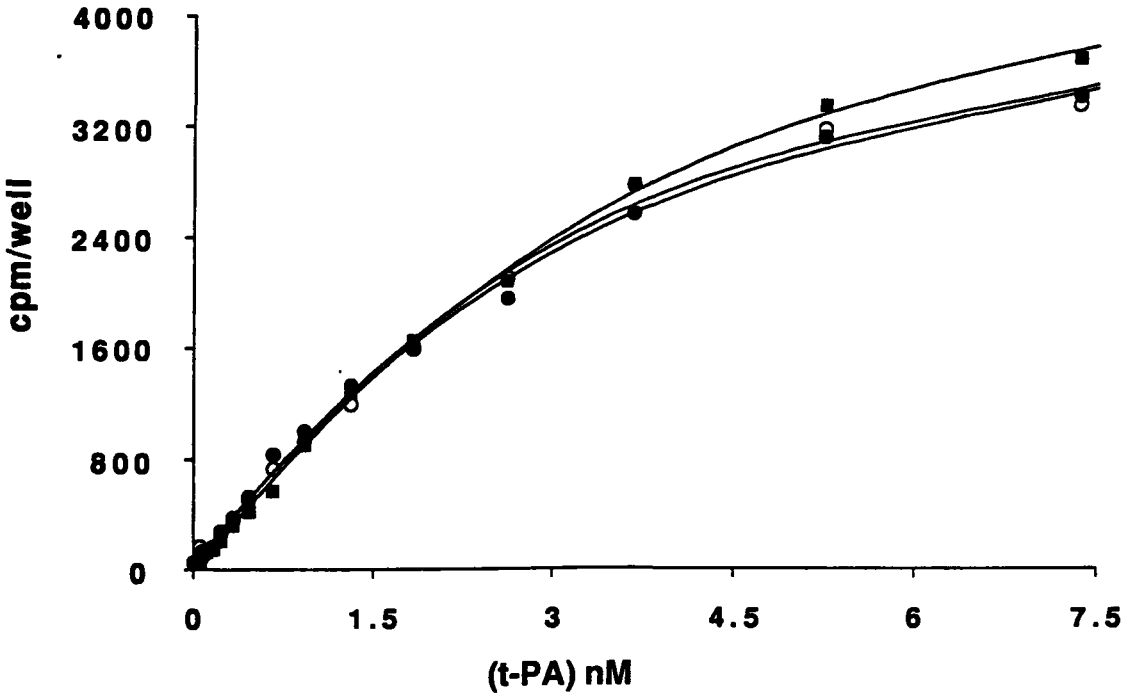

Figure 3. Binding of t-PA to solid-phase fibrin prepared with fibrinogen from II–3 (●), I–2 (■), and the control (○). Fifty microlitres per well of t-PA at varying concentrations (0–7.5 nM) in mass buffer were incubated for 1 h at 37°C with the fibrin surface. Unbound proteins were then eliminated by washing with binding buffer. Fibrin-bound t-PA was detected by incubation for 1 h at 37°C with a goat anti-human t-PA IgG (500 000 counts/min/50 ml). After washing, each well was cut and counted in a γ-radiation counter. Fibrin bound t-PA expressed in counts/min/well is plotted against the concentration of added t-PA.

