Fibrinogen Dusart: Electron Microscopy of Molecules, Fibers and Clots, and Viscoelastic Properties of Clots

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ABSTRACT Ultrastructural perturbations resulting from defects in polymerization of fibrinogen Dusart, a congenital dysfibrinogenemia with the amino acid substitution Aα 554 arginine to cysteine, were investigated by a variety of electron microscope studies. Polymerization of this mutant fibrinogen on addition of thrombin is impaired, producing clots with decreased porosity and increased resistance to fibrinolysis, resulting in thrombotic complications in the family members with this dysfibrinogenemia. Electron microscopy of rotary-shadowed individual molecules revealed that, in contrast to control fibrinogen, most of the αC domains of fibrinogen or fibrin Dusart appeared to be free-swimming appendages that do not exhibit intra- or intermolecular interactions either with each other or with the central domains. The location of albumin on the αC domains was demonstrated by electron microscopy using anti-albumin antibodies. Electron microscopy of negatively contrasted fibrin Dusart fibers indicated that they were less ordered than control fibers and had additional mass visible. Electron microscopy of freeze-dried, unidirectionally shadowed fibers showed that they were twisted with a shorter pitch. Scanning electron microscopy revealed that intact clots were made up of thin fibers with many branch points and very small pore sizes. The viscoelastic properties of Dusart fibrin clots measured with a torsion pendulum indicated a marked increase in stiffness consistent with the structural observations.

INTRODUCTION

Fibrinogen is made up of three pairs of nonidentical polypeptide chains with a total molecular mass of 340 kDa. All amino-terminal ends are linked together to form the central nodule; the carboxyl-terminal ends of the Bβ and γ chains comprise the end globular domains, which are connected to the central region by three-chain, α-helical coiled coils. The carboxyl-terminal ends of the Aα chains, known as the αC domains, are folded back so that they interact with each other and with the central region of fibrinogen (Veklich et al., 1993; Gorkun et al., 1994). Clotting occurs when the fibrinopeptides are cleaved from the central region of the molecule, exposing binding sites that are complementary to sites that are always exposed at the ends of the molecule. The fibrin monomers thus interact in a half-staggered manner to form two-stranded protofibrils, which then aggregate laterally to yield fibers. Fibers grow in size and branch to produce a three-dimensional network that functions to impede bleeding.

The organization of fibrin in a blood clot is a result of a complex assembly process affected by the ratio of thrombin to fibrinogen and other factors (Blombäck et al., 1992; Weisel and Nagaswami, 1992). The structure of the fibrin clot is an important factor influencing the balance between clotting and fibrinolysis, thus affecting any tendency toward thrombosis and cardiovascular disease. Aspects of the mechanisms of assembly of the fibrin clot can be understood through analysis of differences in the structures formed by variant fibrinogens that have specific molecular defects. Several variants have been associated with thromboembolism in vivo (Ebert, 1994), and the analysis of differences in fibrin clot structures observed in such defects may be crucial to our understanding of variations in susceptibility to thrombosis.

Dusart syndrome is a severe thrombotic disorder with a high incidence of thromboembolism and abnormal fibrin polymerization, related to a congenital dysfibrinogenemia with the same name (Soria et al., 1983). The molecular defect is located in the αC domain and consists of a substitution of cysteine for arginine at position 554 in the Aα chain, with consequent binding of albumin (Koopman et al., 1993; Siebenlist et al., 1993). This thrombotic disorder was first attributed to an abnormal clot thrombolysis with reduced plasminogen binding to fibrin and defective plasminogen activation by tissue-type plasminogen activator (Soria et al., 1983; Lijnen et al., 1984). Later studies showed that the fibrin Dusart clot structure is very abnormal, consisting of a rigid network with many thin fibers, resulting in greatly decreased gel porosity (Collet et al., 1993; Koopman et al., 1993; Siebenlist et al., 1993). Both the clot structure and the rate of fibrinolysis were restored almost to normal by the addition of dextran before clotting, which increases the fiber diameter and pore size, thus providing evidence that the increased rigidity and resistance to thrombolysis arise from the abnormal architecture of the Dusart fibrin network (Col-

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let et al., 1993). Other studies have shown a correlation between low gel porosity in plasma clots and premature coronary heart disease (Fatah et al., 1992).

The structure of fibrin clots formed in vitro is largely determined by the kinetics of polymerization (Weisel and Nagaswami, 1992). The clotting time for fibrinogen Dusart is longer than normal (Soria et al., 1983; Koopman et al., 1993), and the rate and extent of lateral aggregation of protofibrils is reduced (Soria et al., 1983; Collet et al., 1993; Siebenlist et al., 1993). Lateral aggregation can be affected by many factors, one of the most significant being intermolecular interactions of the αC domains. Monoclonal antibodies specific for particular regions of the αC domains have dramatic effects on polymerization (Cierniewski and Budzynski, 1992), as do αC fragments (Veklich et al., 1993) or smaller portions of the αC domains (Lau, 1993). Different preparations of fragment X, missing the αC domains, also have impaired lateral aggregation (Mosesson et al., 1967; Hasegawa and Sasaki, 1990; Gorkun et al., 1994). Several other dysfibrinogenemias with amino acid substitutions in the αC domains, including fibrinogen Caracas II (Aα Ser-434 to *N*-glycosylated Asn (Maekawa et al., 1991)), fibrinogen Marburg (missing Aα 461–611 (Koopman et al., 1992)), and fibrinogen Chapel Hill III (which has the same molecular defect as fibrinogen Dusart (Wada and Lord, 1994)), all appear to be defective in lateral aggregation.

It has been demonstrated that fibrin fibers are twisted, and this twisting has been suggested as a mechanism for limitation of the lateral growth of fibers (Weisel et al., 1987). Therefore, a mutation affecting the molecules so that they are less flexible, or that they polymerize with a different amount of twist, could also contribute to differences in lateral aggregation.

The present study investigates the effect of the Dusart mutation on the morphology of the αC domains of fibrinogen molecules, including the ultrastructural location of the albumin within the αC domain. The distribution of the abnormal Aα chains among the fibrinogen molecules was also determined, because this mutation is heterozygous (Soria et al., 1983; Koopman et al., 1993). The detailed ultrastructure of Dusart fibrin fibers was examined to ascertain whether there were any differences in molecular packing induced by the presence of the abnormal αC domains and to determine the effect of the mutation on the degree of twisting. The three-dimensional arrangement of the fibers within the Dusart clot was visualized using scanning electron microscopy to determine qualitatively the network density along with the extent of branching and average pore size. Histograms were produced from measurements of fiber bundle diameters, showing quantitatively the decreased lateral aggregation. Because there are indications that the mechanical properties of fibrin clots formed in vitro may be correlated with the clinical symptoms of thrombosis and thromboembolism (Fatah et al., 1992; Scrutton et al., 1994), the viscoelastic properties of Dusart clots were also measured. The abnormalities observed at all levels of structure indicate how the amino acid substitution and associated albumin in fibrinogen Dusart affects fibrin polymerization, and emphasize the significance of the contributions of the αC domains to clot assembly and hence their effects on the properties of the final gel network.

