BioMed Research International

Clot Structure and Fibrinolysis in Thrombosis and Hemostasis

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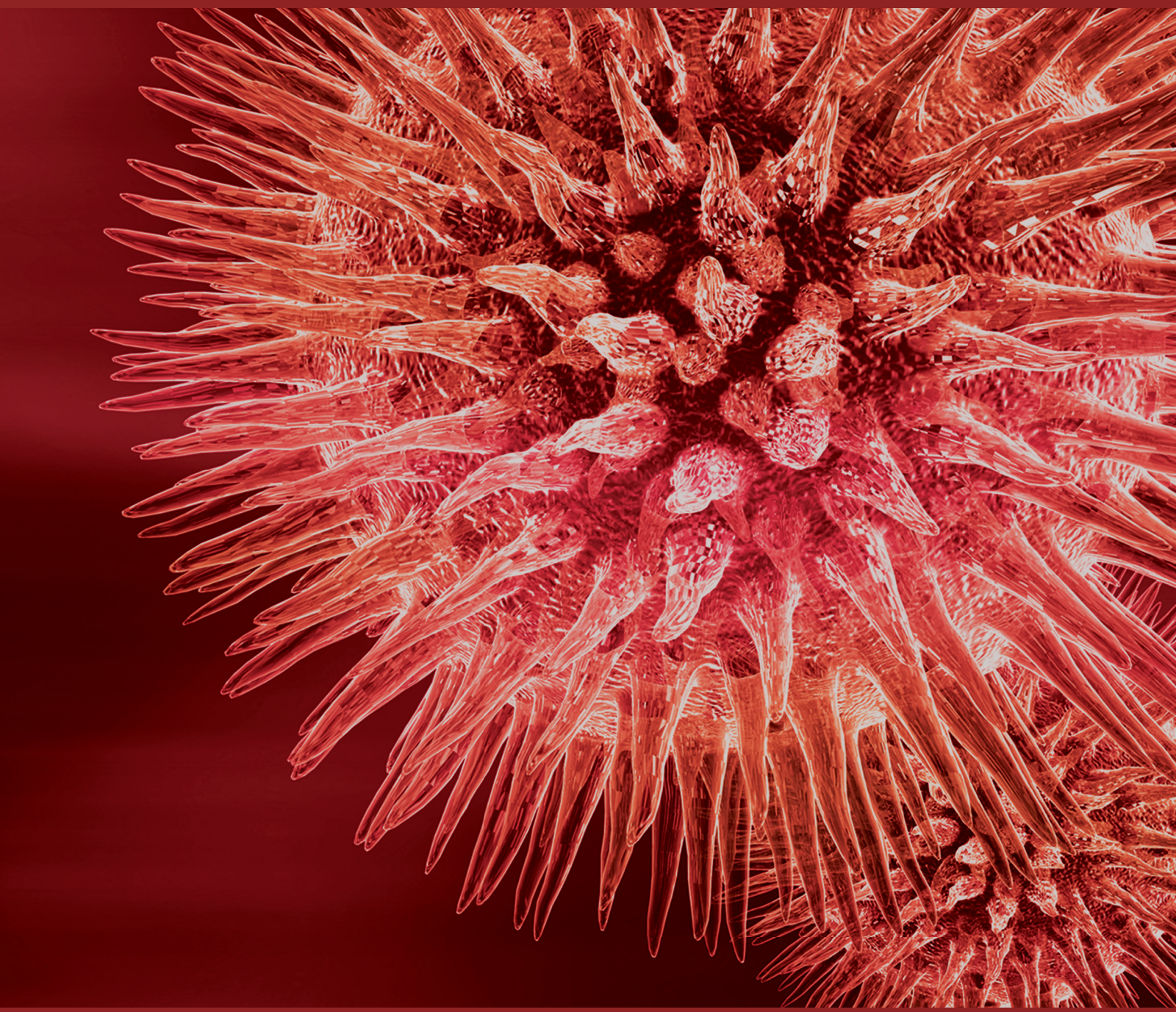

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Editorial

Clot Structure and Fibrinolysis in Thrombosis and Hemostasis

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"Inimicum quamvis humilem metuendum est" ("An enemy, however small, is to be feared").

Today, thrombotic disorders are major contributors to the global burden of disease [1]. The formation of a thrombus, however small, is the common pathology underlying devastating illnesses including ischemic heart disease, ischemic stroke, and venous thromboembolism. Due to the high rates of mortality and morbidity associated with arterial and venous thromboembolism, great efforts have been made in the past years to expand our knowledge on the biological, physical, and chemical features of the blood clot. In this special issue, original research articles and reviews focus on most recent advances on fibrin clot characteristics, mechanisms of clot formation/dissolution, and related clinical conditions.

Fibrin fibers constitute one of the major structural components of the blood clot, providing the clot with a three-dimensional polymeric protein network that imparts considerable elastic strength, with the mechanical and structural properties thereof influencing morbidity and mortality rates associated with thrombotic events. Surprisingly, the process of lateral association of protofibrils into fibrin fibers is still poorly understood. In the research paper by W. Li et al., the authors aim to gain insights into the relatively less studied internal structure of fibrin fibers and describe a novel fiber model. Based on experiments using fluorescence intensity and force (modulus) measurements, they propose that protofibrils are densely packed at the fiber center, but are sparse and loosely connected towards the fiber periphery. The clinical implications of abnormal fibrin clot properties are described in a review article by A. Undas. It has been known for some time that fibrin clots that are composed of thinner, highly branched fibers are less permeable, more rigid, and less susceptible to dissolution by fibrinolytic proteins. A. Undas provides a timely update on the so-called prothrombotic fibrin clot phenotype and its relevance to venous thromboembolism. Listing experimental and clinical studies, the author describes a number of clot structure modifiers implicated in the occurrence of the prothrombotic fibrin clot phenotype and the resulting clot characteristics accounting for a potential risk for thromboembolism.

Not only the formation, but also the dissolution of the fibrin clot is dependent on biochemical and biophysical processes. The review article by N. E. Hudson highlights the importance of understanding fibrinolysis in the light of fibrin biophysical characteristics. The review considers the impact of several structural and mechanical parameters on lytic rates. The author summarizes the newest findings with the expectation that improved understanding of the connection between the biophysical aspects of fibrin and fibrinolytic rates could lead to novel strategies in the development of future fibrinolytic therapies. As an example, the original research by A. Tanka-Salamon et al. describes a novel thrombolytic

approach. The purpose of the work was to prepare a phospholipid-based thermosensitive nanocarrier, in which trypsin is attached to the inner leaflet of the bilayer shell of the liposome, and to characterize this new tool in terms of structure and proteolytic efficiency. The authors show that the lytic efficiency of trypsin depends on heat-dependent release from thermosensitive liposomes. The fibrinolytic efficiency of these liposomes was found to be improved in the dynamic fibrinolytic assay under conditions of permeation-driven fibrinolysis. Because intravascular thrombi are exposed to permeation forces, these properties of the construct suggest that it could be a successful candidate as a therapeutic tool, the utility of which deserves further investigation.

A key player in the inhibition of fibrinolysis is activated factor XIII (FXIIIa). An interesting review by D. C. Rijken and S. Uitte de Willige focuses on the impact of this transglutaminase on fibrinolysis. The inhibitory effects of FXIIIa-mediated cross-links on fibrinolysis are summarized and differences in the crosslinking of purified fibrin, plasma, or whole blood clots are highlighted. The potential effect of clot compaction and clot retraction on the inhibition of lysis by FXIIIa and the pathophysiologic aspects of FXIIIa-mediated cross-links are also explained. The catalytic A subunit of FXIII (FXIII-A) is present not only in plasma but also intracellularly in several human cells, suggesting effects of this protein are not restricted to hemostasis. Understanding the roles of FXIII-A outside of the coagulation system is an intriguing area of research with implications for many clinical conditions and pathophysiologic processes including wound healing, angiogenesis, atherosclerosis, and malignancies. In their review paper, L. Paragh and D. Törőcsik summarize current knowledge on intracellular FXIII-A in wound healing, angiogenesis, and various dermatopathologic conditions.

The pathophysiology of increased thrombotic risk in various clinical conditions was studied in two original papers in this issue. N. K. Tóth et al. examined local, intracardiac hemostasis and fibrinolysis abnormalities that are associated with atrial fibrillation (AF) and increase the risk of thromboembolism. After measuring a comprehensive set of fibrinolytic and hemostasis proteins in intracardiac and peripheral blood samples from AF patients and non-AF controls, the authors concluded that AF patients have elevated factor VIII and von Willebrand factor levels, present in the intracardiac and peripheral environment as well, possibly contributing to the increased thrombotic risk associated with this disorder. S. C. Meyer et al. investigated whether antiplatelet factor 4 (PF4)/heparin antibodies contribute to the increased thrombotic risk observed in myeloproliferative neoplasms. They conclude that thrombotic risk increases in anti-PF4/heparin IgG-positive polycythemia vera, reflecting potential clinical implications and calling for larger, confirmatory cohorts.

Clot formation *in vivo* occurs in a complex environment. Whole blood clots contain cells and cell-derived components that greatly influence thrombus size, composition, and stability. In their timely and comprehensive review, J. Kappelmayer and B. Nagy Jr. summarize central function of selectins and their ligands and their roles as key mediators in cellular events during the development of thrombotic and malignant conditions. The role of platelets in clot formation, including thrombin generation and clot contraction/retraction, is another major focus in hemostasis and thrombosis research. R. Hudák et al. describe the effect of the phosphatase inhibitor calyculin-A on various platelet activation mechanisms contributing to clot formation, clot retraction, and thrombin generation and conclude that this inhibitor could serve as a useful tool in experimental studies.

We are confident that papers in this special issue will be of interest and relevance to those involved in experimental and clinical fields related to thrombosis and hemostasis. We hope that the published articles will provide ideas and inspiration to those dedicated to understand the pathophysiologic mechanisms of clot formation and lysis, and will inspire new ideas for diagnosing, treating, and ultimately preventing thrombotic disorders in the future.

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Research Article

Nonuniform Internal Structure of Fibrin Fibers: Protein Density and Bond Density Strongly Decrease with Increasing Diameter

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The major structural component of a blood clot is a meshwork of fibrin fibers. It has long been thought that the internal structure of fibrin fibers is homogeneous; that is, the protein density and the bond density between protofibrils are uniform and do not depend on fiber diameter. We performed experiments to investigate the internal structure of fibrin fibers. We formed fibrin fibers with fluorescently labeled fibrinogen and determined the light intensity of a fiber, I, as a function of fiber diameter, D. The intensity and, thus, the total number of fibrin molecules in a cross-section scaled as $D^{1.4}$. This means that the protein density (fibrin per cross-sectional area), ρ_p, is not homogeneous but instead strongly decreases with fiber diameter as $D^{-0.6}$. Thinner fibers are denser than thicker fibers. We also determined Young's modulus, Y, as a function of fiber diameter. Y decreased strongly with increasing D; Y scaled as $D^{-1.5}$. This implies that the bond density, ρ_b, also scales as $D^{-1.5}$. Thinner fibers are stiffer than thicker fibers. Our data suggest that fibrin fibers have a dense, well-connected core and a sparse, loosely connected periphery. In contrast, electrospun fibrinogen fibers, used as a control, have a homogeneous cross-section.

1. Introduction

1.1. Fibrinogen. Fibrinogen is a key protein in blood, which upon activation by thrombin polymerizes into a meshwork of fibrin fibers. Fibrin fibers form the major structural component of blood clots. In hemostasis, they stem blood flow in the event of injury and trauma, and they are involved in the initiation of wound healing [1]. In thrombotic disease, aberrant formation of blood clots is the underlying pathology of such diseases as myocardial infarction, ischemic strokes, deep vein thrombosis, and pulmonary embolisms. Fibrinogen is a 340-kDa glycoprotein, with an elongated, trinodular shape; it is 45 nm in length and 4.5 nm in diameter. It is composed of two distal D regions and one central E region, which are connected by two triple-helical coiled coils [2–4].

1.2. Protofibril Formation. The first step in fibrin fiber formation is the formation of protofibrils. It is initiated as thrombin proteolytically removes fibrinopeptide A from the central E region of fibrinogen, thus converting fibrinogen to desA fibrin. Knob "A", which remains after cleavage of fibrinopeptide A, fits into hole "a", located in the terminal D region. Fibrin monomers assemble via these A:a interactions in a half-staggered fashion into double-stranded protofibrils. The interactions between abutting D interfaces, called D:D interactions, are also important for protofibril assembly. It is well accepted in the field that the key interactions for protofibril assembly are the A:a and D:D interactions. If these interactions are blocked, protofibril formation is severely impaired, which is not the case for any of the other interactions.

1.3. Lateral Aggregation. The two-stranded protofibrils then assemble laterally (radially) into mature fibrin fibers [5–7]. However, the interactions for this lateral assembly are much less understood, and the internal arrangement of protofibrils within a mature fiber is not clear. Some studies suggest a relatively regular internal structure, in which the protofibril is packed in a semicrystalline fashion [6, 8] while others suggest that it is packed randomly [9–11]. A commonly used method to investigate the properties and the structure of fibrin clots is turbidimetry and light scattering, which can provide information about fiber size [12, 13] and fiber (protein) density [14, 15]. There are numerous papers about the mechanical properties and structures of a whole blood clot [16, 17]. However, the literature on the emerging field of single fibrin fiber mechanical properties is more limited [18–27]. Moreover, scattering techniques which draw conclusions about single fibers take data that are averaged over many fibers, and then information about single fibers is extracted; in other words, the information about single fibers is indirect [28]. If there are significant differences in single fibers, that difference may be lost in averaging methods. When measuring single fibers, differences between individual fibers can be resolved.

1.4. Fibrin Fiber Lateral Structure. The lateral aggregation of fibrin fibers is poorly understood. In the past, most models assumed a uniform cross-sectional density [6, 8]. Recently there has been some evidence that the internal structure is not homogeneous [28, 29]. Protein density is a good indicator of the fiber structure. Neutron scattering and light scattering are most frequently used to indirectly determine protein density. These techniques have been used to show that protein content inside a fibrin density is only 20–30% (70–80% solvent) (some theoretical studies tried to explain this phenomenon [30, 31]) and the lateral structure of fibrin fibers may be fractal [28]. Similar results were found by high-resolution atomic force microscope (AFM), as it was observed that molecule packing inside the fiber might be denser and tighter than that on the surface [29, 32]. Early rupture force measurements on dry fibrin fibers suggested that the cross-section of fibrin fibers may have a fractal dimension of 1.3 [9].

In the presented work, we used fluorescently labeled fibrinogen to obtain a relationship between fiber light intensity, I, which is proportional to the number of monomers in a fiber cross-section, and fiber diameter, D. We found $I \propto D^{1.4}$, suggesting a denser core and a less dense periphery. For a fiber with uniform density, the number of fibrin molecules would increase as the square of the diameter, D^2.

In addition to the fibrin and protofibril packing inside a fiber, the bonds involved in lateral aggregation are also not well understood. Bonds are important because they hold the fiber together. In the past, there has been no method to determine bond density inside a fiber. The stretching force of a single fiber is proportional to the number of bonds inside the fiber. For a fiber with homogeneous bond density, the stretching force would increase as D^2 (since the cross-sectional area of a fiber increases as D^2). However, our data show the stretching force, $F \propto D^{0.6}$; that is, it increases with a significantly lower power than D^2. This agrees with our proposed model that the inside of a fibrin fiber is not homogeneous.

This relationship was found for fibrin fibers formed from diverse sources: purified fibrinogen and plasma from white older males, white middle-aged males, and black middle-aged females with and without diabetes. Since this relationship was found for all samples, it appears to be a general property of fibrin fibers, regardless of the source.

Combining our light intensity and force data, we propose a novel model for the internal structure of fibrin fibers. In this model, they have a dense core of well-connected protofibrils that becomes less dense and less well connected as more protofibrils aggregate onto the outside of the fiber.

2. Materials and Methods

2.1. Plasma Collection. Blood samples were from several different population groups. Blood was collected from healthy white males from two different subgroups: five healthy middle-aged individuals (40–50 years old) and five healthy older individuals (>70 years old). Blood samples were collected into citrated tubes and then centrifuged in a Beckman model TJ-6 centrifuge at 3700 rpm (2000g) for 15 minutes at room temperature to remove cells and large particles; the remaining plasma was stored at −80°C until further use. These samples were covered under WFU IRB protocol 00012738.

Blood was also collected from black, female South Africans from three different subgroups: five controlled diabetic patients (Type II Diabetes) (receiving insulin treatment), five uncontrolled diabetic patients (Type II Diabetes), and a control group of five healthy (nondiabetic) subjects [18]. These samples were a random subset of a larger sample [33]. Blood from these groups was centrifuged at 2000g at 4°C for 15 minutes. The remaining plasma was stored at −80 until further use. All subjects signed informed consent and ethical approval was obtained from the ethics committees of both the University of Pretoria and the North-West University, South Africa. [33].

2.2. Striated Substrate Preparation. The striated substrates were prepared as previously reported [18–22]. Briefly, a drop of optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed on top of a coverslip. A rectangular polydimethylsiloxane (PDMS) stamp was then pressed into the optical glue to form a striated substrate with 6.5 μm wide ridges and 13.5 μm wide grooves. The optical glue was then cured under 365 nm UV light (UVP 3UV transilluminator, Upland, CA) for 1.5 minutes, at which point it was ready to be used.

2.3. Formation of Electrospun Fibrinogen Fibers and Fibrin Fibers

2.3.1. Electrospun Fibrinogen Fibers. 100 mg/ml lyophilized bovine fibrinogen (Sigma-Aldrich Chemical Co.) was prepared with a solution of 9-part 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) and 1-part minimum essential medium

(MEM, 10x MEM, Gibco, Invitrogen cell culture). 3.17 mg *Rhodamine 6G* fluorophore (Eastmen Kodak, Rochester, NY; molecular weight 479 g/mol) was then added to the mixture to get a final ratio of 15 fluorophores per fibrinogen molecule. The electrospun fibrinogen fibers were spun onto a cover glass slide (number 1.0, 24 mm × 60 mm, Fisherbrand, Pittsburgh, PA) as described in [34].

2.3.2. Fibrin Fibers from Plasma Samples. Samples were prepared as described in [18]. All chemicals were from Sigma-Aldrich unless otherwise noted. Fibrin fibers were formed from the human, platelet-poor plasma samples described above. 2 μl of 0.1 NIH units/ml thrombin was added to an 18 μl mixture of plasma and 20 mM $CaCl_2$ that was first pipetted onto the striated substrate. The sample was kept in a wet atmosphere at room temperature for one hour. After that, the top layer of the fibrin clot was gently rinsed away using calcium free fibrin buffer (140 mM NaCl, 10 mM, Hepes, pH 7.4), and 20 nm carboxyl coated fluorospheres (Invitrogen, Carlsbad, CA) were added to the substrate. The sample was incubated in fibrin buffer before use.

