# Fibrinogen Alès: a homozygous case of dysfibrinogenemia ( $\gamma$ -Asp<sup>330</sup> $\rightarrow$ Val) characterized by a defective fibrin polymerization site "a"

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Congenital homozygous dysfibrinogenemia was diagnosed in a man with a history of 2 thrombotic strokes before age 30. His hemostatic profile was characterized by a dramatically prolonged plasma thrombin clotting time, and no clotting was observed with reptilase. Complete clotting of the abnormal fibrinogen occurred after a prolonged incubation of plasma with thrombin. The release of fibrinopeptides A and B by thrombin and of fibrinopeptide A by reptilase were both normal. Thrombin-induced fibrin polymerization was impaired, and no polymerization occurred with reptilase. The polymerization defect was characterized by a defective site "a," resulting in an absence of interaction between sites A and a, indicated by the lack of fragment D<sub>1</sub> (or fibrinogen) binding to normal fibrin monomers depleted in fibrinopeptide A only (Des-AA fm). By SDS-PAGE, the defect was detected on the  $\gamma$ -chain and in its fragment D<sub>1</sub>. The molecular defect determined by analysis of genomic DNA showed a single base change (A $\rightarrow$ T) in exon VIII of the  $\gamma$ -chain. The resulting change in the amino acid structure is  $\gamma$ 330 aspartic acid (GAT)  $\rightarrow$  valine (GTT). It is concluded that the residue  $\gamma$ -Asp<sup>330</sup> is essential for the normal functioning of the polymerization site a on the fibrinogen  $\gamma$ -chain. (Blood. 2000;96:3473-3479)

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## Introduction

Fibrinogen is a disulfide-bridged protein of 340 000 d comprised of 2 symmetric half-molecules, each having 3 different polypeptides, designated A $\alpha$ -, B $\beta$ -, and  $\gamma$ -chains. During coagulation, thrombin cleaves the amino-termini of the Aa- and BB-chains of fibrinogen, releasing fibrinopeptides A and B, respectively, and converting fibrinogen to fibrin monomers. The spontaneous polymerization of fibrin monomers initiates fibrin clotting with the formation of protofibrils. The newly exposed chain amino-terminus (GPR) of one fibrin molecule, which is referred to as the "A" polymerization site, binds noncovalently to a complementary polymerization pocket, termed the "a" site, in the carboxyl terminus region of the  $\gamma$ -chain of a second fibrin(ogen) molecule. This A-a interaction aligns the fibrin monomers into half-staggered, 2-stranded protofibrils. Subsequently, the growing fibrils aggregate in a lateral fashion to form fibers, and this presumably involves interactions between the amino terminus of the  $\beta$ -chain, the "B" polymerization site, and a complementary site, "b," whose location is uncertain.<sup>1-5</sup> Under physiological conditions, the release of fibrinopeptide B follows the release of fibrinopeptide A and correlates with lateral aggregation.<sup>1</sup> This sequential release of fibrinopeptides, so that the A site appears before the B site, may control the final clot structure.6

During the early stages of the fibrin polymerization process, protofibrils are stabilized by the factor XIIIa-catalyzed formation of cross-links between the  $\gamma$ -chains of the assembling fibrin molecules, and, in a slower process, multiple cross-links form between  $\alpha$ -chains.<sup>7</sup>

More than 240 cases of inherited dysfibrinogenemia have been

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reported,<sup>8</sup> and most were detected because of a prolonged thrombin clotting time related to a defect in the conversion of fibrinogen to fibrin. Many of these molecules have abnormalities located in the carboxyl terminus of the  $\gamma$ -chain and are characterized by a defective fibrin polymerization.<sup>9</sup>

In this study, we report a homozygous case of congenital dysfibrinogenemia, designated fibrinogen Alès, which is characterized by amino acid substitution of Asp for Val at position 330 in the  $\gamma$ -chain. This mutation is responsible for impaired fibrin polymerization owing to defective fibrin polymerization site a, which does not interact with its complementary polymerization site A.

## Materials and methods

## **Routine hemostasis studies**

Blood was collected into 0.13 mol/L trisodium citrate (1 part citrate and 9 parts blood). Plasma was obtained by centrifugation at 3000g for 20 minutes at 4°C and stored at  $-80^{\circ}$ C.

Whole blood clotting time, recalcification time, 1-stage prothrombin time, and coagulation factor II, V, and VII + X quantification were performed by conventional methods reported in Caen et al.<sup>10</sup> Thrombin and reptilase clotting times were determined using several concentrations of reagents diluted either in 150 mmol/L NaCl or in 25 mmol/L CaCl<sub>2</sub>. Fibrinogen cofactor activity in adenosine diphosphate–induced platelet aggregation was determined using normal washed platelets prepared according to Patscheke and Worner.<sup>11</sup> Plasma fibrinogen concentration was determined by a functional assay according to Clauss,<sup>12</sup> by an immunologic assay method according to Mancini et al,<sup>13</sup> and by heat precipitation at 56°C

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as described previously.<sup>14</sup> Fibrinogen-related antigen in serum was determined by enzyme-linked immunosorbent assay as described by Soria et al.<sup>15</sup> Levels of known risk factors recommended by the International Society of Thrombosis and Haemostasis (ISTH)<sup>16</sup> (protein C, protein S, antithrombin III, homocysteine, lupus anticoagulant, lipid analysis) were determined in routine laboratories. Genetic analysis for factor V Leiden was performed as described by Bertina,<sup>17</sup> and prothrombin gene 20210 mutation was conducted as described by Poort et al.<sup>18</sup> Apolipoprotein genotype was performed by gene amplification according to Hixson and Vernier.<sup>19</sup>

#### **Fibrinogen purification**

Fibrinogen was purified according to Blombäck and Blombäck.<sup>20</sup> Fibrinogen preparations were undegraded as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions.<sup>21</sup> The purified fibrinogen (98% purity) was dissolved in 150 mmol/L NaCl and stored at  $-80^{\circ}$ C.

