Resistance of $\gamma$A/$\gamma'$ Fibrin Clots to Fibrinolysis*

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Elevated plasma fibrinogen levels are a major risk factor for thrombosis. This report shows two mechanisms by which fibrinogen can affect the fibrinolysis rate in vitro and thus may lead to thrombosis. First, the lysis rate of fibrin decreases as the initial concentration of fibrinogen increases. Second, a minor variant form of fibrinogen decreases the rate of fibrinolysis. This variant, $\gamma$A/$\gamma'$ fibrinogen, has one altered $\gamma$ chain and is known to bind to factor XIII zymogen. In a fibrinolysis assay containing purified thrombin, fibrinogen, tissue-type plasminogen activator, and plasminogen, clots from $\gamma$A/$\gamma'$ and $\gamma$A/$\gamma'$ fibrinogen lysed at similar rates. However, when factor XIII was added, slower lysis was seen in $\gamma$A/$\gamma'$ fibrin clots when compared with $\gamma$A/$\gamma$ fibrin clots. A D-dimer agglutination assay showed that the $\gamma$A/$\gamma'$ clots were more highly cross-linked than the $\gamma$A/$\gamma$ clots. The lysis rates of $\gamma$A/$\gamma'$ clots were similar to $\gamma$A/$\gamma$ clots in the presence of $N$-ethylmaleimide, a specific inhibitor of factor XIII. The $\gamma$A/$\gamma'$ fibrin clots made in the presence of factor XIII showed increased proteolytic resistance to both plasmin and trypsin. Clots made from afibrinogenemic plasma reconstituted with $\gamma$A/$\gamma'$ fibrinogen also showed significant resistance to lysis compared with $\gamma$A/$\gamma$ fibrinogen. These data demonstrate $\gamma$A/$\gamma'$ fibrin is resistant to fibrinolysis, possibly as a result of concentrating factor XIII on the clot. The total fibrinogen concentration and the amount of $\gamma$A/$\gamma'$ fibrinogen increase clot stability in vitro and thus may contribute independently to the risk of thrombosis in humans.

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Fibrinogen is a 340,000-Da dimeric glycoprotein composed of three polypeptide chains, $\alpha$ ($M_r = 65,000$), $\beta$ ($M_r = 56,000$), and $\gamma$ ($M_r = 47,000$) linked by disulfide bonds. Fibrinogen is converted to fibrin through limited proteolysis by thrombin, which exposes polymerization sites in fibrinogen (1). The fibrin monomers spontaneously associate with each other to form the web-like fibrin clot (2). A plasma transglutaminase, factor XIII, strengthens the fibrin clot by forming covalent bonds between adjacent fibrin monomers (3).

Plasma factor XIII is a 320,000-Da tetrameric protein composed of two polypeptide $a$ chains ($M_r = 83,000$) and two polypeptide $b$ chains ($M_r = 80,000$) (4). Factor XIII normally circulates as an inactive proenzyme until it is activated by thrombin cleavage of a 4000-Da activation peptide from each $a$ subunit, which is followed by the dissociation of the $b$ subunits. Activated factor XIII, or XIIIa, catalyzes the formation of $\gamma$-glutamyl-$\epsilon$-lysine bonds between polypeptide chains in fibrin (5). These cross-links strengthen the fibrin clot (6) and increase its resistance to lysis (7–9).

Recent evidence has revealed an association between a variant form of fibrinogen and factor XIII that may play a role in modulating the stability of a fibrin clot. This fibrinogen variant, referred to as $\gamma$A/$\gamma'$ fibrinogen, peak II fibrinogen (10), $\gamma$A-$\gamma$B fibrinogen (11), or $F_9^{360}, 57.5$ (12), contains an altered $\gamma$ chain termed $\gamma'$ (13), $\gamma$B (14), or $\gamma'^{7.5}$ (12), and comprises approximately 7–15% of the total fibrinogen found in plasma (10). The $\gamma'$ chain arises from alternative processing of the $\gamma$ chain mRNA (15, 16) that leads to the translation of a polypeptide with a 20-amino acid sequence substituted for the carboxy-terminal four amino acids of the $\gamma$ chain. The physiological function of the $\gamma'$ chain remains unclear; however, recent studies have shown that the $\gamma'$ chain binds directly to the zymogen form of factor XIII (17). This suggests that the $\gamma'$ chain of fibrinogen may serve as a carrier for factor XIII to increase the local concentration of factor XIII at the fibrin clot.

