Dynamic Changes of Fibrin Architecture during Fibrin Formation and Intrinsic Fibrinolysis of Fibrin-rich Clots*

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Clotting and fibrinolysis are initiated simultaneously in vivo, and fibrinolysis usually occurs without any individualized lysis front (intrinsic fibrinolysis). We have developed a novel model to assess whether morphological changes resulting from intrinsic fibrinolysis are similar to those previously reported at the lysis front using externally applied lytic agents. Fibrin assembly and fibrinolysis were followed in real-time by confocal microscopy using gold-labeled fibrinogen molecules. An increase in fiber absorbance (30%, p < 0.01) and a decrease in fiber diameter (60%, p < 0.01) due to the ongoing accumulation and packing of fibrin molecules were the most significant detectable features occurring during fibrin assembly. Similar features with a similar magnitude were observed during fibrin dissolution, but in the reverse order and with a 3-fold increase in duration. Then, lysing fibers were progressively transected laterally, and thinner fibers were cleaved at a 2.5-fold faster rate than thicker fibers (p < 0.001). Frayed lysing fibers were seen to interact progressively with adjoining fibers (agglomeration), leading to a 76 and 88% increase in the network pore diameter (p < 0.05) and fiber diameter (p < 0.01), respectively. At the maximum decrease in fiber absorbance (46%, p < 0.05), the network suddenly collapsed with the release of large fragments that gradually vanished. Morphological changes of fibrin that occur during intrinsic fibrinolysis are similar as those observed next to the lysis front, although they are not restricted spatially to the clot/surrounding milieu interface but are observed through the entire clot.

A wide variety of studies of fibrinolysis have been carried out, but most of them have utilized the same basic model of adding fibrinolytic enzymes to a pre-formed clot. This approach is very important clinically, because removal of the fibrin matrix from occlusive thrombi using externally applied lytic agents is the major goal of pharmacological thrombolysis to achieve vessel patency (1–4). However, without external administration of lytic agents, common events in the vasculature that trigger clotting also initiate fibrinolysis at the same time, which is called intrinsic or internal fibrinolysis. Thus, fibrin formation and fibrinolysis usually occur simultaneously. However, this dynamic system is more difficult to simulate in vitro, and only a few studies have been carried out by mixing fibrinogen, plasminogen, and tPA1 with thrombin and using turbidity to follow clot formation and lysis (5–7).

Recent structural studies of fibrinolysis have focused on the diffusional transport of fibrinolytic agents and have given us a good picture of events that occur at the lysis front. The lysis front is a very dynamic region, with movement of partially lysed fiber ends and greatly increased binding of plasminogen and tPA (8, 9). Individual fibers are generally cut across laterally rather than by progressive cleavage uniformly around the fiber. In addition, thin fibers were found to be cleaved at a significantly faster rate than thick fibers. Interestingly, the lysis front velocity of plasma cross-linked fibrin clots made of thin fibers organized in a tight network was always found to be slower than that of clots made of thicker fibers organized in a coarse configuration, whatever the amount of lytic agents (9, 10). However, there is essentially no such dynamic and structural information on the process of intrinsic fibrinolysis, even though it is likely to be the most common physiological mode of fibrinolysis.

In the present work, we developed a dynamic approach to investigate simultaneously the morphological features of fibrin formation and fibrin dissolution. For that purpose, fibrinolytic agents were added before the initiation of clotting, therefore simulating the effect of physiological intrinsic fibrinolysis. Our main purposes were to visualize early clot formation and compare morphological changes occurring at the fiber level and the network level resulting from the presence of lytic agents throughout the whole clot with those previously reported at the lysis front when lytic agents are introduced by diffusion.

MATERIALS AND METHODS

Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Unconjugated colloidal gold solution for light microscopy was from British Biocell International (Cardiff, UK). The average particle size was 5 nm, and the particle concentration was 5 × 1012/ml. Recombinant tissue plasminogen activator (rtPA) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Glu-plasminogen was purchased from American Diagnostica (Greenwich, CT). Calcium chloride was from Sigma. Purified fibrinogen and factor XIII were gifts from O. Gorkun (Chapel Hill, NC).

