Influence of Fibrin Network Conformation and Fibrin Fiber Diameter on Fibrinolysis Speed
Dynamic and Structural Approaches by Confocal Microscopy


Abstract—Abnormal fibrin architecture is thought to be a determinant factor of hypofibrinolysis. However, because of the lack of structural knowledge of the process of fibrin digestion, relationships between fibrin architecture and hypofibrinolysis remain controversial. To elucidate further structural and dynamic changes occurring during fibrinolysis, cross-linked plasma fibrin was labeled with colloidal gold particles, and fibrinolysis was followed by confocal microscopy. Morphological changes were characterized at fibrin network and fiber levels. The observation of a progressive disaggregation of the fibrin fibers emphasizes that fibrinolysis proceeds by transverse cutting rather than by progressive cleavage uniformly around the fiber. Plasma fibrin clots with a tight fibrin conformation made of thin fibers were dissolved at a slower rate than those with a loose fibrin conformation made of thicker (coarse) fibers, although the overall fibrin content remained constant. Unexpectedly, thin fibers were cleaved at a faster rate than thick ones. A dynamic study of FITC–recombinant tissue plasminogen activator distribution within the fibrin matrix during the course of fibrinolysis showed that the binding front was broader in coarse fibrin clots and moved more rapidly than that of fine plasma fibrin clots. These dynamic and structural approaches to fibrin digestion at the network and the fiber levels reveal aspects of the physical process of clot lysis. Furthermore, these results provide a clear explanation for the hypofibrinolysis related to a defective fibrin architecture as described in venous thromboembolism and in premature coronary artery disease. (Arterioscler Thromb Vasc Biol. 2000;20:1354-1361.)

Key Words: fibrin ■ fibrinolysis ■ confocal microscopy

The fibrin matrix has a much more complicated role than that of providing the scaffolding of the thrombus or being the target of fibrinolysis. Abnormal fibrin structure in vitro has been related to in vivo premature coronary artery disease in young patients and to severe venous thromboembolic disease in patients with dysfibrinogenemias. In those situations, an abnormal fibrin matrix made up of abnormally thin fibers has been shown to promote hypofibrinolysis and embolization. Although much is known about the molecular basis of fibrinolysis, relationships between fibrin conformation and fibrinolysis need to be clarified.

Fibrin actively regulates its self-dissolution through numerous interactions with fibrinolytic and antifibrinolytic components. Activation of plasminogen by tissue plasminogen activator (tPA) that is initiated on the conversion of fibrinogen into fibrin is a critical step that is affected by fibrin structure. The theory of a decrease of plasminogen binding to fibrin has been strengthened from observations showing that clots with a fine fibrin (tight) conformation display a slower lysis rate than those with a coarse fibrin (loose) conformation. So far, neither a molecular nor a structural basis has been detected for these differences. Moreover, a recent report demonstrates that under other conditions clots made of thin fibers may be lysed faster.

Recent structural studies have emphasized that fibrin digestion proceeds locally by transverse cutting across fibers rather than by progressive cleavage uniformly around the fiber and that changes of the fibrin network structure are spatially restricted to a zone in which high accumulation of fibrinolytic components takes place. However, none of these findings provide sufficient conclusions regarding the impact of the fibrin network structure and the fibrin fiber diameter on fibrinolysis speed.

In the present work, a dynamic approach using confocal microscopy was designed to assess the impact of fibrin network conformation and fibrin fiber diameter on the speed of fibrinolysis. Simultaneously, dynamic measurements of the distribution of fibrinolytic components in thin and coarse fibrin types were made. This work reveals that tight fibrin networks are dissolved at a slower rate than loose ones but
that thin fibers are cleaved at a faster rate than thick (coarse) ones. Fibrin network architecture rather than fibrin fiber diameter regulates the distribution of fibrinolytic components during the course of fibrinolysis and may account for this apparent paradox.

Methods

Materials

Human thrombin was purchased from Enzyme Research Laboratories Inc and stored at 1000 IU/mL. Unconjugated colloidal gold solution for light microscopy was from British Biocell International. Average particle size was 5 nm, and the particle concentration was 5×10^13/mL. Recombinant tPA (rtPA) was purchased from Boehringer-Ingelheim. FITC was purchased from Biocell. Calcium chloride was from Sigma Chemical Co.