Elevated fibrinogen levels are a major risk factor for thrombosis. Several mechanisms have been proposed to explain the correlation between fibrinogen levels and thrombosis (18, 19). Fibrinogen may contribute to thrombosis by its role in atherosclerotic plaque formation. Fibrinogen binds to platelet membranes through glycoprotein IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) and thereby mediates platelet aggregation (20, 21). Fibrin degradation products stimulate the proliferation of vascular smooth muscle cells (22, 23), which may contribute to narrowing of blood vessels during atherosclerosis. In addition, once fibrinogen is incorporated into the atherosclerotic plaque, it binds low density lipoprotein and sequesters more fibrinogen (18), thus enhancing plaque formation. Another mechanism by which fibrinogen may lead to thrombotic disorders is by increasing blood viscosity. Increased blood viscosity favors the hypercoagulable state (24). Furthermore, increased fibrinogen levels lead to increased fibrin deposition (25–27). However, the causal role of these potential factors in thrombosis is still unclear.

The present report demonstrates that increases in total fibrinogen concentration result in increased clot stability toward fibrinolysis in vitro, suggesting a mechanistic explanation of how elevated fibrinogen levels per se may cause an increased risk of thrombosis. In addition, clots made from $\gamma$A/$\gamma'$ fibrinogen are more stable to fibrinolysis in vitro than clots made from $\gamma$A/$\gamma$ fibrinogen. Taken together, these findings suggest that both the total amount of fibrinogen and the amount of $\gamma$A/$\gamma'$ fibrinogen in plasma may be independent risk factors for thrombosis.

EXPERIMENTAL PROCEDURES

Purification of $\gamma$A/$\gamma$ and $\gamma$A/$\gamma'$ Fibrinogen—Plasminogen-free human plasma fibrinogen (Calbiochem) was dissolved in 39 mM Tris-PO₄,
pH 8.6, containing 5 mM e-aminoacapric acid (EACA) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and dialyzed exhaustively into the same buffer at 4 °C. Insoluble residue was removed by centrifugation at 10,000 × g for 30 min at 4 °C. The γA/γ and γA/γ' forms of fibrinogen were separated using DEAE-cellulose as described previously (29). Briefly, the fibrinogen solution was adsorbed to a column of DEAE-cellulose (6 × 20 cm) and eluted with a 1200-mL exponential gradient generated in a 600-mL constant volume mixing chamber from the starting buffer (39 mM Tris-PO4, pH 8.6, 5 mM EACA, 0.2 mM PMSF) to the final buffer (193 mM Tris-PO4, pH 4.6, 5 mM EACA, 0.2 mM PMSF). The absorbance was monitored at 280 nm, and 11-mL fractions were collected. The elution profile showed two peaks: γA/γ fibrinogen composed the first peak and γA/γ' fibrinogen composed the second smaller peak (data not shown).