Preparation of Fibrin-rich Clots—To a volume of 0.10 ml of purified fibrinogen (1.3 10−5 M final concentration in 20 mM/liter HEPES, pH 7.4, 0.15 mol/liter NaCl) was added 5 mM CaCl2 (final concentration), 80 μl of the colloidal gold solution, 10 nm rtPA, and 2 μg/ml Glu-plasminogen. After 1 min of incubation, 10 μl of thrombin from the stock solution was added to obtain a final concentration of 0.5 IU/ml. Mixing and incubation were conducted in polypropylene tubes at room temperature, and the solution was rapidly soaked up into glass microchambers designed for confocal microscopy (10). Clots were cross-linked by adding activated factor XIII (10 μg/ml final concentration) prior to clotting.

The abbreviations used are: tPA, tissue plasminogen activator; rtPA, recombinant tPA.
Laser-scanning Confocal Microscopy—After being filled up, the microchambers were scanned immediately with a LSM 510 interactive laser cytomter (Carl Zeiss) linked to a Zeiss inverted microscope equipped with a 63× water immersion objective using the reflection mode. The selected area of scanning was picked up at random. Scanning was performed every 2 min during a total of 46 min. At each time point, 20 scans were taken 1 μm apart. Optical sections were then projected and combined into one image, generating a two-dimensional projection. A total of 10 fibrin-clots were processed for both microscopy and morphological analysis.

Morphological Analyses—Each micrograph was processed with the same algorithm using the Visieng software (V5.03, Noesis, development kit) (11–14). After normalization of the scale for the gray level (between 0 and 255), a first morphological filter was applied to remove the noise spikes while maintaining the original contrast ratio between fibers and background. A second filter selected the most contrasted areas leading to a binary mask where the fibers are separated from the background of the images, allowing counting and accurate measurements (Fig. 1). Four different morphological parameters were assessed, permitting a dynamic analysis of fibrin assembly and fibrin dissolution.

At the fiber level, fiber absorbance (mean OD/pixel) and fiber diameter (μm) were recorded at each time point on the fibrin fiber binary mask. Fiber absorbance reflected the average optical density in the binary mask of the fibers and varied according to the progressive aggregation of new fibrin polymers to a preformed fiber or to the ongoing fibrin dissolution. Fiber diameter was measured on a two-dimensional projection of the optical sections using the distribution of the optical density along a transverse line drawn perpendicular to the long axis of the fiber. Each fiber was given a single measurement on the same spot at each time point. An average of ten measurements (10 fibers) performed on the same micrograph at each time point was considered for further analysis. Measurements at the 2-min time point were performed on thinner two-dimensional projections (5 μm instead of 20 μm) to avoid the effect of shifting of the fiber, which occurred during the early step of fibrin formation. Given the fact that a single fiber can be completely visualized within 5 μm of depth, two fibers were picked up in each projection for the measurement of the fiber diameter.

To assess early modifications of the network conformation, the fibrin porosity (in μm) was measured at each time point. For that purpose, the most frequent pore diameter was calculated using a specific morphological algorithm (11, 12, 14), allowing the distribution of the surface areas of the pores according to their diameters. A pore mask was first obtained as the symmetry of the fiber binary mask. Geometric structures (dodecagons) of increasing diameter were shifted inside the binary mask of the pores (Fig. 1, panel 8c). At each step, the areas in which the dodecagons could not fit were eliminated. The diameter of the dodecagon that eliminated the largest area was chosen as the most frequent pore diameter.

Finally, the number of disconnected fiber bundles was recorded at each time point on the fibrin fiber binary mask. This number corresponds to the number of disconnected areas within the whole fibrin network. This parameter was chosen to quantify fibrin formation, during which individual fibers merged, but also to identify and quantify lateral transection of individual fibrin fibers, which occurred during fibrin dissolution (10).

Effects of Beads on Fibrin Formation—The potential impact of colloidal beads on fibrin assembly was assessed by continuous monitoring of the elastic modulus of fibrin-rich clots using the RM-2 analyzer (Hemodyne, Richmond, VA). The same conditions of clotting were used as above. Beads were replaced by buffer in the controls.

Generated Plasmin Activity—The generation of plasmin activity within the clot was monitored with the probe S2251 (Chromogenic, Mölndal, Sweden). Plasmin activity was expressed as optical density and recorded using the same conditions of clotting as above.

Statistical Analysis—Statistical analyses were performed with StatView software (Version 5.0, Abacus Concepts, Inc.). Continuous variables were expressed as mean ± S.E., and differences between time points were determined by analysis of variance (ANOVA). An α level of 0.05 was accepted as significant.