#### Analysis of the prolonged thrombin time

Fibrin polymerization curves were made according to Bouvier and Gruedlinger.<sup>22</sup> Aggregation of fibrin monomer was studied as described by Belitser et al.<sup>23</sup> The release of FpA and FpB after thrombin addition to purified fibrinogen (and that of FpA after reptilase addition) was examined by high-performance liquid chromatography (HPLC) as described by Kehl et al.<sup>24</sup> Cross-linking of fibrin ( $\gamma$  dimerization and  $\alpha$  polymerization) was analyzed by SDS-PAGE according to Weber and Osborn.<sup>21</sup> Fibrin stabilized by factor XIIIa was obtained according to Schwartz et al.<sup>25</sup>

#### Plasmin digests of fibrinogen

Plasmin degradation of fibrinogen was performed in the presence of 5 mmol/L CaCl<sub>2</sub> or 10 mmol/L EDTA, according to Haverkate and Timan.<sup>26</sup> The samples were loaded onto a 10% or 7.5% SDS-PAGE gel according to Laemmli.<sup>27</sup>

## Binding of purified fragment D<sub>1</sub> (or of fibrinogen) to immobilized normal fibrin monomers depleted in fibrinopeptide A (Des-AA fm)

**Purification of fragment**  $D_I$ **.** Purified normal fibrinogen and fibrinogen Alès were degraded with plasmin (final concentration, 2 U/mL) in the presence of 5 mmol/L CaCl<sub>2</sub> for 4 hours at 37°C. The digests were chromatographed on Sepharose–Lysine (Pharmacia, Uppsala, Sweden) to remove plasmin. After an extensive dialysis against 100 mmol/L Tris-HCl, pH 8.6, the fragment D<sub>1</sub> was isolated by DEAE cellulose chromatography as described by Nilehn.<sup>28</sup> The purity of fragment D<sub>1</sub> was tested by SDS-PAGE, and its concentration was calculated from absorbance at 280 nm (A, 1%; 1 cm = 11.20).

**Preparation of Des-AA fm.** Normal human fibrinogen (Kabi AB, Stockholm, Sweden) at 5 mg/mL was clotted with 0.2 U/mL reptilase in the presence of 5 mmol/L EDTA for 30 minutes at 37°C. The fibrin clot was collected, washed extensively with 150 mmol/L NaCl, and dissolved in 4 mol/L urea or in 25 mmol/L acetic acid. The concentration of Des-AA fm was calculated from absorbance at 280 nm (A, 1%; 1 cm = 15.0).

Binding of fragment  $D_1$  to immobilized Des-AA fm. Des-AA fm (or fibrinogen) solution at 5 µg/mL in urea 4 mol/L was immobilized on glutaraldehyde-treated 96-well microplates, as described by Stanilawski et al.<sup>29</sup> To the coated wells, 100 µL of a solution of fragment  $D_1$  at concentrations ranging from 1.5 to 25 µg/mL were added. After an incubation period of 2 hours at 37°C, the plates were washed 4 times. Then, in each well, 100 µL of a monoclonal antibody that specifically reacts with an antigenic neo-epitope on fragment  $D_1$  (anti-D neo-F2C5), previously described by Mirshahi et al,<sup>30</sup> was added. After a 2-hour incubation period, the plates were washed, and bound anti-D neo-F2C5 was detected by adding 100 µL of 0.1% diluted peroxidase-labeled rabbit immunoglobulin anti-mouse to each well (Amersham France SA, Les Ulis, France). All the dilutions were performed in phosphate-buffered saline containing 0.05% Tween 20 and 0.1% serum bovine albumin. After an incubation period of 1 hour at 37°C, the plates were washed, and the amount of bound peroxidase was quantified by adding orthophenylene diamine and perhydrol (Sigma Chemical, St Quentin Fallavier, France) as described by Wolters et al.<sup>31</sup>

Binding of fibrinogen to immobilized Des-AA fm. Microplates were coated with 100  $\mu$ L per well of normal Des-AA fm solution at 5  $\mu$ g/mL in 25 mmol/L acetic acid for 18 hours at 4°C. The plates were washed. Then 100  $\mu$ L normal fibrinogen or fibrinogen Alès, at concentrations ranging from 0.1 to 10  $\mu$ g/mL in Tris-buffer saline-Tween (TBST) containing 1 mmol/L CaCl<sub>2</sub>, were introduced into each well and incubated for 2 hours at room temperature. Identical incubations were performed using 10  $\mu$ g/mL fibrinogen solution containing Gly-Pro-Arg-Pro peptide at concentrations ranging from 25 nmol/L to 1500 nmol/L. The amount of fibrinogen bound to Des-AA fm was measured using a peroxidase-conjugate monoclonal antibody Y18-peroxidase (Gaubius Laboratory, Leiden, The Netherlands) that reacts with FpA. Immunoconjugate bound to the plates was determined with tetra methyl benzidine and perhydrol as the chromogen (Organon Teknika, Boxtel, The Netherlands).

