Journal of Structural Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Nano-thrombelastography of fibrin during blood plasma clotting

Tímea Feller, Miklós S.Z. Kellermayer, Balázs Kiss *

Department of Biophysics and Radiation Biology, and MTA-SE Molecular Biophysics Research Group, Semmelweis University, 1094 Budapest, Tűzoltó u. 37-47, Hungary

ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 3 April 2014
Accepted 4 April 2014
Available online xxxx

Keywords:
Fibrin
Elasticity
Atomic force microscopy
Thrombelastography
Thrombolysis
Streptokinase

ABSTRACT

Hemostasis is a complex process that relies on the sensitive balance between the formation and breakdown of the thrombus, a three-dimensional polymer network of the fibrous protein fibrin. Neither the details of the fibrinogen–fibrin transition, nor the exact mechanisms of fibrin degradation are fully understood at the molecular level. In the present work we investigated the nanoscale-changes in the viscoelasticity of the 3D-fibrin network during fibrinogenesis and streptokinase (STK)-induced fibrinolysis by using a novel application of force spectroscopy, named nano-thrombelastography. In this method the changes in the bending of an oscillating atomic-force-microscope (AFM) cantilever in human blood–plasma droplet were followed as a function of time. Whereas the global features of the time-dependent change in cantilever deflection corresponded well to a macroscopic thrombelastogram, the underlying force spectra revealed large, sample-dependent oscillations in the range of 3–50 nN and allowed the separation of elastic and viscous components of fibrin behavior. Upon STK treatment the nano-thrombelastogram signal decayed gradually. The decay was driven by a decrease in thrombus elasticity, whereas thrombus viscosity decayed with a time delay. In scanning AFM images mature fibrin appeared as 17-nm-high and 12–196-nm-wide filaments. STK-treatment resulted in the decrease of filament height and the appearance of a surface roughness with 23.7 nm discrete steps that corresponds well to the length of a fibrinogen monomer. Thus, the initial decay of thrombus elasticity during fibrinolysis may be caused by the axial rupture of fibrin fibers.

1. Introduction

Thrombi normally form at the site of vascular injury to stop bleeding, but can also appear at undesired locations occluding blood vessels thus causing heart attack or stroke. The mechanical properties of the thrombus play crucial role in proper wound healing. The thrombus needs to be mechanically resistant but at the same time flexible in a wide load range depending on the fluid dynamics characteristic to the vessel type. Thrombi are capable to withstand varying shear stress in a relatively wide range from 0.1 Pa (1 dyn/cm^2) in the venous system up to 7 Pa (70 dyne/cm^2) in arteries (Malek et al., 1999). The most important scaffold of the thrombus is the three-dimensional fibrin network, which, though constituting only 0.25% of the clot (Weisel, 2004), is the main determinant of the mechanical properties, strength and stability (Hudson et al., 2010; Weisel, 2004). Changes in the composition of fibrin network, chemical environment (e.g., ionic strength) and the effect of other platelet-derived effector molecules may modulate the structural and mechanical properties of fibrin. The fiber network becomes denser under low ionic strength conditions by decreasing the interprotofibril distance and increasing the length of the individual fibers (Yeromonahos et al., 2010). In the presence of increased thrombin and Ca^{2+} concentrations longer fibers and greater number of branching points are observed (Weisel, 2004). Increasing thrombin concentration results in thick, straight filaments (Piechocka et al., 2010).

Fibrin forms a unique, three-dimensional network built up of fibers interconnected with each other via trimolecular branching points (Weisel, 2004). The mechanical properties of a polymer network are typically determined by two factors: the properties of the individual fiber and the architecture of the clot (Guthold et al., 2004). To understand fibrin-fiber mechanics, individual, fluorescently labeled fibers (diameter: ~3–6 nm) have been analyzed by using nanomanipulation. The fibers displayed viscoelastic behavior with an elastic modulus of 1–3 MPa (Brown et al., 2009). Combined fluorescence microscopy/atomic force microscopy (AFM) experiments revealed that individual fibrin fibers are highly extensible

Abbreviations: AFM, atomic force microscopy; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IU, international unit (of enzyme activity); SEM, Standard Error of the Mean; STK, streptokinase.

* Corresponding author. Fax: +36 1 266 6656.
E-mail address: kiss.balazs@med.semmelweis-univ.hu (B. Kiss).