2.3.3. Fibrin Fibers from Purified Fibrinogen and Fluorescently Labeled Fibrinogen. Fibrin fibers were formed from purified unlabeled fibrinogen (Enzyme Research Laboratories, South Bend, IN) and purified fibrinogen that was labeled with Alexa-546 fluorophore (about 15 fluorophores per fibrinogen monomer; Life Technologies, Grand Island, NY). 18 μl of fibrinogen solution (13.5 μl nonlabeled and 4.5 μl fluorescently labeled fibrinogen, both at 1.5 mg/ml) and 2 μl of 0.1 NIH units/ml thrombin were pipetted onto a cover glass slide (number 1.0, 24 mm × 60 mm, Fisherbrand, Pittsburgh, PA). The sample was kept in a wet atmosphere at room temperature for one hour. After this period, the top layer of the fibrin clot was gently rinsed away with fibrin buffer, and the sample was kept in fibrin buffer (140 mM NaCl, 10 mM Hepes, and 5 mM $CaCl_2$, pH 7.4).

We performed turbidity experiments with unlabeled fibrinogen and 3 : 1, 1 : 1, and 1 : 3 ratios of unlabeled fibrinogen to Alexa-546-labeled fibrinogen (see Supplementary Figure S1 in the Supplementary Material available online at https://doi.org/10.1155/2017/6385628). Our preparation of Alexa-546-labeled fibrinogen had about 15 dye molecules per fibrinogen molecule; Alexa-Fluor-546 NHS Ester has a molecular weight of 1160 g/mole. The turbidity experiments were performed with the same buffer conditions as the fluorescence intensity experiments (same salt concentrations and pH, same fibrinogen concentrations and ratios). The experiments showed that Alexa-546-labeled fibrinogen does impair fibrin fiber formation in a dose-dependent manner. Specifically, the slope is less steep and the maximum absorption is lower. The lowest dose of labeled fibrinogen—the 3 : 1 ratio we used in the fluorescence intensity experiments—still has some impairment on fibrin fiber formation. However, we believe these fibers are still a reasonably good representation of natural fibrin fibers. This assessment is based on the appearance of the fibers in AFM and fluorescence images and on the overall shape of the turbidity curve, which looks normal. In our intensity experiments, we had to find a compromise between enough labeling, so that the fibers would be clearly visible in the fluorescence images, and too much labeling, which impairs fibrin fiber formation. We believe the 3 : 1 ratio is a good compromise between these two competing requirements.

2.3.4. Fibrin Fibers from Unlabeled Purified Fibrinogen for Force Measurement. Fibrin fibers were formed as described above, except that they were formed from nonfluorescently labeled, purified fibrinogen on a striated substrate, and labeled with 20 nm carboxyl coated fluorospheres after polymerization (Invitrogen, Carlsbad, CA) as described above.

2.4. Fluorescence Microscopy (Optical Microscopy). We used an inverted fluorescence microscope (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany) in these experiments. Fluorescence images were taken with a 40x lens (NA of 0.7), with an excitation source from a short arc mercury lamp (Osram HBO 103W/2, Atlanta Light Bulbs Co., Atlanta, GA). The exposure time was 200 ms. The field of view (image size on computer) was 180 μm × 180 μm. In order to get the same photobleaching for all the fibers in a particular image, fluorescence images of each area were taken only once, under the same conditions. Fluorescence images were collected with a Hamamatsu EM-CCD C9100 camera (Hamamatsu Photonics, KK, Japan) with IPLab software (Scanalytics, Fairfax, VA).

2.5. Fibrin Fiber Measurements and Manipulation

2.5.1. Fluorescence Intensity Measurement. Fibrin fibers (or electrospun fibrinogen fibers) were formed on a coverslip and fluorescence images of the fibers were taken immediately after illuminating the fibers to limit photobleaching as much as possible. Since the diameter of fibrin fibers is below the resolution limit of light microscopy, the AFM was used to obtain images of the same location as the fluorescence image (Figure 1). Assuming a cylindrical fiber, fiber height as determined from AFM topography images was taken as the fiber diameter (fiber width was not used because it is significantly exaggerated in AFM due to the tip broadening effect). ImageJ software (https://imagej.nih.gov/ij/) was used to determine the light intensity of the cross-section of single fibers. The lowest point was used as the baseline to normalize intensity and the total intensity was obtained by summing the intensities of each pixel.

2.5.2. AFM Imaging of Fibrin Fibers. All fibrin fibers were imaged in buffer (same buffers as described above under fibrin fiber preparation), using a combined atomic force microscope (AFM) (Topometrix Explorer, Veeco Instruments, Woodbury, NY) and inverted fluorescence microscope (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany). The fiber sample was placed between the AFM and optical microscope using a customized stage which allows the sample to be moved independently of either microscope [18–22]. Fibers were imaged in tapping mode with any one of the three cantilevers of AFM probe CSC-38 (MikroMasch, Wilsonville, OR) or an equivalent AFM probe (similar k

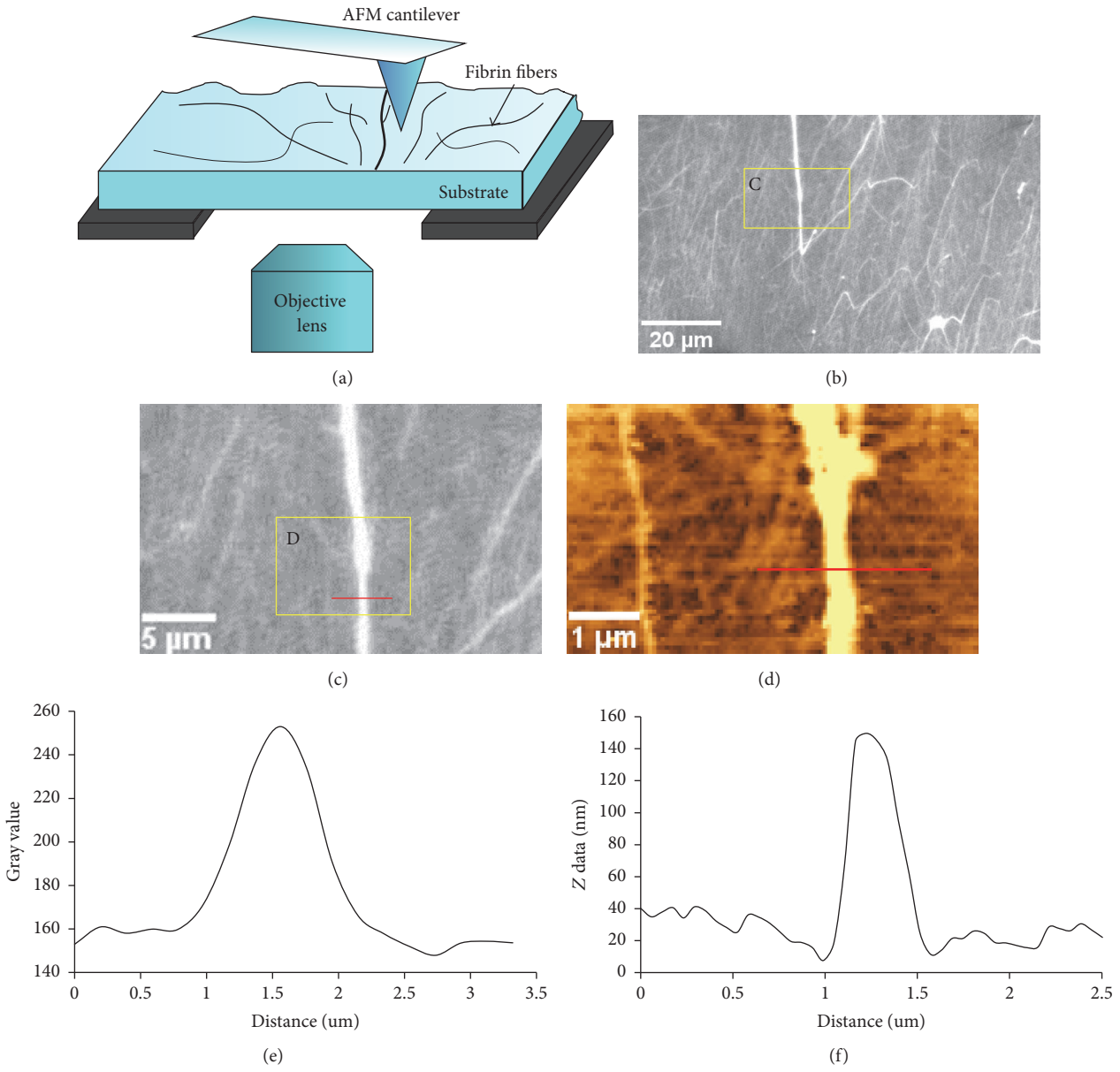

Figure 1: Experimental setup of fluorescence intensity measurement. (a) Schematic lateral view of setup. The AFM is above the sample, and the objective lens of the inverted fluorescence microscope is below the sample. ((b), (c)) Fluorescence images of fibrin fibers. (d) AFM topography image of the same region (yellow box) of fibrin fibers as in (c). (e) Fluorescence intensity distribution of the cross-section (along the red line in (c)) of a single fiber; total fluorescence intensity of this fiber corresponds to the area under this curve. (f) Height distribution of the cross-section along the red line of the fiber in (d), which is the same fiber as in (c). Even though the fiber in (c) looks very bright, it was far below saturation as the gray value limit is 16384 (2^{14}).

and f). The spring constant, k, ranged from 0.03 N/m to 0.09 N/m, and the resonance frequency, f, ranged from 10 kHz to 20 kHz. Fibers were typically imaged at a 50% set point (50% of maximum free amplitude); the set point was adjusted so that the probe exerted the smallest possible normal force on the sample, while still making good contact with the surface. Feedback gains were adjusted as high as possible, without causing ringing. The fiber diameter, D, was determined by AFM imaging the fiber either on top of the ridge adjacent to where the fiber was manipulated (for the force measurements) or on the glass slide (for the fluorescence intensity measurements). The fiber cross-section was calculated assuming a cylindrical cross-section.

2.5.3. Force Data Measurement. Fibrin fiber mechanical properties were determined using a combined fluorescence microscope and atomic force microscopy (AFM) technique, as described before [18–20, 22]. Briefly, as shown in Figure 2, fibers were formed on the striated substrate. The AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY)

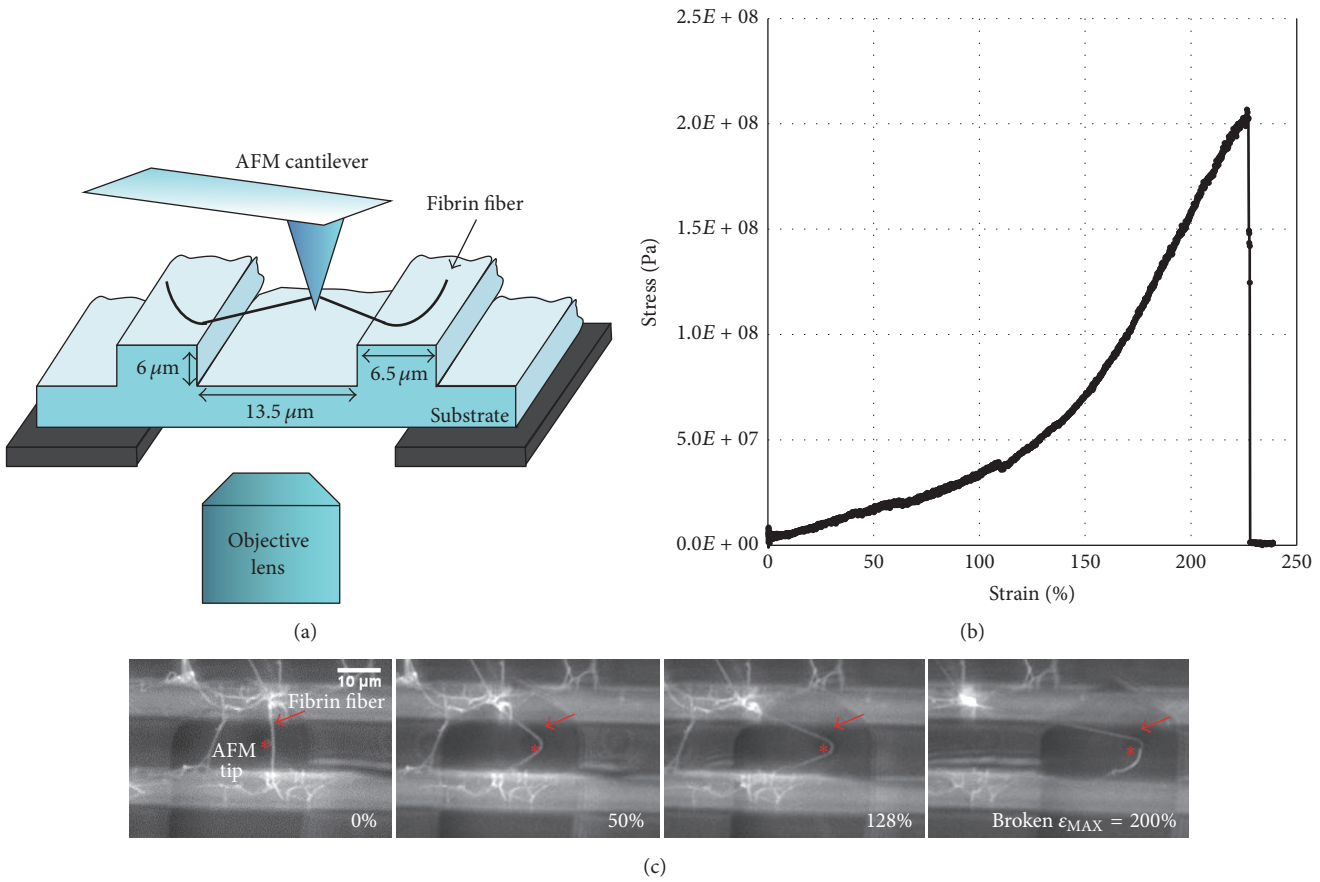

Figure 2: Experimental setup for force measurement. (a) Schematic lateral view of setup. (b) Representative single fibrin fiber stress versus strain plot. The fiber broke at a strain of 225%. The modulus corresponds to the slope of this plot. We used the initial slope, before strain hardening at about 100% strain. (c) Fluorescence images of a fiber being stretched and broken. The large dark object is the AFM cantilever and the AFM tip is marked by an asterisk. The fiber in (c) broke at a strain of 200%. Figures 2(a) and 2(c) are adopted from [18].

tip is located above the sample for manipulation, while the inverted fluorescence microscope (Axiovert 200 or Observer D, Zeiss, Göttingen, Germany) can image the sample from below.

Single fibers, suspended over the 13.5 μm grooves in the striated substrate, were laterally pulled causing the AFM cantilever to torque. NanoManipulator software (3rd Tech, Chapel Hill, NC) provided precise control of the AFM tip and collected force and position data during fiber manipulations. Fiber diameters were measured on the ridges of the striated substrate using the AFM tapping mode (and assuming a cylindrical fiber). From these data, the actual force on the fiber was calculated; and stress and strain were determined as $\sigma = F/A$ and $\varepsilon = \Delta L/L_{\text{init}}$, where A is the fiber cross-sectional area, L_{init} is the initial length of the fiber, and ΔL is the change in fiber length. Young's (elastic) modulus, Y, is then calculated via $Y = \sigma/\varepsilon$ from the initial part of the stress-strain curve. Possible fiber compression effects where the AFM probe contacts the fiber were ignored. Since the fiber is 13.5 μm long and the tip contacts the fiber over a length of only about 500 nm (the very end of the AFM tip is used for manipulations), this contact length is less than 4% of the entire fiber length.

2.6. Statistical Analysis. The significant difference between two groups was determined by using a two-tailed t-test. The significant difference between two slopes was calculated by analysis of covariance (ANCOVA).

3. Results

The goal of our work was to investigate the internal structure of fibrin fibers; in particular we aimed to determine how the number of fibrin monomers and the number of bonds inside a fibrin fiber vary as a function of fiber diameter. These data will provide insights into the structural arrangement of fibrin monomers and bonds inside a fibrin fiber. We used two types of experiments to determine these quantities. (1) Fluorescence intensity measurements of single fibers formed from fluorescently labeled fibrinogen: these data provide information on the number of fibrin monomers in a fiber. (2) Stretching force measurements on single fibers: these data provide information on the number of bonds in a fibrin fiber.

3.1. Fluorescence Intensity as a Function of Fiber Diameter. In these experiments, fibrin fibers were formed on a flat coverslip from fibrinogen that was labeled with Alexa-546.

The fiber diameter, which is beyond the resolution limit of light microscopy, was determined from an AFM topography image of the same area (Figure 1). A cylindrical fiber shape was assumed and the fiber height was used as the diameter of the fiber. We used the height because it can be determined accurately, with nanometer precision. In AFM imaging, the height of the sample can sometimes be compressed by the force exerted on the sample by the AFM tip. However, this effect is typically small. Comparing our diameter values to values obtained by other techniques suggests our height measurements are correct and that it is reasonable to assume the fibers are cylindrical. In particular, our diameter values determined from the height of the fibers agree with the values obtained by SEM [18, 33]. Our diameter values also generally agree with the diameter values obtained by light scattering [28]. The lateral dimensions in atomic force microscopy images are always exaggerated by the tip broadening effect, as the width of the AFM probe gets added to the width of the sample [36]. Therefore, the fiber width in AFM images is not an accurate representation of the fiber diameter.

Photobleaching was minimized by having the shortest possible exposure times and by taking images as rapidly as possible. Fluorescence images of each area were taken only once, under the same conditions, so that residual photobleaching was the same for all fibers in a particular image. Moreover, to average out small fluctuations, we analyzed four closely spaced cross-sections of each fiber (see Figure 1) and took the average. We did the same four-measurement averaging procedure to determine the diameter of each fiber from the AFM scans.

3.2. Dry Electrospun Fibrinogen Fiber (Mixed with Rhodamine 6G). As a control experiment, we first determined the fluorescence intensity of electrospun fibers (Figure 3). Electrospun fibrinogen fibers have about the same diameter as fibrin fibers, on the order of 100 nm [35]. Dye was mixed into the spinning solution. For a homogeneous cross-section with an evenly distributed dye, the fluorescence intensity should increase as D^2, since the cross-sectional area of a fiber (assuming it is cylindrical) is $(\pi/4)D^2$. The molar ratio of fluorophore to fibrinogen monomer in the spinning solution was 15 : 1.

In this control experiment, we found that the total light intensity, I, increases with increasing diameter as $I \propto D^{1.9\pm0.1}$, which means that the number of fluorophores, N_f, also increases as $N_f \propto D^{1.9\pm0.1}$ (Figure 3(e)). This experiment was performed in triplicate (for images and additional data, see Supplementary Figure S2). If we assume a circular cross-section of the electrospun fiber, $A = (\pi/4)D^2$, the fluorophore density $\rho_f = N_f/A$ is constant, as would be expected for a homogeneous fiber with an even distribution of fluorophores (Figure 3(f)).