Procedures

Preparation of Cross-Linked Plasma Fibrin Clots

Blood from healthy informed volunteers was anticoagulated with trisodium citrate (1 vol of 0.13 mol/L citrate for 9 vol of blood). Platelet-poor plasma was obtained by centrifugation of the blood samples at 10000 g for 15 minutes. A volume of 0.10 mL of plasma was recalcified up to a final concentration of 20 mmol/L. After a 1-minute incubation, 10 μL of thrombin was added. Thrombin from the stock solution was diluted in TNE buffer (0.15 mol/L NaCl and 0.01 mol/L Tris-HCl, pH 7.4). Final concentration was adjusted to 0.9 IU/mL and 0.09 IU/mL to obtain tight and loose cross-linked plasma fibrin clots, respectively. Mixing and incubation were conducted in polypropylene tubes. A final volume of 0.12 mL of the mixture, as described above, was soaked up into a glass microchamber designed for flow measurements. Clotting was allowed for at least 20 minutes in a moist atmosphere at 37°C.

Preparation of FITC-tPA

FITC-rtPA was prepared as previously described. To a stock solution of 2 mg/mL rtPA in TNE buffer (140 mmol NaCl and Tris-HCl 20 mmol, pH 7.4) was added FITC dissolved in 0.01 mol/L Tris, 0.1 mol/L NaCl, and 1 mmol/L EDTA, pH 8, at a final concentration of 50 μg/mL. After 1 hour of incubation, free FITC was removed by gel filtration on a G-25 Sephadex column (3.5 mL) equilibrated with TNE buffer. The absorbance ratio of 494 nm to 280 nm in preparations of FITC-tPA was 1.6, corresponding to 2 FITC molecules per molecule of rtPA.

Reflection Laser Scanning Microscopy

Microchambers holding plasma fibrin clots were connected to a reservoir, and the clots were extensively washed with 500 μL of TNE buffer. Then, regular TNE buffer was replaced by 200 μL of TNE buffer containing 5-nm-diameter colloidal gold particles at a final concentration of 2.5×10^13/mL. The excess of beads that did not bind to fibrin was washed out with 500 μL of TNE buffer.

Labeled specimens were scanned with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Inc) linked to a Zeiss inverted microscope equipped with a Zeiss ×63 water immersion objective. A 5-W argon ion laser was used in combination with a 488-nm band-pass filter for the excitation. The microscope was automatically set up in reflection mode by replacing the dichroic filter, typically used in fluorescence mode, by a 80/20 beam splitter that directed ~20% of the reflected light by the sample to the detector. The computer equipped with standard Carl Zeiss software (version 1.5) was used for operating the system and for the processing of images that were collected in a format of 512×512 pixels, with 1024 gradations of intensity. Optical sectioning was achieved by closing the pinhole in front of the detector to 120 μm and collecting the reflected light of multiple optical planes in the z direction. Detector gain and pinhole aperture were automatically adjusted. Twenty optical sections were collected at intervals of 1.0 μm in the z-axis. Collecting a single scan took 1.5 seconds. Optical resolution in the x–y–axis was 0.5 μm and ~0.7 μm in the z-axis. These sections were then projected at 6 different angles 10° apart and combined into 1 image, generating 6 different 3D reconstructed images of the fibrin network.

The design of the present system, especially the reflection mode, allowed images to be collected periodically during ongoing lysis within a very short interval because bleaching was avoided.

Lysis Experiments

Two different types of experiments were conducted. In the first set of experiments, gold-labeled plasma fibrin clots were loaded with 10 μL of rtPA, which was dissolved in platelet-poor plasma. After 15 minutes of incubation in a moist atmosphere, the edge of the thrombus was processed for 20 scans with the confocal microscope set up in the reflection mode. At the network level, scanning was performed at low magnification every 2 minutes, and the lysis-front velocity of the 2 different types of fibrin conformation was recorded at different rtPA concentrations (1, 2, 5, and 10 nmol/L). At the fiber level, scanning was performed at a higher magnification every 30 seconds and with lower rtPA concentration (1 nmol/L). Other experiments had shown that colloidal gold particles did not affect lysis when fibrinogen was labeled before clot formation (data not shown).

In a second set of experiments, fresh native plasma fibrin clots held in microchambers were carefully loaded with 10 μL of a solution containing FITC-tPA at a final concentration of 5 nmol/L. After 15 minutes of incubation, the edge of the clot was located by using the transmitted light mode, and then scanning was started every 2 to 5 minutes in the regular fluorescence mode. The binding-front velocity of FITC-tPA and the lysis-front velocity of the clot were simultaneously recorded in different types of fibrin clots.

