A Role for Platelets and Thrombin in the Juvenile Stroke of Two Siblings With Defective Thrombin-Adsorbing Capacity of Fibrin(ogen)

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Binding of iodine-125-labeled thrombin to fibrin clots from two siblings with juvenile stroke was 30% of normal, and abnormally high amounts of the radioligand (not adsorbed by fibrin) were found in the supernatant. In concordance with this finding, supernatants from the patients' fibrin clots caused abnormal enhancement of platelet aggregation, ATP secretion, and binding of 125I-fibrinogen to platelets exposed to subthreshold concentrations of ADP or epinephrine. Hirudin suppressed the enhancing effect of the patients' supernatants, and substitution of γ-thrombin for α-thrombin led to normalization of platelet responses. Under some experimental conditions, degradation of the patients' fibrinogen by plasmin was impaired. However, the euglobulin lysis time, the rate of fibrin degradation by plasmin, and the lysis of the patients' plasma clots by human melanoma tissue-type plasminogen activator were normal. Patients' plasmas, as well as purified fibrinogen, showed a prolonged thrombin time (partially corrected by 10 mM CaCl₂) and an impaired release of fibrinopeptide A in response to thrombin. However, the release in response to reptilase was normal, and the reptilase, ancord, and thrombin coagulase times were within control (normal) values. In addition, the patients' fibrinogen showed normal polymerization of preformed fibrin monomers, normal sialic acid content, and normal binding to ADP or epinephrine-stimulated platelets. Our studies support the concept that thrombin and platelets play an important role in the occurrence of stroke in these patients and suggest a direction to be followed to identify the mechanism(s) contributing to thrombosis in subjects with abnormal fibrinopeptide release. (Arteriosclerosis and Thrombosis 1991;11:785-796)

In 1958, Imperato and Dettori first called attention to an inherited coagulation defect characterized by hypofibrinogenemia and fibrinopenia. Since that report, the number of congenital dysfibrinogenemias identified has steadily increased, and the nature of the associated coagulation abnormalities has been delineated. Most of these qualitatively abnormal fibrinogenemias are clinically silent or are associated with bleeding problems or defective wound healing. However, congenital dysfibrinogenemias in patients with a thrombotic tendency have also been reported. In some of these patients, an impaired binding of thrombin by fibrin has been found, and venous thrombosis has been suggested to occur as a consequence of excessive fibrin formation by free thrombin. Up to the present, only a few cases of arterial thrombosis associated with inherited dysfibrinogenemias have been reported, and the mechanism by which arterial thrombosis may occur in such cases is poorly understood. We report here on two siblings with congenital dysfibrinogenemia and juvenile stroke whose fibrin...
has a defective thrombin-adsorbing capacity. We suggest a role for thrombin and platelets in the occurrence of thrombosis in these patients.

Several lines of evidence indicate that the thrombin-adsorbing capacity of fibrin(ogen) involves site(s) of thrombin and fibrin(ogen) distinct from those playing a direct role in the cleavage of fibrinopeptides.\textsuperscript{15-20} Our data support these concepts.

Methods

**Materials**

Na\textsubscript{2} EDTA, basic fuchsin, and 2-dithiothreitol were obtained from Fisher Scientific Co., King of Prussia, Pa. (NH\textsubscript{4})\textsubscript{2}S\textsubscript{2}O\textsubscript{8}, acrylamide, N,N'-methylenebis-acrylamide, molecular weight standards, and the Mini Protean II apparatus were from Bio-Rad Laboratories, Richmond, Calif. Bovine serum albumin (Pentex, fraction V) was from Miles Laboratories, Inc., Elkhart, Ind. Sialic acid (N-acetylneuraminic acid, type IV, crystalline), sodium dodecyl sulfate (SDS), thiobarbituric acid, ATP and ADP sodium salts, aspirin (crystalline), lactoperoxidase (70–100 units/mg protein), and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid were from Sigma Chemical Co., St. Louis, Mo. Epinephrine (epinephrine HCl, isotonic solution) was from Elkins-Sinn, Inc., Cherry Hill, N.J. Carrier-free iodine-125–labeled Na\textsubscript{1} was from New England Nuclear, Florence, Italy. Iodo-Gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was from Pierce Eurochemie, Oud Beijerland, The Netherlands. Thrombin was obtained from Parke-Davis and Co., Detroit, Mich. In selected experiments, highly purified human α-thrombin and γ-thrombin were employed. These two reagents were kindly provided by J. Fenton, New York State Department of Health Laboratories, Albany, N.Y., and were stored in small aliquots at −70°C (we thank E. Tremoli and M.B. Donati for supplying this material). D-Phenylalanyl-L-propyl-L-arginine chloromethyl ketone 2HC\textsubscript{1} (PPACK) was from Calbiochem Co., La Jolla, Calif., and was kindly provided by A. Bini. Reptilase was from Pentapharm, Basel, Switzerland; ancord was kindly provided by P. Gaffney, National Institute for Biological Standards and Controls, London U.K.; thrombin coagulase was from Boehringer-Biochemia, Milan, Italy; and human melanoma tissue-type plasminogen activator (t-PA), as well as human plasminogen, was obtained from D. Collen, Centre for Thrombosis and Vascular Research, Leuven, Belgium. Plasma was prepared by incubating plasminogen (0.5 mg/ml) with streptokinase (1,000 units/ml) for 20 minutes at 37°C. Silicone oils (methyl silicone 1.0 DC 200 and Hexphenyl silicone 125 DC 550) were purchased from W.F. Nye, Specialty Lubricants, New Bedford, Mass. Mixtures of DC 200 and DC 550 were prepared as previously reported.\textsuperscript{21} Test tubes for the enzyme-linked immunosorbent assay (ELISA) determination of fibrinopeptide A (FPA) (Maxisorp Nunc 11129-Bynatech) were from Intermed, Roskilde, Denmark.