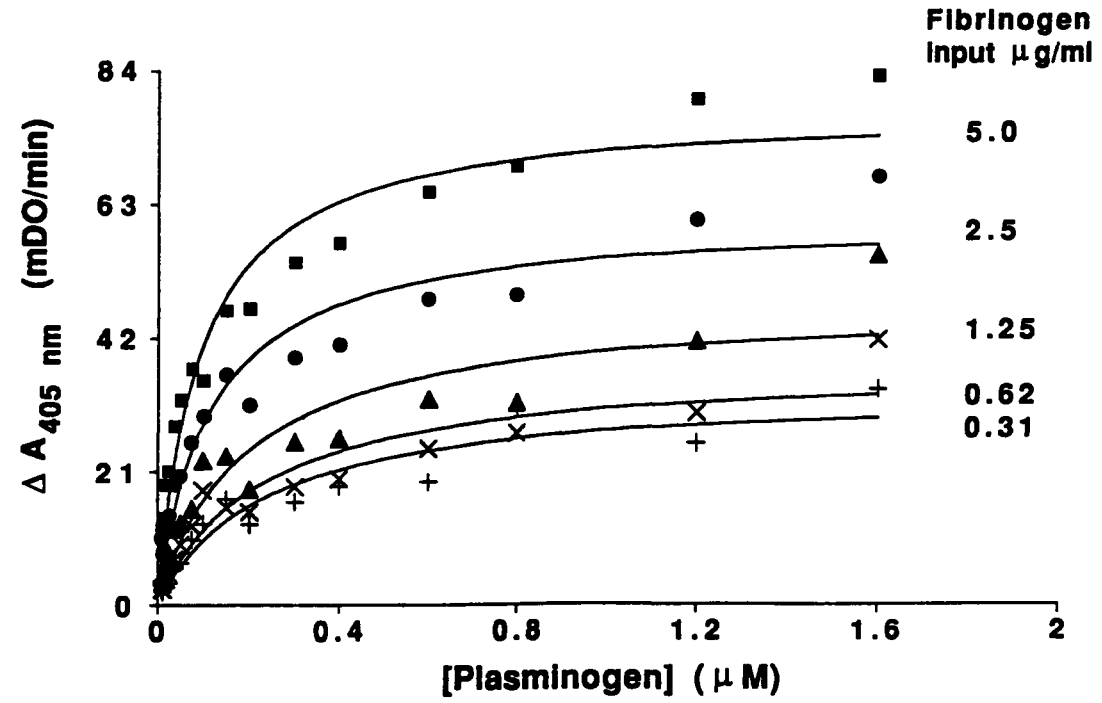

Figure 4. Plasminogen activation by t-PA bound to fibrin surfaces prepared with varying concentrations of normal fibrinogen. Fibrin surfaces containing varying amounts of fibrin were prepared with various concentrations of normal fibrinogen as indicated in Methods. A constant amount of t-PA (30 IU/ml in mass buffer) was then incubated with fibrin for 1 h at 37°C, and the activation of different concentrations of plasminogen was determined as indicated in Figure 5.

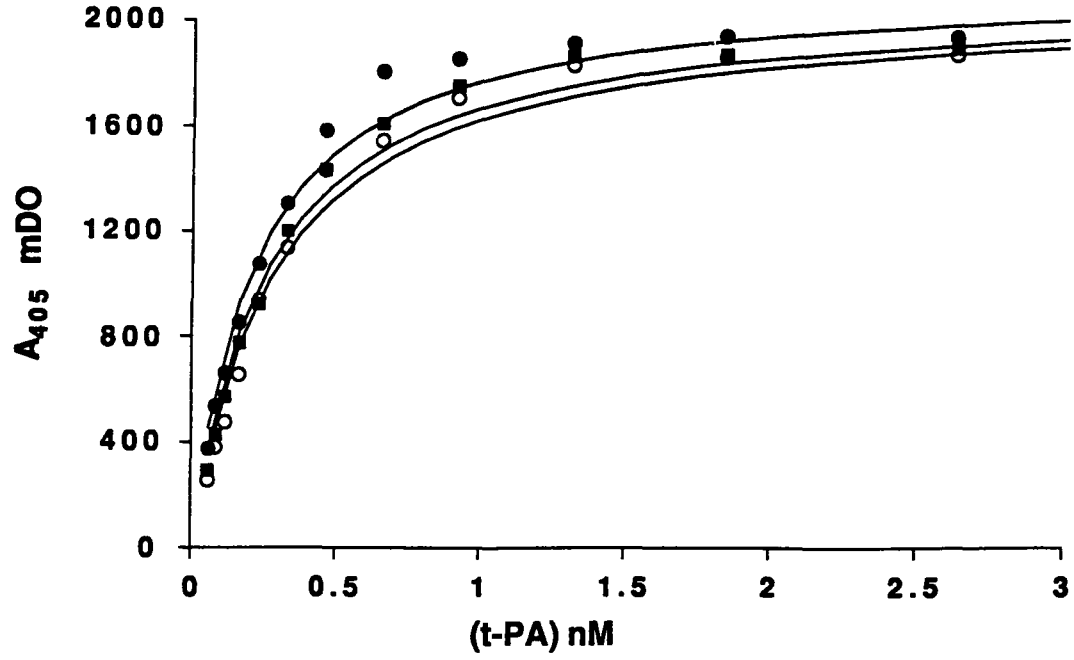

Figure 5. Plasminogen activation by fibrin-bound t-PA. Various amounts of t-PA in mass buffer were incubated for 1 h at 37°C with fibrin plates from II–3 (●), I–2 (■), and the control (○). After washing, fibrin bound t-PA was tested for its ability to activate plasminogen by adding activation mixture containing 200 nM Glu-plasminogen and a chromogenic substrate selective for plasmin (S-2251, 1 mM). Absorbance readings at 405 nm were plotted *versus* the concentration of t-PA.