Image Analysis

Morphological Properties of Tight and Coarse Fibrin Conformations

Average fibrin fiber diameters (n=150) in loose and tight fibrin conformations were determined on high-magnification images by use of the image analysis software package that came with the microscope workstation. These measurements were determined on reconstructed images. Fiber branches and branching point densities were determined at lower magnification in tight and loose fibrin conformations. Branching points were very carefully distinguished from crossing fibers by using the series of reconstructed images at different angles and by the analysis of each scan.

Image Analysis of Lysis Experiments

Measurements of the lysis-front velocity, of the rate of fiber digestion, and of the fiber shape modifications (area in micrometers) were performed on the 2 different types of fibrin conformation and at different rtPA concentrations with the use of gold-labeled specimens.

Measurements of binding-front velocity and lysis-front velocity were also conducted with the 2 different types of native plasma fibrin clots.

Statistical Analysis

Conventional tests were used for calculation of means and standard deviations. Group differences in continuous variables were determined by 1-way ANOVA. A risk of error of 0.05 was accepted to evaluate the statistical significance. Equality of variances between groups was first evaluated by the F test. When the overall F statistic was significant, the Bonferroni test was used to ascertain these differences in normal and logarithmic scales.

Results

Morphological Properties of the 2 Different Fibrin Types

Fibrin networks visualized with confocal microscopy consisted of straight rodlike elements organized in a 3D network (Figure 1). A branch point usually looked like a forked-shape element with a small angle between the 2 fibers emerging.
coarse plasma fibrin network

Figure 1. Three-dimensional reconstruction images of loose (coarse fibrin, A to C) and tight (fine fibrin, D to F) plasma fibrin networks obtained by scanning confocal laser microscopy (73×73 μm²). Native plasma fibrin was labeled with gold particles. Loose and tight fibrin network architectures were obtained by adding 0.09 and 0.9 IU/mL of human thrombin to recalcified platelet-poor plasma. Fibers (arrow) and branch point (arrowhead) are highlighted. Progression in real time of the lysis front in loose (B and C) and tight (E and F) plasma fibrin networks are also shown (146×146 μm²). Lysis was initiated by rtPA (5 nmol/L) loaded at the edge of the clot. Time interval between each micrograph is 5 minutes.

from a third fiber (Figure 1A, arrow). Branches were first detected on single scans and then visualized on the reconstructed images. Crossing fibers were recognized when the 2 fibers were sliding past each other instead of rotating with the same angle when looking at the dynamic tilting of the reconstructed images projected at 6 different angles.

Confocal microscopy in the reflection mode allowed the accurate determination of the morphological properties of loose and tight fibrin conformations without the fading that accompanies a fluorescent signal. Figure 1 shows dramatic differences of the fibrin network architecture between loose (Figure 1A) and tight (Figure 1D) fibrin networks, although the total fibrin amount remains identical. Measurements of fibrin fiber diameter, fiber length, fiber density, and branch point density in loose and tight conformations are summarized in the Table. Tight fibrin clots are made of thinner fibers with significantly higher densities of both branches and fibers than a coarse fibrin network (Table). Repartition of the fibrin fiber–diameter histogram shows that >50% of the fibrin fibers display a diameter >350 nm in the loose conformation, whereas 75% of the fibers display a diameter <350 nm in the tight fibrin conformation.

Fibrinolysis at the Network Level

Lysis-Front Velocity According to Fibrin Conformation
Lysis progresses as a straight and sharp front moving across the entire area of scanning, indicating a homogeneous layer-by-layer elimination of the clot. Following the motion of the lysis front in real time was possible because use of the colloidal gold labeling avoided fading while the specimen was scanned (Figure 1). As seen in Figure 2, lysis-front velocity increases with rtPA concentration in a linear fashion, with an average rate of 3.5 μm · min⁻¹ · nmol⁻¹ rtPA. A significant reduction of the lysis velocity was observed in

<table>
<thead>
<tr>
<th>Fibrin Conformation</th>
<th>Coarse</th>
<th>Fine</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average fiber diameter, nm</td>
<td>376±104</td>
<td>299±70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fiber density, 10⁻³ μm⁻³</td>
<td>4.8±0.3</td>
<td>9.8±1.1</td>
<td>0.019</td>
</tr>
<tr>
<td>Branching density, 10⁻³ μm⁻³</td>
<td>1.4±0.15</td>
<td>2.8±0.46</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are mean±SD.
clots with a tight fibrin conformation, namely, an average rate of $0.58 \, \mu m \cdot min^{-1} \cdot nmol^{-1}$ tPA ($P<0.01$).