MATERIALS AND METHODS

Materials

Plasma was prepared from the blood of the subject and a normal individual, as previously described (Collet et al., 1993). Fibrinogen was purified from the plasma by standard methods (Blombäck and Blombäck, 1956). Dextran (mol wt 40,000) was supplied by Assistance Publique (Paris, France). Goat anti-human albumin polyclonal immunoglobulin G (IgG) antibody was purchased from Bethyl Laboratories (Montgomery, Texas).

Transmission electron microscopy of individual fibrinogen and fibrin molecules

Rotary-shadowed samples were prepared by spraying a dilute solution of fibrinogen or fibrin molecules in a volatile buffer (0.05 M ammonium formate at pH 7.4 or 0.125% (v/v) acetic acid at pH 3.5) and 30% (v/v) glycerol onto freshly-cleaved mica and shadowing with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) (Fowler and Erickson, 1979; Weisel et al., 1985; Veklich et al., 1993). In some experiments, the albumin attached to fibrinogen Dusart was labeled with anti-human albumin antibodies before spraying and shadowing as described previously for other studies with fibrinogen and antibodies (Veklich et al., 1993). Samples at neutral pH were prepared by rapid dilution with continual stirring of concentrated protein solution with 0.05 M ammonium formate at pH 7.4 and 30% (v/v) glycerol to a final concentration of ≈25 μg/ml, and those at acid pH were prepared by dilution into 0.125% (v/v) acetic acid at pH 3.5 with 30% (v/v) glycerol. These specimens were examined in a Philips 400 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ), usually operating at 80 kV and a magnification of 60,000×.

Electron microscopy of negatively contrasted fibrin fibers

Samples were prepared from fibrinogen with added thrombin as described previously (Weisel, 1986a). After the initiation of polymerization, aliquots were taken at specific times and a drop of the mixture was immediately placed on a grid covered by a carbon film and negatively stained with 1–2% (w/v) uranyl acetate. For observations of fine structural details with this technique, it is important to use a very thin carbon film and to clean it by glow discharge before application of the sample. In some cases, preformed clots were applied to 300-mesh, glow-discharged carbon-coated Formvar grids and then negatively contrasted with uranyl acetate. All of these specimens were also examined in a Philips 400 electron microscope generally operating at 80 kV and a magnification of 36,000×.

Electron microscopy of freeze-dried, unidirectionally shadowed fibrin fibers

Samples were prepared by adding thrombin to fibrinogen as described previously (Weisel et al., 1987) with the following modifications. Just as the solution began to gel after the addition of thrombin to Dusart fibrinogen, the clot was dispersed with a Pasteur pipette and applied to a small sheet of glass (microscope coverslip), which had been precoated with a thin carbon film. The samples were washed with phosphate-buffered saline buffer and stabilized by the addition of 1% uranyl acetate and rinsed with distilled water and a solution of 30% ethanol. The samples were covered

with another coverslip of the same size (without any carbon film) arranged in a half-staggered manner, and then the drying process was started using blotting paper. When only a very thin film of liquid remained, the upper coverslip was removed and the sample was quickly frozen in liquid nitrogen. The thin carbon film prevented squashing of the fibers onto the surface of the glass.

Scanning electron microscopy of plasma clots

Scanning electron microscope experiments were carried out on clots that were fixed, dehydrated, critical point dried and sputter-coated with gold, and then observed using an Amray 1400 microscope (Amray Inc., Bedford, MA)(Langer et al., 1988; Weisel and Nagaswami, 1992). Plasma clots were made by recalcification of citrated plasma (one volume of 0.13 M citrate for nine volumes of blood) prepared by centrifugation at 2500 × g for 20 min. Plasma samples were recalcified with 0.5 M $CaCl_2$ to a final concentration of 25 mM. Thrombin was then added to a final concentration of 0.2 IU/ml, and 0.1 ml of the mixture was used to fill pre-etched plastic tubes in which one end was sealed with parafilm. This procedure, originally developed for permeation experiments (Collet et al., 1993), provided a mechanism for thorough rinsing of the clots. Clotting was allowed to proceed to completion for 8 h in a moist atmosphere. Plasma clots were rinsed by permeation of 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4 buffer through the gels after clotting but before fixation to remove all soluble plasma proteins. Washed clots were then prepared for microscopy as described above.

Measurement of viscoelastic properties of plasma clots

Plasma was clotted under the conditions described above. Clots with a volume of 0.115 ml were formed between 12 mm diameter glass coverslips in a Plazek torsion pendulum similar to that described by Janmey and others (Plazek et al., 1958; Gerth et al., 1974; Janmey, 1991). A momentary impulse was applied to the pendulum at room temperature, causing free oscillations with strains of less than a few percent. The dynamic storage modulus, G', loss modulus, G'', and loss tangent, tan δ, were calculated from recordings of the oscillations (Janmey, 1991).

RESULTS

Electron microscopy of individual fibrinogen and fibrin molecules

A previous study of fibrinogen Dusart molecules visualized by electron microscopy using platinum-shadowed specimens showed the presence of additional globular domains (Siebenlist et al., 1993). However, a statistical analysis of the localization of these nodules on the fibrinogen Dusart and their distribution among molecules was not reported. In the present study, fibrinogen Dusart molecules were rotary-shadowed with tungsten before examination by electron microscopy, a method that results in slightly enhanced resolution of the molecular substructure compared with platinum (Erickson and Fowler, 1983; Slayter, 1983; Williams, 1983) and allows individual αC domains of fibrinogen to be consistently observed for statistical analysis (Veklich et al., 1993). Some examples are shown in Fig. 1 *A–D*. Particular attention was paid to the location of the αC domains, which are associated with the central part of most control fibrinogen molecules but appear as free nodules in fibrin monomer (Veklich et al., 1993; Gorkun et al., 1994). The results

