Influence of γ’ Fibrinogen Splice Variant on Fibrin Physical Properties and Fibrinolysis Rate


Objective—A splice variant of fibrinogen, γ’, has an altered C-terminal sequence in its gamma chain. This γA/γ’ fibrin is more resistant to lysis than γA/γA fibrin. Whether the physical properties of γ’ and γA fibrin may account for the difference in their fibrinolysis rate remains to be established.

Methods and Results—Mechanical and morphological properties of cross-linked purified fibrin, including permeability (Ks, in cm²) and clot stiffness (G’, in dyne/cm²), were measured after clotting γA and γ’ fibrinogens (1 mg/mL). γ/γ’ fibrin displayed a non-significant decrease in the density of fibrin fibers and slightly thicker fibers than γA/γA fibrin (12±2 fiber/10⁻⁵nm³ versus 16±2 fiber/10⁻⁵nm³ and 274±38 nm versus 257±41 nm for γ’/γ’ and γA/γA fibrin, respectively; P=NS). This resulted in a 20% increase of the permeability constant (6.9±1.7 10⁻⁵ cm² versus 5.5±1.9 10⁻⁵ cm², respectively; P=NS). Unexpectedly, γ’ fibrin was found to be 3-times stiffer than γA fibrin (72.6±2.6 dyne/cm² versus 25.1±2.3 dyne/cm²; P<0.001). Finally, there was a 10-fold decrease of the fibrin fiber lysis rate.

Conclusions—Fibrinolysis resistance that arises from the presence of γA/γ’ fibrinogen in the clot is related primarily to an increase of fibrin cross-linking with only slight modifications of the clot architecture.

Key Words: coagulation fibrin fibrinogen fibrinolysis thrombosis

High plasma fibrinogen is an independent predictor of cardiovascular events.1 The resulting hypercoagulable state and the decrease of fibrinolysis rate related to the greater amount of fibrin formed are thought to be the major underlying mechanisms of thrombotic events.2–4 Abnormal fibrin structure in vitro has been related to premature coronary artery disease in young patients and to severe thromboembolic disease, irrespective of the plasma fibrinogen concentration.5–8 This so-called thrombogenic fibrin consists of numerous thin fibers organized in a tight three-dimensional network in which resistance to lysis arises directly from its architecture.9,10

Recent in vitro investigations have revealed an association between defective fibrinolysis and the amount of γA/γ’ fibrinogen incorporated into the clot.4 This variant fibrinogen contains an altered γA chain termed γ’ and constitutes approximately 7% to 15% of the total fibrinogen found in plasma.11 The γ’ chain, which arises from alternative processing of the γA chain mRNA, serves as a carrier for factor XIII.12,13 Therefore, it has been suggested that γA/γ’ affects the stability of the clot formed in vitro by concentrating and increasing the rate of factor XIII activation, which catalyzes the formation of isopeptide bonds between α and γ chains of polymerizing fibrin strands.4,11 The resulting extensive cross-linking is thought to be responsible for the lysis resistance and may account in part for the role of γA/γ’ fibrinogen as an independent risk factor for coronary artery disease.14,15 In addition, a recent study of γA/γ’ fibrinogen levels in patients undergoing coronary angiography also showed that γA/γ’ fibrinogen levels were higher, on average, in coronary artery disease patients than in patients without coronary artery disease; this association was independent of total fibrinogen levels.15 More recently, clotting of γA/γA and γA/γ’ fibrinogens purified from plasma has been studied.16 There was delayed release of fibrinopeptide B from γA/γ’ compared with γA/γA, and turbidity and scanning electron microscopy showed that clots formed from plasma γA/γ’ fibrinogen were made of thinner fibers with more branch points than those from γA/γA fibrinogen.

In the present study, we compared the physical characteristics, including mechanical and morphological properties, of γA/γA and γ’/γ’ fibrin. A dynamic and structural approach

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using confocal microscopy was also used to assess fibrinoly-
sis of γA/γA and γ'/γ' fibrin.