3.3. Wet Fibrin Fiber Labeled with Alexa-546 Fluorophore. A 3 : 1 mixture of unlabeled fibrinogen and fibrinogen that was labeled with 15 Alexa-546 fluorophores per fibrinogen monomer was used to form fibrin fibers on a flat glass substrate. For these fibrin fibers, fluorescence intensity also increased with increasing diameter; however it only increases as $I \propto D^{1.4\pm0.2}$ (Figure 4(a)). Since the total fluorescence intensity, I, of a fiber cross-section is proportional to the number of monomers in a cross-section, N_m, this implies that $N_m \propto D^{1.4\pm0.2}$. This is a much lower exponential power than 2, which was seen in the electrospun control fibers and which would be expected for homogeneous fibers (these experiments were carried out in triplicate; for other plots, see Supplementary Figure S3). To obtain the diameter dependence of the fibrin density (protein density), we divide by the cross-sectional area (assuming a cylindrical cross-section, $A = (\pi/4)D^2$). Thus, the protein density scales as $\rho_p \propto D^{-0.6}$; it decreases strongly with increasing diameter (Figure 4(b)). This means that thinner fibers have a higher protein density than thicker fibers. It suggests that the cross-section of a fibrin fiber is not homogeneous and that fibrin fibers have a higher density in the core and a lower density at the periphery.

Dry fluorescently labeled fibrin fibers showed a similar relationship, as the intensity, I, varied as $I \propto D^{1.21\pm0.14}$ (details in Supplementary Figure S4).

3.4. Fiber Modulus as a Function of Fiber Diameter. The number (and strength) of parallel, longitudinal bonds inside a fiber is proportional to the force that is required to stretch the fiber. Therefore, determining the stretching force as a function of diameter can provide information on the bond density of a fiber cross-section. Before stretching a fiber, we first used the AFM in imaging mode to determine the diameter of the fiber (on the anchoring ridge). We then used the AFM tip to pull the same fiber to obtain a stress-strain curve. This procedure is described in detail in [20, 35]. Young's modulus (stiffness), Y, of the fiber corresponds to the slope of the stress-strain curve. The fibrin fibers in these force measurements were formed from plasma from various groups (see methods) and also from purified fibrinogen.

In all of our force experiments, for every sample, we took at least 20 measurements, so there are at least 200 data points in each group. The three different groups are plasma samples from white males, from black females, and from purified fibrinogen. We saw very similar relationships between the modulus and fiber diameter for fibers from all groups. The modulus, Y, decreased strongly as fiber diameter increases with an exponential power of about −1.5; that is, $Y \propto D^{-1.5}$. This appears to be a general property of fibrin fibers, since we saw a similar relationship for all groups.

Specifically, for plasma samples from white males, the slope of this relationship was −1.4 ± 0.1 on a log-log scale, while the slope for plasma samples from black females was −1.6 ± 0.2. The slope for samples from purified fibrinogen was −1.4 ± 0.3 (Figure 5).

Young's modulus may be related to the bond density, ρ_b, inside a fiber. To make this connection, we assume that the lateral bonds will also carry (transmit) some of the longitudinal stress, when a fiber is strained. This is a reasonable assumption for current models of fibrin fibers, in which the protofibrils are connected to each other by lateral bonds. There is evidence that these lateral bonds include

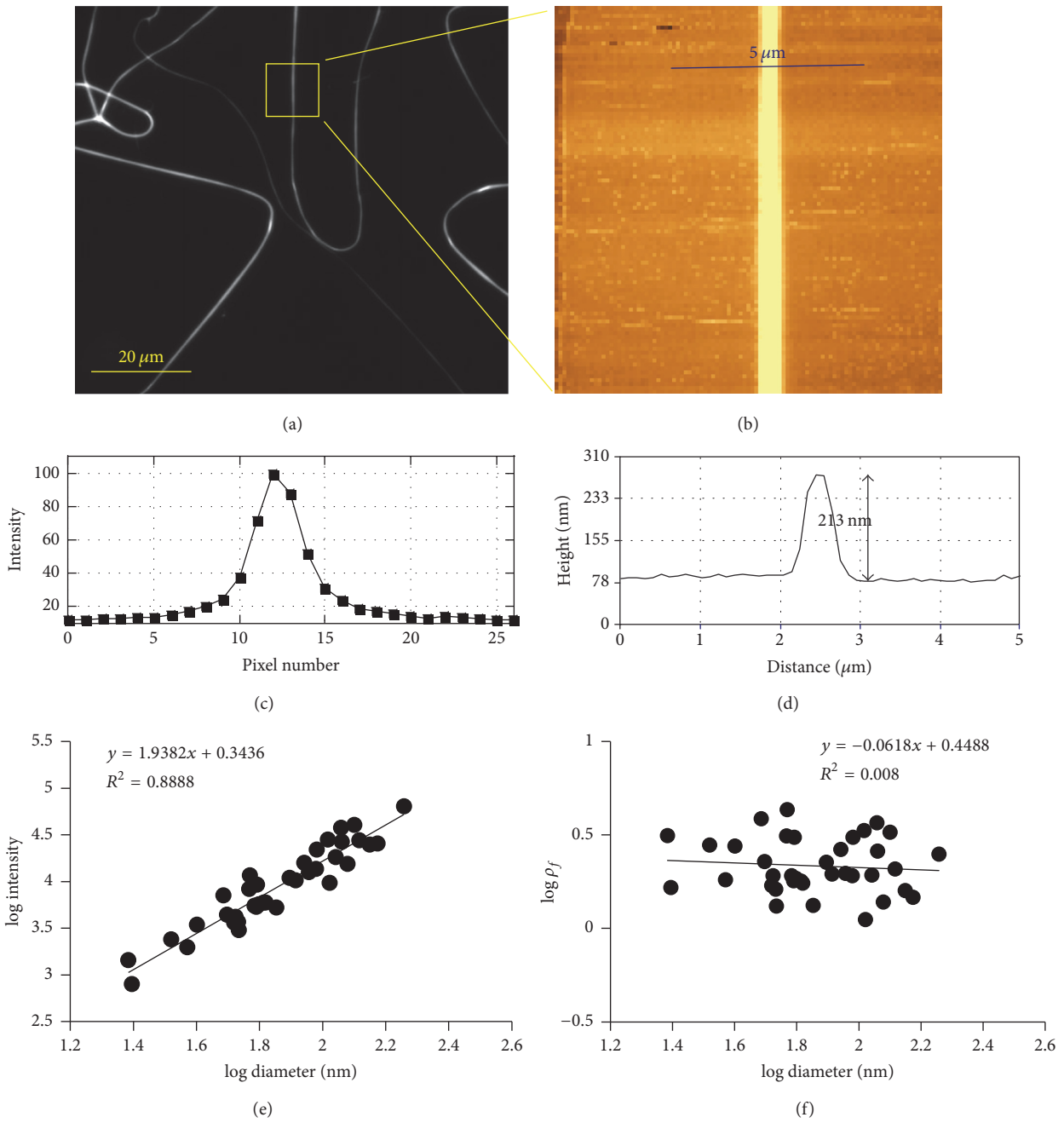

FIGURE 3: Fluorescence intensity of electrospun fibrinogen fiber cross-section as a function of diameter. (a) Fluorescence image (subsection of 180 μm × 180 μm field of view) of electrospun fibrinogen fibers. (b) AFM image of the fiber in the yellow box. (c) Fluorescence intensity of the fiber in the yellow box along the blue line drawn in (b). One pixel is 200 nm (40x objective lens). (d) Height measurement along the indicated 5 μm long blue line in (b). (e) The slope of the relationship between light intensity and fiber diameter is 1.9 on a log-log scale, close to the value of 2.0 expected for a homogenous fluorophore distribution within a cross-section. (f) Fluorophore density $\rho_f = N_f/A$ is independent of fiber diameter, as would be expected for a homogeneous fiber. Each data point represents four measurements.

linked αC regions and perhaps B:b interactions [23, 24]. For the longitudinal stress to be transmitted from one protofibril to the next, these lateral bonds would have to carry (transmit) the longitudinal stress. In other words, the lateral bonds connect the protofibrils. This would put the lateral bonds in a series connection with the protofibrils. In a longitudinal pull, since the protofibrils and the αC regions are in series, they would both experience the applied force. This means that the αC regions would unravel and the fibrin monomers could partially unfold (α-helical to β-sheet transition of the α-helical coiled coils and some other domains could unfold).

In this fiber model, the modulus is then proportional to the bond density, ρ_b, that is, the number (and strength) of bonds per unit cross-sectional area. For example, a doubling

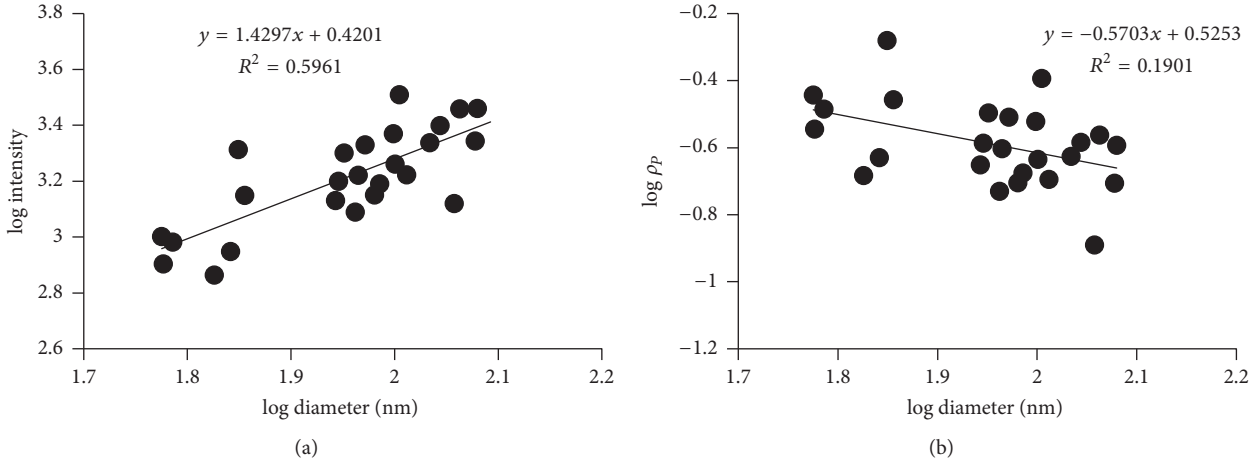

Figure 4: Fluorescence intensity of fibrin fibers (in buffer) as a function of diameter. (a) The slope of the relationship between light intensity and fiber diameter is 1.4 on a log-log scale. (b) The slope of the relationship between protein density and fiber diameter is about −0.6 on a log-log scale. Each data point represents four measurements.

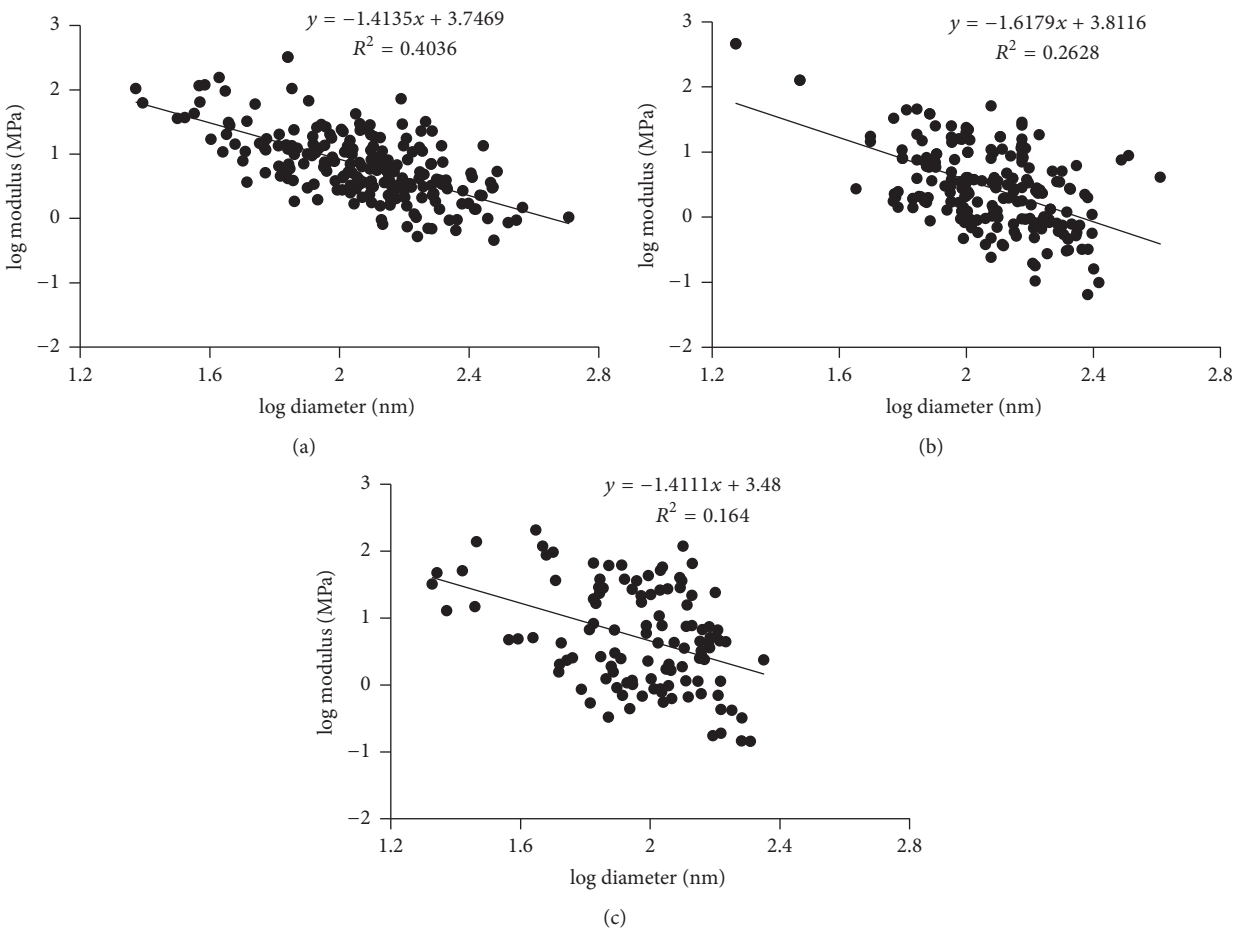

Figure 5: Fibrin fiber modulus as a function of diameter. (a) For plasma samples from white males, the slope was −1.4 ± 0.1 on a log-log scale. (b) For plasma samples from black females, the slope was −1.6 ± 0.2. (c) For samples from purified fibrinogen, the slope was −1.4 ± 0.3. Figures 5(b) and 5(c) are adopted from [18].

of the bonds per unit area would result in a doubling of the modulus. Since $Y \propto D^{-1.5}$, the bond density, ρ_b, also scales as $\rho_b \propto D^{-1.5}$. This means that the bond density decreases drastically with increasing diameter, and thin fibers have a much higher bond density than thick fibers. Since fibers grow from the inside out, by adding more protofibrils to the outside, this finding also implies that the bond density in a given fibrin fiber is not uniform. Our data imply that the bond density strongly decreases as the fiber diameter increases—fibrin fibers have a much higher bond density in the center than at the periphery.

4. Discussion

In our study, we found that the light intensity, I, which is proportional to the number of fibrin molecules in a fiber depends on fiber diameter, D, as $I \propto D^{1.4}$ for wet fibrin fibers. This implies that the protein density, which is proportional to the intensity divided by the cross-sectional area, $A = (\pi/4)D^2$, decreases as $\rho_p \propto D^{-0.6}$. That is, fibrin fibers have a higher protein density in the center than at the periphery. In electrospun fibrinogen fibers, which were used as control fibers with a homogeneous cross-section, the light intensity increased with fiber diameter $I \propto D^{1.9}$, close to the expected D^2 for a constant density fiber. In the force measurements on different plasma and purified fibrinogen samples, the fiber modulus, Y, decreased with increasing fiber diameter D as $Y \propto D^{-1.5}$. This implies that the bond density (bonds per cross-section) also strongly decreases as $\rho_b \propto D^{-1.5}$, even stronger than the protein density, which decreased as $\rho_p \propto D^{-0.6}$. For a homogeneous fiber, the density would be independent of D ($\rho_b \propto D^0$).

4.1. Crosslinking. We did not inactivate FXIII or remove it from our samples. Thus, it is likely that residual FXIII in the plasma samples and the purified fibrinogen solution [37] was activated by thrombin and that the fibrin samples were, at least partially, crosslinked. The finding that all samples showed the same diameter dependence suggests that crosslinking does not have a significant effect on the internal arrangement of fibrin fibers. This is consistent with experiments showing that crosslinking does not have a significant effect on the internal arrangement of fibrin fibers, other than some slight reduction in fiber diameter [38].

4.2. Electrospun Fibrinogen Fibers. The main objective of the experiments with the electrospun fibers was to perform a control experiment with fluorescent nanofibers, in which the intensity would increase proportional to D^2, as would be expected for a homogeneously labeled fiber. We used Rhodamine because it has a similar structure and spectrum as Alexa-546 and because it was soluble in the hexafluoropropanol solvent used for electrospinning.

4.3. Model of Fibrin Fiber Internal Structure. There is some evidence that the internal structure of fibrin fibers has some regularity and some crystallinity. In SEM images, a clear banding pattern with a spacing of 22.5 nm—half the length of a fibrin monomer—can often be seen [17, 39]. This fits well with the regular, half-staggered arrangement of the fibrin monomers in protofibrils and suggests a somewhat regular arrangement of protofibrils inside a fibrin fiber, as proposed in Yang et al.'s multibundle model [6]. However, this seemingly regular arrangement of protofibrils might have been partly induced by the vacuum conditions inside the SEM chamber. A similar, though less pronounced banding pattern with ~22.5 nm spacing, was also seen in some AFM images, though the pattern seems to disappear for larger fibers [29, 32].

Energy dispersive X-ray diffraction (EDAD) [8] and small angle X-ray scattering (SAXS) [28] were used to probe the internal structure of fibrin fibers. Peaks corresponding to lateral periodicity of 19 nm were broad and weak [28], indicating only weak ordering in the lateral (radial) direction. SAXS and light scattering data point to a fiber with a protein content of only 15% and a very porous cross-section that becomes increasingly porous as the diameter increases [28].

Most of these methods and techniques, like neutron scattering and light scattering [40, 41], only give indirect and averaged information about single fibrin fiber internal structure. Our experiments provide a more direct examination of fibrin fiber internal structure.

Our data are consistent with a model in which fibrin fibers do not have a homogeneous structure but rather a dense, well-connected core and a sparse, poorly connected periphery.

To explain our model, we should briefly discuss our data in the context of two simpler models. First, for a homogeneous cross-section of uniformly connected molecules, the intensity (number of molecules) and number of bonds would increase quadratically with diameter; the protein and bond density, and Young's modulus, would not depend on diameter. Second, for a model in which the cross-section looks like the spokes of a bicycle wheel, the intensity (number of molecules) and number of bonds would increase linearly with diameter; the protein and bond density, and Young's modulus, would decrease as D^{-1}.

We observed that the relationship between the number of molecules and fiber diameter is $N_f \propto D^{1.4}$, thus, somewhere between a homogeneous fiber and the bicycle spokes model.

The situation for the bonds (connections that hold a fiber together) is somewhat different. We observed that the number of bonds only increases as $N_b \propto D^{0.5}$, thus, less than for the bicycle spokes model. This could be interpreted that fibrin fibers have a well-connected core but that connections drop off strongly toward the outside of the fiber.