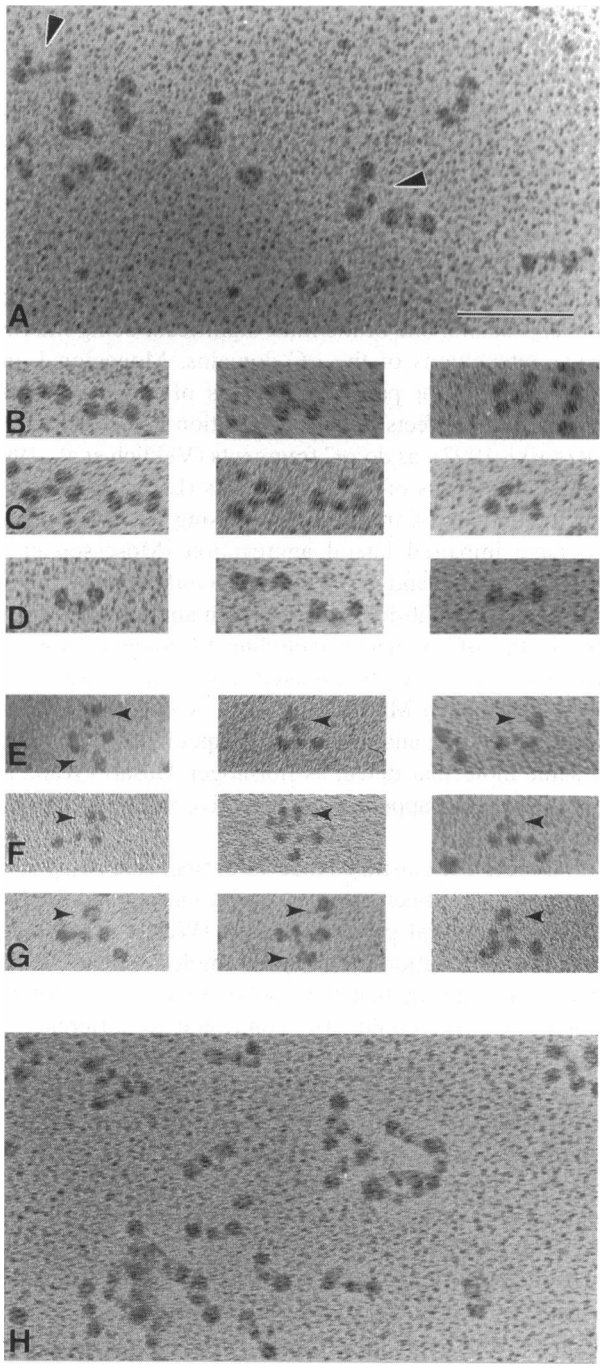

FIGURE 1 Electron microscopy of individual rotary-shadowed molecules. (*A–D*) Fibrinogen Dusart at pH 7.4. (*A*) Field of fibrinogen Dusart molecules, showing examples with one or two extra large nodules (some of which are indicated by arrows) and examples that are trinodular with no apparent extra nodules. (*B–D*) Gallery of fibrinogen Dusart molecules, again indicating the variety of appearances. (*E–G*) Fibrinogen with IgG antibodies to human albumin bound. Each trinodular fibrinogen molecule has one or two three-lobed IgG molecules (*arrows*) attached to nodules (representing albumin) adjacent to the backbone of fibrinogen. (*H*) Field of fibrin Dusart monomer molecules. Many molecules show one or more nodules adjacent to the rest of the molecule; some fibrin molecules appear to be interacting via their αC domains. Bar = 100 nm.

of observations of many molecules in several preparations are summarized in Table 1; the results were similar in repeat experiments and in classification by different individuals. Individual molecules were classified according to our previously published scheme (Veklich et al., 1993; Gorkun et al., 1994) as trinodular, trinodular with one additional large nodule adjacent to the center of the molecule, or trinodular with either one or two small additional nodules located away from the backbone. At least 34% of fibrinogen Dusart molecules displayed one or two additional nodules that were often near the ends. About 8% of fibrinogen Dusart molecules had a single large additional nodule, with the remainder (58%) being trinodular with no additional mass visible. In contrast, 79% of control fibrinogen molecules were trinodular with no additional mass, 15% had a single large additional nodule, and 6% had one or two smaller additional nodules.

Because it has been demonstrated that fibrinogen Dusart has albumin attached via a disulfide bond to cysteine 554 (Koopman et al., 1993; Siebenlist et al., 1993), we used an anti-human albumin antibody to localize the albumin by electron microscopy of individual molecules. The protein concentrations were dilute enough that it was unlikely that an antibody could lie near a fibrinogen molecule by chance. Shadowed IgG molecules appeared as three small lobes in a variable but often roughly triangular configuration. Antibodies were seen binding to the additional nodules that were located near the molecules but not directly in contact with the backbone (Fig. 1, *E–G*). There were one or two antibody molecules bound per fibrinogen, consistent with the presence of an albumin on at least one of the two αC domains.

Fibrin monomer was prepared from fibrinogen Dusart and examined by electron microscopy. At pH 3.5, 79% of the molecules had one or more small additional nodules, often near the molecular ends (Table 1). About 9% of molecules displayed a single large additional nodule, and 12% were simply trinodular with no other visible mass. As expected, results with fibrinogen Dusart at pH 3.5 were similar with 73% of molecules having one or two small additional nodules, 11% with one large additional nodule, and 16% being simply trinodular. Both of these experiments at acidic pH are controls, inasmuch as that the αC domains of normal fibrinogen are mostly dissociated from each other under these conditions (Table 1) (Veklich et al., 1993).

Fibrin Dusart monomer was brought to neutral pH and quickly prepared for electron microscopy so that most molecules had not yet started to polymerize. Here 17% of molecules were simple trinodular structures, whereas 37% had a single large additional nodule and 46% had one or two small additional nodules (Table 1; Fig. 1 *H*). In contrast, normal fibrin monomers at neutral pH were 22% simple trinodular structures, whereas 71% had a single large additional nodule and 7% had one or two small additional nodules (Veklich et al., 1993).

TABLE 1 Shapes observed for Dusart fibrinogen and fibrin under various conditions

Sample	No. molecules examined	Percentage of Molecules with the Appearance		
Dusart fibrinogen, pH 7.4	343	58	8	34
Control fibrinogen, pH 7.4	409	79	15	6
Dusart fibrinogen, pH 3.5	346	16	11	73
Control fibrinogen, pH 3.5*	134	16	0	84
Dusart fibrin, pH 3.5	114	12	9	79
Control fibrin, pH 3.5*	378	13	4	83
Dusart fibrin, pH 7.4	319	17	37	46
Control fibrin, pH 7.4*	344	22	71	7

*Data from Veklich et al. (1993) for comparison.