The reagents for the ELISA determination of the D-dimers were from Ortho General Diagnostics, Milan, Italy, while the chromogenic substrate Chromozym TH and the reagents for the determination of FPA were from Boehringer Mannheim, GmbH Diagnostica, Mannheim, FRG. The ELISA determinations as well as the studies in which the chromogenic substrates were employed were performed according to the manufacturers’ recommendations.

**Purification and Iodination of Fibrinogen**

Blood was collected from 10 normal donors and the two patients on different occasions. Nine volumes of blood (total, 135 ml) were collected from the antecubital vein via a 19-gauge scalp vein needle into one volume (15 ml) of 3.8% trisodium citrate. Samples were immediately centrifuged at 2,500g for 15 minutes. The platelet-poor plasma (PPP) was removed and tested immediately or was stored in small aliquots at −70°C. Fibrinogen was purified by the method of Martinez et al.,\textsuperscript{22} and the concentration of protein present in the purified material was measured spectrophotometrically at 280 nm using a 1% molar extinction coefficient of 15.\textsuperscript{23} The recovery of fibrinogen by this method is 40–60%, and the fibrinogen is 96% clottable. Both control and patients’ purified fibrinogens were labeled with \textsuperscript{125}I by the Iodo-Gen technique.\textsuperscript{24} The specific activity of fibrinogen labeled in this manner was 0.3–0.6×10\textsuperscript{6} cpm/μg protein. Unlabeled and labeled fibrinogens showed the characteristic Acr, Bβ, and γ peaks without any degradation products when reduced with 0.2 M 2-dithiothreitol, electrophoresed on 7.5% SDS polyacrylamide gels,\textsuperscript{25} and stained with Coomassie blue. When periodic acid–Schiff was employed,\textsuperscript{26} only the Bβ and γ peaks could be visualized. PPACK-inhibited thrombin was prepared according to the manufacturer’s recommendations by incubation of 10 units/ml (34 nM) of radiolabeled thrombin for 15 minutes at 37°C with a 20-fold molar excess of PPACK diluted in phosphate-buffered saline.

**Coagulation Tests and Related Studies**

Prothrombin time, partial thromboplastin time, assays of coagulation factors, and thrombin and reptilase clotting times of normal and patients’ purified fibrinogen or citrated plasma, as well as the studies of aggregation of fibrin monomers of normal and patients’ purified fibrinogen, were determined according to Palascak and Martinez.\textsuperscript{27} For fibrin monomer aggregation studies, 1 mg/ml of control or patients’ fibrinogen dialyzed against 0.06 M phosphate buffer, pH 6.8, was incubated at 37°C for 3 hours with 0.12% EDTA, 500 units/ml aprotinin, and 10 units/ml thrombin. After a 60-minute incubation at 4°C, the clot, recovered on a glass rod, was washed in phosphate-buffered saline and dissolved in 0.5 ml 0.02 M acetic acid solution containing 250 units/ml aprotinin. The concentration of monomers in the solution was then adjusted to 1 mg/ml; 0.12-ml aliquots of the samples were added to 1.1 ml 0.06 M
phosphate buffer whose ionic strength was adjusted to 0.12 or 0.24 with NaCl; the aggregation was then read as a function of time at 350 nm. The rate and pattern of total fibrinopeptide release (i.e., FPA and FPB) were studied according to Gralnik et al.28 by measuring trichloroacetic acid–soluble arginine at time intervals between 5 and 60 minutes after addition of reptilase (100 μl of a 20 μg/ml solution) or thrombin (50 μl of a 12.5 units/ml solution) to a 2.8 mg/ml solution of control or patients' fibrinogen. The specific release of FPA was determined by an ELISA method essentially as recommended by the manufacturer, with the following modifications. After incubation of control or patients' fibrinogen with buffer or CaCl₂ (10 mM), concentrations of thrombin or reptilase similar to those employed to release soluble arginine were added, and at time intervals between 1 and 60 minutes, fibrinogen was precipitated twice with bentonite. The supernatants were then allowed to react with a rabbit antiserum against FPA, and after a 1-hour incubation at room temperature, aliquots of the immune complex were transferred to test tubes precoated with FPA. After an additional hour, appropriate amounts of goat anti-mouse immunoglobulin G coupled with peroxidase were added, o-phenylenediamine was employed as a chromogen, and the amount of FPA released was evaluated spectrophotometrically at 492 nm. Total sialic acid contents of normal and patients' fibrinogen were measured by the thiobarbituric acid method after acid hydrolysis of the purified protein.29 Plasma fibrinogen concentrations were determined functionally by the Clauss method (FPT-Dil, Bonomelli, Hommel Farmaceutici, Dolzago, Italy) and immunologically by the latex particle agglutination inhibition test (Diagen Test, Mascia-Brunelli, Milano, Italy) or by rocket immunoelectrophoresis.30