In some experiments, γA/γ and γA/γ' fibrinogen were further purified using a glycine-L-proline-L-arginine-L-proline-L-cysteine (GPRPC)-agarose affinity resin (29). Briefly, the resin was prepared by reacting 10 mg of glycine-L-proline-L-arginine-L-proline-L-cysteine peptide (Howard Hughes Medical Institute Biopolymer Laboratory, Seattle, WA) with 10 mL of 5-thio-2-nitrobenzoate-agarose (Pierce) according to the manufacturer’s protocol. The dialyzed γA/γ or γA/γ' fibrinogen pool from DEAE-cellulose was adsorbed to a column (3 mL) of GPRPC-agarose, washed with 100 mM NaCl, 50 mM Tris-PO4, pH 7.8, 5 mM EACA, 0.2 mM PMSF, and then washed with 2 mM NaBr, 50 mM Tris-PO4, pH 7.8, 5 mM EACA, 0.2 mM PMSF. The fibrinogen was eluted in 1-mL fractions with 2 mM NaBr, 20 mM citrate, pH 5.3, 5 mM EACA, 0.2 mM PMSF and immediately neutralized with 0.02 volume of 2 M Tris-HCl, pH 8.0. The fibrinogen fractions were dialyzed into 137 mM NaCl, 2.7 mM KCl, 10 mM HEPES, pH 7.4, 1 mM CaCl2 and stored at −70 °C.

Fibrinolysis Assay Using Purified Components—Microtiter plate fibrinolysis assays were carried out as described previously by Jones and Meunier (30) using 96-well assay plates (Corning 25-880-96). Fibrinogen and Lys-plasminogen (Calbiochem) were added to an intermixing plate containing assay buffer (0.1 M glycine-L-proline-L-arginine-L-proline-L-cysteine; PMSF, phenylmethylsulfonyl fluoride; NaCl, 30 mM NaHCO3, 4 mM KCl, 1 mM CaCl2, 1 mM NaHPO4, 0.3 mM MgCl2, 0.4 mM MgSO4, 10 mM HEPES, pH 7.4, 0.01% Polysorbate 80). A separate assay plate contained α-thrombin (a generous gift from Dr. Walter J. Kisiel, University of Wisconsin Medical School, Milwaukee Clinical Campus), tissue plasminogen activator. The final concentrations of reagents were NaCl, 30 mM NaHCO3, 4 mM KCl, 1 mM CaCl2, 1 mM NaHPO4, 0.3 mM MgCl2, 0.4 mM MgSO4, 10 mM HEPES, pH 7.4, 0.01% Polysorbate 80, and α-thrombin was added to the wells to initiate clotting. The final concentrations of the reagents were 1.0 mg/ml fibrinogen, 10 μg/ml factor XIII, and 13.2 NIH units/ml α-thrombin in a total volume of 100 μL. The clots were incubated at room temperature until maximum turbidity was reached. When clotting was complete, 50 μL of 11.7 μg/ml trypsin were added to the clots. The turbidity of the clots was measured every 5 min at 405 nm. The optical density was converted to percent lysis as described above.

Whole Plasma Fibrinolysis Assay—The γA/γ and γA/γ' forms of fibrinogen were iodinated with Na125I (DuPont NEN) according to the method of Fraker and Speck (31) to a specific activity of 85,000–120,000 cpm/ng as described previously (29). The plasma fibrinolysis assays were carried out using the method of Wun and Capuano (32). Briefly, the iodinated γA/γ or γA/γ' fibrinogen in 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl2, 10 mM HEPES, pH 7.4, was added: 1 (v/v) to fibrinogen-deficient human plasma (George King Biomedical) at a final concentration of 1.25 mg/ml on ice. The plasma contained less than 0.1 mg/ml residual fibrinogen.) Clotting of the samples was initiated with 1 NIH unit/ml α-thrombin and the clots were incubated at 37 °C in the presence of 0.02% NaNO2. Samples were taken daily for 7 days and centrifuged for 5 min in a microcentrifuge to pellet the insoluble clot. 20 μl aliquots of the supernatant were counted in a gamma counter to measure the soluble 125I-fibrin degradation products. The percentage of clot lysis was calculated using the following equation and plotted versus time.

\[
% \text{lysis} = \frac{A_{405} \times 100}{A_{405 \text{ maximum}}} \tag{Eq. 1}
\]

and plotted versus time; the data were then fit to a sigmoidal curve-fitting routine. For clarity, a maximum of 15 data points per set are shown in each graph.