#### Isolation of genomic DNA, amplification, cloning, and sequencing

Genomic DNA was isolated from blood cells according to the manufacturer's instructions using genomic DNA isolation kit (Gentra System, Minneapolis, MN). A 385-bp fragment encoding exon VIII (amino acids 259 to 350) and an 823-bp fragment encoding the exon IX (amino acids 351 to 427) of the  $\gamma$ -chain were amplified by polymerase chain reaction (PCR) with the following primer pairs: VIII $\gamma$ R 5'-AACTTGGAATCTAAGAAAG-GAAAACTACC-3', VIII $\gamma$ F 5'-GAAGCATCCTACGAAAGAGGG-3' and IX $\gamma$ R 5'-AAAAAAGGAATTCTCTTTTGAAACGGTC-3', IX $\gamma$ F 5'-GA-AGCATCCTACGAAAGAGGG-3'.

Amplification was performed in a 50-µL reaction volume containing 0.3 µg genomic DNA, 5 U Taq polymerase (Gibco BRL), 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Orsay, France), and 0.2 mmol/L each of primer VIII $\gamma$ R and VIII $\gamma$ F or IX $\gamma$ R and IX $\gamma$ F in reaction buffer (20 mmol/L Tris-HCl, pH 8.4, at 25°C, 50 mmol/L KCl, and 2.5 mmol/L MgCl<sub>2</sub>). Samples were heated at 94°C for 4 minutes and were incubated for 37 cycles of 1 minute at 95°C, 1 minute at 57°C, and 2 minutes at 72°C. Twenty microliters amplified DNA mixture was run on a 1.5% (wt/vol) agarose gel (Boehringer Mannheim, Mannheim, Germany). Bands of the appropriate size, as predicted from the genomic DNA of the  $\gamma$ -chain sequence,<sup>32</sup> were cut out and isolated by Jetsorb kit (Genomed, Uithoorn, The Netherlands). The purified double-stranded DNA fragment was then cloned into *Eco*RV-digested pMOSBlue T-vector (Amersham, Orsay, France), and individual clones were sequenced using a T7 DNA sequencing kit (Pharmacia).

#### Determination of endogenous thrombin potential

Endogenous thrombin potential determination was performed according to Hemker et al<sup>33</sup> on the basis of the time course of amidolytic activity using chromothrombin (Stago, Asnières, France) after the triggering of thrombin generation by calcium. Experiments were performed using both native citrated plasma and normal plasma that was depleted in its fibrinogen by removing the clot formed by the addition of reptilase.

## Results

#### **Clinical and laboratory data**

Fibrinogen Alès was discovered in a 25-year-old man admitted to the hospital for multiple traumas. During this period there were no signs of any unusual bleeding or thrombotic tendency. However, at the age of 30, the propositus suffered 2 episodes of thrombotic stroke, as demonstrated by angiography. No history of hemorrhage or thrombotic tendency was observed in the propositus' parents or in his sister (35 years old). The known risk factors for thrombosis, suggested by ISTH,<sup>16</sup> were absent in the propositus and his sister. Levels of protein C, protein S, and antithrombin III were within the normal range; levels of triglycerides, total cholesterol, and LDL/

Table 1. Routine coagulation studies from the propositus and the members of his family

	Propositus	Sister	Father	Mother	Contro
Prothrombin time (sec)*	>180	>180	16.5	15.5	13
Thrombin time (sec)					
Without CaCl <sub>2</sub>	300	380	30	30	20
With CaCl <sub>2</sub>	300	380	ND	ND	17
Reptilase time (sec)	Unclottable	Unclottable	ND	ND	20
Reptilase (×10)	Unclottable	Unclottable	ND	ND	10
Fibrinogen (g/L)					
Functional assay	0.20	0.20	0.80	0.80	2 ñ 4
Heat precipitation	3.15	3.30	3.70	4.00	2 ñ 4
Immunologic assay	3.05	3.02	4.00	4.40	2 ñ 4
Fibrinogen-related					
antigen in serum					
(μg/mL)	20	20	ND	ND	20



HDL cholesterol were normal; lupus anticoagulant and anticardiolipid findings were negative. The level of homocysteine was also found to be within the normal range. Neither the factor V Leiden nor prothrombin gene 20210 G $\rightarrow$ A polymorphisms were present in the propositus or his sister. Apo E genotype was found to be epsilon 3/epsilon 4 for the propositus and his sister.

Results of the coagulation screening tests of the propositus and his family members are summarized in Table 1. He and his sister had the same profile. Prothrombin time was markedly prolonged, whereas the blood factors II, V, and VII + X were within normal range. Thrombin time was markedly prolonged, resulting in a small and weak clot. Reptilase time was infinite; no clot was observed with this enzyme. The presence of calcium chloride (from 25 to 100 mmol/L) did not induce any correction of the thrombin time. Polymerization curve, performed with the plasma of the propositus, did not show any increase of turbidity when thrombin was added to plasma diluted 1:10. However, using a lower dilution of the plasma (1:3), a prolonged lag time and a low polymerization rate were demonstrated in comparison with the polymerization curve of the normal plasma diluted to 1:10 (Figure 1).