Fiber substructure as determined by electron microscopy of negatively contrasted and freeze-dried unidirectionally shadowed specimens

The molecular packing in fibrin fibers was examined by electron microscopy of negatively contrasted fibers. As observed previously with samples prepared by other methods (Collet et al., 1993; Siebenlist et al., 1993), fibrin Dusart fibers were very thin. Because the exclusion of stain by protein is directly related to the brightness of the striations, analysis of the fibrin band pattern can reveal aspects of the protein shape and molecular packing. Control fibrin fibers have a characteristic band pattern with a repeat of 22.5 nm (Fig. 2 *A*) (Weisel, 1986a). Some examples of the band pattern of fibrin fibers made by addition of thrombin to fibrinogen Dusart are shown in Fig. 2, *B–F*. The repeat was 22.5 nm, and the appearance of the band

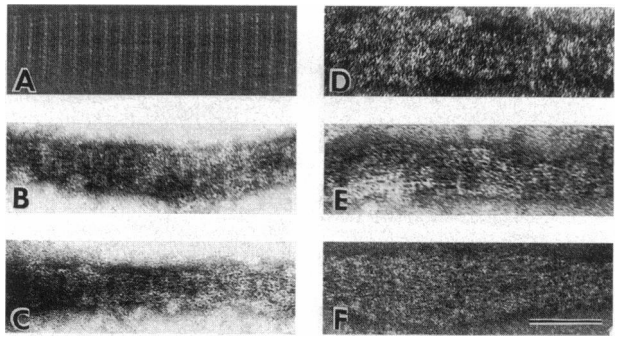

FIGURE 2 Electron micrographs of negatively contrasted fibrin fibers. (*A*) Control fibrin that has a distinctive band pattern with a 22.5 nm repeat. (*B–F*) Fibers made from fibrinogen Dusart. These fibers, which are arranged from better to less well ordered, have the same band pattern and repeat but are less well ordered. Some of the bright, stain-excluding spots probably arise from the attached albumin molecules. Bar = 100 nm.

pattern was generally similar to that of control fibrin fibers, but the Dusart fibers were nearly always much less ordered. The edges of the fibers are commonly very rough compared with control fibrin fibers. In addition, there are areas that show bright, stain-excluding nodules, although the disorder in the cross-striations makes it difficult to correlate these bright areas with any particular band making up the striations. In some cases (Fig. 2, *E* and *F*), longitudinal strands are visible, corresponding to individual protofibrils making up the fibers.

Freeze-dried fibers, unidirectionally shadowed with platinum, were prepared so that we could look at the surface structure of the fibers. As expected, the fibers were very thin. The surface of the fibers usually appears to be rough, probably a result of the same structures that give the edges of the negatively contrasted fibers a frayed appearance. These fibers sometimes had fine striations at a slight angle to the longitudinal axis of the fiber (Fig. 3), indicating that the fibers were made up of protofibrils that were twisted around each other. However, these striations were difficult to see because the fibers were thin and the distance over which they could be observed, i.e., between two successive branch points, was short. At each branch point, the surface appearance changes because of the difference in direction of shadowing. As a result, it was only possible to measure the pitch of a few fibers. Nevertheless, it appears that the pitch of the twisting may be shorter than that of control fibers. The mean pitch was calculated from the measured angles of the fine striations to be 770 ± 400 nm (mean ± SD), as compared with 1930 ± 280 nm for control fibers (Weisel et al., 1987).

Fibrin Dusart clot structure as observed by scanning electron microscopy

Clots formed from Dusart plasma have been studied previously by electron microscopy of specimens prepared using a freeze-etching technique (Collet et al., 1993). Although a general picture of the alterations in clot structure induced by the Dusart mutation was obtained by this technique, accurate measurements of average fiber bundle diameters were difficult because of limitations in the depth of etching. Therefore, the clots in the present study were prepared by critical point drying, sputter-coated with gold, and examined by scanning electron microscopy. For these experiments, clots were made by addition of calcium and thrombin to citrated plasma. Plasma clots from Dusart plasma are shown in Fig. 4 *A*, and those from a control subject are in Fig. 4 *B*. Dusart plasma clots were made up of thin fibers, with closely spaced branch points to form a uniform mesh with very small pores (Fig. 4 *A*). Average fiber bundle diameters were 19 ± 8 nm (mean ± SD), compared with a mean of 133 ± 38 nm for normal fibers from the control plasma clots formed at the same time under the same conditions. Histograms of fiber bundle diameters show that the distribution of diameters is also much narrower for the Dusart clots (Fig. 5 *A*) than for the control (Fig. 5 *B*). The mean distance between branch points was estimated to be 290 ± 150 nm, in comparison with an average of 5000 ± 1400 nm for control clots.

Plasma clots were also prepared in the presence of dextran, which has been shown to produce a more normal network structure from Dusart fibrin (Collet et al., 1993). Clots formed from Dusart plasma in the presence of 15 mg/ml dextran 60 had average diameters of 81 ± 24 nm (Fig. 4 *C*), whereas in the presence of 30 mg/ml dextran 60, diameters were 120 ± 41 nm. Statistical analysis by the unpaired *t*-test gave results of at least $p < 0.99$ for all of these comparisons. Histograms of the fiber bundle diameters in Dusart clots formed in the presence of 30 mg/ml dextran (Fig. 5 *D*) are similar to those of control clots (Fig. 5 *B*), and those of Dusart clots with 15 mg/ml dextran (Fig. 5 *C*) are intermediate between those with the higher dextran concentration and Dusart clots without dextran. Control plasma clots are not much affected by the presence of dextran (Fig. 4 *D*).

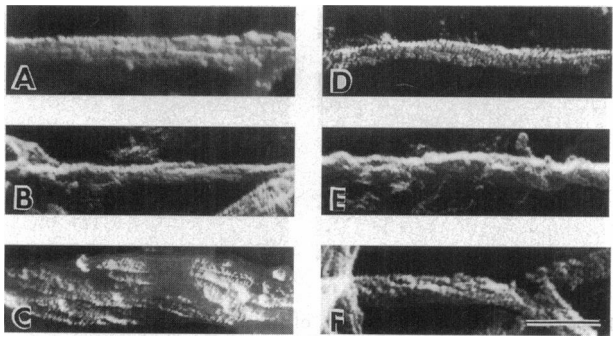

FIGURE 3 Electron micrographs of unidirectionally shadowed fibrin Dusart fibers. The fine striations at a slight angle to the fiber axis arise because the fibers are made up of protofibrils twisted around each other. Bar = 100 nm.