RESULTS

The present in vitro setting allowed investigation of the dynamics of both the fibrin assembly and the fibrin dissolution of fibrin-rich clots (without platelets). Accurate determination of the morphological properties either at the network level or the fiber level without the fading that usually accompanies a fluorescent signal after extensive scanning was made possible by the use of a rapid confocal microscope in the reflection mode.

Fibrin Assembly—Fibrin-rich clots consisted of straight rod-like elements organized in a three-dimensional meshwork (Fig. 1, panels 8a–8c) as reported previously (10). The labeling conditions, under which there were ~20 fibrinogen molecules per gold particle, did not affect fibrin assembly. Indeed, the addition of colloidal beads prior to clotting had no significant effect on the kinetics of fibrin assembly and fibrin dissolution or on clot properties, as shown by the monitoring of fibrin’s mechanical properties (Fig. 2), which is likely the most sensitive measure of fibrin structure/properties.

Individually labeled molecules were not visible because structures need to be at least 50 nm in size before they can be detected in this system. Monitoring of the early stages of fibrin assembly was difficult, because fibers were extremely mobile, leading to a fuzzy appearance of the two-dimensional reconstructed micrographs (Fig. 1, panel 2). What appear to be multiple views of the same fiber adjacent to each other probably arise from lateral motion of these fibers that are not yet firmly anchored in a gel. A structured scaffold appeared between 2 and 4 min after the initiation of clotting, and fiber mobility decreased dramatically after this time. As a conse-
sequence, fibers became sharper, and observation of fiber ends was a common feature between 4 and 8 min after clotting initiation (Fig. 1, arrowheads). Examination of consecutive sections demonstrated that these features were really fiber ends and not just fibers that passed out of a particular section. Although fibers still appeared thicker and bent, appearing as if a few fibers were fusing into a single one, the average apparent fiber diameter was significantly reduced (33%, \( p = 0.01 \)) between 2 and 4 min after clotting initiation (Fig. 3A).

An increase in fibrin absorbance was a constant feature observed at all time points during fibrin formation (29.7% increase between 2 and 8 min, \( p < 0.05 \)) (Figs. 1 and 3B), indicating that fibers were becoming thicker via the permanent aggregation of additional protofibrils to already formed or forming fibers. The optical absorbance of fibers as well as the elastic modulus of the whole clot peaked at the 8-min time point, indicating that fibrin formation was completed (Figs. 2 and 3B).

A lengthening of existing fibrin stands was observed once these strands had coalesced into a linear structure (Fig. 1, arrowheads). Propagation of the fibers extended until contact was made with another growing fiber. One consequence of the phenomenon of the joining of two fibers into one was a 46% decrease in the number of connected fiber bundles between 4 and 8 min after the initiation of clotting (Fig. 4A). Within the same time interval, additional fibrin fibers also appeared in pores that were unoccupied previously (Fig. 1, arrows). The appearance of new fibers was further supported by the measured decrease of the fibrin network porosity (Fig. 4B).

**Fibrinolysis**—The same major features as those described above for fibrin assembly were observed, although in a different time scale and in the reverse order. The first detectable morphological change was a decrease in optical absorbance of fibers, which culminated before major changes in the network architecture occurred (between the 8- and 15-min time points) (Figs. 3B and 5, panels 8a and 16a). The other remarkable morphological change was a complete dissolution of the thinner fibers (<400 nm of diameter) (Fig. 5, panels 8a and 16a, arrows), which displayed faster lysis rates than the thicker fibers (9.5 ± 1.2 min. versus 21.4 ± 1.9 min, respectively, \( p < 0.001 \)). Thicker fibers underwent surface erosion without any major detectable modifications in their shape. Generated plasmin underwent an exponential increase just after the completion of fibrin formation (Fig. 1), which was expected from other experimental data on the generation of plasmin by the action of fibrin-bound tPA on plasminogen.