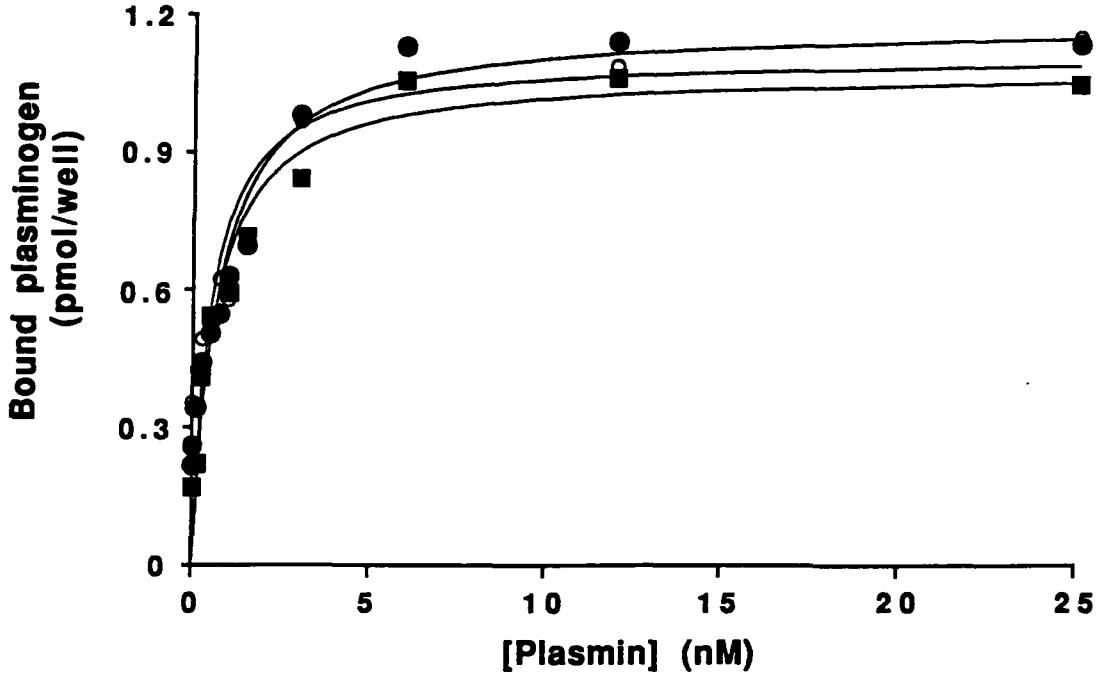

Figure 6. Binding of ^{125}I-Glu-plasminogen to plasmin degraded fibrin. Solid-phase fibrin surfaces from II–3 (●), I–2 (■), and control (○) were degraded with increasing amounts of plasmin (0–25 nM) as described in Methods. After elution of plasmin with AMCHA and washing, 50 ml of mass buffer containing 500 nM of Glu-plasminogen supplemented with trace amounts of ^{125}I-Glu-plasminogen (500 000 counts/min/well) were incubated for 18 h at 4°C. After washing, the plates were cut off and the wells counted in a γ-radiation counter. Fibrin-bound Glu-plasminogen expressed in pmol/well is plotted against the concentration of plasmin used to degrade fibrin.

by fibrin bound t-PA was measured by the release of pNA from the plasmin-selective chromogenic substrate S-2251. As shown in Figure 4, a diminution of no more than 50% in the concentration of normal fibrin affects the activation of plasminogen in this model system. Although the fibrinogen purified from the affected members of this family contained about 50% abnormal γ chains, no significant differences on the generation of plasmin by t-PA bound to fibrin from the control and the propositus were observed (Figure 5). We can therefore conclude that both the stimulation of t-PA activity and the binding and activation of plasminogen by the abnormal fibrin were normal.