Viscoelastic properties of Dusart fibrin clots

It has been suggested that greater clot stiffness, i.e., resistance to deformation, may increase the propensity of individuals to thrombotic disease (Fatah et al., 1992; Scrutton et al., 1994). The stiffness and other viscoelastic properties of clots can be measured directly by use of a torsion pendulum, in which the recovery of a specimen from deformation induced by an applied torsional stress is monitored. In one mode of operation, the stress initiates a series of damped harmonic oscillations, from which some of the viscoelastic properties of the clot can be derived. Plasma clots from the subject with fibrinogen Dusart were prepared by addition of thrombin to recalcified plasma and application to the plates of the torsion pendulum before clotting. Control clots were formed from the plasma of a normal subject. Fibrinogen concentrations were 2.3 mg/ml for Dusart plasma and 2.4 mg/ml for the control. The frequency of free oscillation was ≈5 rad/s for Dusart clots and ≈2 rad/s for the control, and results are summarized in Table 2. The measured storage

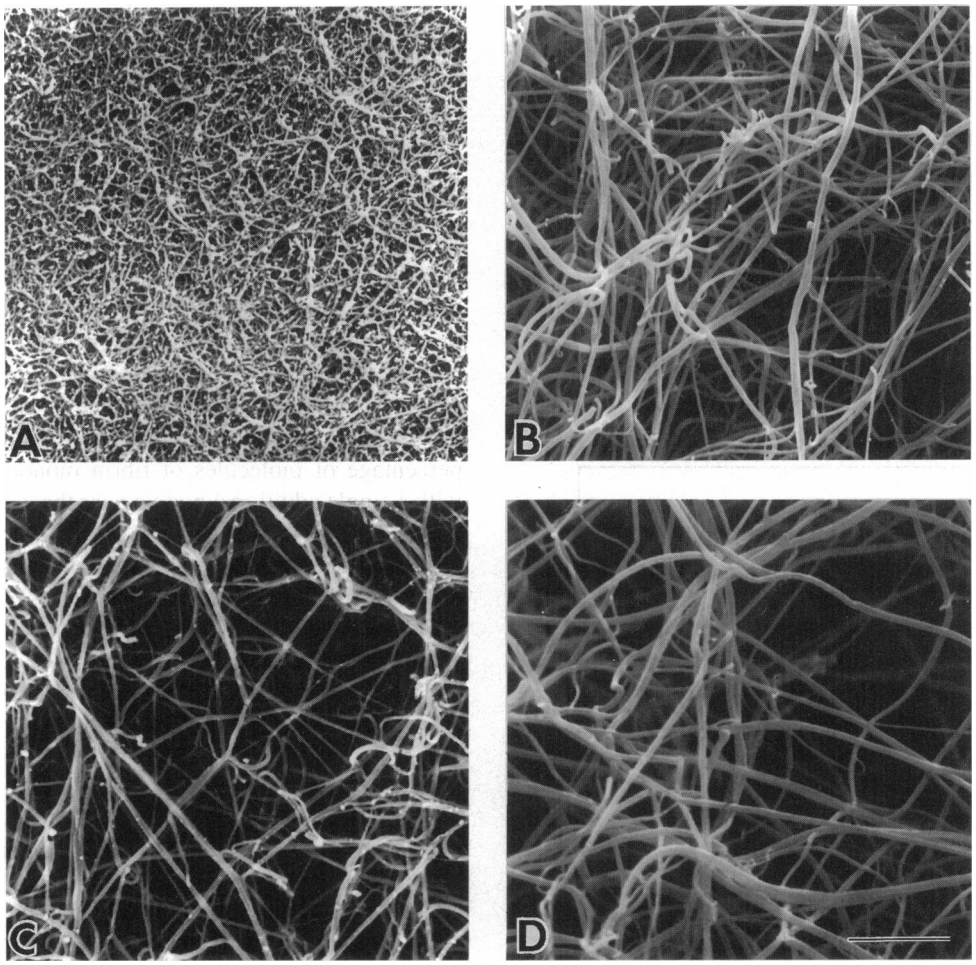

FIGURE 4 Scanning electron micrographs of plasma fibrin clots. The magnification is the same for all images (bar = 3 μm). (*A*) Clot made from fibrinogen Dusart plasma. (*B*) Clot made from control plasma. (*C*) Clot made from Dusart plasma with 15 mg/ml dextran. (*D*) Clot made from control plasma with 15 mg/ml dextran.

moduli (G') were 7240 ± 950 dyn/cm^2 (mean ± SD) for Dusart clots vs. 1220 ± 120 for control clots; the loss moduli (G'') were 570 ± 80 dyn/cm^2 vs. 94 ± 16 for Dusart and control clots, respectively. The loss tangents ($\tan \delta = G''/G'$) were 0.079 and 0.077 for the Dusart and control clots, respectively.

DISCUSSION

Localization of the αC domains and albumin on individual fibrinogen and fibrin Dusart molecules

Because the molecular defect in fibrinogen Dusart has been identified as Aα 554 (Arg → Cys) (Koopman et al., 1993), the αC domains were examined. Although the presence of albumin on the αC domains of fibrinogen Dusart molecules sometimes makes these nodules appear larger than normal, there was insufficient resolution to enable the size of the individual αC domains to be used as a criterion in the classification scheme. Therefore, only the location of the αC domains, as indicated by the presence or absence of additional nodules, was used in the analysis of images of the shadowed molecules (Veklich et al., 1993). If no additional nodules were observed, it was assumed that the αC domains were bound to the central region of the molecule, although in a small percentage of cases they may be away from the backbone but just not stand out sufficiently from the background. The observation of a single large additional nodule adjacent to the central region was interpreted as indicating that the two αC domains had been released from the trinodular backbone, but remained bound to each other. Small additional nodules located away from the backbone were interpreted as being αC domains that were not associated with either the central domain or the other αC domain. Some examples of individual shadowed fibrinogen Dusart molecules have been reported previously, but without any statistical analysis (Siebenlist et al., 1993).