Plasma fibrinogen level determined by the functional assay of Clauss<sup>12</sup> was markedly decreased, but when it was determined by heat precipitation and the immunological method, the results were normal. Platelet count and platelet aggregation levels were both normal. The fibrinogen-related antigen, which remained in serum



Figure 1. Coagulation profiles of fibrinogen. Normal plasma and plasma from the propositus, used at the same immunologic concentration of fibrinogen (4 mg/mL), were diluted in 150 mmol/L NaCl. After thrombin addition, the optical density (OD) was recorded at 350 nm. Normal plasma diluted to 1:10  $(\bigcirc)$ , plasma from the propositus diluted to 1:10  $(\spadesuit)$ , and plasma from the propositus diluted to 1:3 ( $\blacktriangle$ ).



**Figure 2.** Polymerization of isolated fibrin monomers. To 2.8 mL phosphate buffer at pH 7.4 was added 0.2 mL fibrin monomers at 6 mg/mL. OD was recorded at 350 nm. Normal fibrin monomers ( $\bigcirc$ ), fibrin monomers from the propositus (●).

obtained after 18-hour clotting of the plasma with thrombin, was normal.

The father and the mother of the propositus, who are cousins, also had abnormal fibrin formation that was less severe than that observed in their children. These data indicate that the patient and his sister are homozygous and that his dysfibrinogenemia is congenital.

### Coagulation of purified fibrinogen

The thrombin clotting time of purified fibrinogen Alès was markedly prolonged compared to normal fibrinogen (165 seconds; control, 15 seconds), and the reptilase time was infinite because fibrinogen Alès remained unclottable even when a high concentration of reptilase was used.

The HPLC profiles of FpA and FpB, released by thrombin from fibrinogen Alès, were normal with respect to both the initial rate and the total amount released. Furthermore, the rate of FpA released after the addition of reptilase to fibrinogen Alès was normal (data not shown).

Fibrin monomer aggregation of Des-AABB-fm (fibrin monomers depleted in fibrinopeptides A and B), derived from the abnormal fibrinogen, did not show any increase of the turbidity (Figure 2). The analysis of the cross-linking profiles of fibrin mediated by factor XIIIa showed that  $\gamma$ -chains from fibrinogen Alès were converted to  $\gamma$ -dimers and that all the  $\alpha$ -chains were converted to  $\alpha$ -polymers (data not shown).

## Analysis of fibrinogen molecule

SDS-PAGE of purified fibrinogen Alès was carried out under reducing conditions according to the method of Laemmli.<sup>27</sup> A  $\gamma$ -chain with higher mobility than that of the normal  $\gamma$ -chain was observed (Figure 3A), and there was no  $\gamma$ -chain with a normal



Figure 3. SDS-PAGE of purified fibrinogen after reduction with 2-mercaptoethanol. (A) 10% SDS-PAGE according to Laemmli.<sup>27</sup> (B) 10% SDS-PAGE according to Weber and Osborn.<sup>21</sup> Lane 1, normal fibrinogen; lane 2, fibrinogen Alès.



Figure 4. Plasmin degradation of fibrinogen analyzed by SDS-PAGE according to Laemmli after reduction with 2-mercaptoethanol. Lanes 1 and 2, plasmin digest of normal fibrinogen in the presence of 10 mmol/L EDTA and 5 mmol/L CaCl<sub>2</sub>, respectively. Lanes 3 and 4, plasmin digest of fibrinogen Alès in the presence of 10 mmol/L EDTA and 5 mmol/L CaCl<sub>2</sub>, respectively.

mobility. In contrast, SDS-PAGE, performed according to the method of Weber and Osborn,<sup>21</sup> showed no difference in electrophoretic migration between  $\gamma$ -chains of normal fibrinogen and those of fibrinogen Alès (Figure 3B).

#### Plasmin digestion of purified fibrinogen

When plasmin digestion was performed in the presence of calcium and the profile of the plasmin digest of fibrinogen Alès was analyzed by SDS-PAGE under reducing conditions, only the  $\gamma$ -chain and the  $\beta$ -chain remnants of fragment D<sub>1</sub> remained, and the Alès  $\gamma$ -chain remnant of fragment D<sub>1</sub> ( $\gamma$ Alès/D<sub>1</sub>) had a higher mobility than that of normal fragment D<sub>1</sub> ( $\gamma$ /D<sub>1</sub>) (Figure 4, lanes 2, 4).

When plasmin digestion was performed in the presence of EDTA, the profile of fibrinogen digest from Alès revealed  $\gamma$ - and  $\beta$ -chain remnants of fragment D<sub>3</sub>. Under this condition, the Alès  $\gamma$ -chain remnant of fragment D<sub>3</sub> did not differ from the normal one (Figure 4, lanes 1, 3).

#### Binding of fibrinogen and its derived fragment D1 to Des-AA fm

In contrast to normal fragment  $D_1$ , the fragment  $D_1$  isolated from fibrinogen Alès did not bind to immobilized normal Des-AA fm (Figure 5). In the same way, fibrinogen Alès did not bind to Des-AA fm (Figure 6). As a control, it was shown that 750 nmol/L of the peptide Gly-Pro-Arg-Pro, (corresponding to the site A), inhibited the binding of normal fibrinogen to Des-AA fm by more than 80%.



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Figure 6. Binding of normal fibrinogen and fibrinogen Alès to insolubilized normal Des-AA fm in the presence of 1 mmol/L CaCl<sub>2</sub>. Fibrinogen bound to immobilized Des-AA fm was evaluated using an antibody against fibrinopeptide A. Normal fibrinogen (open circles), fibrinogen Alès (filled circles).