Methods

Human thrombin was purchased from Enzyme Research Laborato-
ries (South Bend, IN) and stored at 1000 IU/mL. Unconjugated
colloidal gold solution for light microscopy was from British Biocel-
International (UK). Average particle size was 5 nm and the particle
concentration was 5×10^12/mL. Recombinant tissue plasminogen
activator (rtPA) was purchased from Boehringer Ingelheim (Ger-
many). Glu-plasminogen was purchased from American Diagnostica
(USA). Calcium chloride was from Sigma. Purified factor XIII was
a generous gift from Dr O. Gorkun (University of North Carolina,
Chapel Hill). Recombinant γA and γ' fibrinogens were prepared as
described.17

Preparation of Fibrin Clots

To a volume of 0.10 mL of purified fibrinogen (4.4 10^-6 Mol/L final
concentration in 20 mmol/L HEPES, pH 7.4, 0.15 mol/L NaCl), 10
μL of a concentrated CaCl2 solution was added to obtain a 5-mmol/L
final concentration, and activated XIII was added to obtain a final
concentration of 1 IU/mL. After 1 minute of incubation, 10 μL of
thrombin from the stock solution was added to obtain a final
concentration of 0.9 IU/mL. Mixing and incubation were conducted
in polypropylene tubes at room temperature. This solution was either
soaked up into glass microchambers designed for flow measurements
or placed between the coverslips of a torsion pendulum. Clotting was
allowed for at least 40 minutes in a moist atmosphere at room
temperature.

Scanning Light and Electron Microscopy

Fibrin clots in the microchambers were permeated with 5-nm
(diameter) colloidal gold particles at a final concentration of
2.5×10^12/mL dissolved in buffer containing 0.15 mol/L NaCl, 0.01
mol/L Tris/HCl, pH 7.4. The excess beads were washed out with 500
μL of the same buffer.10 Labeled specimens were scanned with LSM
510 interactive laser cytomter (Carl Zeiss) linked to a Zeiss inverted
microscope equipped with a Zeiss 63X water immersion objective
using the reflection mode. Optical sections were then projected and
combined into one image, which generated three-dimensional recon-
structed images.10

For scanning electron microscopy, the same clots were fixed,
dehydrated, critical-point dried, and coated with gold palladium.18
Specimens were observed and photographed digitally using a Philips
XL20 scanning electron microscope (Philips Electron Optics, Eind-
hoven, The Netherlands).

Morphological Analysis

Average fibrin fiber diameter and fiber and branching point densities
were determined on reconstructed images of laser scanning confocal
microscopy taken at high and low magnification, respectively.10
Fiber diameters were measured on digitized micrographs obtained
from the scanning electron microscopy experiments.

Clot Mechanical Properties

Permeability index (Ks) of fibrin clots was measured using the
permeation technique. Briefly, clots formed in thin glass microcham-
bers (250 μm) were permeated with 0.15 mol/L NaCl, 0.01 mol/L
Tris/HCl, pH 7.4, at different gradients of pressure. The calculated
Ks index (cm^3) provides information on the fibrin network archite-
cture (shape and size of the pores). It represents the surface of the gel
allowing flow and is obtained with the following equation: Ks=Q×L×η/ρ×A×ΔP×L, where Q is the volume of liquid (mL)
having the viscosity η (10^-2 poise) flowing through the fibrin gel
with length (L) (2.2 cm) and cross-section (A) (0.03 cm^2) in a given
time (t) (seconds) under a differential pressure (ΔP) (range: 4000 to
10 000 dyne/cm^2).19

Clots of a constant width of 1 mm were formed between two
12-mm-diameter glass coverslips in a torsion pendulum device as
previously described.20 A momentary impulse was carefully applied
to the torsion pendulum arm (air pressure), causing free oscillations
of this arm with strains <3%. The frequency of these free oscilla-
tions and the rate at which they are damped are functions of the
elastic and viscous properties of the clots and are independent of the
amplitude of the initial displacement of the arm. The rigidity index
G’ in dyne/cm^2, which reflects the clot’s elastic properties, was
calculated from the recordings of these oscillations on a chart
recorder. The loss modulus (G’’), which represents the energy
dissipated by non-elastic viscous processes was measured from
damping of the oscillations. The loss tangent (G’/G’’), which is a
measure of the energy dissipated by non-elastic viscous processes
relative to the energy stored by elastic processes, was also assessed.
This investigation was performed with and without activated factor
XIII.