Degradation of Purified Fibrinogen and Fibrin by Plasmin

For these studies, 5 mg/ml purified fibrinogen was incubated at 37°C in plastic tubes with plasmin (final concentration, 0.05 μM), and at intervals between 0 and 240 minutes after addition of the proteolytic enzyme, aliquots of the mixture (about 20 μl) were removed, diluted in SDS buffer with or without the reducing agent, boiled for 10 minutes, and electrophoresed on 7.5% SDS polyacrylamide minigels (5% for unreduced samples). For these experiments, fibrinogen was tested either after dilution in citrate buffer or after overnight dialysis against 0.02 M tris(hydroxymethyl)aminomethane (Tris), 0.15 M NaCl, pH 7.4. The latter preparation was proteolyzed with plasmin both before and after addition of Ca²⁺ (1–5 mM). For the degradation of non–cross-linked fibrin, fibrinogen (5 mg/ml) dissolved in citrate or in Tris buffer was mixed at 37°C with Na₂ EDTA (final concentration, 4 mM), plasmin (final concentration, 0.05 μM), and thrombin (final concentration, 20 units/ml). Then, aliquots of the supernatant were removed at intervals between 1 and 4 hours and processed as described above for fibrinogen.

Tissue-Type Plasminogen Activator–Induced Lysis of Plasma Clots

Citrated plasma (300 μl) was mixed with t-PA (final concentration, 37.5 IU/ml), CaCl₂ (final concentration, 25 mM), and thrombin (final concentration, 100 units/ml). The mixture was immediately transferred to siliconized tubes (4-mm i.d.) and allowed to clot at 37°C. After 15 minutes, a 1-cm portion of the clot was transferred to a petri dish, washed for 15 minutes in cold phosphate-buffered saline, and incubated at 37°C in autologous plasma. At timed intervals between 1 and 24 hours, 50-μl aliquots of the supernatant were collected, and the amount of D-dimers released from the clot was determined by an ELISA method. At each time and for each sample, the extent of the lysis was calculated from the amount of fibrin split products released from the clot and was expressed as a percentage of the D-dimer concentration measured at the completion of lysis.

Binding of Thrombin by Fibrin

The binding of radiolabeled thrombin by normal or patients' fibrinogen (both at a final concentration of 10 mg/ml) was performed essentially as described by Liu et al.31 with modifications. Briefly, purified α-thrombin (specific activity, 4,645 US units/mg protein) was adjusted (with a 0.02 M sodium barbital buffer, pH 7.5, containing 0.13 M NaCl) to an ion strength of 0.15 and labeled with 125I by the lactoperoxidase method.32 The labeled protein had a specific activity of 5–10⁶ cpm/nmol and was similar to the unlabeled enzyme in its ability to clot fibrinogen. For binding studies, 50 μl of increasing concentrations of 125I-thrombin (final concentration, 5–35 nM, corresponding to 1.5–10.5 units/ml, respectively) were added at 37°C to separate Eppendorf tubes containing 200 μl of either control or patients' fibrinogen (both at a final concentration of 0.05 μM). The samples were centrifuged for 1 minute at 12,000g, the clot was washed twice with barbital buffer, and the radioactivity present in the clot and in the supernatant was counted separately. Free thrombin was also measured by the Chromozym TH method. In another series of experiments, the binding of normal or PPACK-inhibited thrombin to the fibrin preformed by reptilase was determined. For these studies, 1 minute after the addition of reptilase (100 μl of a 20 μg/ml solution) to 200 μl fibrinogen (final concentration, 10 mg/ml) in 300 μl barbital buffer, increasing concentrations (1–2.5 units/ml) of thrombin were added, and the amounts of free and bound thrombin were determined 1 minute later. With the exception of samples in which PPACK-inhibited thrombin was employed, the extent of the binding was also evaluated by the
Chromozym TH method. The studies in which the patients' PPP was substituted for purified fibrinogen were performed in a similar fashion except that to reduce the time-dependent inhibition of thrombin by antithrombin III, thrombin and reptilase (or buffer) were added simultaneously, and the radioactivity present in the clot and in the supernatant was separated 1 minute later. In addition, in selected experiments reptilase was omitted. The PPP samples in which γ-thrombin was substituted for α-thrombin were handled in a similar fashion.