In characterizing images of individual molecules quantitatively, we found that 42% (8% with one large extra nodule plus 34% with one or two smaller extra nodules) of molecules of fibrinogen Dusart displayed additional mass away from the backbone of the molecule, compared with 21% (15% with one large extra nodule plus 6% with one or two

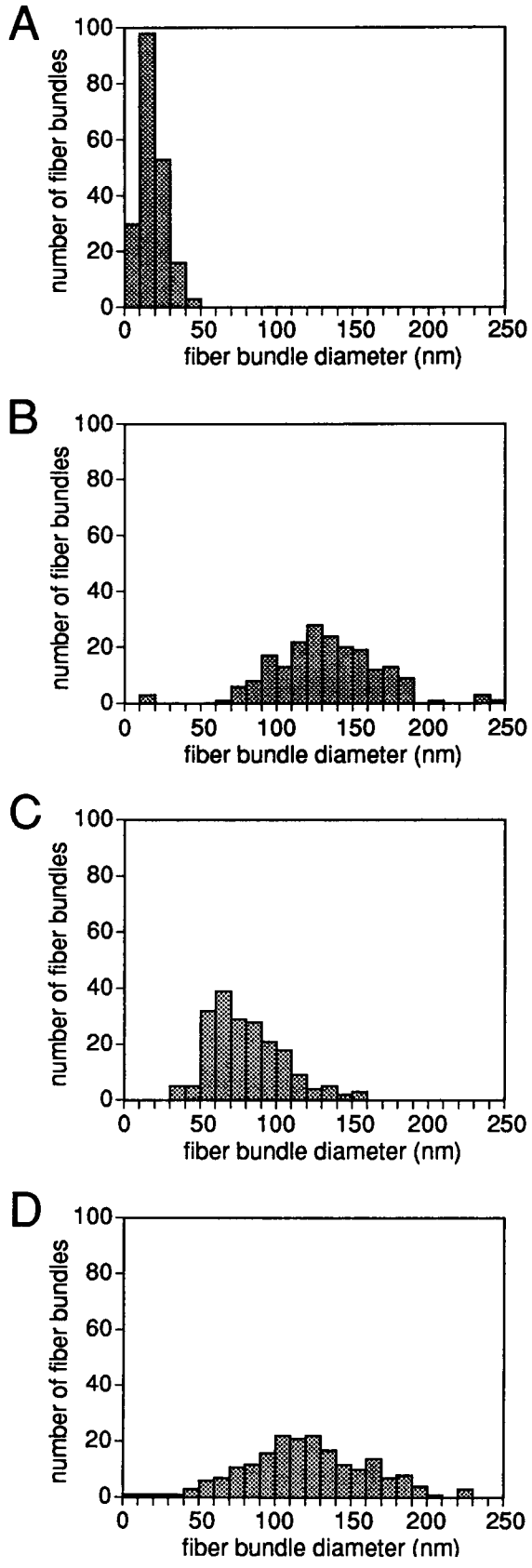

FIGURE 5 Histograms of fiber bundle diameters. (*A*) Clot from Dusart plasma. (*B*) Control plasma clot. (*C*) Dusart plasma clot with 15 mg/ml of dextran 60. (*D*) Dusart plasma clot with 30 mg/ml of dextran 60.

TABLE 2 Viscoelastic properties of fibrinogen dusart plasma clots

Parameter	Dusart	Control
G′, storage modulus (dyn/cm^2)	7240 ± 950	1220 ± 120
G″, loss modulus (dyn/cm^2)	570 ± 80	94 ± 16
tan δ, loss tangent (G″/G′)	0.079	0.077

small extra nodules) for control fibrinogen. In the present study, the results obtained from quantitative characterization of individual molecules suggest that there is a marked reduction in the interactions of the αC domains of fibrinogen Dusart with the central nodule of the molecule, compared with those of control fibrinogen. Furthermore, the low percentage of molecules of fibrin monomer at neutral pH with a single additional nodule near the central region of the molecule (37% compared with 71% for control fibrin) suggests that intramolecular interactions of the modified αC domains are also adversely affected.

Because fibrinogen is heterozygous, half of the αC chains should be normal. If the distribution of abnormal and normal chains is random, 25% of the molecules would be completely normal, 25% would have two defective chains, and the rest (50%) would have a single defect. In this case, if the defective chains cannot interact with the central region of fibrinogen, only 25% of the molecules would display a normal trinodular structure with no additional mass, but a much higher percentage (58%) of simple trinodular structures was observed in fibrinogen Dusart. Therefore, even allowing for the fact that this value for the percentage of molecules with no extra nodules may be slightly larger because of the failure to detect some free αC domains, the distribution of observed structures seems to be inconsistent with the hypothesis of a random distribution of normal and defective chains among the molecules.

On the other hand, if the Dusart molecules consist of either two normal or two defective chains, then 50% of the molecules should exhibit a simple trinodular structure, which is much closer to the observed value of 58%. Thus, our results are more consistent with the hypothesis that these molecules consist of either two normal or two defective chains and not one of each. Such a segregation of normal and abnormal polypeptide chains, so that apparently there are no heterodimers, has also been observed for several other dysfibrinogenemias (Galanakis et al., 1983; Galanakis and Hultin, 1990; Galanakis, 1991; Lee et al., 1991), but heterodimers have been reported in some other cases (Siebenlist et al., 1988; Galanakis et al., 1989).

It has previously been shown that fibrinogen Dusart has albumin covalently bound to it (Koopman et al., 1993). At least 90% of the albumin is bound to the αC domains (Siebenlist et al., 1993). In our study, an antibody to albumin was used to localize spatially the albumin molecules on the αC domains of fibrinogen Dusart. Because the bound antibodies were generally located away from the trinodular backbone of the fibrinogen molecule, it appears that the albumin is bound to the globular portion of the αC domain.

Substructure of fibers from Dusart fibrin

The measured repeat and substructure of the band pattern of fibers prepared by addition of thrombin to fibrinogen Dusart is normal, although the fibers generally appear to be less ordered. The pattern of striations of negatively contrasted fibrin is a reflection of the protein density along the fiber, and hence is directly related to the molecular structure and packing. The fact that this pattern of striations is not affected by the mutation indicates that the fundamental molecular packing is unchanged. On the other hand, the observed disorder indicates that not all molecules or protofibrils are in register. A reduced degree of order of the fibers is probably to be expected, given that the band pattern is not clearly visible in other thin fibers such as those produced by control fibrinogen clotted with thrombin at high salt concentration (Weisel, 1986b). However, the decrease in order with fibrinogen Dusart is so extreme that it seems most likely that the albumin interferes with the intermolecular interactions, with the main effect being observed in the lateral aggregation of protofibrils. Because half of the chains will contain a bound albumin molecule, there will be considerable additional mass that should show up in the fibrin band pattern. If these albumin molecules were always in the same location with respect to the globular domains of fibrin, there would be an increase in density in the appropriate region(s) of the fibrin band pattern; this region would become more effective at excluding stain and would appear brighter. Regions with greater density were observed in some fibers, although the locations of these regions could not be correlated in a convincing way with any particular cross-striations; this suggests that either the αC domains, and consequently the albumin molecules, are not arranged in an orderly manner in fibrin, or the localization is not readily discernible because of the disorder of the molecular packing in the fibers.