Lysis Experiments

At the edge of the gold-labeled fibrin clots in the glass microcham-
ers,10 μL of a solution containing rt-PA (150 mmol/L) and
Glu-plasminogen (2.5 μg/mL) was loaded. After 15 minutes of
incubation in a moist atmosphere during which rt-PA and Glu-plas-
minogen were allowed to diffuse within the fibrin network, the edge
of the thrombus was scanned with the laser scanning confocal
microscope set in the reflection mode. Scanning was performed at
low magnification every minute, and 20 scans were performed 1 μm
apart along the z-axis for each time point. The lysis front velocity and
the rate of fiber digestion were determined for each type of fibrin
using reconstructed images.10

Statistical Analysis

Conventional tests were used for calculation of means and standard
deviations. Group differences in continuous variables between
γA/γA and γ'/γ' fibrins were determined by ANOVA (Version 5.0;
Abacus Concepts). A risk of error of 0.05 was accepted to evaluate
the statistical significance.

Results

Fibrin Physical Properties

Although the clots were formed under similar fibrinogen
concentrations and clotting conditions, dramatic differences
were demonstrated between the physical properties of γA/γA and
γ'/γ' fibrin.

Both types of fibrin network consisted of straight
rod-like elements organized in a three-dimensional net-
work. The spatial organization of the fibrin fibers was found to be homogeneous in both types of fibrin clots
using laser scanning confocal microscopy and scanning
electron microscopy (Figure 1). However, γ'/γ' fibrin
structure appeared less compact than γA/γA fibrin, with a
25% decrease of the fibrin fiber density (P=NS) and a 7%
increase of the average fibrin fiber diameter (P=NS)
(Table 1). Interestingly, a 3-fold difference in the average
fiber diameter was found between laser scanning confocal
microscopy and scanning electron microscopy, indicating
a possible shrinkage of the clots during processing for
scanning electron microscopy and/or an overestimation of
the fiber diameter related to the limitation of the optical
microscopy spatial resolution (Table 1). Consistent with
morphological studies, permeability of γ'/γ' fibrin was
increased by 20% compared with γA/γA fibrin, indicating
a lower fibrin fiber density and larger pores (Table 2).

Besides these slight structural differences, striking differ-
ences were demonstrated between viscoelastic properties of
γA/γA and γ'/γ' fibrin (Table 2). The γ'/γ' fibrin was found
to be 3-times stiffer than γA/γA fibrin, as shown by the difference in the storage moduli G' (dyne/cm²). The energy dissipated by non-elastic viscous processes (loss modulus G'') was 1.6-times lower in γA/γA fibrin compared with γ'/γ' fibrin. As a consequence, the energy dissipated by viscous processes relative to the energy stored by elastic processes (tan δ = G''/G') was decreased by 40% in γ'/γ' fibrin compared with γA/γA fibrin. Of interest, there was no significant difference in the storage moduli G' of uncross-linked γA/γA and γ'/γ' fibrin (10.1±2.1 dyne/cm² versus 11.8±1.9 dyne/cm²; P=NS).

Fibrinolysis

The dynamic and structural approaches of laser scanning confocal microscopy were used to assess fibrinolysis of both γA/γA and γ'/γ' clots. Lysis was found to progress as a straight and sharp front moving across the entire area of scanning (Figure 2). However, unexpectedly, the lysis front velocity of γ'/γ' fibrin was found to be 8-fold slower than for γA/γA fibrin, although only slight morphological differences were found between γA/γA and γ'/γ'. At the fiber level, similar changes were observed in both types of fibrin. Fibers underwent progressive disaggregation by transverse cutting with a transient increase in their diam-

**Table 1. Morphological Properties of Recombinant γA/γA and γ'/γ' Fibrin Using Confocal Microscopy**

<table>
<thead>
<tr>
<th>Fibrin Type</th>
<th>Fibrin Diameter (nm) (LSCM)</th>
<th>Fibrin Diameter (nm) (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γA/γA</td>
<td>276±11</td>
<td>93±23</td>
</tr>
<tr>
<td>γ'/γ'</td>
<td>274±38</td>
<td>108±24</td>
</tr>
</tbody>
</table>

**Table 2. Mechanical Properties of Recombinant γA/γA and γ'/γ' Fibrin**

<table>
<thead>
<tr>
<th>Fibrin Type</th>
<th>G' in dyne/cm² (n=3)</th>
<th>G'' in dyne/cm² (n=3)</th>
<th>Tan δ</th>
<th>Ks in cm² 10⁹ (n=3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>γA/γA</td>
<td>25.1±2.3</td>
<td>2.7±0.1</td>
<td>0.10</td>
<td>5.5±1.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>γ'/γ'</td>
<td>72.6±2.6</td>
<td>4.4±0.4</td>
<td>0.06</td>
<td>6.9±1.7</td>
<td>0.03</td>
</tr>
</tbody>
</table>