D-Dimer Assay—A D-dimer agglutination assay kit (Sigma) was used to measure D-dimer content in lysed fibrin clots. Clots were formed from γA/γ or γA/γ' fibrinogen using 1.0 mg/ml fibrinogen, 13.2 NIH units/ml α-thrombin, 16 mg/ml tissue-type plasminogen activator, 30 μg/ml Lys-plasminogen, and 10 μg/ml factor XIII and allowed to lyse completely. After lysis, 50 μl of the lysed clots were serially diluted in the manufacturer’s proprietary buffered saline solution, pH 7.3, containing 0.02% sodium azide. 20 μl of the diluted sample were then mixed with 20 μl of a suspension of latex beads coated with mouse monoclonal antibody (MA-8D3) specific for the fibrin D-dimer on the test card. The test card was rocked back and forth, and agglutination was scored after 3 min on duplicate samples.

Tryptic Digest of Fibrin Clots—γA/γ or γA/γ' fibrinogen and factor XIII were added to a 96-well assay plate containing assay buffer (0.1 M

The abbreviations used are: EACA, e-aminoacapric acid; GPPRC, glycine-L-proline-L-arginine-L-proline-L-cysteine; PMSF, phenylmethylsulfonyl fluoride.

![Figure 1](image)

**FIG. 1.** Fibrinolysis rates decrease with increasing fibrinogen concentrations. Fibrinolysis assays using 13.2 NIH units/ml α-thrombin, 16 ng/ml tissue-type plasminogen activator, 30 μg/ml Lys-plasminogen, and 10 μg/ml factor XIII were carried out at room temperature using the following concentrations of fibrinogen: 1 mg/ml, 2 mg/ml, 3 mg/ml (○), 4 mg/ml (□). Clot turbidity was monitored by absorbance at 405 nm and expressed as percent lysis. Each point is the average of duplicate determinations. Inset, the clot lysis t½ was determined from each curve and graphed versus fibrinogen concentration.
results were seen in the absence of factor XIII (data not shown). These results are consistent with other published data (30) and demonstrate that fibrin clots made at high fibrinogen concentrations are more resistant to lysis, suggesting a mechanistic explanation for the role of fibrinogen as a risk factor for thrombosis (33).

γA′/γ′ Fibrin Clots Are Resistant to Fibrinolysis—A second potential risk factor for thrombosis is the amount of γA′/γ′ fibrinogen in plasma. This form of fibrinogen has been shown to act as a carrier protein for factor XIII by binding directly to the γ′ chain (17). We hypothesized that this binding may serve to concentrate factor XIII locally at the surface of the growing fibrin clot, thereby resulting in a more highly cross-linked and stabilized clot. Fibrinolysis assays were therefore carried out using purified γA′γA or γA′γ′ fibrinogen. As seen in Fig. 2, both the rate of clotting and the rate of lysis were significantly decreased in γA′/γ′ fibrin clots. Clots made from γA′/γ′ fibrinogen in the presence of factor XIII clotted more slowly and subsequently lysed more slowly (Fig. 2, inset). Clots formed from γA′γA fibrinogen in the presence of physiological factor XIII concentrations (10 μg/ml) showed a lysis t½ of 72 ± 0 min, whereas the clots formed from γA′γ′ fibrinogen had a t½ of 179 ± 4 min. Clot stability was enhanced further in the presence of supraphysiological concentrations of factor XIII. In experiments where the factor XIII concentration was increased to 100 μg/ml, the γA′γA fibrinogen clots showed a lysis t½ of 99 ± 1 min, whereas the clots formed from γA′γ′ fibrinogen had a t½ of 207 ± 8 min. These data suggest that factor XIII increases the clotting time as well as decreases the fibrinolytic rates of γA′γ′ fibrin. In the absence of added factor XIII, fibrin clots made from γA′γA and γA′γ′ fibrinogen clotted and lysed at similar rates, with a lysis t½ of 50 ± 1 min for γA′γA fibrinogen and 54 ± 1 min for γA′γ′ fibrinogen (Fig. 3), demonstrating that the resistance of γA′γ′ fibrin to fibrinolysis is dependent on the presence of factor XIII. Furthermore, when factor XIIIa was inhibited as it was formed during clotting by the prior addition of N-ethylmaleimide, the fibrinolytic rates of γA′γ′ fibrin clots were similar to clots made without factor XIII (data not shown). These data show that active factor XIII is required for the decreased fibrinolytic rate of the γA′γ′ fibrin.