Electron microscopy of unidirectionally shadowed fibers from fibrinogen Dusart demonstrated that these are twisted structures. Like control fibers, they are made up of protofibrils twisted around each other (Medved' et al., 1990). It appears that normally this twisting may limit the extent of lateral aggregation of fibers so that their diameters reach a plateau (Weisel et al., 1987; Weisel, 1988). Protofibrils are added to a growing fiber until the energy of binding from the addition of a new protofibril is exceeded by the energy required to stretch that protofibril because of the increasing diameter. Experimentally, the pitch of the twist of fibrin Dusart fibers was difficult to measure because the fibers are so thin and the distance between branch points is very short. However, it appears that the pitch may be somewhat smaller than that of control fibers.

Because twisting appears to play a role in determining the diameters of fibrin fibers (Weisel et al., 1987) and fibers from fibrinogen Dusart are much thinner, any possible effects of this mutation on twisting should be considered. The main determinant of the pitch of twisting of a protofibril, and hence presumably of fibers as well, is the location of the complementary binding sites at the ends and the middle of each fibrin molecule. The pitch will be shorter if the sites at the ends of the molecule are farther off the longitudinal axis of the molecule. It seems unlikely that a mutation in the carboxyl-terminal Aα chain would perturb the locations of these complementary binding sites, and it has been shown that removal of the αC domains, which would probably not affect the complementary binding sites, does appear to correct the defect in this mutation (Siebenlist et al., 1993). Thus, with this assumption, the protofibrils would have the same pitch as usual. However, the observations of negatively contrasted fibers demonstrating that the fibers are disordered indicates that interactions between protofibrils are not as regular as normal, probably because interactions between them are weaker. Protofibril interactions could be weaker because there are fewer αC-αC domain interactions, because there is a mixture of normal and abnormal molecules. The pitch of the fibers is more irregular, as indicated by the larger standard deviation, but also appears to be shorter, perhaps because weaker αC-αC interactions allow an added protofibril, with a right-hand twist, to interact preferentially with the next protofibril in the right-hand direction, causing a decrease in pitch. Furthermore, it should be noted that the longitudinal disorder in these fibers means that protofibrils at the fiber periphery need not be stretched as much, and hence interactions leading to greater twisting, or shorter pitch, are less hindered.

Thus, it seems that the fibers are probably thinner primarily because of weaker intermolecular interactions between αC domains, not because of an intrinsic limitation arising from stretching of fibrin molecules. On the other hand, the limitation on the pitch at the lower end (i.e., one standard deviation below the mean, or $\approx$370 nm) could arise from the limits to the flexibility of fibrin, given that the calculated elongation for a fiber of 20 nm diameter would be 1.014 ($e = ((2\pi r/p)^2 + 1)^{1/2}$), which is about the same as that previously determined as the limiting value of 1.013 for the growth of control fibers (Weisel et al., 1987; Weisel, 1988).

The abnormal molecular and fiber structure of Dusart fibrin may account for both the reported resistance to plasmin and the defective activation of plasminogen to plasmin by tissue-type plasminogen activator (t-PA), leading to the hindrance to thrombolysis observed in Dusart clots. Plasminogen binds less to fibrin Dusart than to control fibrin (Lijnen et al., 1984). The abnormal αC domains and the extra mass of the albumin may play a critical role, inasmuch as these changes could modify the interaction of plasminogen with its binding site on fibrin (Weisel et al., 1994). On the other hand, it may be that the observed changes in fiber structure, such as thinner, less well-ordered fibers with a shorter pitch, affect plasminogen binding. In either case, anything that affects plasminogen binding could be important, because this appears to be a limiting factor in fibrinolysis (Hoylaerts et al., 1982; McDonagh, 1994). These results, demonstrating an increased resistance to fibrinolysis by thin fibers, are also in agreement with experiments

involving the effect of fibrin structure on the rate of fibrinolysis (Gabriel et al., 1992). These studies demonstrated that the thickness of fibrin fibers determines the pattern of lysis; namely, with thin fibers, there is a reduced rate of conversion of plasminogen to plasmin by t-PA and a reduced rate of plasmin cleavage; hence, clots made of thin fibers are lysed more slowly than those made of thicker fibers. Thus, the results of fibrinolysis of fibrin Dusart are consistent with these other studies, although the exact mechanisms of resistance to plasmin remain incompletely defined.

Structure and mechanical properties of fibrin Dusart clots

Whole clots can be examined by scanning electron microscopy, and clots prepared by the methods described here are probably better preserved than those prepared by other techniques. Clots from fibrinogen Dusart are dramatically different than control clots. It is immediately apparent that the clots are made up of thin fibers with very short distances between branch points; the fibers are very thin, 19 nm vs. 133 nm for control fiber diameters. Dextran, which has previously been shown to modify normal fibrin polymerization such that thicker fibers are formed (Dhall et al., 1976; Carr and Gabriel, 1980; Blombäck et al., 1992), also increases the diameters of Dusart fibers such that histograms of fiber bundle thickness are similar to those from control plasma without dextran, although the molecular mechanism involved is not known.

It seems that the Dusart fiber diameters are even thinner than would be expected from loss of the intermolecular interactions of the αC domains. However, this is not a strong conclusion, because experiments involving polymerization of various fragment X-like preparations missing the carboxyl-terminal α chain, and other related experiments, yield variable results depending on the experimental conditions (Mosesson et al., 1967; Holm et al., 1985; Medved' et al., 1985; Hasegawa and Sasaki, 1990; Maekawa et al., 1991; Cierniewski and Budzynski, 1992; Koopman et al., 1992; Gorkun et al., 1994). It is likely that the extra mass of the albumin molecules covalently linked to the α chain interferes with the kinetics of polymerization. Lateral aggregation would be more affected than earlier steps because of the accumulation of additional mass from many abnormal molecules in the protofibrils. Evidence supporting this interpretation comes from experiments involving the addition of αC fragments to fibrin monomer (Veklich et al., 1993; Gorkun et al., 1994). In these studies, where intermolecular interaction via the αC domains is blocked by the αC fragments, the decrease in final turbidity, which is indicative of thin fibers, is much more striking than with fragment X monomer, where the αC domains are merely missing.