As new technology developed in recent years, more detailed information about the internal structure of fibrin fiber was reported. Yeromonahos et al. used light scattering and small angle X-ray scattering measurements to investigate fibrin fiber internal structure [28]. Their data are consistent with a fractal fiber cross-section that would also have decreasing protein density with increasing diameter. High-resolution AFM imaging showed that thinner fibers are denser than thicker fibers and that molecular packing decreases as the fiber becomes thicker [29, 32]. Our data are also consistent

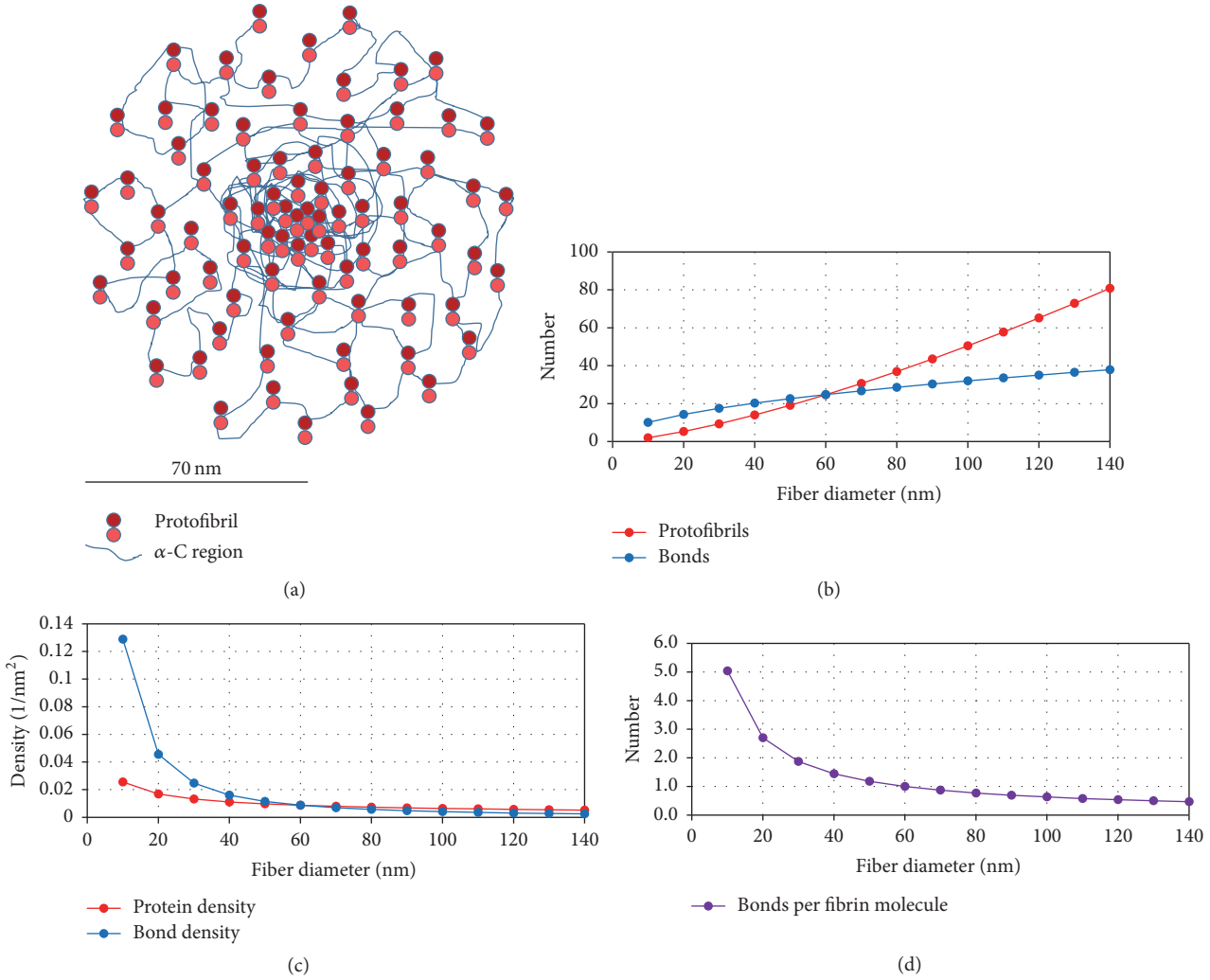

FIGURE 6: (a) Schematic cross-section of a hypothetical 140 nm diameter fiber with 81 protofibrils (approximately to scale). The fiber has a dense core of closely packed and well-connected protofibrils; the density then decreases toward the periphery. In this simplified depiction, protofibrils are connected by αC region interactions, ignoring other interactions (see main text). (b) Plot of the diameter dependence of the total number of protofibrils and the total number of bonds in a cross-section versus fiber diameter for the fiber depicted in (a). The number of protofibrils increases as $D^{1.4}$ (same as the diameter dependence of fibrin monomers in a cross-section). The number of bonds increases as $D^{0.5}$, and the molecules in the center of the fiber are assumed to have 5 bonds on average, which drops to less than 0.5 bonds for molecules at the periphery. These are only the bonds *between* protofibrils (interprotofibrillar bonds), and we do not count the classical intraprotofibrillar A:a and B:b bonds into this interprotofibrillar bond count. (c) Plot of the protein density and the bond density decreasing as $D^{-0.6}$ and $D^{-1.5}$, respectively. The schematic fiber in (a) corresponds to this protein density. (d) The number of interprotofibrillar bonds per monomer, starting with a hypothetical 5 bonds per monomer, dropping as $D^{-0.9}$ ($D^{0.5}/D^{1.4}$). Fibrin molecules at the periphery have less than 0.5 interprotofibrillar bonds, on average. It should be noted that because protofibrils contain about 15 fibrin monomers, a protofibril, whose fibrin monomers have less than 0.5 bonds per monomer, can still be attached to a fiber. It should also be noted that the starting point of 5 interprotofibrillar bonds per monomer (in addition to the A:a and B:b interactions) is only an estimate. The estimate is based on (i) estimating how many interaction points the two large αC regions of a fibrin monomer might have and (ii) having about 0.5 bonds at the periphery.

with an earlier paper suggesting that a fibrin fiber cross-section has a fractal dimension of 1.3 [9].

Combining our light intensity and modulus data, we propose a possible internal structure model of a fibrin fiber, in which the fiber has a densely packed, well-connected core and a less dense, loosely connected periphery (Figure 6).

It has to be pointed out that this is a simplified model. The key feature, and only feature in this model that is supported by our data, is the power law of the decreasing protein and bond density. In the depicted model, the protofibrils have a parallel spatial arrangement along the fiber axis, and the protofibrils are held together only by the interactions of the αC region. There is good evidence that the αC region is important for lateral assembly [24, 42–46]. However, fibers can still form without this region [42]; thus, they are not absolutely required, and there are likely other lateral interactions. The model does not depict other possible interactions, such as the B:b interactions, which might be involved in lateral

aggregation, and possible D region interactions [6]. It is also possible that the protofibrils, and the two fibrin strands within a protofibril, might not be arranged in the depicted parallel arrangement. In fact, Rocco et al. recently suggested the Y-ladder model for fibrin fiber assembly, based on coupled time-resolved X-ray and light scattering data [11]. In this model, fibrin monomers initially form (nonparallel) Y-ladder polymers, in which only one of the two A:a knob:hole interactions is engaged. Before the engagement of the second A:a bond between two fibrin units to form the classic double-stranded protofibrils, the Y-ladder polymers could allow the relatively frequent formation of branch points via off-axis binding of another activated monomer to the not-yet engaged a-site, leading to secondary chain growth. Therefore, many of these polymers may be interconnected before they coalesce to form thicker fibrin fibers. A possible consequence of the Y-ladder model is that the internal structure of fibrin fibers would be open, with some random internal branch points between fibrils. Fibers may contain some interconnected single-stranded fibrils and the "classical" double-stranded, half-staggered fibrils. Such an internal structure may also be consistent with the decreasing protein and bond density emerging from our data.

4.4. Possible General Assembly Mechanisms from Modeling. Computational modeling may also provide insights into the lateral assembly of fibrin fibers. A mechanism that may result in the observed nonuniform fiber with a denser core and a less dense periphery is diffusion-limited aggregation (DLA) of rods into fibers, under the condition that the rods will not move significantly after assembly, as shown in a recent modeling paper [47]. Before activation, a fibrinogen solution is essentially a colloidal solution of noninteracting fibrinogen molecules (particles), which upon activation quickly aggregate into a fibrin network. Diffusion-limited aggregation implies that diffusion is the rate-limiting step with most particle encounters resulting in an aggregation event, whereas reaction-limited aggregation implies that a reaction upon an encounter is the rate-limiting step. Song and Parkinson showed in their DLA modeling work that rods will assemble into fibers with an open, fractal-like cross-section [47], very much resembling the model in Figure 6 of our paper. A similar mechanism, activation-limited aggregation, was used by Curtis et al. to model fibrin fiber assembly [48]. Activation-limited aggregation is based on diffusion-limited aggregation, except that some monomers are active (can aggregate), whereas others are inactive (cannot aggregate). Their models also resulted in a clot with an open, fractal structure, and these results may be transferable to single fiber assembly.

It might be thought that thicker fibers would have a higher bond density, because of prevailing lateral aggregation over linear elongation during the fiber formation process. However, our data show the opposite—thicker fibers have a lower bond density. This implies that explaining fiber thickness by just considering the kinetic rates of longitudinal bond formation versus lateral bond formation is not the correct approach. Rather, it could be that fiber thickness depends on how many fibers, and especially how many branch points, are formed early in the aggregation phase, which then sets the scaffold for the later, lateral aggregation phase. Fiber and branch point formation in the early aggregation phase may only depend on how quickly and to what extent fibrinogen gets converted to fibrin. If most fibrinogen is quickly converted to fibrin, it will form many early protofibrils and small fibers with many branch points; if less fibrinogen is converted, it will form fewer fibrils and fibers with fewer branch points. In the later, lateral aggregation phase, protofibrils are then just added to the outside of the already formed, early scaffold. Fewer early fibers with fewer branch points ultimately result in a mesh with thicker fibers. The number (and quality) of lateral bonds in any given fiber may not so much depend on how fast the lateral bonds form, but on other factors like steric hindrance. These other factors may lower the number and quality of lateral bonds toward the periphery of fibrin fibers.

5. Conclusion

The internal structure of fibrin fibers, and especially the packing of protofibrils, has been unclear. In this paper, we tried to gain insights into the internal structure of fibrin fibers by using fluorescence intensity and force (modulus) measurement. In the fluorescence intensity measurement, we found that the light intensity, I, of a fiber cross-section depends on fiber diameter, D, as $I \propto D^{1.4\pm0.2}$ ($N_f \propto D^{1.4\pm0.2}$), and the protein density depends on D as $\rho_p \propto D^{-0.6}$. In the force (modulus) measurements, we obtained a relationship between modulus and fiber diameter as $Y \propto D^{-1.5}$, indicating that the bond density of bonds connecting fibrin subunits (protofibril) together decreases dramatically as fiber diameter increases as $\rho_b \propto D^{-1.5}$. These relationships suggest that protofibrils are densely packed and well connected in the center and become sparse and loosely connected toward the periphery.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Review Article

Factor XIII Subunit A in the Skin: Applications in Diagnosis and Treatment

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The role of factor XIII subunit A (FXIII-A) is not restricted to hemostasis. FXIII-A is also present intracellularly in several human cells and serves as a diagnostic marker in a wide range of dermatological diseases from inflammatory conditions to malignancies. In this review, we provide a guide on the still controversial interpretation of dermal cell types expressing FXIII-A and assess the previously described mechanisms behind their accumulation under physiological and pathological conditions of the human skin. We summarize the intracellular functions of FXIII-A as well as its possible sources in the extracellular space of the dermis with a focus on its relevance to skin homeostasis and disease pathogenesis. Finally, the potential role of FXIII-A in wound healing, as a field with long-term therapeutic implications, is also discussed.

1. Introduction

Factor XIII (FXIII), fibrin stabilizing factor, is an enzyme consisting of two subunits: an A (FXIII-A) and a B subunit (FXIII-B) that can form a tetrameric complex in the plasma (pFXIII-A) with two of each subunit (FXIII-A_2B_2). As a member of the transglutaminase family, FXIII crosslinks fibrin residues to produce the mature clot in blood coagulation [1]. In addition to being a pivotal member of the coagulation cascade, FXIII-A is also found intracellularly (cFXIII-A) in various cells all around the body. Besides megakaryocytes and platelets, monocytes [2], macrophages [3], dendritic cells (DCs) [4], fibroblasts [5], mast cells [6], and sebocytes [7], all with an important role in the homeostasis of the skin, have been listed as FXIII-A-producing cells. Thus, FXIII-A is of interest in not only the fields of hemostasis but also cellular expression and skin biology. Therefore in this review, besides giving a general overview on FXIII-A production and its presence in the intracellular and extracellular space, we also aim to summarize our current knowledge on the role of FXIII-A in the (patho)physiology of the skin and discuss its possible cutaneous therapeutic applications. (List of abbreviations of certain forms of factor XIII used in the article is shown in Abbreviations.)

2. FXIII-A in the Circulation

FXIII, as mentioned before, is a member of the transglutaminase family. In the plasma, FXIII is present as a tetramer where FXIII-A is the catalytic enzyme and FXIII-B is a glycoprotein responsible for inhibiting FXIII-A upon binding to it. Only approximately 1% of FXIII-A exists in a free form in the plasma. FXIII-A upon activation (FXIII-$A_2{}^*$) crosslinks the γ-glutamyl and ε-lysyl residues of connected polymerized fibrin chains to produce the mature clot during hemostasis [1]. For more details regarding their interaction and role in blood coagulation, we refer to the excellent reviews [8–10].

While FXIII-B is exclusively produced by the liver [11] and is only present in the circulation, FXIII-A can also be found in the extracellular space and in the cytoplasm of various cells. Therefore, we are focusing our review on the role of FXIII-A. pFXIII-A has long been thought to originate in bone marrow, mainly produced by megakaryocytes and platelets. However, the findings of Poon et al. suggested that other cells might also contribute to pFXIII-A levels. During bone marrow ablation, they noted only a 25% reduction in pFXIII-A levels in contrast to a 90% reduction in platelet count. The reduction of pFXIII-A levels was also less than

expected in thrombocytopenic patients, further supporting the presence of a nonthrombopoietic source of pFXIII-A [1, 2, 12]. Studies by Griffin et al., using a model where mice floxed in coding exon 7 of the FXIII-A gene *(F13A1)* were crossed with mice transgenic for *Pf4-Cre*-recombinase (thrombopoietic deletion) or *Cd11b-Cre*-recombinase (myeloid deletion), raised the possibility that a unique Pf4-dependent progenitor cell is the major source of the plasma pool, which was independent of thrombopoietin receptor. While, in *F13A1*$^{fl/fl}$*-Pf4-cre* mice, FXIII-A plasma activity was decreased by 85% and absent in platelets, in *F13A1*$^{fl/fl}$*-CD11b-cre* mice plasma activity decreased by 40%. Interestingly, *F13A1* mRNA levels also decreased in the aorta (91.6%) and heart (99.2%) of *F13A1*$^{fl/fl}$*-Pf4-cre* mice but showed no reduction in the heart and a 54.6% reduction in the aorta tissue samples of *F13A1*$^{fl/fl}$*-CD11b-cre* mice [13].

3. Extracellular FXIII-A

Because FXIII-A lacks a signal sequence for secretion, for a long time, the only possible explanation was that ecFXIII-A either was from the circulation or originated from dying cells. This was supported by in vitro findings that FXIII-A could not be detected in the culture medium when it was expressed in baby hamster kidney cells [10]. However, Cordell et al. suggested nonclassical secretion by showing that FXIII-A in macrophages was associated with podosomes and was present in intracellular vesicles associated with Golgi matrix protein-130 (GM-130), which is involved in the delivery of proteins to the plasma membrane [14]. Interestingly, under specific conditions, FXIII-A can also appear on the cell surface where it could exert its transglutaminase activity and play a role in modulating cell adhesion [15].

Based on these findings, FXIII-A-positive macrophages were implicated in the elevation of ecFXIII levels both in the bronchoalveolar lavage fluid of patients with asthma [16] and in tissue samples of patients with chronic rhinosinusitis with nasal polyps [17]. Considering the known substrate profile of FXIII-A, such as fibronectin, vitronectin, osteopontin, thrombospondin, and certain adhesive glycoprotein components of the extracellular matrix (ECM), which will be discussed later in this review, the alterations in the ecFXIII-A levels might contribute to tissue repair in these diseases. Although the correlation between the levels of ecFXIII-A and the increased number of FXIII-A-positive macrophages corroborated that cFXIII-A could be a potent contributor to ecFXIII-A levels [15], questions as to which of the FXIII-A producing cell types could indeed contribute to ecFXIII-A and the stimuli behind its possible secretion are yet to be answered. Based on these findings, future studies are also needed in the field of dermatology to examine the correlation of ecFXIII-A with FXIII-A-positive cells within various skin lesions. While using mouse models with selective ablation of the FXIII-A-positive skin-resident macrophages, their contribution to ecFXIII-A (and perhaps to pFXIII-A) levels could be assessed as well.

4. Intracellular FXIII-A

Whereas, in early embryonic life, the cells positive for cFXIII-A are the mesenchymal histiocytes and hepatocytes [18], in adult life, in addition to monocytes and tissue macrophages [3], cFXIII-A was detected also in DCs [4], fibroblasts [5], sebocytes [7], and mast cells of the skin and recently in the subcutaneous preadipocytes [19]. Although it is outside the scope of this review, osteoblasts, chondrocytes [20, 21], and cornea cells [22] should also be listed here as FXIII-A-producing cells [23, 24]. Confirming that the intracellular Ca^{2+} concentration is sufficient for its activation within the cell as shown in platelets and monocytes [8], cFXIII-A was also suggested to play a role in various intracellular (intracytoplasmic and intranuclear) processes [25].

4.1. Macrophages. Being the first cell type in which cFXIII-A was detected more than 30 years ago [26], the circulating blood monocytes/macrophages provide an excellent model for the examination of the possible roles of cFXIII-A. It was shown that cFXIII-A is present from a very early stage of monocyte differentiation (monoblasts in the bone marrow) [27, 28] to the macrophages of connective tissue and serous cavities [29]. Based on these findings, FXIII-A became interpreted as a marker protein of the cell line. Actin and myosin [30], the two major elements of the cell cytoskeletal locomotory system, were the first identified intracellular substrates, followed by vinculin [31], the small heat shock protein HSP27 [32], and thymosin beta-4 [33]. All these molecules have a significant role in cytoskeletal remodeling [8]. cFXIII was also suggested to participate in the phagocytosis of certain particles. Fcg and complement receptor-mediated uptake of sensitized erythrocytes and complement-coated yeast particles were greatly diminished in monocytes of FXIII-deficient patients. The phagocytic functions of cultured monocytes/macrophages showed changes alongside FXIII-A mRNA expression and protein synthesis [34]. In addition, cFXIII-A can also induce the activation and mobilization of monocytes from the splenic reservoir in response to angiotensin II binding its receptor (AT1), which can activate cFXIII-A that in return dimerizes AT1 [35].