Binding of plasminogen to fibrin surfaces

The binding of plasminogen to fibrin surfaces prepared with fibrinogen from the propositus, his mother and the control was further investigated using surfaces with different states of fibrin degradation. These surfaces are an equivalent of the state of fibrin during ongoing fibrinolysis and were obtained by treatment with varying concentrations of plasmin. The binding of plasminogen to these surfaces (Figure 6) was saturable and specifically inhibited by 6-AHA or by treatment of the degraded surface with carboxypeptidase B as previously described.[16] Results obtained with fibrin surfaces prepared with fibrinogen from II–3, I–2 and the control were identical, indicating that the binding of plasminogen to carboxy-terminal lysine residues produced by plasmin was not modified by the mutation.

DNA sequence analysis of both strands of the amplification products demonstrated that one of the fragments contains a single base substitution in the codon Asn_{308} (AAT) of the γ chain, changing this to the codon for Lys (AAG). No other changes were found. Direct sequence determination of the amplified fragment shows both the normal and the abnormal sequence, indicating that the patient is heterozygous for this mutation.

Discussion

More than 200 dysfibrinogenaemias have been reported.[1] Thromboembolic manifestations are observed in 18% of patients[1] and in five cases the dysfibrinogenaemia affected the fibrinolytic system.[2,5,20] The C-terminal end of the γ chain was affected in 19 cases.[1] The abnormality of fibrinogen Bicêtre II was also localized in the γ chain and was detected in a young male patient with spontaneous thrombophlebitis complicated with pulmonary embolism.

A substitution in Asn_{308}→Lys of the γ chain of fibrinogen Bicêtre II was identified by DNA sequence analysis. The abnormal γ chain represented about 50% of the total and migrated to an anodic position corresponding to an M_r of 48 000. A doublet in SDS–PAGE performed under reducing conditions was therefore observed. The change in the migration of the affected γ chain after electrophoresis according to Laemmli, may be explained by a change in local conformation affecting the degree of hydrophobicity and thereby the mobility of proteins in the presence of SDS.[21] This phenomenon is not specific for mutations at position 308 of the γ chain; it has been found also in fibrinogens Vlissingen,[22] Baltimore III[23] and Tochigi.[24]

A similar mutation has been previously reported for dysfibrinogenaemia Kyoto I.[25,26] Yoshida *et al.*[25] have shown an accelerated digestion of fragment D1 of fibrinogen Kyoto I resulting from a new cleavage site between Lys_{308} and Gly_{309} that increases the accessibility of γ Lys_{356}–Ala_{357} and γ Lys_{302}–Phe_{303} peptide bonds to plasmin. Since fibrinogens Kyoto I and Bicêtre II share a similar mutation in the γ chain, the interactions of t-PA and plasminogen with intact and degraded fibrin generated from fibrinogen Bicêtre II, respectively, have been characterized in the present study.

No significant differences in the binding of t-PA to fibrin from the propositus and to fibrin from a control were observed, despite the proximity of the mutation with a new t-PA binding site localized on the γ 311–379 remnant.[27] Fibrin prepared from the abnormal fibrinogen stimulated the activity of t-PA to a similar extent as fibrin prepared from a control fibrinogen.

The binding of plasminogen to carboxy-terminal lysine residues of degraded fibrin is the main pathway for the amplification of fibrinolysis. An increase or decrease in the number of such sites may modify this mechanism. Despite the presence of an extra lysine residue in position 308 of dysfibrinogen Bicêtre II, the number of binding sites for plasminogen after degradation of the intact fibrin surface with plasmin, was not modified. The absence of increase in the binding of plasminogen suggests (i) that the new mutation does not modify the accessibility of buried lysine bonds in fibrin, and (ii) that cleavage of the Lys_{308}–Gly_{309} bond may not occur in fibrin.

The absence of thrombotic episodes in the mother of the propositus despite three pregnancies and the isolated thrombotic event of the patient, together with the normal fibrinolytic tests described here, suggest that this dysfibrinogenaemia may not be associated with a thrombotic tendency. Further investigations of fibrinogen interactions with either platelets or other haemostatic proteins are, however, necessary to explain the thrombotic event of the propositus.

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