Effect of Residual Free Thrombin on Interaction of Patients' Fibrinogen With Platelets

Preparation of platelet-rich plasma (PRP), as well as suspensions of washed platelets, and studies of platelet aggregation and secretion of ATP were performed as previously reported. The interaction of patients' fibrinogen with platelets, as well as the ability of residual free thrombin to affect this interaction, was determined in aggregation and binding studies. For aggregation studies, 500 μl washed platelets was resuspended in control or patients' PPP or in Tyrode's buffer (pH 7.4) containing normal or patients' purified fibrinogen (500 nM). In each case, the number of platelets was adjusted to 3x10^9/ml. ADP or epinephrine was added, and the minimum concentrations of these agents required to cause 50% light transmittance within 3 minutes were determined. In studies of the effect of residual free thrombin on platelet aggregate, ADP or epinephrine was employed at concentrations that, when tested alone, caused only 10% light transmittance. Microliter amounts of buffer or the thrombin-containing supernatant were added simultaneously with each of these aggregating agents, and aggregation and secretion of ATP were monitored. The binding of patients' 125I-fibrinogen to platelets was determined as described for normal fibrinogen. In studies in which thrombin-containing supernatant on the abnormal protein from the three siblings are similar (References 11 and 33 and this article).

Case Histories

The patients are the offspring of a consanguineous marriage, the father and mother being first cousins. Both parents are asymptomatic and show normal coagulation parameters. In contrast, of the four children, three experienced thromboembolic complications. The propositus L.V. was admitted to the Department of Neurology of Naples University Medical School in 1976 at the age of 26 because of a left-sided hemiparesis. Her medical history was uneventful until the time of admission, and she denied smoking or taking oral contraceptives. Clinical and laboratory studies excluded abnormalities of lipid or carbohydrate metabolism, and her blood pressure was normal. Angiography revealed an occlusion of the right internal carotid artery just at its origin. L.P. is the younger brother of L.V. He was admitted to the Department of Neurology in 1978 at the age of 21 because of sudden weakness of the left limbs and impaired superficial sensation. A diagnosis of left-sided hemiparesis was made, and like his sister he did not have hypertension or abnormalities of liver or kidney function or of lipid or carbohydrate metabolism. At that time, the patient smoked five cigarettes per day. Transfemoral angiography was then performed, and several days after the examination, the patient developed a severe motor weakness of the lower limbs. A new angiographic study showed obstruction of the abdominal aorta just below the renal arteries. The patient was then transferred to the Division of Vascular Surgery for an aortobifemoral bypass operation. Coagulation studies showed similar abnormalities in both patients, and the abnormal fibrinogen identified had been designated as fibrinogen Naples. Since then, both patients have been treated with Coumadin (Crinos Farmacobiologica, Villa Guardia, Italy) and have had no other thrombotic problems. Another sibling was admitted to the Institute of Internal Medicine of Milan University in 1983 at the age of 32 with symptoms of deep vein thrombosis. These symptoms occurred 3 weeks after abdominal surgery. The majority of the findings on the abnormal protein from the three siblings are similar (References 11 and 33 and this article).

Results

Coagulation Tests and Related Studies

Patients' plasma partial thromboplastin time, antithrombin III, plasminogen, and single coagulation factors, as well as platelet aggregation in response to ADP, epinephrine, or collagen, were all normal. The template bleeding time of both patients was normal. Table 1 shows that the prothrombin time was twice the normal value, while the reptilase time, the ancrod time, and the thrombin coagulase time were normal. The thrombin time in response to 5 units/ml thrombin was higher than 300 seconds. Addition of increasing concentrations of thrombin resulted in progressive correction of thrombin time. When the thrombin time was determined in the presence of 10 mM

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CaCl₂ 20 units/ml gave a clotting time of 22 and 20 seconds for fibrinogen from L.P. and L.V., respectively. Similar coagulation abnormalities were observed when the patients' purified fibrinogen was substituted for the patients' plasma. Addition of increasing concentrations of patients' plasma (25–75 μl) to proportionally decreasing amounts (75–25 μl) of control plasma resulted in a progressive prolongation of the thrombin time that did not occur when heat-defibrinated patients' plasma was used. The euglobulin lysis time and the dilute whole-blood clot lysis time were normal, and the patients' clots were not soluble in 5 M urea (Table 1). The rate and extent of total fibrinopeptide released in response to reptilase were normal, while both these parameters were 50% of normal values when thrombin was used (Figure 1, upper panel). On the other hand, polymerization of preformed fibrin monomers was normal at low and high ionic strength (Figure 1, lower panel). The abnormal fibrinopeptide released in response to thrombin was analyzed further. Control and patients' purified fibrinogen was incubated with thrombin or reptilase, and the release of FPA was measured by the ELISA method. When thrombin was employed, the rate and the extent of FPA released from patients' fibrinogen were markedly lower than normal (Figure 2), and the abnormality was quantitatively and qualitatively similar when CaCl₂ (10 mM) was added to the system (data not shown). When reptilase was used, maximal release from normal or patients' fibrinogen occurred within 10 minutes, and in three separate determinations, the amount released from patients' fibrinogen was within control values (5.2±0.5 μg/mg for control fibrinogen, 4.7±0.7 for the protein from L.P., and 5.7±0.9 for that from L.V.; p always >0.05). The sialic acid residues of the fibrinogen molecule, measured after acid hydrolysis, were normal (5.9 sialic acid residues/molecule in the fibrinogen from L.P., 6.2 in that from L.V., and 5.4–6.5 in that of normal fibrinogen). Plasma fibrinogen concentrations measured by the Clauss method were 279 mg/dl for L.P. and 176 mg/dl for L.V., whereas the values were twice as high when determined by the latex particle agglutination inhibition test or by rocket immunoelectrophoresis. Binding of patients' fibrinogen to platelets exposed to 10 μM ADP was normal: 5.38±0.60 pmol fibrinogen/3x10⁸ platelets with the protein from L.P., 6.03±0.15 with that from L.V., and 6.12±0.21 with normal fibrinogen (p always >0.05). Likewise, binding in response to 20 μM epinephrine was normal: 3.96±0.67 pmol fibrinogen/3x10⁸ platelets with the protein from L.P., 4.52±0.28 with that from L.V., and 4.16±0.42 with normal fibrinogen (p always >0.05). The binding was also normal when lower concentrations of ADP (see below) or epinephrine (not shown) were employed. Other coagulation tests and related studies (e.g., percent clottability of the abnormal protein, release of FPB, etc.) gave results similar to those reported in the other sibling.¹¹