The viscoelastic properties of the Dusart plasma clots were strikingly different than those of the control with respect to both the storage modulus (G') and the loss modulus (G''), suggesting that this dysfibrinogenemia may provide a good example for furthering our understanding of how fibrin structure relates to the mechanical properties of a clot. G' for the Dusart clots, which is a measure of their elastic properties or stiffness, was about six times higher than that of the control clots, even though the fibrinogen concentrations were similar in both of the plasma samples. Although the mechanisms responsible for the elasticity of fibrin clots are not entirely known (Ferry, 1988), in general, an increase in G' could arise from an increase in the number of branch points or an increase in the stiffness of the fibers (Gerth et al., 1974; Janmey et al., 1991). Comparison of the scanning electron microscope images of the clots from Dusart and control plasmas (Fig. 4, *A* and *B*) shows a dramatic increase in the number of branch points (nearly 20-fold) for the dysfibrinogenemic subject, which would be expected to contribute to a large increase in the storage modulus. However, this effect is probably partially offset by a decrease in stiffness of the Dusart fibers, given that they are both thinner and less ordered than those in control clots. The very thin fibers making up fine clots formed from purified fibrinogen at high pH and ionic strength, for example, result in structures with lower G' values than those of clots formed under physiological conditions with thicker fibers (Gerth et al., 1974; Roberts et al., 1974; Nelb et al., 1976).

In addition, the loss modulus (G''), which represents the energy dissipated by nonelastic, viscous processes, was also increased in Dusart plasma clots, even though its thinner fibers would be expected to give rise to a smaller G'' (Roberts et al., 1973, 1974; Gerth et al., 1974; Nelb et al., 1976). These experiments have also suggested that the viscous processes represented by G'' appear to arise primarily from slippage between protofibrils in the fibers. Thus, the greater G'' for Dusart clots is consistent with the looser and less ordered structure of the fibers that was observed in negatively contrasted preparations (Fig. 2). On the other hand, the loss tangent ($\tan \delta = G''/G'$), which is a measure of the energy dissipated by viscous processes relative to the energy stored by elastic processes, is essentially the same for both Dusart and control clots. This result is compatible with the foregoing discussion, because it would be expected that the thinner fibers of the Dusart clots would cause $\tan \delta$ to be lower than that of the control were it not for the weaker interactions between protofibrils that would increase G'' relative to G'.

The dramatic increase in the stiffness of Dusart clots, as indicated by G' and by the compaction coefficients measured previously (Collet et al., 1993), may be a contributing factor to the tendency toward thrombosis and thromboembolism in subjects with this dysfibrinogenemia. The enhanced resistance of Dusart clots to mechanical deformation means that these clots are more likely to retain their form under hydrostatic pressure and thus restrict blood flow. Evidence to support this suggestion comes from a study in which a correlation was established between the in vitro formation of a tight, rigid gel network and a propensity toward premature cardiovascular disease (Fatah et al., 1992). Furthermore, one possible contributing factor toward

fibrinogen as a cardiovascular risk factor is the logarithmic increase in stiffness of plasma clots with increasing fibrinogen concentration (Scrutton et al., 1994).

Mechanisms of abnormal polymerization of fibrinogen Dusart

There is now considerable evidence (referenced in the Introduction) that the αC domains of fibrin are involved in lateral aggregation. The αC domains are associated with each other and with the central domain in both control fibrinogen and fibrin monomer desA, but interact intermolecularly in desAB fibrin (Veklich et al., 1993; Gorkun et al., 1994). Since cleavage of the B fibrinopeptides usually occurs after polymerization to form protofibrils, the αC domains would then interact to bring protofibrils together, i.e., to increase the diameter of fibers.

In these as well as previous studies (Collet et al., 1993; Koopman et al., 1993; Siebenlist et al., 1993), the fibrinogen Dusart mutation in the αC domain has been demonstrated to have striking effects on the clot structure. The fibers are greatly reduced in diameter, an effect that probably arises because the altered αC domains do not interact with each other in a normal way, as reported in the present study. It should be noted that studies in which the αC domains were cleaved from fibrinogen Dusart showed that the clot structure was closer to normal (Siebenlist et al., 1993). Altogether, these results provide strong evidence on the importance of the αC domains in fibrin assembly.

The very short distance between branch points may be a result of increased branching because of fewer αC-αC interactions. However, increased branching also seems to be correlated with the growth of thin fibers. A quantitative study of clot structures under various conditions has demonstrated that the distance between branch points decreases with fiber diameter, so that clots made up of very thin fibers will have a great many branch points (Baradet et al., 1995).

The high degree of branching in Dusart plasma clots results in a greatly reduced average pore size, which is consistent with the observation that the flow required to transport proteins or other components of blood into a clot or thrombus is greatly reduced (Collet et al., 1993). This aspect of clot structure probably has a direct bearing on the balance between clotting and fibrinolysis in the subjects who carry this dysfibrinogenemia, providing an additional contribution to their strong tendency toward thrombosis and thromboembolism.

The Dusart syndrome may provide a model of thrombotic disorders that could be a useful aid for the development of new antithrombotic strategies, especially with the finding of similar (although perhaps not so extreme) clots formed from the plasma of patients who have had myocardial infarction at an early age (Fatah et al., 1992). Modulation of the fibrin gel architecture toward a coarser network could represent a way to improved fibrinolytic therapy. The direct involvement of the αC domains in lateral aggregation demonstrated in earlier studies is dramatically reinforced by fibrinogen Dusart, suggesting that this part of the fibrinogen molecule may be an appropriate target for attempts to alter clot structure to decrease thrombosis.

CONCLUSIONS

1) The αC domains of fibrinogen Dusart do not interact intramolecularly with each other or with the central region of the molecule, and the αC domains of Dusart fibrin do not interact intermolecularly to promote lateral aggregation. 2) The covalently linked albumin molecules are located on the globular portion of the αC domain. 3) Fibrin fibers from this mutant fibrinogen are much less ordered than control fibers and are twisted structures with a pitch that appears to be smaller than normal. 4) The fibers are much thinner than those of control clots, as a result of the defective αC domains and the extra mass from the bound albumin, both of which affect the kinetics of lateral aggregation. 5) These defective properties of fibrinogen and fibrin lead to the formation of an extremely abnormal three-dimensional network with much shorter distances between branch points, resulting in clots with very small pores. 6) The viscoelastic properties of Dusart clots indicate a dramatic increase in stiffness, consistent with the observed structural differences.

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