#### Determination of the $\gamma$ -chain defect in fibrinogen Alès

Considering the abnormal migration of the  $\gamma$ -chain as shown by SDS-PAGE and the localization of the abnormality to the carboxyl terminus part of the  $\gamma$ -chain by plasmic digest, 2 DNA fragments comprising exons VIII and IX encoding for amino acids 259 through 350 and 351 through 427 of the  $\gamma$ -chain were amplified by PCR. The DNA sequence of the amplified exon IX from our patient was indistinguishable from the DNA normal sequence. DNA sequence analysis of exon VIII from Alès demonstrated a single base substitution in the codon for Asp<sup>330</sup> (GAT), changing this for the codon for Val (GTT) (Figure 7). Sequence analysis of 10 clones derived from DNA of the patient showed only the abnormal sequence, indicating that this patient is homozygous.

## Analysis of endogenous thrombin potential

Endogenous thrombin potential (ETP), the area under the thrombin generation curve, was higher in normal fibrinogen-depleted plasma than in the corresponding untreated plasma. Thrombin measured at the peak from 5 normal healthy volunteers was 50% to 63% of that measured using the corresponding reptilase-treated plasmas, indicating that 50% to 37% of thrombin was bound to the clot.

In contrast, ETP in Alès plasma (experiment performed in triplicate) was found to be much higher than that observed using the pool of 5 normal untreated plasmas. Both plasmas, however, have the same immunologic level of fibrinogen. Furthermore, ETP



Figure 5. Binding of purified normal fragment D1 and of purified fragment D1 from fibrinogen Alès to insolubilized normal Des-AA fm. Fragment D bound to immobilized Des-AA fm was evaluated using an anti-D neo-monoclonal antibody. Normal fragment D1 (○), fragment D1 from fibrinogen Alès (●).

Figure 7. DNA sequence analysis of a mixture of clones containing the amplified exon VIII of the  $\gamma$ -chain gene coding for fibrinogen Alès. An adenine was substituted by a thymine (arrow indicates the mutation), changing the triplet GAT (Asp) to GTT (Val).



Figure 8. Endogenous thrombin potential. Time-course of amidolytic activity using chromothrombin (Stago, Asnières, France) after adding calcium to the plasma to trigger thrombin generation. Normal plasma ( $\bigcirc$ ), reptilase-treated normal plasma ( $\triangle$ ), Alès plasma ( $\bullet$ ), reptilase-treated Alès plasma ( $\bullet$ ).

was found to be similar in Alès samples, regardless of whether they were treated by reptilase (Figure 8). The coefficient of variation in thrombin evaluation measured at the peak was 8% in Alès plasma, 13% to 15% in untreated normal plasma, and 10% in reptilase-treated plasma (due to experimental error in sampling time).

## Discussion

Homozygous dysfibrinogenemia was described in 2 members of the same family (the propositus and his sister), denoted as fibrinogen Alès. Each of the parents, who are cousins, had a heterozygous form of the anomaly. The abnormal fibrinogen of the propositus was unclottable by reptilase, and the thrombin-clotting time was dramatically delayed. Fibrinogen concentration was decreased when measured by functional assay, but it was normal when determined by heat precipitation or immunologic assay. Prolongation of the thrombin clotting time was not related to the defective release of FpA or FpB. In the same way, the absence of clotting by reptilase did not result from an abnormal release of FpA. Fibrin polymerization performed by adding thrombin to plasma was markedly impaired, and the aggregation of fm derived from thrombin-treated fibrinogen Alès did not show any increase in turbidity. In addition, with plasma diluted 1:10, there was no polymerization.

SDS-PAGE of purified fibrinogen Alès indicated that the structural defect was located in the y-chain. Furthermore, because the anomaly was also detected in the  $\gamma$ -chain remnant of fragment  $D_1$  but not in the  $\gamma$ -chain remnant of fragment  $D_3$ , it was evident that the anomaly was located in the peptide removed during the conversion of fragment D<sub>1</sub> to fragment D<sub>3</sub>.<sup>34</sup> Based on these results, genomic DNA of the propositus encoding exons VIII and IX of the  $\gamma$ -chain were amplified by PCR. Sequence analysis of the amplified fragments demonstrated that the propositus was homozygous for a single base substitution (GAT $\rightarrow$ GTT) in the codon  $\gamma$ -Asp<sup>330</sup>, converting this residue to a Val. The absence of normal  $\gamma$ -chain in fibrinogen Alès is consistent with the genetic analysis, which demonstrated that the patient was homozygous for the mutation. The difference in apparent molecular weight observed by SDS-PAGE<sup>27</sup> between the  $\gamma$ -chain from Alès and that of the normal  $\gamma$ -chain was unexpected, but this has been reported<sup>35</sup> previously for other fibrinogens with a single amino acid substitution. This change has been ascribed to an alteration in hydrophobicity or a local conformation that then affects the mobility of the protein-SDS

The A-a interaction occurring between the reptilase-activated E domain of one molecule of fibrin monomer and the D domain of another plays an essential role in fibrin formation because the cleavage of FpA alone is sufficient to induce the fibrin assembly process.<sup>36</sup> In fibrinogen Alès, this type of interaction does not occur because fragment D<sub>1</sub> from Alès, which contains the site a, does not bind to immobilized normal Des-AA fm, which has the site A available. Under the same conditions, normal fragment D<sub>1</sub> binds to Des-AA fm. Similarly, intact fibrinogen Alès does not bind to normal Des-AA fm, whereas normal fibrinogen does. As a control, it was shown that this binding process was inhibited by the addition of GPRP peptide (corresponding to the site A), which is known to compete for binding of normal fibrinogen or normal fragment D<sub>1</sub> to Des-AA fm.

