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Plasmin-driven fibrinolysis in a quasi-two-dimensional nanoscale fibrin matrix☆

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ABSTRACT

Fibrin plays a fundamentally important role during hemostasis. To withstand the shear forces of blood flow and prevent embolisation, fibrin monomers form a three-dimensional polymer network that serves as an elastic scaffold for the blood clot. The complex spatial hierarchy of the fibrin meshwork, however, severely complicates the exploration of structural features, mechanical properties and molecular changes associated with the individual fibers of the clot. Here we developed a quasi-two-dimensional nanoscale fibrin matrix that enables the investigation of fibrin properties by topographical analysis using atomic force microscopy. The average thickness of the matrix was ~50 nm, and structural features of component fibers were accessible. The matrix could be lysed with plasmin following rehydration. By following the topology of the matrix during lysis, we were able to uncover the molecular mechanisms of the process. Fibers became flexible but retained axial continuity for an extended time period, indicating that lateral interactions between protofibrils are disrupted first, but the axial interactions remain stable. Nearby fibers often fused into bundles, pointing at the presence of a cohesional force between them. Axial fiber fragmentation rapidly took place in the final step. Conceivably, the persisting axial integrity and cohesion of the fibrils assist to maintain global clot structure, to prevent microembolism, and to generate a high local plasmin concentration for the rapid, final axial fibril fragmentation. The nanoscale fibrin matrix developed and tested here provides a unique insight into the molecular mechanisms behind the structural and mechanical features of fibrin and its proteolytic degradation.

1. Introduction

Fibrin is the three-dimensional scaffold of the clotting blood. Fibrin is produced by proteolytic cleavage from fibrinogen followed by spontaneous polymerization. Fibrinogen is a 340 kDa polypeptide circulating in blood, built up of three pairs of polypeptide chains, Aα, Bβ and γ. The protease thrombin cleaves fibrinopeptide-A and -B from fibrinogen monomers (Doolittle, 1984), which yields fibrin molecules with three pairs of chains, α, β and γ. Fibrin monomers bind *via* side-to-side and end-to-end interactions to form half-staggered, double-stranded protofibrils with a twisted configuration (Weisel and Litvinov, 2013). The bonds of the protofibrils are mostly D-D interactions of the γ-chains or A-A knob-hole interactions resulting from the cleavage of fibrinopeptide-A (Litvinov et al., 2005). Protofibrils then associate to form a fibrin fiber. From these polymeric structures fibrinopeptide-B is cleaved and a B-B knob-hole interaction is formed, resulting in lateral aggregation and formation of fibers (Litvinov et al., 2007). Fibers may differ in width and height, and they branch to form a three-dimensional network. The fibrin molecule also has Ca^{2+}-binding sites that are important for maintaining structure and function (Weisel, 2005). Factor XIIIa (fXIII) produces post-translational isodipeptide cross-links that stabilize the network (Lorand, 2005). The ionic strength during clotting influences the size of the fibers: diameter increases with decreasing ionic strength (Yeromonahos et al., 2010). The applied thrombin level also has an effect on the diameter: the lower the thrombin concentration, the larger the fibers (Ryan et al., 1999). The structure of the fibrin fibers and the network determine the global physical properties of the clot. Not only the width, height or protein content of the fibers may vary, but they also have an impact on clot stability and the resistance of the network to proteolysis (Collet et al., 2000). The structure of the network has been investigated with various methods such as electron-microscopy (Weisel et al., 1981), light- and X-ray scattering (Ferri et al., 2001; Yeromonahos et al., 2010), confocal microscopy (Collet et al., 2000), fluorescence microscopy and atomic force microscopy (AFM) (Liu et al., 2010). AFM carries the advantage that proteins may be imaged under aqueous conditions in which physiological structure and

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function are retained.

As soon as the blood clot is formed, its dissolution begins upon the action of plasmin, a 83 kDa serine-protease. Plasmin cleaves fibrin fibers at specific locations, one of which is at the C-terminus of the α-chain, called the αC domain. The αC domain plays important role in the intermolecular interactions and the lateral association within the fiber (Weisel and Medved, 2001). In fibrinogen the αC domain is loosely bound intramolecularly, and only after the cleavage of fibrinopeptide-B can it associate to other protofibrils (Medved et al., 1985). Fibers lacking αC domain produce a clot with reduced stiffness which is more susceptible to plastic deformations and is lysed faster (Collet et al., 2005). Plasminogen binding affinity of this region is relatively high, with a K_d of 16–33 nM (Tsurupa and Medved, 2001). Accordingly, as fibrinolysis proceeds, αC domains are cleaved off first (Doolittle, 2008; Longstaff and Kolev, 2015). Subsequently, the intramolecular coiled-coil connection between the D and E domains is cleaved, which is followed by the scission of the β-chain at the N-terminus (Longstaff and Kolev, 2015). Fibrinolysis is influenced not only by the clot architecture but also by mechanical stress, that is, by clot stretching and distortion (Collet et al., 2006). Stressed clots are more resistant to plasmin-driven fibrinolysis (Varju et al., 2011), and stretched fibrin fibers tend to elongate rather than become lysed when treated with plasmin (Li et al., 2017a). Fibrin fibers formed at reduced thrombin concentrations have an increased diameter and elongate rather than being fragmented (Bucay et al., 2015).