Interestingly, FXIII-A was also shown to translocate to the nucleus of differentiating macrophages where it may also exert its enzymatic activity. It still needs to be elucidated if this is behind the altered gene expression profile of FXIII-A-deficient macrophages or a cytoplasmic activity targeting unidentified proteins. The significant changes in genes involved in wounding, immune processes, and ECM formation show that cFXIII-A is involved in a wide range of cellular processes, including participating in gene expression regulation of the key functions of macrophages [15]. However, we also showed that the presence of cFXIII-A was not ubiquitous in macrophages but marked a specific activated status, the so-called alternative activation. Such activated macrophages are characteristic to the healthy skin and dominant in pathological conditions where inflammation is not related to infection. These are conditions, such as wounding or tumor development, that display ECM formation and where cFXIII-A expression could have a significant diagnostic value that we

will introduce in more detail later in the dermatopathology section. The key cytokine driving macrophages toward the alternative polarization is IL-4. IL-4 was shown to be the strongest inducer of FXIII-A gene expression and protein levels within macrophages so far [15]. On the contrary, in infectious lesions, such as tuberculosis, TNFα and IFNγ are the cytokines behind the classical activation of macrophages and in parallel inhibit the expression of cFXIII-A [34]. This interesting finding could explain, at least partially, why FXIII-A-deficient patients have no reported increased susceptibility to infectious diseases.

4.2. Dendritic Cells. In addition to macrophages, FXIII-A was identified in a great variety of cell types. However, the question of antibody specificity was put forward in many cases. The validity of the term "FXIII-A dendrocytes," which has been widely accepted and is still used [36], is one of the prime examples of such debates. A detailed characterization of dermal FXIII-A-positive cells by Zaba et al. highlighted that FXIII-A could be detected only in macrophages. Cells capable of antigen presentation in the skin, which is the hallmark of dendritic cells (DCs), lacked FXIII-A [37]. A possible explanation for the discrepancies between ex vivo and in vitro findings could be that IL-4 in combination with GM-CSF is a widely used but also challenged [38] cytokine combination for in vitro DC differentiation. In such models, FXIII-A-deficient DCs showed a reduced chemotactic response to CCL19 and impaired cell motility [39].

Based on these data, further studies are needed to determine if the presence of cFXIII-A is just a "side-product" in in vitro differentiated DCs, due to its previously described induction by IL-4, or if there are certain DC subsets expressing FXIII-A also under in vivo conditions.

4.3. Mast Cells. Recently, interest in mast cells as FXIII-A-producing cells [6] that could contribute to ecFXIII-A and perhaps to pFXIII levels has grown. Using immunogold labeling and electron microscopy, the double staining for FXIII-A and tryptase showed that, in sections of drug-induced acute urticaria, the two proteins colocalized within the granules of mast cells. Interestingly, the granules were also detected in dermal DCs and in endothelial cells of postcapillary venules, suggesting a mast cell, dermal dendrocyte/endothelial cell interaction in urticaria [40, 41]. Additionally, after IgE mediated activation of bone marrow-derived cultured mast cells (BMCMCs), cFXIII-A was found to be one of the most abundant proteins in the proteome. However, in mast cell-deficient mice, pFXIII and activity levels were increased in correlation with reduced bleeding times. The root of these changes was that the human chymase and mouse mast cell protease-4 (the mouse homologue of human chymase) could downregulate FXIII-A via proteolytic degradation. Thus, deficiency of the chymase led to increased FXIII-A amounts and activity and reduced bleeding times in homeostatic conditions and during sepsis. Mast cells are ever-evolving cell types not just in urticaria but also in other dermatological conditions such as psoriasis. It is of crucial importance to further confirm the role of mast cell-derived FXIII-A in human disease settings [6].

4.4. Fibroblasts, Sebocytes, and Keratinocytes. FXIII-A positivity of fibroblasts [5], keratinocytes, and sebocytes turned out to be dependent on the applied antibody. A recent study found that the clone AC-1A1 mouse monoclonal antibody for FXIII-A was a useful tool for staining sebocytes with increased proliferative activity. It suggested that, in the future, this antibody can be used in the diagnosis of sebocyte-related malignancies [7]. Using the same antibody, keratinocytes were also positive showing a correlation with their maturation. However, using different antibodies for FXIII-A, we did not detect FXIII-A-positive sebocytes or keratinocytes in the examined specimens, which is in line with other authors.

4.5. (Pre)adipocytes. FXIII-A was also shown to negatively regulate adipogenesis in mouse white adipose tissue and in differentiating 3T3-L1 preadipocytes. FXIII-A on the surface of the studied preadipocytes contributed to cell-matrix interactions by promoting the assembly of fibronectin from plasma into the preadipocyte extracellular matrix. Modulation of cytoskeletal dynamics induced the proliferation of the cells and at the same time inhibited their differentiation into lipid-accumulating mature adipocytes. FXIII-A, via the same pathway, might also be crucial in the transformation of embryonic fibroblasts into adipocytes [19]. More and more evidence supports that subcutaneous adipose tissue is in direct communication with the dermis through altering local inflammation and defense mechanisms against pathogens [42]. It is of interest to address subcutaneous adipose tissue and its FXIII-A levels in various inflammatory skin lesions.

5. Application of FXIII-A in Dermatopathology

Shortly after the discovery that cFXIII-A is expressed in macrophages of various origin throughout the body [26, 29, 43], its detection has been implicated in dermatological diagnosis and a heterogenic population of FXIII-A-positive dermal dendritic cells was also described [36, 44].

Cerio et al. detected "FXIII-A dendrocytes" both in the neonatal foreskin and normal adult skin mainly in the upper dermis and in the papillary dermis surrounding vessels [45]. Due to the limits of the available functional testing of such cells both in vivo and ex vivo at that time, the term "FXIII-A dendrocyte" was derived from the spindle shape appearance of such cells rather than from their potential to present antigens. As we have previously detailed, with the improvement of available tools and in our knowledge of the differences between macrophages and DCs, it is more likely that FXIII-A positivity indicates alternative activation of macrophages [34, 37].

In the next section, we aim to systematically describe diseases with FXIII-A-positive cells, using the terms for the identified cell types according to original publications.

5.1. FXIII-A in Granulomatous Diseases. Granulomas are characteristic to diseases in which the immune system is incapable of eliminating the antigen that can be of pathogen or of noninfectious origin such as lipids or foreign bodies. Although T cells and DCs are also involved in granuloma formation, the key cells leading to the well-recognized histological changes are the macrophages.

Our findings that FXIII-A marks the alternative activation pathway in macrophages is best reflected in diseases of granuloma annulare (GA) and necrobiosis lipoidica (NL), which are the prime examples of noninfectious, nonmalignant inflammatory skin diseases with granuloma formation. Although the activating stimuli behind the symptoms are not known, (connective tissue disorder, impaired local circulation, and lack of anti-inflammatory signaling affecting the macrophages), there is a remarkable change in ECM remodeling. Our results demonstrated that the macrophages in both diseases, expressed both FXIII-A and CD206 (also a widely accepted marker for detecting alternatively activated macrophages). Moreover, we also found that FXIII-A-positive cells formed distinct populations from both CD11c and CD1a expressing cells in GA and in NL [25], in which markers were convincingly shown to detect DCs in the healthy skin [37]. Altogether, besides demonstrating that FXIII-A is a useful tool to mark alternatively activated macrophages also in pathological conditions such as GA and NL, we also proposed to integrate the classification by Zaba et al. for macrophages and DCs into dermatopathology [25]. Importantly, further characterization of FXIII-A-positive cells revealed that the cells expressing FXIII-A also expressed the macrophage marker CD163, whereas only 60% coexpressed CD68, a marker that has been also widely used to detect macrophages. To explain these findings, it should be kept in mind that the skin is a dynamic system and therefore it would be an oversimplification to search for such polarized conditions as described in in vitro conditions in any given disease. Therefore these findings further highlight the importance of multiple labeling in cases where the characterization of the macrophage populations is essential in setting up a diagnosis. To allow further conclusions and new perspectives in its dermatopathological application, we emphasize the importance of using multiple immunohistochemical markers in the description of FXIII-A-positive cells. We have collected the markers that have been used so far in combination with FXIII-A in Table 1.

As we introduced previously, on the other extreme of macrophage polarization is classical activation, which marks the presence of TNFα and/or IFNγ in the inflammatory environment. These cytokines are typical in pathogen-associated lesions such as tuberculosis granulomas caused by *Mycobacteria* but are also the key cytokines in sarcoidosis, a disease lacking any pathogen. Supporting our previously detailed in vitro findings, macrophages were FXIII-A negative in the inflammatory foci of both tuberculosis and leprosy, just as in sarcoidosis [46]. Interestingly, FXIII-A-positive macrophages could still be found near the granulomas. This finding can be explained with the possible commitment of FXIII-A-positive resident macrophages to keep their FXIII-A expression, just as the ongoing tissue damage and reorganization might be an inducer for a subset of alternatively activated macrophages.

It is important to emphasize that FXIII-A is also not correlated with the presence of a pathogen but with the microinflammatory environment. This finding is best reflected in chronic suppurative granulomatous mycoses. An increased number of hypertrophied FXIII-A-positive DCs with prominent dendrites were seen in paracoccidioidomycosis [47] and in chromoblastomycosis, of which some were colocalized with the pathogen itself [48].

In systemic histiocytic disorders, such as juvenile xanthogranuloma, xanthoma disseminatum, Erdheim-Chester disease, and dendrocytomas, FXIII-A, along with other markers (e.g., S100, CD1a, CD68, fascin, CD207, and CD35), is also used as a key diagnostic tool [49–51]. As reported previously, FXIII was coexpressed with CD68 in these diseases, allowing the conclusion that these cells are mostly phagocytes. However, the mechanisms behind its induction and the question as to whether FXIII-A has any role in these diseases remain unanswered.

5.2. FXIII-A in Neoplasms. The expression of FXIII has also been studied in solid tumors, but the specificity of the antibodies used for FXIII-A and the lack of multiple colabeling to characterize the FXIII-A-expressing cells might also lead to misinterpretation of these results with time.

Kaposi's sarcoma is a lymphangioproliferative disease caused by an HHV8 infection; it has 4 clinical presentations: classic, African endemic, iatrogenic immunosuppression related, and acquired immunodeficiency syndrome (AIDS) related. It was one of the first malignancies where FXIII-A became introduced as a diagnostic tool. The spindle-shaped FXIII-A-positive DCs around the small blood vessels suggested that they might play a role in the angioproliferative process [52]. Confirming the importance of these findings, FXIII-A is still a widely used marker in the diagnosis of Kaposi's sarcoma, but without any association to AIDS.

Another disease in which FXIII-A has stood the test of time and is used in the diagnosis is dermatofibroma (DF) where the spindle-shaped cells are FXIII-A positive but negative for CD34, in contrast to dermatofibrosarcoma protuberans (DFSP) where the cells are negative for FXIII-A and CD34 positive [53, 54]. The CD34 and FXIII-A-positive (CDa1−) marker combination has also been described in medallion-like dermal dendrocyte hamartomas, which is a benign congenital dermal lesion [55].

FXIII-A was also suggested to be a useful tool in differentiating between schwannomas neurofibromas and neurotized melanocytic nevi, which are common benign cutaneous neoplasms. In contrast to S100 protein, which was found to be expressed by tumor cells in all three diseases, FXIII-A was only found in neurofibromas (30–70% of cells within the tumors were positive for FXIII-A) [56]. However, whether the same cells were expressing S100 and FXIII-A proteins or FXIII-A positive cells were distinct ones infiltrating the tumor were not confirmed.

The expression of FXIII was examined in patients with tuberous sclerosis as well, where it was present in the stromal

Table 1: Markers used most often during immunohistochemistry to distinguish cells in the dermis and epidermis and their coexpression with FXIII in normal skin and dermatological conditions.

Marker	Coexpression with FXIII+/− (%)	In dermatology	Function	Expressing cells	References
CD11c (CR4)	−	Normal skin	(i) Type I transmembrane glycoprotein (ii) Integrin αX (ITGAX) forms with ITGB2 a leukocyte specific integrin receptor (CR4)	(i) Myeloid dendritic cells	[25, 37, 45, 62]
	−	NL			
	−	GA			
	−	AD			
	−	Psoriasis			
	−	Stromal cell cc.			
CD1c (BDCA-1)	−	Normal skin	(i) Type I membrane glycoprotein (ii) Mediating the presentation of nonpeptide antigens to T cells	(i) Myeloid dendritic cells	[25, 37, 63]
	−	NL			
	−	GA			
	+ (25%)	MF			
	+ (25%)	Psoriasis			
CD45	+	Normal skin	(i) Protein tyrosine phosphatase (iii) Required for differentiation, growth, mitosis	(i) Bone marrow derived cells	[37, 45, 64, 65]
	−	AD			
	−	Psoriasis			
HLADR	+++ (85%)	Normal skin	(i) MHC class II receptor (ii) Antigen presentation to T cells	(i) Antigen presenting cells	[37, 45, 49, 62]
	+	AD			
	+	Psoriasis			
	−	Histiocytosis			
CD34	−	JLS	(i) Single chain transmembrane glycoprotein (ii) Regulating cell-cell adhesion and inhibits hematopoietic cell differentiation	(i) Hematopoietic progenitor cells (ii) Endothelial progenitor cells (iii) Vascular endothelial cells	[54, 66–69]
	−	Self-healing papular mucinosis			
	−	DF			
	−	DFSP			
	−	Oral lichen planus			
CD11b (MAC-1α)	−	Normal skin	(i) Integrin αM chain (ii) Part of C3 complement receptor (iii) Receptor for C3bi, fibrinogen, FX	(i) Myeloid cells (ii) Monocytes (iii) NK cells (weak)	[45]
CD14	+ (22%)	Normal skin	(i) Lipopolysaccharide receptor	(i) Monocytes (ii) Macrophages (iii) Myeloid dendritic cells	[45, 62]

Table 1: Continued.

Marker	Coexpression with FXIII+/− (%)	In dermatology	Function	Expressing cells	References
CD83	− − −	Normal skin NL GA Histiocytosis	(i) Immunoglobulin receptor (ii) Antigen presentation, T cell activation	(i) Mature dendritic cells	[25, 49]
CD205 (DEC205)	−	Normal skin	(i) C type lecithin type I membrane protein, part of the macrophage mannose receptor family (ii) Participates in antigen endocytosis	(i) Mature dendritic cells (ii) Thymic epithelial cells (iii) Monocytes	[37]
CD209 (ICAM-3, DC-SIGN)	+++ (98.8%) +++ (96%) +++ (93.5%)	Normal skin NL GA	(i) C type lecithin receptor type II (ii) Adhesion receptor, connection between DC-T cells and DC-endothelial, antigen receptor	(i) Tissue dendritic cells (ii) Macrophages (iii) Immature dendritic cells	[25]
CD208 (LAMP-3, DC-LAM)	− − −	Normal skin NL GA	(i) Type I lysosome associated membrane glycoprotein (ii) Maturation marker (upregulated by GM-CSF, IL-4, TNFα)	(i) Mature dendritic cells	[25, 37]
CD123 (IL3Rα chain)	− − −	Normal skin Tuberculoid leprosy Lepromatous leprosy	(i) Forming high affinity IL3 receptor (ii) Cell growth and differentiation	(i) Plasmacytoid dendritic cells (ii) Basophil granulocytes	[37, 70, 71]
CD68	+++ (85.5%) ++ (64.33%) ++ (62.3%) − − − − +++	Normal skin NL GA AD Psoriasis Lichen planus Chronic GVHD Dermal dendrocytomas	(i) Type I lysosomal/endosomal associated membrane glycoprotein (ii) Binding low-density lipoprotein	(i) Monocytes (ii) Tissue macrophages (iii) Dendritic cells	[25, 48, 49, 53, 65, 72, 73]
CD1a (Leu-6)	− − − − − − − − −	Normal skin NL GA AD Psoriasis Lichen planus Chronic GVHD Leprosy LCH/DC histiocytosis	(i) Type I membrane glycoprotein (ii) Lipid and glycolipid antigen presentation	(i) Langerhans cells (ii) Dendritic cells (iii) Cortical thymocytes	[25, 45, 49, 63, 65, 70–72]

Table 1: Continued.

Marker	Coexpression with FXIII+/− (%)	In dermatology	Function	Expressing cells	References
CD207 (langerin)	− −	Tuberculoid leprosy LCH	(i) Type II C-type transmembrane lecithin receptor (ii) Capturing antigens and inducing Birbeck granule formation	(i) Langerhans cells (ii) Dendritic cells	[49, 70]
CD36	+++ (92%)	Normal skin	(i) Class B scavenger receptor (ii) Binding long chain fatty acids, oxidized LDL, collagen types I, IV and V, and thrombospondin, as well as for apoptotic cells	(i) Endothelial cells (ii) Erythrocytes (iii) Platelets (iv) Monocytes (v) Macrophages (vi) Macrophage-derived dendritic cells	[45]
CD54 (ICAM-1)	− +	Normal skin Lichen planus	(i) Type I transmembrane glycoprotein (ii) Adhesion molecule, ligand for integrin	(i) Monocytes (ii) Macrophages (iii) Lymphocytes (iv) Activated endothelial cells (v) Granulocytes (vi) Dendritic cells	[45, 69]
CD163 (M130)	+++ (97.8%) +++ (77.1%) +++ (85.5%)	Normal skin NL GA	(i) Single chain transmembrane protein, hemoglobin/haptoglobin complex scavenger receptor, (ii) Signal transduction for proinflammatory cytokine production	(i) Mature tissue macrophage (ii) Blood monocyte	[17, 25, 37]
CD206 (MMR)	+++ (94.8%) ++ (52.2%) ++ (74.7%)	Normal skin NL GA	(i) Type I membrane protein, macrophage mannose receptor (ii) Mediating antigen endocytosis/phagocytosis	(i) Macrophages (ii) Dendritic cells (iii) Endothelial cells	[25]
S100	− − − − + (?) −	LHC/DC histiocytosis Chromoblastmycosis DF DFSP Neurofibroma Schwannoma	(i) Ca^{2+} binding protein regulation of protein phosphorylation, transcription factors (ii) Ca^{2+} homeostasis, the dynamics of cytoskeleton constituents, enzyme activities, cell growth and differentiation, and the inflammatory response	(Neural crest cells) (i) Langerhans cells (ii) Dendritic cells (iii) Macrophages (iv) Keratinocytes (v) Adipocytes (vi) Myoepithelial (vii) Chondrocytes	[48, 49, 54, 56, 68, 74]

NL = necrobiosis lipoidica; GA = granuloma annulare; AD = atopic dermatitis; GVHD = graft versus host disease; DF = dermatofibroma; DFSP = dermatofibrosarcoma protuberans; LCH = Langerhans cells histiocytosis; DC = dendritic cell; JLS = juvenile localized scleroderma; − < 20% expression, 20% < + < 50%, 50% < ++ <75% expression, +++ > 75% expression; ?: possible coexpression.

cells of the skin lesion [57]. It was suggested that cFXIII-A also takes part in fibrous tumor formation as an inducer of growth, such as a growth factor, based on its expression in fibrokeratomas, angiofibromas (adenoma sebaceum of Pringle), and oral fibrous hyperplasia [5].

A study of patients with oral squamous cell carcinoma suggested that the Leu allele of the FXIIIVal34Leu polymorphism, which decreases FXIII-A activity, is associated with an increased risk of squamous cell carcinoma, although without the increase in disease progression. Possibly both of these findings are due to a less porous fibrin network of thinner fibers that may facilitate tumor stroma formation and tumor cell proliferation but harbor tumor cells less effectively during metastasis formation [58]. In addition to this finding, in basal cell carcinoma and superficial malignant melanoma, an increase in the density of FXIII-A-positive DCs was associated with a low proliferative rate [59].