Dynamic changes in fiber shape and network conformation were identified later (≥10 min after clotting initiation). Fibers were first progressively transected laterally, giving rise to fuzzy and fragmented clusters of fiber pieces with progressive bending of the whole fiber, which remained attached to the scaffold (Fig. 5, panel 16b). The disaggregation of the fibers led to a significant increase in the average apparent fibrin diameter (88% increase between the 8- and 25-min time points, \( p = 0.0016 \)) (Fig. 3A). After complete transection occurred, fibers became frayed and splayed into a lace-like mesh, but also tended to merge with adjacent fibers (Fig. 5, panel 16a, arrowheads). These modifications led to a significant increase in the number of disconnected fiber bundles (59% increase between the 8- and 15-min time points, \( p < 0.05 \)) (Fig. 4A), as observed when comparing panel 8b with panel 16b of Fig. 5.

At the network level, a progressive augmentation of the pore diameter (76%, \( p < 0.05 \)) was apparent between the 8- and 25-min time points as a consequence of “fiber agglomeration” or merging (Fig. 4B). This led to the formation of dense, poorly defined areas (Fig. 5, panel 24a). The whole network started to collapse after the maximum decrease of the fiber absorbance was reached (46%, \( p < 0.05 \)). This resulted in the progressive release of pieces of material into the surrounding milieu (Fig. 5, panel 24b). These big agglomerates displayed an initial high optical density and then retracted very fast into small and very dense particles that vanished within a few minutes, leaving an empty area without visible structures.

The addition of factor XIII prior to clotting did not modify the fibrin formation step. Fibrin stabilization delayed the overall fibrinolysis process without any changes in the morphological events (data not shown).

**DISCUSSION**

Recent structural studies have emphasized that fibrin digestion proceeds locally by transverse cutting across fibers, leading to the progressive disaggregation of the whole fiber struc-
ture rather than by progressive cleavage uniformly around the fiber as suggested previously (2, 9, 10, 15). Although thin fibers are cleaved at a much faster rate than thick fibers, the fibrin network configuration and especially the number of fibers per volume have been shown to have a more dramatic impact on the fibrinolysis rate than fiber diameter per se. In particular, a fibrin network made of a high number of thin fibers has been shown to bind rtPA that is diffusing into the clot at a slower rate as compared with a network made of a smaller number of thicker fibrin fibers. These unexpected dynamic and structural differences were observed at the plasma/clot interface (i.e. the lysis front), because lytic agents were introduced at one edge of the clot and diffused into it. In some particular conditions, including high concentrations of rtPA and extremely coarse fibrin configuration, rtPA underwent a convective transport, and fiber digestion was shown to occur simultaneously throughout the whole clot without any individualized lysis front (10). However, because fiber digestion was fast in these specific conditions, morphological analyses were not possible. In the present study, we have simulated the physiological situation by adding lytic agents prior to the initiation of fibrin assembly and then following fibrin assembly and lysis in real-time. In this case, clotting and lysis were initiated at the same time, and then each proceeded at different time scales. It is important to note that labeling of the fibrinogen molecules before the initiation of coagulation neither affected the morphological aspect of the fibrin clot nor modified its mechanical properties. In addition, rtPA concentration was adjusted so that lysis started after the first 10 min after the initiation of clotting, allowing fibrin to form and stabilize.

This study provides the first direct visualization of the dynamic process of some of the early stages of fibrin formation and is consistent with previous investigations using a different approach, i.e. turbidity and light scattering (16, 17). Although the resolution of the light microscope is not sufficient to detect oligomers or protofibrils, small fibers were readily apparent. Initially, these fibers were fuzzy, indicating that they were very mobile. Hence, each optical section took 0.8 s to collect, indicating that the motion was relatively rapid (0.07 μm/sec). They grew rapidly by addition at their ends until meeting another fiber, at which point the two precursor fibers usually fused. A structured scaffold appeared early in the time sequence, at which point most fiber motion ceased. This is the point commonly known as the gel point, obtained from visual observation. Then, fiber diameters increased dramatically over time as measured by the increase in fiber absorbance, which is consistent with the well known finding from studies of fibrin polym-

![Graph A](image1)

**Fig. 4.** Graph A shows the variation of the average disconnected areas during fibrin assembly and fibrin dissolution. Values of p refer to the difference between the 2- and 8-min time points and the 10- and 25-min time points, respectively. Graph B shows the variations of the most frequent pore diameter of the fibrin network during fibrin assembly and fibrin dissolution. Values of p refer to the difference between the 10- and 25-min time points.