Therefore, these results demonstrate that the mutation of the residue  $\gamma$ -Asp<sup>330</sup> to Val perturbs the conformation of the fibrin polymerization site a required for fibrin polymerization and leads to a nonfunctional polymerization site a in fibrinogen Alès. This same mutation was already described by Reber et al<sup>37</sup> in fibrinogen Milano I, but in this family, the affected patients were heterozygous.

Another mutation at the same position,  $\gamma$ -chain 330, was reported in a different patient with heterozygous congenital dysfibrinogenemia, fibrinogen Kyoto III (Asp $\rightarrow$ Tyr),<sup>38</sup> and was also characterized by impaired fibrin polymerization. The comparison of the sequence of the  $\gamma$ -chains of human,<sup>32</sup> rat,<sup>39</sup> and lamprey<sup>40</sup> fibrinogens showed that  $\gamma$ -Asp<sup>330</sup> is totally conserved, suggesting that this residue is essential for the function of fibrinogen.

Furthermore, in determining by crystallography the structure of the complex GPRP-fragment D<sup>41</sup> or that of the complex GPRPrFbgC30 (recombinant 30-kd protein, corresponding to the carboxyl terminus region of the  $\gamma$ -chain), it has been shown that  $\gamma$ Asp<sup>330</sup> forms a salt bridge with Arg of GPRP, providing an interaction essential for D:E binding. It has also been shown that the carboxyl group of Asp<sup>330</sup> is hydrogen-bonded to the side chain of His<sup>340</sup>, amino acid, which also forms an additional hydrogen bond with the peptide amino terminus.<sup>42</sup> Therefore, it was hypothesized that the replacement of Asp<sup>330</sup> by an uncharged amino acid residue such as Val would alter the electrostatic environment of the polymerization pocket and also the extensive and precise network of hydrogen bonds within this pocket.

The absence of interaction between the polymerization sites A and a explains the unclottability of fibrinogen Alès by reptilase. More interesting, the observation that thrombin induces clotting of fibrinogen Alès suggests that polymerization can occur only through B-b interaction because the polymerization site B is unmasked in Des-AABB fm and masked in Des-AA fm. This should be compared to the Metz fibrinogen data, reported by Soria et al,<sup>43</sup> a homozygous abnormal fibrinogen characterized by a whole blood clot formation occurring only through B-b interaction, because the addition of thrombin to fibrinogen Metz induces only the release of FpB. Therefore, because FpA is not released, this leads to an unavailability of site A. This situation represents "the other side of the coin" to fibrinogen Alès. Given that no hemorrhagic disease was observed in the propositus or in his sister, it is assumed that the clot resulting from the interaction B-b is sufficient to stop bleeding.

With respect to the 2 episodes of arterial thrombosis it seems likely that, because clotting by thrombin is dramatically delayed in the patient, thrombin generated in the Alès sample is not trapped in fibrin, allowing platelet aggregation to occur. This hypothesis is consistent with endogenous thrombin potential, which has been proposed as a parameter for plasma-based hypercoagulability, being much higher in Alès plasma than in control. This difference was attributed to the fact that 37% to 50% of thrombin formed after calcium addition was trapped into the normal forming clot. The percentage of thrombin bound to the clot was deduced from the difference between thrombin activity in recalcified defibrinated plasma and that in recalcified plasma. Because clotting was dramatically delayed in Alès plasma, the thrombin generated remained in solution and its activity was similar to that found with fibrinogen-depleted normal plasma. Because reptilase added to plasma from Alès did not induce any clotting, it was shown, as a control, that endogenous thrombin potential was identical in Alès native and reptilase-treated plasmas. In all samples, a decay of thrombin activity that resulted from the neutralization of thrombin by antithrombin III was observed after 120 seconds, as already reported.44

However, it is important to know whether thrombin bound to Alès fibrin monomers is still accessible for platelet aggregation. In fact, it is well established that fibrin contains independent sites having affinity for thrombin. One of these sites, located in the E domain of the fibrin monomer, interacts with the anion binding site of thrombin. This binding exosite of thrombin is implicated in both fibrin and platelet binding. It has been shown that an equilibrium exists between thrombin bound to fibrin monomers and thrombin bound to platelets.<sup>45,46</sup> Therefore thrombin bound to fibrin monomers is still active for platelet aggregation, but its activity is reduced in comparison to free thrombin. Thus, thrombin bound to fibrin monomers remains partly available for platelet aggregation. Furthermore, it has been reported that clot-associated thrombin induces platelet aggregation.<sup>47,48</sup>

In 2 other patients with congenital dysfibrinogenemia, an arterial thrombotic risk is reported that can be related to a defective thrombin binding to the clot, responsible for platelet aggrega-

tion.<sup>49,50</sup> However, the sister of the propositus, though also homozygous for dysfibrinogenemia, is asymptomatic. We tried to determine whether the thrombotic risk could be linked to other risk factors associated with dysfibrinogenemia. The known risk factors for thrombosis, recommended by ISTH, were absent in the propositus and in his sister. The propositus is a smoker, but his sister is not. Both the patient and his sister also have apoE  $\epsilon$  3/ $\epsilon$  4 genotype, more frequent in stroke patients (21.1%) than in stroke-free controls (8.9%; P < .05), indicating that apoE  $\epsilon$  4 is a prominent lipid predictor for ischemic stroke among other risk factors.<sup>51</sup> It is therefore suggested that the onset of thrombotic disease in this patient was multifactorial and was linked to a synergistic effect of dysfibrinogenemia, apoE  $\epsilon$  3/ $\epsilon$  4 genotype, and smoking.