According to studies by our laboratory with the newest marker combinations and antibodies, in the various examined skin malignancies, FXIII-A-positive cells were almost exclusively tumor-associated macrophages. Tumor-associated macrophages are also polarized via the alternative pathway fitting into the concept that FXIII-A-positive macrophages are associated with tissue remodeling processes, thus being the prime pathological features in most of the malignancies. Importantly, FXIII-A-positive cells were found in high numbers in close association with fibrin deposits around tumor cells suggesting that ecFXIII-A with a possible macrophage source might be involved in tumor matrix formation [9, 60]. In line with this finding, an interesting study demonstrated that FXIII contributes to hematogenous metastasis formation, as the genetic elimination of FXIII-A significantly decreased lung metastasis formation in mice. As a possible explanation, FXIII was shown to enhance the early survival of embolic tumor cells by impeding natural killer cell function and protecting the newly localized tumor cells from natural killer cell-mediated lysis where the crosslinked fibrin matrices might be crucial [61].

Taking these data into consideration, to answer in full detail whether FXIII-A-positive macrophages support or inhibit tumor growth is an interesting topic for future studies.

5.3. FXIII-A in T-Cell Dermatoses. FXIII-A-positive cells showed increased numbers in skin samples from atopic eczema, psoriasis, lichen planus, spongiotic dermatitis, chronic graft versus host disease (GVHD), pityriasis alba, and mycosis fungoides. Introducing these diseases in more detail is beyond the scope of this review. In summary, these inflammatory diseases all have a notable accumulation of T cells, which led to the hypothesis that FXIII-A-positive cells might be able to cross talk with T cells through their dendrites and could present certain antigens. A possible FXIII-A-positive cell, lymphocyte interaction was observed in atopic dermatitis at the site of spongiosis and vesicle formation in the epidermis, while, in psoriatic plaques, an expanding population of FXIII-A-positive DCs was accompanied by endothelial and T lymphocyte expansion [64].

Deguchi et al. compared the number of antigen-presenting cells in atopic dermatitis, psoriasis, lichen planus, and GVHD. They found that there was only minimal change in the number of CD68+ macrophages in all samples compared to healthy skin. However, there was a significant increase in the amount of CD1a+, FXIII-A-positive cells in lichen planus, spongiotic dermatitis, and chronic GVHD, both in the dermis and in epidermis. In psoriasis, even though the number of FXIII-A-positive cells increased, it did not reach statistical significance, and, in acute GVHD, there was a significant decrease in these cells [72]. In a murine model, Yoo et al. demonstrated that, during acute GVHD, FXIII-A-positive DCs migrated to the superficial dermis and became hypertrophic and highly branched [75]. Additionally, in drug-induced toxic epidermal necrolysis (TEN), decreased numbers of FXIII-A-positive DCs were detected [65].

An increased number of FXIII-A-positive DCs were also described in pityriasis alba, suggesting that it could mark a subtle inflammation behind the postinflammatory hypopigmentation, but other causes besides FXIII accumulation were not excluded [76].

5.4. FXIII-A in Sclerotic Disorders. Scleroderma, and its localized form, morphea, is characterized by fibrosis involving the dermis and the subcutaneous tissue, with prominent ECM reorganization. Characterizing the cells involved in the lesions, it was found that FXIII-A positivity was lost in the papillary dermis but became prominent in the reticular dermis at the site of fibrosis. Interestingly, preceding the appearance of intense fibrosis, a reduction was also found in FXIII-A-positive cell numbers. This finding pointed to the presence of FXIII-A-positive cells that varied according to different stages of disease pathogenesis [77] and the fact that these cells might be important in fibrosis by crosslinking the newly formed ECM also found in scleroderma [66].

Interestingly, in a case of self-healing papular mucinosis, which is a milder form of sclerodermoid lichen myxedematosus, CD34+ or FXIII-A-positive DCs have been described next to mast cells, suggesting that these cells together could contribute to the dermal mucin deposition of the lesions [67].

6. FXIII-A in Wound Healing and Angiogenesis

Wounding is the most commonly observed pathological condition, marked by the presentation of damage-associated molecular patterns (e.g., ATP, HSP, HMGB1, hyaluronan fragments, DNA, and heparin sulfate). It can be the result of trauma/injury, impaired circulation, or necrosis with an infectious background. In the complex process of wound healing, cells and enzymes work together in a finely tuned system. Wound healing is divided into three stages: the inflammatory phase, proliferative phase, and maturation phase. During the inflammatory phase (1–3 days of wound healing), the clot forms, the inflammatory cascade is initiated, and neutrophils and macrophages are drawn to the wound site. Around day 4, fibroblasts, vessel formation, and ECM production overtake the scenery, and the transition to the

proliferative phase occurs. From week 3, myofibroblasts, collagen remodeling, and wound contraction become characteristic of the maturation phase [78, 79].

The strongest piece of evidence supporting the role of FXIII-A in wound healing is that 14–36% of patients with FXIII-A deficiency experience impaired wound healing and abnormal scar formation [8]. Such impaired wound healing was also confirmed by using FXIII-A transgenic mice (deletion of the exon encoding the active site cysteine of the mouse FXIII-A). Inbal et al. found significant differences in maturity and organization of the wound between the controls, the FXIII-A-deficient and the FXIII-A-deficient recombinant FXIII- (rFXIII-) treated mice. Whereas the controls and the group treated with rFXIII had a normal mature wound healing process, the FXIII-A-deficient mice without treatment showed delayed reepithelialization, irregular fibrotic scars with ill-defined edges, discoloration, and necrotized fissures on histological slides [80]. A study of myocardial infarction (MI) in mice showed that FXIII correlates with predicted healing time and ventricular wall thickness. Nonetheless, in a different study, the association of decreased FXIII levels with wall rupture in patients after MI and its beneficial effect in decreasing vascular permeability during myocardial ischemia were noted [81–84].

These findings all support the complex roles of FXIII-A and strongly suggested that the observed differences were not simply related to the blood coagulation defect accounting for the acute, excessive hemorrhage of these patients. The most significant observation was that the impaired wound healing could be almost completely rescued with rFXIII-A. Therefore, we focus on pFXIII-A and ecFXIII-A to address the pathways in which FXIII-A could play a role. Nevertheless, it is important to emphasize that FXIII-A expressing alternatively activated macrophages [34, 37] are important in the clearance of wound debridement, with an increased number of scavenger receptors and the binding of apoptotic cells [3, 78, 85].

After tissue injury, pFXIII is rapidly activated by thrombin and calcium. Besides binding fibrin polymers together to form a mature clot, FXIII-A_2^* interacts with complement C3, integrating it in the fibrin clot and with this delaying fibrinolysis [23]. Importantly, FXIII-A_2^* can crosslink matrix components as well such as fibronectin, vitronectin, osteopontin, thrombospondin, collagen VI, and von Willebrand factor, which could further impact cell attachment [8, 86, 87] and migration [88]. In addition, FXIII-A_2^* could mediate endothelial cell-platelet interaction through $\alpha_v\beta_3$-integrin (vitronectin receptor), regardless of its transglutaminase activity [89], thus making FXIII-A important in new vessel formation and remodeling. Inbal and Dardik described that the binding of FXIII-A_2^* to endothelial integrin $\alpha_v\beta_3$ leads to $\alpha_v\beta_3$-VEGFR-2 cross-linkage and a consequent VEGFR-2 activation. VEGFR-2 activation increased cell proliferation and survival via multiple pathways by upregulating Erg-1 and cJun transcription factors and downregulating thrombospondin-1 (TSP-1) on mRNA and protein levels [90]. TSP-1 is an antiangiogenic protein that by binding CD36 on the surface of epithelial cells induces their apoptosis. In transgenic TSP-1-overexpressing mice, delayed granulation tissue formation and delayed wound healing were observed [91]. Importantly, Dardik et al. also found that an antibody for $\alpha_v\beta_3$-integrin (vitronectin receptor) blocked the binding of FXIII-A_2^* to fibroblasts, showing this receptor to be the target of the factor also on fibroblasts suggesting its role in the antiapoptotic effect of FXIII [92].

As demonstrated above, FXIII plays a central role in all stages of wound healing: whether it is clot formation, extracellular matrix formation, macrophage/monocyte activation, enhancement of fibroblast, and epithelial cell migration, FXIII is an essential part of the pathway. These findings make it a promising future treatment option in wound healing, which we will discuss later to a fuller extent.

7. Future Perspectives for FXIII-A in the Field of Dermatology

Based on the variety of processes in which FXIII-A is considered to have a role (Table 2), translating basic research into clinical practice is an interesting field with potential pathological and therapeutic relevance, which we aim to address in the following section.

7.1. Understanding FXIII-A-Positive Cells in Skin Lesions. Detection of FXIII-A in inflammatory and malignant diseases will unquestionably keep its place in dermatological diagnosis. However, more data and relevant experiments are needed to answer the long-lasting question as to whether FXIII-A is just a marker or also contributes to disease pathogenesis. This question is of particular importance in malignancies, where answering the level of contribution to the function of tumor-associated macrophages is as important as the role of ecFXIII-A in tumor matrix remodeling and could give FXIII-A a place in predicting tumor progression.

More and more data are anticipated regarding the origin, the functional properties, and the gene expression profiles of FXIII-A-positive cells with the improvement of the methods and techniques to work on ex vivo dermal immune cells. These techniques may allow us to study them, perhaps even at a single-cell level, without affecting their marker profiles and behaviors during their isolation and separation, such as antigen presentation.

7.2. Wound Treatment. Application of FXIII is the most promising in wound management, with a potential breakthrough already. Administration of rFXIII has been tested as a treatment option in various diseases, such as Crohn's disease, and in microvascular surgeries. During vascular grafting, the addition of rFXIII to the fibrin sealant resulted in a more desirable outcome than with the fibrin alone [93]. Moreover, topical application of rFXIII on heterotopic neonatal mouse heart allografts produced higher numbers of new vessels and increased contractility compared to the untreated mice, suggesting that rFXIII could potentially have a therapeutic effect in diseases where restoring circulation is essential [94]. It could have importance in dermatological diseases such as chronic venous and neuropathic ulcers, NL, and pyoderma gangrenosum, an autoinflammatory disease

Table 2: Previously described/suggested mechanisms of action of factor XIII related to skin homeostasis and diseases.

Affected cells by factor XIII					
ECM	Monocytes	Macrophages	Dendritic cells	Fibroblasts	Endothelial cells
Mechanism of action					
(i) Crosslinking ECM components and complement C3 (ii) Crosslinking bacterial surface components (iii) Fibrin matrix formation around tumor cells (iv) Inducing tumor cell exit to the vasculature	(i) Dimerization of AT1 and mobilization of cells (ii) Facilitating entry into the artery wall (iii) Inducing receptor mediated phagocytosis (iv) Antiapoptotic (v) Promoting proliferation migration	(i) Translocation to the nucleus and macrophage activation (ii) Gene expression regulation (iii) Released cFXIII-A by damaged macrophages	(i) Induction of antigen presentation (ii) Induction of cytokine production (iii) Induction of mucin deposition	(i) Enhancing adherence and migration (ii) Antiapoptotic (iii) Promoting proliferation migration (iv) Homeostasis of collagen production	(i) Endothelial-platelet interaction (ii) Antiapoptotic (iii) Promoting proliferation and migration
References					
[1, 8–10, 23]	[1, 8–10, 23]	[1, 8–10, 23, 25, 37, 43, 62]	[1, 8–10, 23, 67, 72, 75–81]	[1, 5, 8–10, 23]	[1, 8–10, 23]

ECM = extracellular matrix.

with severe ulceration. In line with these findings, the studies conducted so far on the application of FXIII as a treatment option in dermal wound treatment are promising. Accelerated wound surface reduction, shorter healing time, improved availability of granulation tissue, and decreased secretion, and bleeding tendencies were all reported following daily local administration of FXIII concentrate on clear, noninfected wounds of chronic venous leg ulcers and on pyoderma gangrenosum lesions [24, 95–99].

However, it should be noted that success is very dependent on the selection of the involved subjects. This selection is in regard to the background of the wound, other comorbidities, and genetic associations. These criteria are best reflected in the case of arterial-venous mixed disease patients [95] where none of the beneficial effects could be observed, which was contrary to chronic venous leg ulcers (CVLU). In CVLU, the Val34Leu, Tyr204Phe, and Pro564Leu polymorphisms of FXIII-A did not shown any relation to the prevalence of the ulcer, but Leu34 and Leu564 alleles were associated with smaller ulcer size [100–103].

7.3. FXIII-A in Connective Tissue Disorders and Rejuvenation. The ability of FXIII-A to affect collagen synthesis and ECM remodeling is also of potential interest. In particular, FXIII-A was already shown to be beneficial in diseases such as scleroderma. After systemic application of rFXIII-A, a decrease in stiffness of the skin and improved musculoskeletal symptoms were observed. This finding could be related to its possible effect in downregulation of collagen synthesis [104]. We also need to mention here that elevated pFXIII levels were found to promote inflammation and degenerative tissue remodeling in rheumatoid arthritis [105, 106]. This finding should be kept in mind and considered in the safety measurements of studies yet to come.

Platelet-rich plasma (PRP) is widely used for its beneficial effects in wound healing and rejuvenation via multiple actions, as it contains several different types of molecules such as growth factors and cytokines [107]. Some of the results from treatment of chronic wounds with PRP [107–109] are similar to those we have discussed in relation to local application of rFXIII in CVLU [95]. Thus far, there have been no reports, at least to our knowledge, about the FXIII-A content of PRP or its contribution to the effects of PRP. Therefore, further investigations are needed to assess complex cellular functions, the substrate profile of FXIII-A, and its involvement in the various steps of wound healing and rejuvenation.

Abbreviations

FXIII: Factor XIII
FXIII-A: Factor XIII subunit A
FXIII-B: Factor XIII subunit B
FXIII-A2: Factor XIII subunit A as a dimer
pFXIII: Factor XIII in plasma
cFXIII: Factor XIII in cells
ecFXIII: Factor XIII in the extracellular space
pFXIII-A: Factor XIII subunit A in plasma as part of the tetramer or in dimer form
cFXIII-A: Factor XIII subunit A in cells as part of the tetramer or in dimer form
ecFXIII-A: Factor XIII subunit A in the extracellular space as part of the tetramer or in dimer form
rFXIII: Recombinant factor XIII
FXIII-A_2^*: Activated factor XIII subunit A.

Conflicts of Interest

The authors have no conflicts of interest.

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Review Article

Inhibition of Fibrinolysis by Coagulation Factor XIII

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The inhibitory effect of coagulation factor XIII (FXIII) on fibrinolysis has been studied for at least 50 years. Our insight into the underlying mechanisms has improved considerably, aided in particular by the discovery that activated FXIII cross-links α2-antiplasmin (α2AP) to fibrin. In this review, the most important effects of different cross-linking reactions on fibrinolysis are summarized. A distinction is made between fibrin-fibrin cross-links studied in purified systems and fibrin-α2AP cross-links studied in plasma or whole blood systems. While the formation of γ chain dimers in fibrin does not affect clot lysis, the formation of α chain polymers has a weak inhibitory effect. Only strong cross-linking of fibrin, associated with high molecular weight α chain polymers and/or γ chain multimers, results in a moderate inhibition fibrinolysis. The formation of fibrin-α2AP cross-links has only a weak effect on clot lysis, but this effect becomes strong when clot retraction occurs. Under these conditions, FXIII prevents α2AP being expelled from the clot and makes the clot relatively resistant to degradation by plasmin.

1. Introduction

Coagulation factor XIII (FXIII) is activated by thrombin into activated FXIII (FXIIIa). FXIIIa is a transglutaminase that catalyzes the formation of isopeptide bonds between the free amine group of a lysine residue and the acyl group at the end of the side chain of a glutamine residue [1]. FXIII was formerly named the fibrin-stabilizing factor, as the formation of the isopeptide bonds in fibrin results in the stabilization of fibrin. Cross-linking makes fibrin clots insoluble in weak acid or urea [2] and strongly modifies the physical properties such as clot stiffness [3]. In addition, cross-linking of fibrin makes the clot more resistant to proteolytic degradation by the fibrinolytic system.

Inherited FXIII deficiency with undetectable FXIII activity is associated with a severe bleeding tendency. Umbilical bleeding a few days after birth is a characteristic feature and occurs in about 80% of the cases, while intracranial hemorrhage at birth constitutes a main threat to life [4]. It is not fully established which mechanisms are primarily responsible for the bleeding tendency. These mechanisms may involve the lack of physical stabilization of fibrin, the enhanced sensitivity to fibrinolysis, and/or even other mechanisms.

In this paper, we will review the effect of FXIII on fibrinolysis, in particular on the extent of inhibition of lysis and the biochemical mechanisms that are involved. There has been a long-running debate on these topics with many apparently conflicting reports. While reviewing the literature, it is helpful to distinguish mechanisms that play a role in purified fibrin clots and mechanisms that play a role in plasma or whole blood clots where cross-linking of other proteins could occur. This paper shows that most important mechanisms are gradually being elucidated. Excellent reviews about FXIII, including its effect on fibrinolysis, have been published earlier [1, 5].

2. The Fibrinolytic System

The conversion of the inactive proenzyme plasminogen into the active enzyme plasmin is the central step in the fibrinolytic system [6]. Plasmin degrades fibrin into soluble fibrin degradation products. Two physiologic plasminogen activators are capable of catalyzing the conversion of plasminogen: tissue-type and urokinase-type plasminogen activator (t-PA and u-PA, resp.). The most important plasminogen activator for fibrin degradation is probably t-PA, although u-PA may

also be involved in a complementary action of t-PA and u-PA [7]. In the absence of fibrin, t-PA is a poor plasminogen activator. However, in the presence of fibrin, the plasminogen activator activity of t-PA is two orders of magnitude higher as a result of the binding of both t-PA and plasminogen to fibrin and the formation of a cyclic ternary complex. This makes t-PA a fibrin-specific agent.

Inhibition of the fibrinolytic system may occur at the level of plasminogen activation, mainly by plasminogen activator inhibitor-1 (PAI-1) [8]. The two chain forms of t-PA and u-PA are also efficiently inhibited by plasminogen activator inhibitor-2 (PAI-2), but the plasma levels of PAI-2 are normally low or even undetectable (except during pregnancy), and other intracellular functions of PAI-2 have been postulated [9]. Inhibition of the fibrinolytic system may also occur at the level of plasmin, mainly by α2-antiplasmin (α2AP) [10]. PAI-1 and PAI-2, as well as α2AP, are members of the serine protease inhibitor (serpin) superfamily. Fibrin degradation is additionally inhibited by thrombin-activatable fibrinolysis inhibitor (TAFI) [11]. TAFI represents a link between coagulation and fibrinolysis. The inhibitor is slowly activated by thrombin, but this activity of thrombin is three orders of magnitude higher in the presence of thrombomodulin. The activated form (TAFIa) is a plasma carboxypeptidase B and has a short half-life of about 10 min under physiologic conditions. The antifibrinolytic activity of TAFIa is based on the elimination of C-terminal lysine and arginine residues from partially degraded fibrin. This results in a strongly reduced binding of plasminogen on partially degraded fibrin and a concomitant reduction of the activation of plasminogen [12].