G', or loss, indicates storage modulus reflects the clot’s stiffness; G'', or loss modulus, represents the energy dissipated by non-elastic, viscous processes. The loss tangent (G''/G') is a measure of the energy dissipated by non-elastic viscous processes relative to the energy stored by elastic processes. Ks is the permeability constant and reflects the porosity of the fibrin network.

**Discussion**

A variant form of the γ chain, the γ' chain, is found in 7% to 15% plasma fibrinogen and is present as a heterodimer with the more common γA chain in γA/γ' fibrinogen. The plasma level of the γA/γ' fibrinogen has been shown to be an independent risk factor for coronary artery disease. In the process of investigating the mechanism responsible for the thrombogenicity of γA/γ' fibrinogen, it has been shown that γA/γ' fibrin was more resistant to lysis than γA/γA fibrin because of the more extensive cross-linking in γA/γ' fibrin. This has been related to a higher rate of factor XIII activation by γA/γ' fibrinogen as compared with γA/γA fibrinogen. Fibrin architecture and fibrinolysis rate of cross-linked plasma fibrin have been shown to be closely related, and whether structural differences between γA/γA and γA/γ' fibrin may account for the difference of fibrinolysis rate between γA/γA and γA/γ' fibrin is an important issue. The present investigation shows that the higher density of cross-links in γA/γ' previously reported accounts for the dramatic

**Figure 2.** Series of micrographs showing the dynamic lysis of γA/γA (A, B, C) and γ'/γ' fibrin with rtPA (150 nmol) and glu-plasminogen (2.5 µg/mL). Lysis-front motion is visualized every minute on the top panel and every 4 minutes (D, E, F). Lysis front progresses as a straight and sharp line with a higher velocity in γA/γA fibrin as compared with γ'/γ' fibrin. Each micrograph is a reconstruction from optical sections representing a volume of 146 x 146 x 20 µm³.
Fibrin actively regulates its self-dissolution through numerous interactions with fibrinolytic and anti-fibrinolytic components. In particular, fibrin fiber diameter and the spatial distribution of fibers are determinants in regulating rtPA binding and fibrinolysis speed. The fibrinolysis resistance of the so-called thrombogenic tight fibrin conformation, made of a high density of thin fibers organized in a tight three-dimensional network, is primarily related to a decrease of rt-PA binding as compared with the coarse fibrin conformation made of fewer fibers that are thicker. In particular, the tight fibrin conformation has been found in young myocardial infarction patients and in patients with severe venous thrombotic disorder. There are several possible reasons for these differences. There are many heterogeneities in plasma fibrinogen, including α-chain splice variants, genetic polymorphisms, and posttranslational modifications such as glycosylation, nitration, oxidation, sulfation, phosphorylation, and proteolytic degradation. The recombinant fibrinogens used in the present study were not subject to these heterogeneities, although the amount of protein available was certainly a limitation in that there was not enough protein for all studies that we wanted to perform. In recognition of the heterogeneities of pooled plasma, Cooper et al did perform some experiments with γA/γ′ and γA/γA fibrinogens purified from a single donor. An alternative explanation may be that the homozygous γ′/γ′ fibrinogen polymerizes differently than the heterozygous form. However, Cooper et al did not check whether there was some factor XIII in their preparations. This is an important issue, because it is established that factor XIII will be to produce clots with thinner fibers, 25 it is therefore possible that their findings are not a direct consequence of the presence of the γ′ chain but rather an indirect result caused by factor XIII that is present without their realizing it.

In the present investigation, recombinant γ′ fibrin was used so that only interactions between identical variant molecules occur rather than the more complex interactions between the more common γA/γ ′ molecules. In this case, only slight differences between the morphological properties of γ′/γ ′ and γA/γA fibrin network were observed. Indeed, γ′/γ ′ fibrin
displayed a slight increase of the fibrin fiber diameter and a slight decrease of the number of fibrin fibers per volume compared with γA/γA fibrin. This is further supported by the permeation experiments. In addition, scanning electron microscopy and laser scanning confocal microscopy showed the same trend, although there was a 3-fold difference in the average fibrin fiber diameter between laser scanning confocal microscopy and scanning electron microscopy. It is obvious that shrinkage of the clots may have occurred during sample processing for the scanning electron microscope, whereas fully hydrated and non-damaged fibers were visualized with the laser scanning confocal microscope. It is also likely that the limitation of the optical resolution of the laser scanning confocal microscope might have overestimated these measurements.