http://dx.doi.org/10.1016/j.jsb.2014.04.002
1047-8477/

up to 2.5 and 3.3 times their original length before rupturing and fiber crosslinking with factor XIII renders fibers less extensible, stiffer, and less elastic (Liu et al., 2010). Viscous properties appear at high shear stress with an irreversible component of structural deformation, but the mechanical properties of the clot remain intact by large with the cessation of stress (Nelb et al., 1981). The observation suggests that a dynamic rebuilding is essential in clot stability (Weisel, 2007). Strain stiffening is also a characteristic feature of individual fibrin fibers (Hudson et al., 2010), which contributes to strain distributing across the fibrin network. Rheological measurements revealed negative normal stress in the case of fibrin: a tendency not to dilate but to contract perpendicular to the applied stress due to the nonlinear force response of the single fibrin fiber (Janmey et al., 2007). Several different studies investigated individual fibrin fibers to find the molecular basis or determinants of the physical characteristics of the thrombus: electron microscopy (Muller et al., 1984) optical tweezers (Collet et al., 2005), AFM studies and discrete molecular dynamics simulations. (Hudson et al., 2010, 2013; Lim et al., 2008). The most widely employed method to assess the global mechanical properties of the whole thrombus is thrombelastography (Hartert, 1951; Mallett and Cox, 1992; Salooja and Perry, 2001). It provides time-dependent data during thrombogenesis and characterizes the complex mechanical response by applying shear stress to the whole clot. During a classical thrombelastography measurement a coupling develops between a standing component (e.g., wall of the vial holding blood plasma) and a rotating component (e.g., thin probe wire) due to fibrin network formation. The fibrin network couples and transmits the rotation, hence causing displacement in the probe wire during thrombogenesis. A classical thrombelastogram thus delivers information about the global mechanical properties of the fibrin network under the influence of shear forces and shear stress. However, thrombelastography results are difficult to compare with other mechanical measurements because of the complexity of *in vivo* clots (Weisel, 2004). Furthermore, miniscule changes in the mechanical response of the 3D fibrin network during thrombogenesis are averaged out in thrombelastograms leading to the loss of detail in the mechanical data. Mechanical studies on fibrinogen monomers and single fibrin protofibrils performed with AFM (Lim et al., 2008) showed that fibrin protofibrils exhibit triphasic response on the force-extension curve finally resulting in an exponential increase in the force before the rupture occurs. Rupture force analysis performed with optical tweezers revealed an average fibrinogen-fibrin binding force between 125 and 160 pN (Litvinov et al., 2005). Although the molecular mechanisms behind the mechanics of individual fibrin fibers are being increasingly explored, the detail behind the time-dependent evolution of thrombus mechanics is still largely unknown.

Disintegration of the fibrin network occurs during thrombolysis. During thrombolysis *in vivo* plasminogen is activated by a tissue-type plasminogen activator which subsequently cleaves fibrin fibers (Cesarman-Maus and Hajjar, 2005). Streptokinase (STK) accelerates fibrinolysis by forming a complex with plasminogen in which all three domains of STK bind to the catalytic domain of plasminogen (Loy et al., 2001). Scanning electron microscopy experiments revealed free fiber ends and gaps in the continuity of the fibers (Veklich et al., 1998). The molecular-level mechanistic detail of thrombolysis, however, is still far from being fully understood.

In the present work we devised an AFM-based dynamic method to reveal the mechanical changes of fibrin during plasma clot formation and thrombolysis. The mechanical data were converted into a nano-thrombelastogram so as to reveal connection between mechanical detail and global thrombus properties. The emerging thrombus displayed viscoelastic behavior with gradually increasing stiffness. The magnitude total AFM cantilever displacement as a function of time correctly reflected the thrombelastographic features.

2. Materials and methods

2.1. Coagulation assay

Normal mixed human plasma, collected at the 1st Department of Internal Medicine of Semmelweis University, Faculty of Medicine, Budapest, Hungary, anticoagulated by sodium citrate (0.11 mol/L, 1:9 volumetric ratio), was used for each measurement. Plasma samples were pooled typically from six healthy individuals. Prothrombin time as a measure of the coagulation status was normal in each sample. Prothrombin time measurements were performed by using a BCS-XP system (Siemens Healthcare Diagnostic products GmbH, Martburg, Germany). Samples were stored between 15 and 25 °C prior to the experiment. Typically 200 µl HEPES buffer containing Ca^{2+} (Ca-HEPES: 10 mM HEPES, 150 mM NaCl and 5 mM $CaCl_2$) was added to 100 µl plasma. The 300-µl samples were incubated at 25 °C either *in situ* on a glass slide or alternatively in a 1-mL microcentrifuge tube.

2.2. Fibrinolysis

Plasma clots were treated with streptokinase (Streptase, CSL Behring, Marburg, Germany) to induce fibrinolysis. The applied STK quantities were calculated according to the mass of the Ca^{2+}-activated plasma sample or the mass of the thrombus which was ~302 mg on average (Light et al., 2000). The applied final enzyme activities varied between 300 IU and 10,000 IU per sample. Fibrinolysis was performed at room temperature (25 °C) and lasted typically for 40–60 min.

2.3. Force spectroscopy and nano-thrombelastography

Calcium-activated plasma sample was applied on the center of a glass slide enclosed by a custom-made Parafilm ring (Pechiney, Chicago, IL, USA) with an internal diameter of 15 mm. The glass slide was then immediately placed on the stage of an inverted microscope combined with a molecular-force-probe AFM (MFP-1D, Asylum Research (Santa Barbara, CA, USA). The cantilever of the AFM was immersed under optical control completely into the plasma droplet without making contact with the substrate surface (Fig. 1a). Typically, the triangular lever E of an MSCT-AUHW cantilever (Bruker, Billerica, MA, USA) was moved vertically inside the plasma sample across a distance of 1 µm with velocities ranging between 0.25 and 2.5 µm/s. Cantilever deflection was measured with a quadrant photodiode, then converted into force data by taking the spring constant of the cantilever into account. Spring constant was calculated from the thermally driven oscillations of the cantilever. Typical spring constants ranged between 0.05–0.2 N/m. For each measurement new cantilevers were used, because after an experiment the clot was not removable without causing damage to the tip or the cantilever. Data analysis was performed by using Igor Pro 5.05A (Wavemetrics, Lake Oswego, OR) and Microsoft Excel 2007 (Microsoft, Redmond, WA, USA).

2.4. Morphology of fibrin network

For morphology measurements samples containing plasma and Ca-HEPES in a volumetric ratio of 1:2 were incubated *in situ* for 10 min on freshly cleaved mica. Unbound fibrin was washed away by rinsing the mica extensively with distilled water, then blowing gently with a stream of high-purity N_2 gas. Non-contact mode (AC mode) AFM images of mica-bound fibrin were acquired with an

Please cite this article in press as: Feller, T., et al. Nano-thrombelastography of fibrin during blood plasma clotting. J. Struct. Biol. (2014), http://dx.doi.org/10.1016/j.jsb.2014.04.002