**Sensitivity of Patients' Fibrinogen and Fibrin to Plasmin**

One hour after the addition of plasmin, the degradation of the Aα and Bβ chains of reduced control fibrinogen was almost complete. In contrast, after the same time, the lysis of patients' fibrinogen handled in a similar manner was essentially limited to the Aα chain. This retarded degradation was little affected by longer exposure to plasmin (4 hours) or by predigestion of the proteins against 0.02 M Tris, 0.15 M NaCl (data not shown). In contrast, sensitivity to plasmin of control and patients' fibrinogen was comparable when Ca³⁺ (1–5 mM) was added to the samples before plasmin was added. The data relative to those from studies in which 2 mM Ca³⁺ were employed are shown in Figure 3. Likewise, the degradation of non-cross-linked patients' fibrin by plasmin was normal (not shown). Finally, the rate of lysis of cross-linked patients' fibrin was within control

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**Table 1. Patients' Coagulation Tests and Studies of Fibrinogen and Fibrin**

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values, as judged by the release of the d-dimers from patients' plasma clots incubated with t-PA (Figure 4).

**Binding of Thrombin by Patients' Fibrin**

As shown in Figure 5, the binding of thrombin by patients' or control fibrinogen was concentration dependent, as was that reported by others. However, at all concentrations tested, the binding of thrombin by patients' fibrinogen was 25–30% of normal values (p always <0.05). Defective binding of thrombin was also observed when FPA was removed from control and patients' fibrinogen by reptilase and when normal or PPACK-inactivated thrombin was added to the mixture. The data obtained with 1 unit/ml thrombin are presented in Table 2. Comparable results were obtained when higher concentrations (2.5 units/ml) of thrombin were employed (data not shown). In other studies, the abnormal binding by patients' fibrin was documented by the Chromozym TH method. In five experiments in which 1 unit/ml nonradiolabeled thrombin (3.4 nM) was added to 200 μl of control or patients' fibrinogen preincubated for 1 minute with reptilase, 1 μl of the supernatant from control fibrinogen contained 10.9±0.3 pmol residual free thrombin; 1 μl of that from L.P. contained 16.2±0.4 pmol; and 1 μl of that from L.V. contained 15.1±0.4 pmol (p always <0.05 control versus each patient). Comparable differences were observed when higher concentrations (2.5 units/ml) of thrombin were used (not shown). A series of studies was finally performed to check whether the defective binding of thrombin by fibrin could still be detected in patients' plasma, that is, in the presence of normal concentrations of antithrombin III. This appeared to be so. In three experiments in which 1 unit/ml thrombin and 100 μl 20 mg/ml reptilase were added simultaneously to 200 μl of control or
patients' plasma, control plasma bound 12.3±1.7% of normal thrombin and 10.7±0.9% of PPACK-thrombin; fibrin from L.P. bound 4.2±1.3% and 4.0±0.5% of normal and PPACK-inhibited thrombin, respectively; and that from L.V. bound 5.0±0.6% and 4.0±0.3%, respectively (p always <0.05 control versus each patient for each agonist). For both control and patients' samples, the binding of thrombin by fibrin in plasma was 70–80% of that observed in experiments with purified fibrinogen.