rtPA Binding-Front Velocity as a Function of Fibrin Network Conformation

The binding-front velocity measures the progressive accumulation of fluorescently labeled rtPA on native fibrin in a given area. After accumulation, lysis starts, and a straight lysis front appears, moving across the fibrin network (Figure 3). Great differences of binding-front and lysis-front velocities could be observed between fine and coarse fibrin networks (Figure 3). Progression of the binding front was $36\pm5$ and $8.66\pm3 \, \mu m/min$ ($P<0.01$) in coarse and fine fibrin networks, whereas lysis-front velocities were $31\pm4$ and $2.15\pm0.5 \, \mu m/min$ ($P<0.01$), respectively.

The highest concentration of rtPA was found within the thin superficial layer of the lysing clot, commonly known as the lysis front or prelysis zone. Thickness of the lysis front was higher in the coarse than in the fine fibrin networks, reflecting higher densities of binding sites for tPA on thick fibers (Figure 3).

Fibrinolysis at the Fiber Level

The unique opportunity of coupling gold labeling and confocal microscopy yielded the possibility to follow in real time the physical process of fibrinolysis of native hydrated fibrin at the fiber level.

Changes in Fiber Shape

Dynamic changes in fiber shape were accurately measured when lysis was initiated with the smallest concentration of rtPA (1 nmol/L, Figure 4). An invariable sequence of events could be seen in those conditions but with a large variety of scenarios, depending either on fiber diameter or fibrin network conformation. A reduction in the gold labeling density could eventually be observed as the first sign of lysis before any detectable change in the fiber shape. However, the most obvious dynamic feature was that fibers were chopped off progressively. Lateral transection of the fibrin fibers could either go all the way across the fiber and release big fibrin

![Figure 2](http://atvb.ahajournals.org/content/10/10/1357/F2.large.jpg)

**Figure 2.** Graph showing lysis-front velocity according to fibrin network conformation and final concentration of rtPA. Conditions of plasma fibrin formation and fibrin labeling are given in the legend to Figure 1.

![Figure 3](http://atvb.ahajournals.org/content/10/10/1357/F3.large.jpg)

**Figure 3.** Dynamic progression of FITC-tPA binding front and FITC-rtPA lysis front observed with confocal microscopy. FITC-rtPA (5 nmol/L) was loaded at the edge of native loose (A to C) and tight (D to F) plasma fibrin networks. Time interval between each micrograph is 16 minutes. Conditions of plasma fibrin formation are given in the legend to Figure 1 ($146\times146 \, \mu m^2$).
chunks or be limited. Incomplete transection occurred within thick fibers, giving rise to a fuzzy and fragmented aspect of the fiber shape (Figure 4). Those very thick fibers usually remained attached to the clot by their proximal portions for a long period. They finally bent and splayed into a lacelike mesh at the edges before splitting and folding up in big chunks. Partially degraded fibers of smaller diameter could either disappear or aggregate laterally, giving rise to thicker fibers. One important consequence of the transection process was that fibrin fibers had a significant tendency to increase in diameter (or area) while being digested by plasmin (Figure 5A).

Measurements of Fiber Lysis Rate According to Fiber Diameter and Fibrin Network Conformation

Substantial differences were observed between thick and thin fibrin fiber digestion within the same fibrin network. Instead of increasing in diameter or being fragmented as a consequence of incomplete transection, thin fibers <450 nm in diameter disappeared suddenly without any detectable increase in diameter before complete lysis (Figures 4 and 5A). In these cases, the time interval may not have been short
Hypofibrinolysis that leads to fibrin accumulation has been related to premature coronary artery disease and venous thrombotic disorders.\textsuperscript{2,11–14} Convincing evidence suggests that thrombi with an abnormal fibrin architecture made of thin and numerous fibers organized in a tight network configuration may be partially responsible.\textsuperscript{1,15} However, relationships between this so-called thrombogenic fibrin architecture and hypofibrinolysis remain unclear, primarily because of the lack of structural information on the process of fibrinolysis. In the present investigation, we have developed a dynamic and structural approach to fibrinolysis with confocal microscopy to correlate fibrinolysis speed with fibrin morphological properties. We found that cross-linked plasma fibrin fibers are transected laterally rather than progressively and uniformly digested from the outside in. Lysis of clots with a loose fibrin conformation made of thick fibers is faster than those with tight fibrin conformation made of thin fibers, although individual thick fibers are cleaved at a slower rate than are thin fibers.