The parents of this propositus and the affected members of the family Milano I are asymptomatic, but they are heterozygous for the mutation with a circulating mixture of normal and abnormal fibrinogens. Therefore, as already reported for other dysfibrinogenemias, the potential risk factor would explain the variability observed in the clinical symptoms associated with this homozygous dysfibrinogenemia because the mutation is associated with severe thrombophilia in the patient and appears to be silent in his sister.

In summary, a structural defect in a variant of fibrinogen, called fibrinogen Alès, was identified. Our results demonstrate that the substitution of the aspartic acid in position  $\gamma$ -330 by valine leads to a nonfunctioning polymerization site a, which is necessary for the normal polymerization of fibrinogen.

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## References

- Blombäck B, Hessel B, Hogg D, Therkildsen L. A two-step fibrinogen–fibrin transition in blood coagulation. Nature. 1978;275:501-505.
- Olexa SA, Budzynski AZ. Evidence for four different polymerization sites involved in human fibrin formation. Proc Natl Acad Sci U S A. 1980;77: 1374-1378.
- Medved LV, Litvinovich SV, Ugarova TP, Lukinova NI, Kalikhevich VN, Ardemasova ZA. Localization of a fibrin polymerization site complementary to Gly-His-Arg sequence. FEBS Lett. 1993;320:239-242.
- Shainoff JR, Dardik BN. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Ann N Y Acad Sci. 1983;408:254-268.
- Weisel JW, Veklich Y, Gorkun O. The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. J Mol Biol. 1993:232:285-297.
- Lewis SD, Shields PP, Shafer J-A. Characterization of the kinetic pathway for liberation of fibrinopeptides during assembly of fibrin. J Biol Chem. 1985;260:10192-10199.
- Francis CW, Marder VJ. Rapid formation of large molecular weight alpha-polymers in cross-linked fibrin induced by high factor XIII concentrations: role of platelet factor XIII. Clin Invest. 1987;80: 1459-1465.

- 8. Ebert RF. Index of variant human fibrinogens. Boca Raton, FL: CRC Press; 1994.
- Côté HC, Lord ST, Pratt KP. Gamma-chain dysfibrinogenemias: molecular structure–function relationships of naturally occurring mutations in the gamma chain of human fibrinogen. Blood. 1998; 92:2195-2212.
- Caen JP, Larrieu MJ, Samama M. L'hémostase: méthodes d'exploitation et diagnostic pratique. Expansion scientifique. Paris, 1975.
- Patscheke H, Worner P. Platelet activation detected by turbidometric shape-change analysis: differential influence of cytochalasin B and prostaglandin E1. Thromb Res. 1978;12:485-496.
- Clauss A. Gerinnungsphysiologishe schnellmethode zur bestimmung des fibrinogens. Acta Haematol. 1957;17:237-242.
- Mancini G, Carbonara A-O, Heremans J-F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry. 1965;2: 235-254.
- Desvignes P, Bonnet P. Direct determination of plasma fibrinogen levels by heat precipitation: a comparison of the technique against thrombin clottable fibrinogen with spectrophotometry and radial immuno-diffusion. Clin Chim Acta. 1981; 110:9-17.
- Soria J, Soria C, Borg JY, et al. Platelet aggregation occurs in congenital afibrinogenaemia despite the absence of fibrinogen or its fragments in

plasma and platelets, as demonstrated by immunoenzymology. Br J Haematol. 1985;60:503-514.

- Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia: report on a study of the SSC Subcommittee on Fibrinogen. Thromb Haemost. 1995;73:151-161.
- Bertina RM, Koeleman BP, Koster T, Rosendaal FR, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369:64-67.
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996; 88:3698-3703.
- Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*l. J Lipid Res. 1990;31: 545-548.
- Blombäck B, Blombäck M. Purification of human and bovine fibrinogen. Arki Kemi. 1956;10:415-443.
- Weber K, Osborn M. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem. 1969;244:4406-4412.
- Bouvier C, Gruedlinger J. Etudes des phénomènes de coagulation et de fibrinolyse par une méthode simple d'enregistrement optique. Schweiz Med Wsch. 1963;93:1441-1443.