Effect of Residual Free Thrombin on Platelet Aggregation, Secretion, and Fibrinogen Binding

The aggregation of platelets is involved in arterial thrombosis.34 Therefore, the possible effect of the defective binding of thrombin by patients' fibrin on platelets was examined. The minimum concentrations of ADP or epinephrine that caused 50% aggregation of platelets resuspended in patients' PPP were similar to those found with PPP from controls (p always >0.05). Likewise, the concentrations of ADP or epinephrine that caused 10% aggregation of washed platelets in the presence of normal or patients' fibrinogen were similar to each other. Addition of microliter amounts of the thrombin-containing supernatants greatly enhanced the sensitivity of platelets to these concentrations of ADP or epinephrine. This enhancement was detected when the supernatant of normal or patients' fibrin was employed. However, the effect of the patients' supernatant was always greater than that of the supernatant from normal fibrin. A typical example of these findings is shown in Figure 6. Ten microliters of the supernatant from the clot obtained from patients' fibrinogen and

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The aggregation of platelets is involved in arterial thrombosis.34 Therefore, the possible effect of the defective binding of thrombin by patients' fibrin on platelets was examined. The minimum concentrations of ADP or epinephrine that caused 50% aggregation of platelets resuspended in patients' PPP were similar to those found with PPP from controls (p always >0.05). Likewise, the concentrations of ADP or epinephrine that caused 10% aggregation of washed platelets in the presence of normal or patients' fibrinogen were similar to each other. Addition of microliter amounts of the thrombin-containing supernatants greatly enhanced the sensitivity of platelets to these concentrations of ADP or epinephrine. This enhancement was detected when the supernatant of normal or patients' fibrin was employed. However, the effect of the patients' supernatant was always greater than that of the supernatant from normal fibrin. A typical example of these findings is shown in Figure 6. Ten microliters of the supernatant from the clot obtained from patients' fibrinogen and

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Figure 4. Line plot of t-PA-induced lysis (%) of clots prepared from control (○) and patient’s (L.P., ◊) plasma as a function of time (hours). t-PA, tissue-type plasminogen activator. Similar results were found when clots were prepared from plasma of patient L.V.

1 unit/ml thrombin (3.4 nM) induced 50% aggregation and 1.5 μM ATP secretion when added to washed platelets in combination with 1 μM ADP. In contrast, 10 μl of the clot supernatant from normal fibrinogen and 1 unit/ml thrombin induced only 20% aggregation and minimum ATP secretion under similar conditions. The amount per microliter of free thrombin measured in the platelet suspension to which 10 μl of the patient’s supernatant was added was 16.2 pmol, while in the one to which 10 μl of the control supernatant was added, the amount of free thrombin was 11.8 pmol. When concentrations of purified thrombin comparable to those found in control and patients’ supernatants were added to platelet suspensions in combination with 1 μM ADP, enhancements of platelet aggregation and secretion comparable to those caused by the supernatants were observed (data not shown). Qualitatively comparable results were obtained when normal or either of the patients’ PPP was substituted for purified fibrinogen in the clotting mixture, as well as when reptilase was omitted (data not shown).

In concordance with platelet aggregation and secretion results, the thrombin-containing supernatants significantly affected fibrinogen binding to platelets exposed to low concentrations of epinephrine or ADP. The data relative to studies with 1 μM ADP are shown in Figure 7 and indicate that, as for platelet aggregation and secretion, the effect of the patients’ supernatants is always greater than that of control. Analysis of the data (Table 3) indicates that the binding in response to ADP or epinephrine used alone is comparable to that observed when these agents are used in combination with the thrombin-containing supernatants. Thus, the supernatants appeared to affect the exposure rather than the affinity of fibrinogen for its platelet receptor. These differences in the effect on platelet aggregation, secretion, and fibrinogen binding were correlated with the amounts of free thrombin detected in the supernatant by the Chromozym TH method. Thus, they suggested that the abnormal enhancement of platelet aggregation and secretion caused by the patients’ supernatants was due to the larger amount of residual free thrombin present in the patients’ supernatants. To confirm the thrombin dependence of the phenomena observed, the effect of hirudin was investigated. Simultaneous addition of this specific thrombin inhibitor and of 10 μl of the thrombin-containing supernatant to the platelets suppressed the enhancement of ADP-induced fibrinogen binding (Figure 7). The enhancement was not restored by amounts of the supernatant as high as 30 μl (not shown). Aspirin is known to inhibit the aggregation and secretion of platelets exposed to low concentrations of thrombin, and this inhibitory effect may be overcome by increasing the concen-
Figure 6. Tracings showing enhancement of ADP-induced aggregation and secretion of washed platelets by thrombin-containing supernatant from patient L.P. Similar results were obtained when fibrinogen from patient L.V. was used, as well as when reptilase was omitted from incubation mixture. Enhancement of aggregation and secretion was also observed when patient’s platelet-poor plasma was substituted for purified fibrinogen.

tion of thrombin used. Therefore, we determined the potentiating effect of the supernatant on platelets preincubated at 37°C with 100 μM aspirin for 30 minutes. Aspirin was as effective as hirudin in suppressing the enhancement of the binding induced by 10 μl patients’ supernatants. However, when 30 μl of patients’ supernatants was employed, aspirin was ineffective (not shown). Finally, to further examine the possibility that the enhancement by the patients’ supernatants was due to defective binding of thrombin by fibrin that would lead to abnormally high amounts of free thrombin in the supernatant, γ-thrombin was substituted for α-thrombin. α-Thrombin has a high affinity for fibrinogen, while γ-thrombin has practically none. Using γ-thrombin, we found that 10 μl normal and patients’ PPP to which 15 nM (final concentration) of γ-thrombin has been added were equipotent (in combination with 1 μM ADP) in causing 50% light transmittance and secretion of 1.5 μM ATP.