Two types of lysis can be distinguished both in vivo and in vitro: external and internal lysis [13]. In internal lysis, all fibrinolytic components are homogeneously distributed within the clot, whereas in external lysis plasminogen activators, t-PA or u-PA, are acting from the outside of the clot. In in vitro experiments, internal lysis is accomplished by adding plasminogen activator to fibrinogen, plasma, or blood before clot formation is induced, while external lysis is obtained by the incubation of a preformed clot in a milieu which is enriched in a plasminogen activator.

3. Effect of Factor XIIIa on Lysis of Purified Fibrin Clots

Cross-linkage of fibrin by FXIIIa results in a rapid formation (within minutes under physiological conditions) of cross-links between γ chains yielding γ chain dimers and in a slower formation (minutes to hours) of cross-links between α chains yielding α chain polymers consisting of a varying number of α chains. β chains are not involved directly in the cross-linking of fibrin [14]. Only a small number of α chain polymers have been formed by the time all the γ chains have been linked, illustrating the difference in reaction rates. A minor amount of cross-linking also occurs between α and γ chains and, upon prolonged incubation (hours to days), between interfibril γ chains resulting in γ chain trimers and γ chain tetramers [15].

Some early fibrinolysis studies did not find an effect of FXIIIa on the lysis of purified fibrin clots [16, 17]. Francis and Marder [18], however, were able to demonstrate that the intramolecular cross-links producing α chain polymers, in particular the very high molecular weight α chain polymers obtained with elevated FXIIIa concentrations, are associated with the inhibition of fibrinolysis. In addition, Siebenlist and Mosesson [19] found inhibition of lysis by FXIIIa and claimed that resistance to fibrinolysis is not induced by α chain polymer formation or γ chain dimer formation, but by γ chain multimer formation. Although the latter two studies disagree about the responsible structures, they agree that inhibition of fibrinolysis occurs particularly after a very strong cross-linking of fibrin. It is quite possible that the studies that found no or only a small effect did not induce such a strong cross-linking. Indeed, recent studies demonstrating small inhibitory effects of fibrin cross-linking on fibrinolysis were not performed under strong cross-linking conditions [20]. The small inhibitory effects of the latter study were also observed with a recombinant mutant of fibrinogen (γQ398N/Q399N/K406R), which does not allow for γ chain cross-linking, suggesting that α chain cross-linking explained the inhibition [21]. This is in line with the above-mentioned conclusion from Francis and Marder [18].

4. Effect of Factor XIIIa on Lysis of Fibrin Clots Prepared from Plasma or Whole Blood

FXIIIa does not only catalyze intramolecular cross-links within fibrin but also catalyze intermolecular cross-links between fibrin and other proteins. In a recent proteomic study, 48 proteins were found to be cross-linked to plasma clots [22]. In particular, proteins with an inhibitory effect on fibrinolysis are of importance for clot stability. These proteins include α2AP, PAI-2, TAFI, and complement C3. Cross-linking of α2AP occurs via Gln14 in α2AP and Lys303 in the α chain of fibrin [23]. Other minor cross-linking sites in α2AP are Gln34, Gln431, and Gln459 [24]. Cross-linking of PAI-2 occurs via Gln83 and Gln86 in PAI-2 and at one of six lysine residues in the α chain of fibrin, but not at Lys303 [25]. Potential cross-linking sites in TAFI are Gln2, Gln5, and Gln292 [26]. Finally, recent studies suggest that complement C3 prolongs fibrinolysis and is cross-linked to fibrin by FXIIIa [27]. The mechanism of the inhibition of fibrinolysis by complement C3 has not yet been elucidated. As described below, the cross-linking of α2AP has the strongest effect on fibrinolysis as compared to the cross-linking of PAI-2, TAFI, and complement C3.

5. Cross-Linking of α2-Antiplasmin to Fibrin

An important discovery for the mechanism of the inhibition of fibrinolysis by FXIII is that FXIIIa cross-links α2AP to fibrin when blood is clotted in the presence of calcium ions [28]. The cross-linked α2AP is fully active and essential for the inhibition of fibrinolysis, particularly of spontaneous fibrinolysis by t-PA-induced plasminogen activation on the fibrin surface [29]. The cross-linking of α2AP to fibrin occurs rapidly; maximal α2AP cross-linking is almost reached when α chain polymerization has just started [30]. An unexplained

phenomenon is that the cross-linking of α2AP stops at about 30% incorporation (corresponding to about one α2AP molecule per 25 molecules of fibrin), whereas the cross-linking of fibronectin to fibrin in the same experiments continues to about 100%. Enhancing the concentrations of FXIIIa increases the rate of α2AP cross-linking but does not change the maximal incorporation [30]. It has been suggested that FXIIIa not only accelerates the cross-linking of α2AP but also accelerates the release of cross-linked α2AP and that the latter activity explains the partial incorporation [31]. However, other mechanisms related to α2AP heterogeneity (see below) or structural hindrance by α chain polymerization should be considered as well, although structural hindrance does not appear to play a role in the cross-linking of α2AP to fibrinogen [32].

It is somewhat remarkable that higher levels of FXIII do not increase the maximal level of α2AP cross-linking [30], whereas the Leu variant of the Val34Leu polymorphism of FXIII, which is more rapidly activated by thrombin than the Val variant, shows a higher incorporation of α2AP than the Val variant. This occurs in plasma samples of healthy subjects [33] as well as in purified systems [34]. The two latter studies utilized the same microtiter plate incorporation assay. This suggests that the assay reflects the rate of α2AP incorporation more than the maximal incorporation.

6. Heterogeneity of α2-Antiplasmin

In the circulation, α2AP undergoes both N-terminal and C-terminal proteolytic modifications that significantly modify its functional properties [10]. Approximately 35% of circulating α2AP lacks the C-terminus containing the binding site for plasmin(ogen) which is crucial for the rapid inhibitory mechanism of α2AP [35]. Although this so-called nonplasminogen-binding form of α2AP has not yet been purified and biochemically characterized, there is evidence that α2AP with an intact C-terminus, also called plasminogen-binding form, is selectively cross-linked to fibrin by FXIIIa [36].

Approximately 70% of circulating α2AP is cleaved at the N-terminus between Pro12 and Asn13 of full-length α2AP which has Met as the N-terminus (Met-α2AP) [37]. The 12-residue shorter form has Asn as the N-terminus (Asn-α2AP) and is cross-linked to fibrin ~13 times faster than Met-α2AP, probably because the cross-linking site Gln14 is more exposed in Asn-α2AP than it is in Met-α2AP [38]. Lysis rates of plasma clots are slower when the plasma samples contain relatively more Asn-α2AP [38]. The enzyme that cleaves Met-α2AP in vitro is named antiplasmin-cleaving enzyme (APCE) and is identical to the soluble form of membrane-bound fibroblast activation protein in the circulation (cFAP) [38]. Correlation studies of circulating cFAP levels and percentage α2AP N-terminal cleavage in the plasma samples of patients and healthy controls confirm that cFAP is also responsible for the cleavage in vivo [39, 40]. The α2AP gene codes for either Arg or Trp as the sixth amino acid. cFAP cleaves Met-α2AP(Arg6) ~8-fold faster than Met-α2AP(Trp6), suggesting that this Arg6Trp polymorphism may be functionally significant [41].

If we assume that the N-terminal and C-terminal proteolytic modifications of α2AP occur independently of each other, we can calculate that 65% × 70% = 45.5% of the α2AP in plasma has a form (Asn-plasminogen-binding α2AP) that is rapidly incorporated in fibrin by FXIIIa. This may partially explain why the maximal incorporation of α2AP is only ~30%.

7. Relative Contributions of Fibrin-Fibrin Cross-Links and Fibrin-α2-Antiplasmin Cross-Links to Inhibition of Lysis

During blood clotting, FXIIIa introduces cross-links within fibrin as well as between fibrin and α2AP. The relative contributions of the two types of cross-links in the inhibition of fibrinolysis by FXIII have been thoroughly investigated. Jansen et al. [42] studied the lysis rate of fresh whole blood clots containing t-PA that was added before clotting in vitro. They reported that fibrin-α2AP cross-linking explains the FXIIIa-induced resistance of blood clots to fibrinolysis, whereas fibrin-fibrin cross-linking has only a small, if any, influence. This was later confirmed by Fraser et al. [43], who showed that the antifibrinolytic function of FXIII in plasma clots prepared in a Chandler loop and incubated in a buffer containing t-PA is independent of fibrin-fibrin cross-linking and is expressed exclusively through α2AP. Reed and Houng [44] studied t-PA-induced fibrinolysis in anesthetized ferrets with pulmonary emboli and found, in contrast to the previous investigators, that both fibrin-fibrin and fibrin-α2AP cross-linking caused resistance to lysis.

Significant inhibition of fibrinolysis by fibrin-fibrin cross-links requires strong cross-linking conditions, resulting in very high molecular weight α chain polymers and/or γ chain trimers and tetramers, whereas significant inhibition of fibrinolysis by fibrin-α2AP cross-links occurs immediately after clotting, as these links are formed rapidly. Therefore, it can be anticipated that the relative contribution of fibrin-fibrin cross-links to the total inhibition of fibrinolysis by FXIII depends on the extent of cross-linking and increases, for instance, with the age of a thrombus.

8. Effect of Clot Compaction and Clot Retraction on Lysis

The inhibition of clot lysis by FXIII is determined not only by the degree of cross-linking but also by the design of the lysis experiments. This was already concluded in 1979 in a paper about the existing controversy concerning the question of whether or not FXIII cross-linking affects fibrinolytic rates [45]. In this connection, Mutch et al. [46] published an interesting observation that model thrombi formed under flow in a Chandler loop and subsequently incubated in a buffer containing t-PA reveal a significant effect of FXIII on fibrinolysis. This effect is also revealed by thrombi prepared from platelet-free plasma. It is suggested that flow is required during clot formation.

We recently studied the effect of clot retraction on the antifibrinolytic effect of FXIII [47], stimulated by preliminary

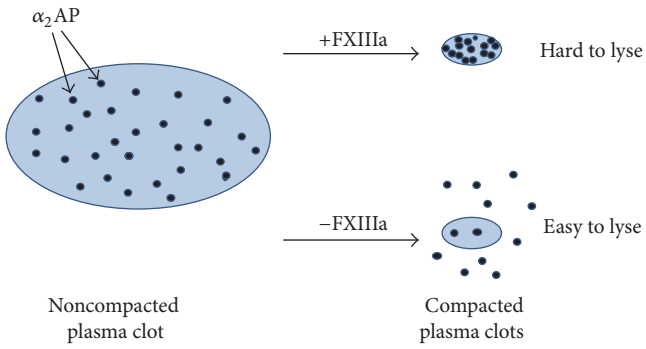

Figure 1: Activated factor XIII (FXIIIa) partially prevents α2-antiplasmin (α2AP) from being expelled from a plasma clot during compaction and thereby strongly inhibits fibrinolysis. Taken from Rijken et al. [47].

data from Sakata and Aoki pointing to the potential importance of clot retraction [29]. Our studies started with platelet-poor and t-PA-containing plasma clots with and without physical compaction by centrifugation, as a model of platelet-mediated clot retraction. Without compaction, FXIII slightly inhibited clot lysis (1.6-fold). With compaction, however, FXIII revealed a strong inhibition of clot lysis (7.7-fold). Because FXIII did not show inhibition with either compacted or noncompacted clots prepared from α2AP-deficient plasma, the inhibition was completely ascribed to the cross-linking of α2AP, in agreement with previous studies [42, 43]. Under these conditions, the fibrin-fibrin cross-links were apparently too limited to play a significant role. The same held true for potential cross-links between fibrin and other proteins such as TAFI and PAI-2. Experiments with platelet-rich plasma clots, with and without platelet-mediated clot retraction, showed similar results as platelet-poor plasma clots with and without compaction. The slight inhibition by FXIIIa of the lysis of noncompacted or nonretracted plasma clots implied that cross-linked α2AP is a somewhat better inhibitor of plasmin that is generated on the fibrin surface than non-cross-linked α2AP. However, this type of inhibition in assays without compaction or retraction is small and probably not easily detectable since various studies in the literature report no inhibition (e.g., [45]). The extra and strong inhibition of the lysis of compacted or retracted plasma clots is easily explained, since FXIIIa partially prevents α2AP from being expelled from the clot during compaction or retraction. This mechanism is illustrated in Figure 1.

The requirement of clot compaction for the full expression of the antifibrinolytic effect of FXIII in clot lysis assays explains the old controversy between Gaffney and Whitaker [48] and Rampling and Flexman [45]. The first authors found a strong inhibitory effect on lysis of plasma clots harvested by winding the clot onto a glass rod (thus with compaction), whereas the latter authors found no inhibitory effect using undisturbed plasma clots (thus without compaction). The requirement of clot compaction also explains why the lysis of Chandler loop thrombi is sensitive to FXIII [46], as Chandler loop thrombi are always compacted. Therefore, compaction and not the formation under flow is the essential feature a clot must show in order to reveal FXIII-dependent clot resistance.

9. (Patho)physiologic Aspects

What is the (patho)physiologic meaning of the mechanism illustrated in Figure 1? It is not easy to answer this question, because our knowledge of platelet-mediated clot retraction is limited. We do not know when, where, and how fast clot retraction occurs in the body. However, it is clear from Figure 1 that in vivo retracted clots are fairly resistant to fibrinolysis owing to the cross-linking of α2AP by FXIIIa. Without FXIIIa, α2AP would be largely expelled from the clot during retraction. It is assumed that platelet-mediated clot retraction can occur in the absence of FXIIIa activity. This is still a matter of debate [49], but in our hands platelet-rich clots do retract in the presence of FXIIIa inhibitor [47]. Full absence of FXIII in patients is rare, but low levels do occur more frequently, for instance, in acquired FXIII deficiency. Studies in purified systems [50] and in platelet-poor plasma [46] indicate that plasma FXIII levels of 50% of normal are required for the optimal inhibition of fibrinolysis. FXIII levels below 50% are associated with a smaller α2AP incorporation and enhanced clot lysis. Platelets and platelet FXIII could contribute to the cross-linking of α2AP to fibrin [51–53]. Hemostasis in FXIII-deficient patients is already normalized at therapeutic FXIII plasma levels of 3–5% [4]. This suggests that the severe bleeding problems of FXIII-deficient patients are not primarily caused by enhanced clot lysis, but by impaired (physical) stability of the clots due to insufficient formation of γ chain dimers and possibly α chain polymers. Enhanced fibrinolysis could contribute, however, to the severity of the bleeding tendency, as recently observed in a subpopulation of patients with von Willebrand disease [54].

While enhanced fibrinolysis may be associated with bleeding, impaired fibrinolysis could result in a thrombotic tendency. Although the cross-linking of α2AP to fibrin seems already to be maximal at a plasma FXIII level of 50%, the incorporation of α2AP is only partial (about 30%) and one could imagine that the incorporation may increase or may become faster in certain physiologic or pathophysiological conditions. One example is the earlier-mentioned increased rate of α2AP incorporation by the Leu variant of the Val34Leu polymorphism of FXIII [33, 34]. Similar increases might be caused by variations in α2AP (for instance, in the N-terminal or C-terminal heterogeneity) or in fibrinogen.

10. Conclusions

The inhibitory effects of FXIII-mediated cross-links on fibrinolysis are summarized in Table 1. Fibrin-fibrin cross-links are responsible for weak effects, although very strong cross-linking conditions in vitro result in a moderate inhibition of fibrinolysis. It is not yet clear to what extent these strong cross-linking conditions occur in vivo. Fibrin-α2AP cross-links are also responsible for only weak effects, but these cross-links result in strong inhibition of clot lysis when clot retraction occurs. We assume that blood clotting in vivo is

Table 1: Summary of inhibitory effects of FXIII-mediated cross-links on fibrinolysis.

Type of cross-links	Inhibitory effect
Fibrin-fibrin cross-links	
(i) γ chain dimers	None
(ii) α chain polymers	Weak
(iii) High molecular weight α chain polymers and/or γ chain multimers	Moderate
Fibrin-α2-antiplasmin cross-links	
(i) In nonretracted fibrin clots	Weak
(ii) In retracted fibrin clots	Strong

often followed by platelet-mediated clot retraction, suggesting that this inhibition observed in vitro is representative of the in vivo situation.

The effects summarized in Table 1 are to a great extent based on internal lysis experiments. In external lysis, fibrinolytic components are transported from the outside to the inside of the clot by diffusion and, in particular in vivo, by flow. This transport may be affected by cross-linking. Although fibrin cross-linking has only minor effects on global network structure, it has a strong effect on individual fibers since they become more compact by cross-linking [55]. This will reduce the transport of, for instance, plasminogen activators through the fibers and reduce external clot lysis.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Structure and Function of Trypsin-Loaded Fibrinolytic Liposomes

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Protease encapsulation and its targeted release in thrombi may contribute to the reduction of haemorrhagic complications of thrombolysis. We aimed to prepare sterically stabilized trypsin-loaded liposomes (SSL_T) and characterize their structure and fibrinolytic efficiency. Hydrogenated soybean phosphatidylcholine-based SSL_T were prepared and their structure was studied by transmission electron microscopy combined with freeze fracture (FF-TEM), Fourier transform infrared spectroscopy (FT-IR), and small-angle X-ray scattering (SAXS). Fibrinolytic activity was examined at 45, 37, or 24°C on fibrin or plasma clots with turbidimetric and permeation-driven lysis assays. Trypsin was shown to be attached to the inner surface of vesicles (SAXS and FF-TEM) close to the lipid hydrophilic/hydrophobic interface (FT-IR). The thermosensitivity of SSL_T was evidenced by enhanced fibrinolysis at 45°C: time to reduce the maximal turbidity to 20% decreased by 8.6% compared to 37°C and fibrin degradation product concentration in the permeation lysis assay was 2-fold to 5-fold higher than that at 24°C. SSL_T exerted its fibrinolytic action on fibrin clots under both static and dynamic conditions, whereas plasma clot dissolution was observed only in the permeation-driven assay. The improved fibrinolytic efficiency of SSL_T under dynamic conditions suggests that they may serve as a novel therapeutic candidate for dissolution of intravascular thrombi, which are typically exposed to permeation forces.

1. Introduction

Thrombolysis based on enzymatic dissolution of fibrin is currently the first-line treatment of ischemic stroke as well as certain selected cases of acute myocardial infarction [1, 2]. Most of the fibrinolytic agents are plasminogen activators, which can be classified as "indirect fibrinolytics," because their enzymatic action is directed towards plasminogen, while, in contrast, fibrinolytics such as plasmin and its derivatives degrade fibrin without any intermediate step of plasminogen activation and are therefore designated as "direct fibrinolytics." Systemic (intravenous) administration of indirect fibrinolytics is accompanied by frequent bleeding side effects related to the large excess of activator at its therapeutic dose over the inhibitor capacity of blood plasma [3]. In contrast, locally administered plasmin exerts its fibrinolytic action in fibrin-bound form being protected from its main inhibitor (α_2-plasmin inhibitor) but is immediately inactivated when entering the circulation, thus preventing bleeding at remote sites of vascular injury (reviewed in [4]). However, catheter directed thrombolysis combined with systemic thrombolysis may be associated with higher risk of pulmonary embolism or intracranial haemorrhage compared to systemic thrombolysis alone [5].