- Belitser VA, Varetskaja TV, Malneva GV. Fibrinogen–fibrin interaction. Biochim Biophys Acta. 1968;154:367-375.
- Kehl M, Lottspeich F, Henschen A. Analysis of human fibrinopeptides by high-performance liquid chromatography. Hoppe Seylers Z Physiol Chem. 1981;362:1661-1664
- Schwartz ML, Pizzo SV, Hill RL, McKee PA. The effect of fibrin-stabilizing factor on the subunit structure of human fibrin. J Clin Invest. 1971;50: 1506-1513.
- Haverkate F, Timan G. Protective effect of calcium in the plasmin degradation of fibrinogen and fibrin fragments D. Thromb Res. 1977;10:803-812.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-685.
- Nilehn JE. Separation and estimation split "products" of fibrinogen and fibrin in human serum. Thromb Diath Haemorrh. 1967;18:487-498.
- Stanilawski M, Marel A, Mitard M, Pene J. Attachment of lymphoid cells to glutaraldehyde activated plasmatic cup: a convenient mean for quantitative estimation of cytoplasmic and membrane Ig. In: Peeters H, ed. Protides and Biological Fluids. New York: Pergamon Press; 1980:479-482.
- Mirshahi M, Soria J, Soria C, Perrot J-Y, Boucheix C. A latex immunoassay of fibrin/fibrinogen degradation products in plasma using a monoclonal antibody. Thromb Res. 1986;44:715-728.
- Wolters G, Kuijpers L, Kacaki J, Schuurs A. Solidphase enzyme-immunoassay for detection of hepatitis B surface antigen. J Clin Pathol. 1976; 29:873-879.
- Rixon M-W, Chung DW, Davie EW. Nucleotide sequence of the gene for the gamma chain of human fibrinogen. Biochemistry. 1985;24:2077-2086.
- Hemker H-C, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of

thrombin decay processes. Thromb Haemost. 1986;56:9-17.

- Southan C, Thompson E, Panico M, Etienne T, Morris H-R, Lane D-A.Characterization of peptides cleaved by plasmin from the C-terminal polymerization domain of human fibrinogen. J Biol Chem. 1985;260:13095-13101.
- Lounes KC, Soria C, Valognes A, Turchini M-F, Soria J, Koopman J. Fibrinogen Bastia (γ 318→Tyr): a novel abnormal fibrinogen characterized by defective fibrin polymerization. Thromb Haemost.1999;82: 1639-1643.
- Shimizu A, Schindlauer G, Ferry JD. Interaction of the fibrinogen-binding tetrapeptide Gly-Pro-Arg-Pro with fine clots and oligomers of alphafibrin: comparisons with alpha beta-fibrin. Biopolymers. 1988;27:775-788.
- Reber P, Furlan M, Rupp C, et al. Characterization of fibrinogen Milano I: amino acid exchange gamma 330 Asp→Val impairs fibrin polymerization. Blood. 1986;67:1751-1756.
- Terukina S, Yamazumi K, Okamoto K, Yamashita H, Ito Y, Matsuda M. Fibrinogen Kyoto III: a congenital dysfibrinogen with a gamma aspartic acid-330 to tyrosine substitution manifesting impaired fibrin monomer polymerization. Blood. 1989;74: 2681-2687.
- Crabtree GR, Kant JA. Organization of the rat gamma-fibrinogen gene: alternative mRNA splice patterns produce the gamma A and gamma B (gamma ') chains of fibrinogen. Cell. 1982;31: 159-166.
- Strong DD, Moore M, Cottrell BA, et al. Lamprey fibrinogen gamma chain: cloning, cDNA sequencing, and general characterization. Biochemistry. 1985;24:92-101.
- Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. Nature. 1997;389:455-462.
- 42. Pratt KP, Côté HCF, Chung DW, Stenkamp RE, Davie EW. The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal γ chain fragment complexed with the

peptide Gly-Pro-Arg-Pro. Proc Natl Acad Sci U S A. 1997;94:7176-7181.

- 43. Soria J, Soria C, Samama M, Caen JP. Study of 10 cases of congenital dysfibrinogenemia: clinical and molecular aspects. In: Henschen AHB, Mc-Donagh JM, Saldeen T, eds. Fibrinogen: Structural variants and interactions. Vol 3. Berlin: Walters de Gruyter; 1985:165-183.
- Beguin S, Kessels H, Dol F, Hemker H-C. The consumption of antithrombin III during coagulation, its consequences for the calculation of prothrombinase activity and the standardisation of heparin activity. Thromb Haemost. 1992;68:136-142.
- Bouton MC, Jandrot-Perrus M, Bezeaud A, Guillin MC. Late-fibrin(ogen) fragment E modulates human alpha-thrombin specificity. Eur J Biochem. 1993;215:143-149.
- Jandrot-Perrus M, Clemetson KJ, Huisse MG, Guillin MC. Thrombin interaction with platelet glycoprotein lb: effect of glycocalicin on thrombin specificity. Blood. 1992;80:2781-2786.
- Gandossi E, Lunven C, Gauffeny C, Roome NO, Berry CN. Platelet aggregation induced in vitro by rabbit plasma clot-associated thrombin, and its inhibition by thrombin inhibitors. Thromb Haemost. 1998;80:840-844.
- Lunven C, Gauffeny C, Lecoffre C, O'Brien DP, Roome NO, Berry CN. Inhibition by Argatroban, a specific thrombin inhibitor, of platelet activation by fibrin clot-associated thrombin. Thromb Haemost. 1996;75:154-160.
- Koopman J, Haverkate F, Lord ST, Grimbergen J, Mannucci P-M. Molecular basis of fibrinogen Naples associated with defective thrombin binding and thrombophilia: homozygous substitution of B beta 68 Ala→Thr. J Clin Invest. 1992;90:238-244.
- Di Minno G, Martinez J, Cirillo F, et al. A role for platelets and thrombin in the juvenile stroke of two siblings with defective thrombin-adsorbing capacity of fibrin(ogen). Arterioscler Thromb. 1991;11:785-796.
- Peng DQ, Zhao SP, Wang JL. Lipoprotein (a) and apolipoprotein E epsilon 4 as independent risk factors for ischemic stroke. J Cardiovasc Risk. 1999;6:1-6.