Exploring the biophysics of fibrinolysis is challenging for several reasons: 1) in contrast to usual biochemical reactions, in which the substrate diffuses to the enzyme's active site, in fibrinolysis it is the enzyme that diffuses towards the appropriate sites of a spatially stationary substrate; 2) the size of plasmin is small, therefore it is difficult to follow the motion of single enzyme molecules in the fibrin meshwork; 3) the dense three-dimensional fibrin meshwork precludes the structural and nanomechanical analysis of individual fibers during lysis. Thus, the sequence of events during plasmin-driven fibrinolysis is still not precisely resolved. It is still unclear whether plasmin digests transversely, thereby cutting an entire fiber (Veklich et al., 1998), or by a progressive and gradual cleavage uniformly along the fiber (Sakharov et al., 1996).

In the present work we developed a method of generating a two-dimensional (2D) footprint of the three-dimensional (3D) fibrin network so that the fibers forming the network become easily accessible for AFM analysis. Use of this quasi-2D nanoscale fibrin network allowed us to identify fiber-level mechanisms of plasmin-dependent fibrinolysis: the cleavage of lateral interactions within the fiber proceeds initially, while the global clot features are retained. The fibers that have thus become more flexible are rapidly chopped up axially in the final steps of fibrinolysis.

2. Materials and methods

2.1. Preparation of the 2D fibrin network

Human fibrinogen (type I from human plasma, F3879, Sigma-Aldrich, Merck, Darmstadt, Germany) was used for each measurement at a final concentration of 0.5 or 1 mg/ml. To prepare the fibrinogen stock solution, the lyophilized powder was completely dissolved in HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) to achieve a concentration of 10 mg/ml. The solution was dialyzed against the same buffer for eight hours in Slide-A-lyzer dialysis Cassettes (Thermo Fischer, Waltham, MA) by changing the dialysis buffer every two hours. The fibrinogen solution was stored in 20 µl aliquots at −20 °C. Thrombin from human plasma (T6884, Sigma-Aldrich, Merck, Darmstadt, Germany) was also diluted in HEPES buffer to a concentration of 1500 IU/ml and stored in 20 µl aliquots at −20 °C. To prepare the 2D network, the fibrinogen sample was diluted and incubated at 37 °C for 30 min. In the meantime, thrombin was also diluted to a concentration of 50 IU/ml and incubated on ice. Immediately prior to mixing thrombin with fibrinogen, $CaCl_2$ was added to the fibrinogen solution to a final concentration of 10 mM. Subsequently, 10 µl each of the fibrinogen-calcium and thrombin solutions were mixed directly on a freshly cleaved mica surface to achieve a final concentrations of 0.5 or 1 mg/ml fibrinogen, 5 mM Ca, 1 IU (maximum) thrombin and 150 mM (maximum) NaCl. Clotting was allowed to proceed for at least 90 min at 25 °C in a humid, closed environment. Then the sample was dried gently with high-purity nitrogen gas, washed with Milli-Q water to remove salts and dried again with a stream of nitrogen gas.

2.2. Fibrinolysis

Human plasmin (P1867, Sigma-Aldrich, Merck, Darmstadt, Germany) was diluted to 6 µM with HEPES buffer and stored, until further use, in 5 µl aliquots at -80 °C. Lysis was induced with different plasmin concentrations. First, the 2D fibrin network sample was rehydrated by pipetting 50 µl HEPES buffer on its surface and incubating at ambient temperature for 10 min. To initiate fibrinolysis, 50 µl plasmin solution (diluted in HEPES buffer to twice the final concentration) was pipetted in addition onto the 2D fibrin network. The sample was immediately loaded into the AFM for analysis. Once lysis was complete, the sample surface was rinsed with Milli-Q water to remove salts and soluble components and dried with a stream of high-purity nitrogen gas for further analysis.

2.3. AFM imaging

AFM images were acquired with a Cypher ES atomic force microscope (Asylum Research, Santa Barbara, CA) in non-contact mode. Scanning of dried samples was carried out by using silicon-nitride cantilevers (OTESPA-R3, Bruker, Billerica, MA) with a relatively high free amplitude (1V) and 0.7 V setpoint. Fibrinolysis was followed in buffer by using silicon nitride cantilevers (BL-AC40TS-C2, Olympus, Tokyo, Japan) with a relatively low, 300 mV free amplitude and 220 mV setpoint. Cantilever oscillation was driven with Blue Drive photothermal excitation. Height-, amplitude- and phase-contrast images were collected at a line-scan rate of 0.5–1.5 Hz.

2.4. Image processing and data analysis

Image analysis was performed by using the software toolkit of the AFM driver program (based on Igor Pro 6.34 A, Wavemetrics, Lake Oswego, OR). Fibrin fiber height was measured on cross-sectional topographical profiles as the distance between the height maximum and the substrate (mica). Fiber width was measured as the full width at half maximum (FWHM) on the cross-sectional profile. The relative error of the width measurement was calculated to be less than 1% (see Supplementary Information). Further data analysis was performed by using Microsoft Excel 2010 (Microsoft, Redmond, WA), OriginPro 8 (Northampton, MA), and GraphPad InStat and Prism 7 (San Diego, CA) softwares. The results shown in this work were collected in 22 independent experiments.

3. Results and discussion

3.1. Topology of the quasi-two-dimensional nanoscale fibrin network

To reveal the structural and mechanical properties of fibers within the blood clot under various conditions, we developed a quasi-two-dimensional fibrin network which is essentially a 2D footprint of the 3D clot (Fig. 1). Preparation of the 2D fibrin network involved the *in situ* mixing of fibrinogen and thrombin on a freshly cleaved mica sheet, clotting for 2 h, and a final stabilization step by drying. The AFM image of a 20 × 20 µm^2 area of the dried 2D fibrin network is shown in Fig. 1B. A meshwork of fibers with different diameter is seen. The