Figure 7. Bar graph showing enhancement of ADP-induced binding of patient’s (L.P.) radiolabeled fibrinogen to platelets (125I fibrinogen bound; pmol/10^8 platelets) by thrombin-containing supernatant and inhibition by hirudin. Both for control and patient, enhancement caused by addition of thrombin-containing supernatant was significant (p always <0.05, ADP+buffer vs. ADP+supernatant). Hirudin significantly affected this enhancement (p<0.05, ADP+supernatant vs. ADP+supernatant+hirudin). Statistical analysis also showed a difference between enhancement obtained using thrombin-containing supernatant from the patient and that from control (p<0.05). Similar results were obtained when thrombin-containing supernatant from patient L.V. was employed. Data are mean±SEM of three determinations.
not associated with thrombotic risk. Thus, it seems that patients' fibrinogen binds to platelets and supports the notion that platelet aggregation is similar to that seen with normal fibrinogen. The ability of the thrombin-containing supernatant to enhance platelet aggregation was correlated with the amounts of residual free thrombin present in the supernatant. Patients' supernatants markedly enhanced the binding of fibrinogen to platelets exposed to low concentrations of ADP. Hirudin, a specific thrombin inhibitor, suppressed the enhancing effect of the supernatant, and this suppression could not be overcome by increasing the amounts of the thrombin-containing supernatant. Normal and patients' supernatants were equipotent in enhancing ADP-induced platelet aggregation when γ-thrombin was substituted for α-thrombin. Binding studies indicated that lower than normal amounts of thrombin were adsorbed by patients' fibrin. Thus, after trauma to blood vessels, the thrombin formed may be poorly adsorbed by patients' fibrin, and abnormally large amounts of unadsorbed free thrombin may have local pathological consequences. Arterial thrombosis occurred in one of the patients after angiography. Under these circumstances, there could be sufficient locally accumulated free thrombin to act synergistically with minimum concentrations of other naturally occurring agents to enhance platelet activation.

Although the main thrust of this report was to describe a possible mechanism contributing to thrombosis in patients with abnormal fibrinopeptide release, these data also have implications for the pathophysiology of the thrombin–fibrinogen interaction. One of the major functions of plasma fibrinogen is its conversion into insoluble fibrin. This process initiates with the binding of thrombin to fibrinogen and the thrombin-catalyzed release of fibrinopeptides (i.e., the cleavage of the arginine<sub>16</sub>-glycine<sub>17</sub> bond of the Aα chain and of the arginine<sub>14</sub>-glycine<sub>15</sub> bond of the Bβ chain). In addition to this unlikely that these defects are involved in the thrombotic tendency of our patients.

Liu et al<sup>15</sup> showed that fibrin binds thrombin. This thrombin-adsorbing capacity of fibrin is commonly referred to as antithrombin I, and its pathophysiological significance remains to be defined. A current concept is that abnormally low binding of thrombin by fibrin may lead to abnormally high fibrin formation by the free enzyme, and this may play a role in the pathogenesis of venous thrombosis associated with this defect.<sup>1</sup> In addition to its role in the conversion of fibrinogen to fibrin, thrombin is a potent inducer of platelet aggregation, and at concentrations far below the ones that cause clotting of patients' fibrinogen, it greatly enhances the sensitivity of platelets to naturally occurring agents.<sup>37</sup> Thrombin-induced activation of platelets occurs within seconds<sup>37</sup>; the aggregation of platelets plays a central role in the pathogenesis of arterial thrombosis,<sup>34,38</sup> and studies in mice show that combinations of aggregating agents act synergistically in vivo to cause platelet aggregation.<sup>39</sup> Therefore, we investigated the relations between platelet aggregation and the impaired thrombin-adsorbing capacity of patients' fibrinogen. The ability of the thrombin-containing supernatant to enhance platelet aggregation was correlated with the amounts of residual free thrombin present in the supernatant. Patients' supernatants markedly enhanced the binding of fibrinogen to platelets exposed to low concentrations of ADP. Hirudin, a specific thrombin inhibitor, suppressed the enhancing effect of the supernatant, and this suppression could not be overcome by increasing the amounts of the thrombin-containing supernatant.

### Table 3. Analysis of <sup>125</sup>I-Fibrinogen Binding to Platelets Exposed to Low Concentrations of ADP Alone or in Combination With Microliter Amounts of Control or Patients' Thrombin-Containing Supernatant

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Kd (µM)</th>
<th>&lt;sup&gt;125&lt;/sup&gt;I-fibrinogen bound (pmol/10&lt;sup&gt;8&lt;/sup&gt; platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (1 µM) + buffer</td>
<td>21.4 ± 5.3</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>ADP (1 µM) + patient's supernatant</td>
<td>23.6 ± 4.7</td>
<td>7.1 ± 0.3*</td>
</tr>
<tr>
<td>ADP (1 µM) + control supernatant</td>
<td>22.7 ± 5.1</td>
<td>3.2 ± 0.4*†</td>
</tr>
</tbody>
</table>

Data presented are mean ± SEM of three determinations in which thrombin-containing supernatant from patient L.P. was employed. * p < 0.05 ADP plus buffer vs. ADP plus supernatant. † p < 0.05 ADP plus patient's vs. ADP plus control supernatant.