A D-dimer agglutination assay was performed to determine if the γA′γ′ fibrin clots were more highly cross-linked than the γA′γA fibrin clots. As seen in Table I, the lysed γA′γ′ fibrin clots showed positive agglutination down to a dilution of 1:300, whereas the γA′γA fibrin clots showed positive agglutination down to a dilution of only 1:128. These data show that the γA′γ′ fibrin clots are more highly cross-linked, possibly because of high local concentrations of factor XIII at the clot surface due to binding to the γ′ chain.

It should be noted that these assays were carried out with γA′γA and γA′γ′ fibrinogen that had been separated on DEAECelullose and further purified by GPRP-agarose chromatography. Factor XIII co-purifies with γA′γ′ fibrinogen on DEAECelullose (17), presumably by binding directly to the γ′ chain in γA′γ′ fibrinogen. We have verified that factor XIII co-purifies with γA′γ′ fibrinogen on DEAECelullose, but is depleted from γA′γ′ fibrinogen purified further on GPRP-agarose (Fig. 4). In experiments using fibrinogen purified only on DEAECelullose, the γA′γ′ fibrin clots were resistant to lysis in the absence of added factor XIII (Fig. 5), presumably due to the contaminating factor XIII.

To ensure that the increased lysis resistance was not due to factor XIII affecting the fibrinolytic system directly, γA′γA and γA′γ′ fibrin clots were formed with thrombin and then digested with trypsin. The γA′γA fibrin clots had a lysis t½ of 5.7 ± 0.2 min, whereas the γA′γ′ clots had a t½ of 39.1 ± 1.8 min (Fig. 6). These data suggest that the increased fibrinolytic resistance of the γA′γ′ fibrin clots is due primarily to proteolytic resistance, although inhibition of the fibrinolytic system cannot be ruled out entirely.

γA′/γ′ Fibrin Clots in Whole Plasma Are Resistant to Fibrinolysis—Fibrinolysis assays were also carried out in whole plasma (32) to ensure that the effect of γA′γ′ fibrinogen on clot lysis was not an artifact of the purified fibrinolysis assay. γA′γA and γA′γ′ fibrinogens were iodinated and added to fibrinogen-deficient plasma obtained from an afibrinogenemic individual. Clotting was initiated by adding thrombin, and samples were assayed daily for 7 days to quantitate the amount of soluble fibrin degradation products (Fig. 7). After 7 days, the clots formed from γA′γA fibrinogen showed complete lysis, with a lysis half-time of 4.3 ± 0.1 days, whereas clots formed from γA′γ′ fibrinogen exhibited no detectable lysis. These results demonstrate that γA′γ′ fibrin clots generated in whole plasma, in which a number of potentially confounding factors are pres-
(particularly protease inhibitors), are also resistant to fibrinolysis.

**DISCUSSION**

Fibrinolytic therapy is a widely used tool in the treatment of thrombosis. Fibrinolytic agents such as streptokinase or tissue-type plasminogen activator are used to activate the fibrinolytic system by converting the protease zymogen plasminogen to active plasmin, which then cleaves the fibrin clot into soluble components. Unfortunately, the administration of these agents often produces secondary hemorrhagic events that may be life-threatening. The development of new fibrinolytic agents with greater clot specificity is an area of intense study, but less attention has been focused on the factors that determine clot stability. If clot stability could be reduced, then lower amounts of these fibrinolytic agents could be used, thus reducing the chance of adverse side effects.