A great number of biochemical studies have provided strong evidence for the molecular basis of fibrinolysis.\textsuperscript{9,16–18} Although much less was known about the physical changes in the fibrin matrix that precede solubilization, it has been assumed that fibrin was digested from the outside in, with products of degradation released layer by layer.\textsuperscript{16,19} This model was based on the characterization of fibrin degradation products released from clots and confirmed later by confocal microscopy showing that plasma fibrin degradation resulted in 2 sequential phases.\textsuperscript{7,8} During the prelysis phase, which is characterized by very few structural changes of the fibrin matrix, plasminogen accumulates on the surface as more C-terminal lysine binding sites are exposed. Then, the fibrin network becomes mobile before collapsing and disappearing during the second (end) stage. Unexpectedly, recent investigations with scanning electron microscopy and transmission electron microscopy (SEM and TEM, respectively) have shown that plasmin digestion proceeds locally by transverse cutting across fibers rather than by progressive cleavage uniformly around the fiber.\textsuperscript{10} However, the impact of fiber diameter and fibrin network architecture on fibrinolysis speed as well as on the detailed dynamic process of fiber digestion was lacking.

Fibrin labeling with gold particles was an essential technique for these experiments. The expected higher resolution obtained with reflected light (because of its shorter wave length and its higher coherence) than obtained with the light emitted by fluorophores was confirmed here.\textsuperscript{20,21} Accurate measurements of fiber diameter and identification of branch points were possible. Discrepancies between measurements obtained from confocal micrographs and scanning electron micrographs can be explained to some extent by the plasma origin of the fibrin clot in the present experiments.\textsuperscript{22} However, another important difference may be also that our experiments dealt with fully hydrated fibrin instead of the dehydrated samples used in SEM. Dehydration leads to shrinkage of the native fibrin that artificially underestimates fibrin fiber diameter and fiber length and overestimates fiber density.\textsuperscript{22} Moreover, the higher degree of stability of reflective samples than of fluorescent samples, which are typically more subject to thermal degradation and photobleaching, allowed us to perform dynamic experiments that were essential for the structural characterization of the fibrinolysis process, especially at the fiber level.

The most obvious feature of fibrinolysis as recently reported with SEM studies was clearly confirmed with this dynamic approach.\textsuperscript{10} Drops of density, gaps, and holes appearing along individual fibers as well as the release of small chunks from free fiber segments were the most obvious evidence of the lateral transection of fibrin fibers by plasmin...
The observed resistance to fibrinolysis of cross-linked plasma fibrin with a tight network architecture. However, opposite results have been reported with purified and non-cross-linked fibrin, depending on the conditions of fibrin formation. Unlike clots made of purified fibrinogen with a tight network obtained under high salt conditions, those obtained by adding contrast media before clotting were found to be more resistant to fibrinolysis than coarse purified fibrin obtained without contrast media. The absence of cross-linking and the different conditions of fibrin formation may account for these differences between purified and plasma fibrin.

The present study was conducted in a very specific set of conditions. The lytic agent rtPA was neither incorporated nor permeated, and there were no other blood elements within the cross-linked plasma clots. This may explain why structural modifications of fibrin were restricted to the lysis-front area. However, changes could eventually occur all over the network in the case of extremely coarse fibrin network digested with a high concentration of rtPA. In these particular conditions, convective transport might have occurred, and the huge area of tPA binding would have led to modifications of the fibers all over the fibrin network so that lysis could start in different places simultaneously without any individualized lysis front. This is more likely to be the case in vivo because, ordinarily, plasminogen and tPA bind simultaneously to the forming fibrin and because pressure-driven permeation markedly enhances lysis. These conditions and the effect of platelets require further investigation.

In conclusion, these structural and dynamic features of fibrinolysis confirm what was suspected from recent SEM experiments and are in accordance with the most recent molecular modeling of fibrinolysis. The crawling of plasmin across fibrin leads to a progressive lateral transection of the fibrin and to the disaggregation of the fibers instead of a progressive and uniform digestion from the outside in, with products of degradation released layer by layer. rtPA binding regulates the lysis speed and is dependent on the fibrin conformation rather than on the fibrin fiber diameter. Finally, these results demonstrate that the so-called thrombogenic fibrin consisting of thin fibers organized in a tight 3D network is thrombogenic because of its resistance to lysis that arises directly from its network architecture.

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References


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