### Discussion

The mechanisms involved in the pathogenesis of arterial and venous thrombosis in dysfibrinogenemias are poorly understood. In a recent comprehensive review<sup>4</sup> on this topic, it was stated that "One perplexing finding in the clinical picture of abnormal fibrinogens is the presence of thrombosis..." and that "... Reactivity toward plasmin by fibrinogen mutants has not been systematically studied and could offer a pathogenesis for both bleeding and thrombotic manifestations." In fibrinogen New York, the deletion of amino acids 9–72 of the Bβ chain is associated with a low binding rate of thrombin by fibrin and a thrombotic tendency that is thought to be related to the impaired fibrinolysis of this mutant.<sup>7,8</sup> Fibrinogen Pamplona, a congenital dysfibrinogenemia with defective binding of thrombin by fibrin, also exhibits an abnormal fibrin-enhanced plasminogen activation.<sup>12</sup> Therefore, we determined the sensitivity of patients' fibrinogen and fibrin to plasmin. The impaired proteolysis of fibrinogen Naples was detected only in Ca<sup>2+</sup>-free preparations and is likely to have little pathophysiological significance, in view of the facts that the degradation of patients' fibrin by plasmin, the euglobulin lysis time, the whole-blood clot lysis time, and the stimulating effect of fibrin on t-PA–induced activation of plasminogen were entirely normal.

Abnormally high binding of fibrinogen to platelets is associated with increased in vitro platelet sensitivity to aggregating agents, and platelet hyperreactivity is present in some patients with an abnormally high tendency to thrombosis (see Reference 34 for a review). Furthermore, an abnormal fibrinogen associated with venous thrombosis has been shown to cause platelet hyperaggregability.<sup>36</sup> Our studies show that patients' fibrinogen binds to platelets and supports the notion that platelet aggregation is similar to that seen with normal fibrinogen. The patients' coagulation tests (Table 1) showed abnormalities that have been reported in several abnormal fibrinogens not associated with thrombotic risk. Thus, it seems
reversible binding, an additional binding between fibrin(ogen) and thrombin was demonstrated almost 50 years ago, and recent reports have defined the major features of this relatively rapid adsorption. It contributes to the thrombin-catalyzed cleavage of fibrinopeptides and involves site(s) of fibrin(ogen) and thrombin that are distinct from those that play a direct role in the cleavage. Our data are consistent with this formulation. After removal of FPA from patients' fibrinogen, the defective binding of thrombin by fibrin could still be detected. Pretreatment with PPACK, a specific inhibitor of the catalytic site of thrombin, minimally affected the impaired binding of thrombin by patients' fibrin (Table 2). The defective thrombin-adsorbing capacity of patients' fibrinogen was associated with an abnormal FPA release in response to thrombin. However, reactivity to reptilase, anacrod, and thrombin coagulase, three stimuli that specifically release FPA, was normal (Table 1). In addition, the cleavage of FPA is known to precede that of FPB, and high-performance liquid chromatography studies of fibrinogen from another affected sibling imply that the defective cleavage of fibrinopeptides from patients' fibrinogen cannot be accounted for by amino acid substitutions in the N-terminal region of the Aa or Bβ chain.

Fibrinogen New York is functionally characterized by a slow release of FPA, a 50% decrease in total release of FPB, and a defective binding of thrombin by fibrin. The biochemical defect is a deletion of amino acids 9–72 of the Bβ chain, a segment that includes part of FPB (amino acids 1–14) and a plasmin-derived peptide (amino acids 1–42). During the Xth Fibrinogen Workshop (Rouen, June 28–30, 1990), J. Koopman, J. Grimbergen, S.T. Lord, F. Haverkate, and P.M. Mannucci reported that they had identified the mutation alanine→threonine in the Bβ chain of fibrinogen Naples, defined the homozygous state of these patients, and related the structural defect to the impaired thrombin-adsorbing capacity of this protein. These similarities between fibrinogen Naples and fibrinogen New York are consistent with a relation between the defective thrombin-adsorbing capacity of fibrin(ogen) and the abnormal cleavage of fibrinopeptides. In addition, they support the concept that an important locus for the binding of thrombin at its secondary fibrin binding site is located in the TV-terminal region of the Aβ or Bβ chain.

In conclusion, our data support the theory that the thrombin-adsorbing capacity of fibrin plays an important role in the pathophysiology of fibrinogen and suggest that platelets and thrombin are involved in the pathogenesis of stroke in these patients.

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References


**KEY WORDS** • dysfibrinogenemia • antithrombin I • platelet aggregation • synergistic effects • thrombin • arterial thrombosis
A role for platelets and thrombin in the juvenile stroke of two siblings with defective thrombin-adsorbing capacity of fibrinogen.

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