![Graph B](image2)

![Top panels](image3)

**Fig. 5.** Top panels are a series of micrographs showing the dynamic dissolution of purified fibrin visualized at 8, 16, and 24 min after the initiation of clotting (numbers refer to time points). Bottom panels are the same series of micrographs showing the detection of disconnected fiber bundles using the Noesis software. Each micrograph is the two-dimensional projection of optical sections representing a volume of 48 × 48 × 20 μm³. Complete dissolution of fibers and merging fibers are indicated by arrows and arrowheads, respectively. Micrographs 8b, 16b, and 24b show the binary mask of the fibrin network obtained at the 8-, 16-, and 24-min time points, which is used to determine the number of disconnected fiber bundles.
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erization using turbidity that the gel point occurs long before the turbidity has risen to any great extent (17).

Great similarities in the dynamic morphological events that occur during fibrin formation and fibrin dissolution were found, although in the reverse chronological order. During fibrin formation, protofibrils and small fibers agglomerate to make fibers that are sharper, straight, rod-like elements, with a progressive decrease in fiber diameter. In contrast, during fibrinolysis, the fibers become progressively disaggregated so that they are more diffuse and more curved. Interestingly, these same events occur at the lysis front, so that it appears that the process is much the same regardless of the mode of transport of the lytic agents (9,10). A constant increase of the fiber optical density is observed throughout fibrin formation, which indicates a persistent aggregation of fibrin molecules even after the network formation is completed, whereas a drop of fiber absorbance is the first detectable feature observed after the initiation of fibrinolysis using either diffusion or pressure-driven permeation of lytic agents (10). Interestingly, the previous reported differences in the rate of fiber digestion according to the fiber diameter (10) are also observed when rtPA is added prior to the initiation of fibrin assembly. In particular, thin fibrin fibers are formed, extend, and are digested at a faster rate than thicker fibers. Finally, we report a similar cascade of morphological events at the fiber level as described next to the lysis front (10). These findings confirm that fibrin digestion proceeds locally by lateral transection rather than progressive cleavage uniformly around the fiber, irrespective of the mode of transport of the fibrinolytic agents.

One of the major dissimilarities between intrinsic fibrinolysis and the diffusion or pressure-driven permeation of lytic agents studied previously is that no release of individual fiber pieces was observed in the present experimental setting. Thin fibers vanished very quickly, giving rise to fibrin degradation products that can easily diffuse, whereas thick fibers tended to aggregate and agglomerate with adjacent fibers. Release of the cleaved pieces of fiber is less likely during intrinsic lysis because there is no lysis front; thus, the cleaved pieces cannot escape and instead become stuck onto adjacent fibers. Aggregation of lysing fibers has been shown to be critical for accelerating fibrinolysis (5,9,15), but to date it has not been very apparent next to the lysis front (10). The present study confirms that agglomeration dramatically promotes fibrinolysis, probably by facilitating the crawling of plasmin across agglomerated fibrin as suggested previously (15). Agglomeration of the fibers also promotes the progressive retraction of the whole network, which later collapses into big particles.

Although thicker fibers are more likely to undergo agglomeration than thinner fibers, no definite conclusion can yet be drawn on whether the fibrin architecture itself modulates the fibrinolysis rate, as was reported previously in the diffusion model (10). The present study was conducted in specific conditions and represents a more complex model system. In particular, the kinetics of fibrin formation directly affects the fibrinolysis rate irrespective of the specific effect of fibrin conformation on fibrinolysis. This is because rtPA is present during fibrin assembly so that lysis can begin at any time. For example, a coarse fibrin network is obtained when lowering the thrombin concentration, which slows down the rate of fibrinopeptidase cleavage so that fibrin monomer formation is slow with respect to lateral aggregation. As a consequence of the slower fibrin polymerization, fibrinolysis is initiated before completion of fibrin assembly (6,7,10,18).

Some crucial fibrinolytic components need to be explored (TAFI, PAI-1, antiplasmin) in future investigations to extend the present findings. In addition, platelets, which generate a heterogeneous architecture of the fibrin network (19–21), dramatically impair the progression of fibrinolysis in vitro (22–28) and in vivo (29,30) and also require further investigation using the present experimental conditions.

In conclusion, the present findings suggest that intrinsic fibrinolysis leads to the same morphological changes of the fibrin architecture as observed during fibrin formation but also next to the lysis front during diffusion of lytic agents. It is likely that both types of fibrinolysis are occurring simultaneously in vitro, because thrombolysis occurs in the presence of continued coagulation.

REFERENCES