Given our data from both morphological analysis and permeation experiments, one would have expected γ′/γ′ fibrin clots to be less stiff than γA/γA clots. However, clot mechanical properties or clot stiffness do not result solely from a balance between a high degree of branching and thicker fibers. These characteristics enhance the network rigidity but are antithetical, because more branching leads to thinner fibers and thicker fibers yield less branching. It should be emphasized that besides fibrin network architecture, fibrinogen concentration and factor XIIIa-induced cross-linking are critical factors in the regulation of fibrinolysis. The present investigation provides a good support for this statement. The 3-fold increase of stiffness of γ′/γ′ fibrin compared with γA/γA fibrin is explained neither by the clotting conditions nor by the morphological properties of the three-dimensional network. Fibrinogen concentration and clotting conditions were similar, and one would have expected a 100-fold increase in both fibrin fiber and branch point densities to account for such a difference in fibrin stiffness. In addition, no difference was found between the stiffness of uncross-linked γ′/γ′ and γA/γA fibrin. As a consequence, the only plausible explanation is a substantial increase in the extent of cross-linking as previously reported.

A unique opportunity provided by laser scanning confocal microscopy was the possibility of measuring the fiber lysis rate with respect to the fibrin network configuration. It has been reported using this kind of dynamic and structural approach to fibrinolysis that fibrin network architecture rather than fibrin diameter regulates both the distribution of the fibrinolytic components and the fibrinolysis speed. In particular, although thicker fibers are digested at a slower rate than thin fibers, networks made of a high density of thin fibrin fibers are much more resistant to lysis than those made of thicker fibers with a lower fibrin fiber density. Our present investigation is contrary to these previous findings. One would have expected only a slight increase in fibrinolysis speed of γ′/γ′ fibrin compared with γA/γA fibrin, whereas the lysis rate was greatly decreased. The present study shows that in addition to fibrin configuration, the extent of fibrin cross-linking is critical and may overwhelm the impact of fibrin architecture itself on fibrinolysis speed. It is important to emphasize that if there is a similar increase in cross-linking of plasma γA/γ′ clots, together with the reported change in

## Table 3: Fibrinolysis Rate of Recombinant γA/γA and γ′/γ′ Fibrin

<table>
<thead>
<tr>
<th>Fibrin Type</th>
<th>γA/γA</th>
<th>γ′/γ′</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis front velocity (μm/min) (n=6)</td>
<td>40.7±17.6</td>
<td>5.4±1.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fiber lysis rate (fiber/min) (n=6)</td>
<td>1903±222</td>
<td>197±24</td>
<td>0.01</td>
</tr>
</tbody>
</table>
fibrin architecture, the effects on lysis rates should be even more dramatic. Several important issues should be addressed in the future. One of the most important questions is whether fibrinogen $\gamma'$ affects the lysis rate of platelet-rich clots. The architecture of platelet-rich clots plays a critical role in determining fibrinolysis speed.27,28 This issue is especially relevant given the fact that plasma $\gammaA/\gamma'\gamma$ fibrinogen levels are a marker of arterial thrombotic activity, and also given that fibrinogen $\gamma'$ chain displays much less binding to the platelet fibrinogen receptor, glycoprotein IIb/IIIa.17,29 Finally, other issues are the interaction between fibrin architecture and the plasma $\gammaA/\gamma'\gamma$ fibrinogen level, as well as the factor XIIa Val34Leu polymorphism, which affects both the physical properties of the fibrin network and platelet deposition in vitro and confers a protective effect on subjects for the occurrence of myocardial infarction.25,30

In conclusion, this investigation provides new insights into the mechanism of the fibrinolysis resistance of $\gamma'/\gamma'\gamma$ fibrin compared with $\gammaA/\gammaA$ fibrin and confirms previous findings regarding the critical role of the extensive cross-linking in $\gamma'/\gamma'\gamma$ fibrin.4,13

References
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