Increased fibrinogen levels are a major risk factor for cardiovascular disease (18, 19). There are many potential mechanisms to explain how high fibrinogen levels may contribute to the disease process. One possible role is that high fibrinogen levels produce a hypercoagulable state that favors thrombosis. It is known that the plasma levels of fibrinogen have a direct influence on the amount of fibrin formed in clots (25–27). The present study suggests two additional mechanisms through which high fibrinogen levels may lead to thrombosis. In agree-

| Dilution | Agglutination γA/γA fibrin | Agglutination γA/γA’ fibrin |
|----------|-----------------------------|-----------------------------|
| 1:16     | +                           | +                           |
| 1:32     | +                           | +                           |
| 1:64     | +                           | +                           |
| 1:128    | ±                           | +                           |
| 1:250    | –                           | +                           |
| 1:256    | –                           | +                           |
| 1:300    | –                           | ±                           |
| 1:350    | –                           | –                           |
ment with previously published data, the results show that fibrinolysis rates in vitro vary inversely with fibrinogen concentration (33). This suggests that in hyperfibrinogenemia, there may not only be a greater propensity for a hypercoagulable state, but the clots that result will also be more resistant to lysis. In addition, the present study shows that γA/γfibrinogen affects the stability of fibrin clots formed in vitro, possibly by increasing the local concentration of factor XIII at the clot. Fibrinolysis assays using purified components revealed that clots formed with γA/γfibrinogen show significantly decreased fibrinolysis compared with γA/γA fibrinogen, but only in the presence of active factor XIII. γA/γ fibrin clots are also more highly cross-linked than the γA/γA fibrin clots, as assessed with a D-dimer agglutination assay. The resistance of γA/γ fibrin clots to fibrinolysis was verified in reconstituted whole plasma. These results suggest that γA/γ fibrinogen levels in plasma may modulate clot stability by affecting fibrinolysis rates.

The physiological function of γA/γ fibrinogen has been elusive for many years. It is clear that the γ chain displays much less binding to the platelet fibrinogen receptor, glycoprotein Iib-IIia (integrin αIIbβ3) (11, 29, 34), but this negative role does not explain the raison d’être for γA/γ fibrinogen. The present studies suggest that the physiological role of γA/γ fibrinogen may be to increase clot stability by localizing factor XIII at the fibrin clot (Fig. 8). In this scenario, unactivated factor XIII is delivered to the growing fibrin clot by γA/γ fibrinogen. Through the action of thrombin, fibrinogen (including γA/γ fibrinogen) is converted to fibrin. Factor XIII that is bound to the newly formed γA/γ fibrin is subsequently activated to XIIIa, since fibrin acts as a positive modulator in factor XIII activation by thrombin (3). Thrombin cleavage dissociates the active a2 dimer from the b subunits of factor XIII, thereby allowing the free a2 dimer to catalyze the cross-linking of the fibrin clot. It is not clear at the present time whether the b subunits remain bound to the γ chain or dissociate upon thrombin activation of the a2 dimer. The subunits after thrombin activation. Thus, the delivery of factor XIII to the fibrin clot by γA/γ fibrinogen could serve to increase the local concentration of factor XIIIa and result in a more highly stabilized clot.

The concentration of γA/γ fibrinogen in plasma may therefore be a risk factor for thrombosis, independent of the total plasma fibrinogen concentration. The molecular mechanisms that affect the ratio of γA versus γ fibrinogen transcription are unknown, although the liver expresses both mRNAs, while other tissues express only the γA mRNA (35). It is clear that the two mRNAs arise by alternative processing of the original transcript (15, 16); it is possible that the relative amounts of the mRNA pools or their relative translation rates in liver vary among individuals, giving rise to different amounts of γA/γA and γA/γ fibrinogen. There are presently no data available regarding the variability of γA/γ fibrinogen levels in human populations. Epidemiologic studies to address this issue are therefore currently underway.

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