Nanomedicine offers approaches for noninvasive and rapid thrombolytic treatment, with the hope of further reducing the morbidity and mortality of occlusive cardiovascular events. Being encapsulated into liposomes, drugs are preserved from metabolization prior to reaching target tissues, and simultaneously they minimize exposure of healthy tissue to the encapsulated drug during its circulation in the blood. The possibility to target liposomes helps in localizing sufficient quantities of thrombolytic agents to the desired thrombus. In vivo results show strong evidence that external

targeting is superior to passive targeting of highly stable long-circulating drug formulations. A promising alternative for external targeting is achieved by temperature-triggered, localized intravascular drug release from thermosensitive liposomes with focused heating ([6], reviewed in [7]).

Trypsin could be a novel candidate for being a liposome-encapsulated thrombolytic drug because of its high fibrinolytic efficiency [8] and about 3.5 times lower molecular mass compared to that of plasmin allowing higher encapsulated enzyme concentration. If administered in a plasma environment, encapsulated trypsin is protected against plasma inhibitors, the most abundant of which is α_1-PI (α_1-protease inhibitor). However, in contrast to plasmin and tPA, trypsin lacks any structural domains for recognition and specific binding to fibrin. Thus, an alternative targeting strategy is required to allow for a local proteolytic action of trypsin in thrombi. Such an option is offered by the temperature-dependent release of the enzyme from the thermosensitive liposomes developed and characterized in the current study, which in vivo could be achieved with focused ultrasound thermal effects. Following the local release of trypsin in thrombi, the availability of the fibrin substrate will protect the enzyme from inactivation by α_1-PI, similarly to the fibrin-mediated protection of other proteases (plasmin and PMN-elastase) against plasma protease inhibitors [9].

The purpose of our work was to prepare trypsin-loaded PEGylated liposomes and characterize them in terms of their structure and proteolytic efficiency in pure fibrin, as well as in plasma environment.

2. Materials and Methods

2.1. Proteins and Reagents. If not otherwise indicated, experiments were performed in HEPES buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.4). Porcine trypsin and bovine thrombin were purchased from Serva Electrophoresis GmbH (Heidelberg, Germany) and the latter was further purified as described in [10] yielding a preparation with specific activity of 2100 IU/mg. Thrombin activity of 1 IU/mL was considered equivalent to approximately 10.7 nM by active site titration [11]. Fibrinogen (human, plasminogen-depleted) was from Calbiochem (San Diego, CA, USA). Hydrogenated soybean phosphatidylcholine, cholesterol, distearoyl-phosphatidylethanolamine PEG2000, and cholesterol sulphate were acquired from Avanti (Birmingham, AL, USA). DPH (1,6-diphenyl-1,3,5-hexatriene) and fluorescamine were from Sigma-Aldrich Kft. (Budapest, Hungary) and Spectrozyme-PL (H-D norleucylhexahydrotyrosyl-lysine-p-nitroanilide) was from Sekisui Diagnostics (Pfungstadt, Germany). Citrated, fresh frozen plasma (fibrinogen concentration: 2.35 g/l) was obtained from the Hungarian Blood Supply Service (Budapest, Hungary).

2.2. Preparation of Trypsin-Loaded Liposomes. Trypsin-loaded (SSL_T) and empty (SSL) sterically stabilized liposomes consisting of hydrogenated soybean phosphatidylcholine, cholesterol, distearoyl phosphatidylethanolamine PEG2000, and cholesterol sulphate at a molar ratio of 15 : 4 : 1 : 1 were prepared by thin-layer evaporation method as follows. Constituents were mixed and dissolved in a chloroform/methanol mixture (95 : 5 volume ratio) at a total phospholipid concentration of 34 mg/ml. Evaporation of the organic solvent was facilitated by a vacuum pump (20 mbar, at 24°C, overnight). The lipid film was hydrated at room temperature with HBS for preparing empty liposomes or 0.1 mM HCl in distilled water containing 10 mg/ml porcine trypsin for trypsin-loaded liposomes and stirred for 10 min at 1200 rpm. Formation of unilamellar vesicles was promoted by sonication (three cycles of 30 s at 50 watt, 20 kHz in a Branson Sonifier 250, Branson Ultrasonics Corp., Danbury, CT, USA) followed by ten freeze-thaw cycles (−78°C; +42°C). SSL_T was then extruded through a 100 nm pore diameter polycarbonate filter in a LiposoFast mini-extruder (Avestin Inc., Ottawa, Canada). Nonencapsulated trypsin and lipid debris were removed from supernatant by centrifugation (3 times for 15 min at 133,000g) with a Beckman Airfuge 340401 ultracentrifuge. The sediment was resuspended in HBS yielding a liposome preparation of neutral pH, with trypsin encapsulated in its inactive form due to the low pH inside of the enzyme loaded vesicles. The concentration of phospholipids in the SSL_T suspension was determined with the fluorescent probe DPH [12] yielding a concentration of about 30 mg/ml. Changes in enzyme activity of the encapsulated trypsin were monitored daily on the chromogenic small peptide substrate Spectrozyme-PL (SpPL) at 405 nm after lysing liposomes by stirring together with 2.5 v/v% Triton X-100 for ten seconds at 1200 rpm. SSL_T was stored at 4°C until use.

2.3. Dynamic, Permeation-Driven Lysis of Fibrin and Plasma Clots. In order to examine the fibrinolytic activity of our liposome preparation under dynamic conditions, fibrin and plasma permeation studies were performed at 24 and 45°C. The inner surfaces of 5 ml pipette tips (Finntip, Thermo Scientific, Budapest, Hungary) were precoated with 1 g/l fibrinogen for 3 h and then air-dried [13]. Fibrin clots were prepared from fibrinogen at 7.5 μM clotted with 16 nM thrombin in the fibrinogen-coated pipette tips. Plasma clots were prepared from citrated, fresh frozen plasma supplemented with fibrinogen to 7.5 μM final concentration and $CaCl_2$ to 12.5 mM and filled in the tips. After 70 min of incubation at 37°C, stable clots were washed thoroughly with HBS to remove unclotted fibrinogen and other plasma proteins. Trypsin-loaded liposomes (50 μl) containing phospholipid at 5 mg/ml and enzyme activity corresponding to that of 300 nM free trypsin, empty liposomes, or HBS were layered over clots of 200 μl volume. After the entry of the liposomal suspension into the clots, HBS was added and continuously supplemented to keep a constant hydrostatic pressure over the clots. The pipette tips were kept at 24 or 45°C during the lytic process. Consecutive fractions of 50 μl were then collected and their protein concentration was determined by the fluorescamine method [14] and plotted against the eluted volume. Fractions with the highest protein content were analyzed with SDS polyacrylamide gel electrophoresis (SDS PAGE) in 4–15% gradient gels under nonreducing conditions, followed by visualization of protein bands with silver staining.

2.4. Lysis of Fibrin and Plasma Clots in a Turbidimetric Assay. Two different experimental setups were designed to measure the fibrinolytic effectiveness of our liposomes under static conditions. First, fibrinogen at 7.5 μM or plasma supplemented with fibrinogen (7.5 μM) and calcium (12.5 mM) were clotted with thrombin (20 nM) at 37°C in 96-well microtiter plates. Clot dissolution was started by layering 50 μl of SSL_T (or empty liposomes or HBS) containing phospholipid at about 30 mg/ml and enzyme activity corresponding to that of 1.8 μM trypsin over the clot ("extrinsic lysis" assay), followed by measuring the light absorbance at 340 nm at 37°C with a Zenyth 200rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) and in parallel at 45°C with a CLARIOstar® microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). In a second ("intrinsic lysis") setup, fibrinogen and plasma clots were prepared as above, but prior clotting SSL_T (or SSL) was homogeneously dispersed in the clotting mixture at 3 mg/ml final phospholipid concentration and 180 nM trypsin. The clotting and the clot dissolution phases were monitored by measuring the light absorbance at 37°C and 45°C in parallel. The CLARIOstar® microplate reader was used in an orbital averaging mode. Using this mode, the measurement takes place on an orbit with definable diameter within each well. It results in higher absorbance values compared to the measurement using a Zenyth 200rt microplate spectrophotometer in a normal mode, when one point is measured in the middle of each well. Therefore, data gained at 37°C with the Zenyth microplate reader were multiplied by a factor of 2.41 to be comparable with those gained at 45°C with the other instrument. For comparison of lytic rates of the two static assays, the time needed to reduce the turbidity of the clots by a given fraction of the maximal value (T_{20}, T_{50}, and T_{80} to reach 0.8, 0.5, and $0.2A_{max}$, resp.) was calculated as a quantitative parameter of fibrinolytic activity. The curve analyzing process and statistical comparison of the T values with Kolmogorov-Smirnov test were performed in Matlab R2016a (The MathWorks, Inc., Natick, MA, USA).

2.5. Transmission Electron Microscopy Combined with Freeze Fracture (FF-TEM). A 1-2 μl droplet of SSL_T or SSL suspension was used for freeze fracturing. The samples were pipetted to the golden sample holder and rapidly frozen in the mixture of liquid and solid Freon, cooled by liquid nitrogen. Fracturing was performed at 173 K in a freeze fracture device (BAF 400D, Balzers AG, Liechtenstein). The fractured surfaces were etched for 30 s at 173 K and then shadowed by platinum and covered with carbon. The replicas obtained were washed with surfactant and water and were finally transferred to 200-mesh copper grids. The electron micrographs were made in a Philips Morgagni 268D electron microscope.

2.6. Fourier Transform Infrared Spectroscopy (FTIR). For FTIR spectroscopic study, the attenuated total reflection (ATR) technique was used, having a penetration depth of infrared light in the samples in the order of one micrometer, so the investigation in bulk aqueous phase was possible, too.

ATR-FTIR spectroscopic measurements were carried out by means of a Varian 2000 (Scimitar Series) FTIR spectrometer fitted with a diamond attenuated total reflection cell (Specac's "Golden Gate" single reflection ATR unit with active area of 600 × 600 μm^2). SSL_T (or SSL) suspension (5 μl) was spread onto the diamond ATR surface. Room temperature spectra (128 scans, resolution of 2 cm^{-1}) were recorded both as suspension using a cap to avoid sample drying and as dry films after slow evaporation of the buffer solvent under ambient conditions. ATR correction was executed after each data collection. All spectral manipulations were performed using GRAMS/32 software package (Galactic Industries Incorporation, USA).

2.7. Small-Angle X-Ray Scattering (SAXS). Small-angle X-ray scattering measurements were performed using CREDO, an in-house transmission geometry setup [15]. SSL_T and SSL samples (at 20 mg/ml phospholipid) were filled into thin-walled quartz capillaries of 1.2 mm average outer diameter. After proper sealing, these were placed in a temperature-controlled aluminium block, which was inserted into the vacuum space of the sample chamber. Measurements were done using monochromatized and collimated Cu Kα radiation (0.1542 nm wavelength), and the scattering pattern was recorded in the range of 0.23–1.03 nm^{-1} in terms of the scattering variable (defined as $q = (4\pi \sin \vartheta)/\lambda$, where 2ϑ is the scattering angle and λ is the X-ray wavelength). The total measurement times were 7.5 hours for each sample. In order to assess sample and instrument stability during the experiment, the exposures were made in 5-minute units, with frequent sample change and reference measurements. The same measurement protocol was used in our previous study executed on artificial nanoerythrosomes [16]. These individual exposures were corrected for beam flux, geometric effects, sample self-absorption, and instrumental background and calibrated into physical units of momentum transfer (q, nm^{-1}) and differential scattering cross section (absolute intensity, $cm^{-1} \times sr^{-1}$). The averages of all the corrected and calibrated scattering patterns for each sample were azimuthally averaged to yield single one-dimensional scattering curves for each sample [17–19].

3. Results and Discussion

3.1. Evaluating the Fibrinolytic Function of SSL_T. According to the trypsin activity measurements, enzyme activity of our SSL_T preparation on the first day (100%) was equivalent to the activity of 1.8 μM free trypsin. Then, following an initial drop of 30% during the first 3 days, it remained unchanged for 10 days stored at 4°C (data not shown).

The fibrinolytic activity of SSL_T was tested under dynamic conditions with permeation of fibrin and plasma clots at room temperature and 45°C in parallel to test the thermosensitivity of the liposome vehicle construct. Trypsin-loaded liposomes were layered over the surface and after their entry into the clots HBS was added and continuously supplemented to keep a constant hydrostatic pressure over the clots. The eluted fluid was collected in fractions and analyzed for protein

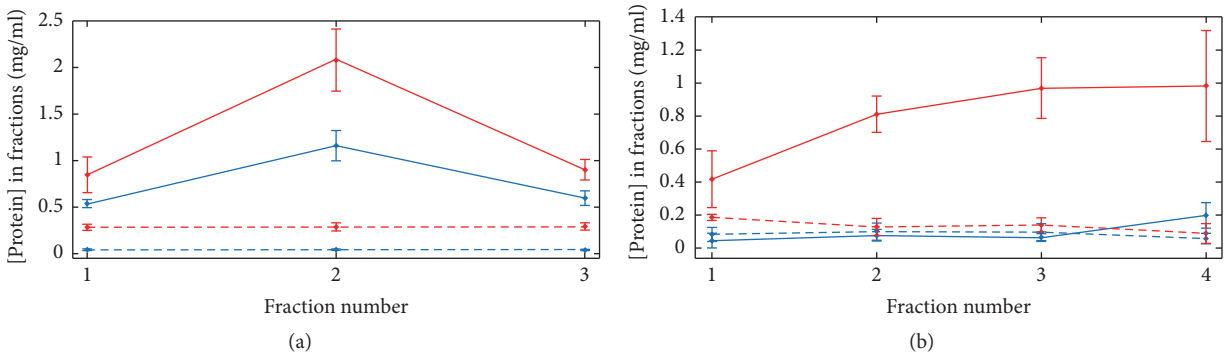

FIGURE 1: Permeation-driven lysis of fibrin and plasma clots. SSL_T (solid lines) or HBS (dashed lines) was layered to the surface of fibrin (a) or plasma (b) clots; thereafter a constant hydrostatic pressure was maintained over the clots and the eluted fluid was collected in fractions of 50 μl each, in which the protein content was measured and is shown as mean ± SEM ($n = 3$). Red and blue colours indicate clot lysis at 45°C and 24°C, respectively.

content, indicating the release of soluble fibrin degradation products (Figure 1).

Elevated temperature during the lytic phase markedly enhanced the lytic efficiency of the trypsin-loaded liposomes. Close to the phase transition temperature enhanced area fluctuations and increased lateral compressibility of the liposome membrane lead to increased likelihood of spontaneous lipid pore formation and therefore to permeation events [20] and the consequent release of the encapsulated enzyme. Thus, increased lytic efficiency at elevated temperatures proves that the applied liposome is a *thermosensitive* construct. The upper limit of mild hyperthermia during cancer therapy is 45°C, while higher temperatures are used for ablation techniques causing serious damage in the tumor tissue, but cells with normal vascularization and normal heat-shock protection mechanisms might be safely subjected to a thermal treatment of 45°C [21, 22]. This local thermal effect could be achieved in thrombi with high intensity focused ultrasound with controlled and selected parameters. The conformal microwave array applicators are also potential hyperthermia applications that may be suitable for heat-assisted thrombolysis [22–25].

Nevertheless, (i) the drug release at 24°C, (ii) the slightly elevated level of detectable protein in fractions of the control sample at 45°C in a fibrin environment (Figure 1(a)), and (iii) the lower detectable protein concentrations in fractions collected during plasma permeation with SSL_T (Figure 1(b)) need further consideration.

(i) Since SSL_T was stored at 4°C before and during trypsin activity measurements, the observed stability of the encapsulated enzyme provides information about drug retention capacity at low temperatures. Therefore, it cannot be excluded that at 24°C protein leakage increases from SSL_T due to changes of the organization in the SSL_T double layer. Furthermore, despite the fact that PEGylation is the most common method for vesicle stabilization and preventing interactions between the particle surface and plasma proteins, interactions between PEGylated nanoparticles and albumin, fibrinogen, IgG, and apolipoproteins have been reported [26]. Moreover, interactions of antimicrobial [27], cytoskeletal [28, 29], and other proteins (calponin [30]) with lipid vesicles have been found to cause vesicle leakage. Since fibrinogen is known to bind phospholipids [31, 32], drug release from SSL_T due to interaction with fibrin(ogen) is also a plausible explanation for detectable fibrin degradation at 24°C during fibrin permeation by trypsin-loaded vesicles.

(ii) Mild disintegration at 45°C due to less stable structure of fibrin clots compared to that of plasma clots containing calcium and FXIII (Figure 1(b), red, dashed lines) may lead to the constant, slightly elevated level of detectable protein in fractions of the control sample in the case of fibrin clots (Figure 1(a), red, dashed lines).

(iii) Lower protein concentrations of the eluted fractions in the plasma clot permeation (Figure 1(b)) compared to the fibrin clot permeation (Figure 1(a)) are presumably the consequence of the plasma clot being more resistant to lysis compared to the fibrin clot due to the possibility to form cross-links during the clotting phase. Furthermore, previous studies have shown that α_1-antitrypsin is noncovalently bound to fibrin clots prepared from plasma, preserving its serine protease inhibitor activity despite extensive washing [33]. The presence of such an effective inhibitor of trypsin may also lead to decreased fibrinolytic efficiency of SSL_T in plasma clots.

SDS polyacrylamide gel electrophoresis was performed to examine the protein size distribution of the fractions with the highest protein concentrations in both cases (Figure 2).

A plasmin-like degradation pattern of fibrin [34] was observed with different types of fibrin degradation products, which evidenced that the protein content of fractions with the highest protein concentrations originated exclusively from fibrin degraded by trypsin. It should be noted that no fibrin degradation was found after layering empty liposomes over the fibrin or plasma clots.

In addition to the permeation studies, the fibrinolytic activity of SSL_T was investigated under static conditions in "extrinsic lysis" assay by liposomes layered over and in "intrinsic lysis" assay by liposomes incorporated into fibrin and plasma clots at 37 and 45°C. In the extrinsic lysis setup,

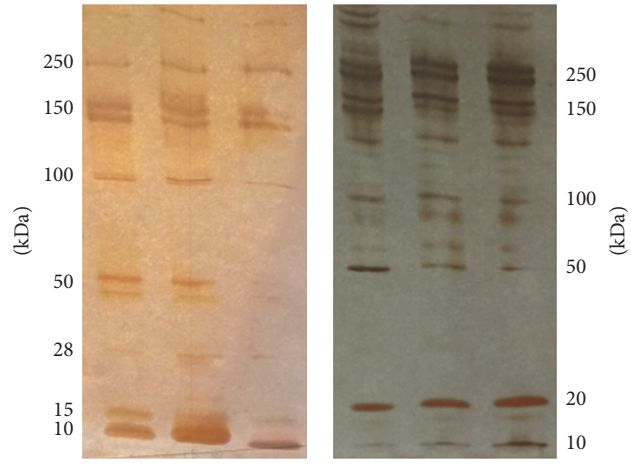

FIGURE 2: SDS polyacrylamide gel electrophoresis of protein fractions with the highest concentrations collected during fibrin and plasma clot permeation by SSL_T. Three parallel fractions collected during fibrin (panel (a), fraction number 2) and plasma (panel (b), fraction number 3) clot permeation with SSL_T.

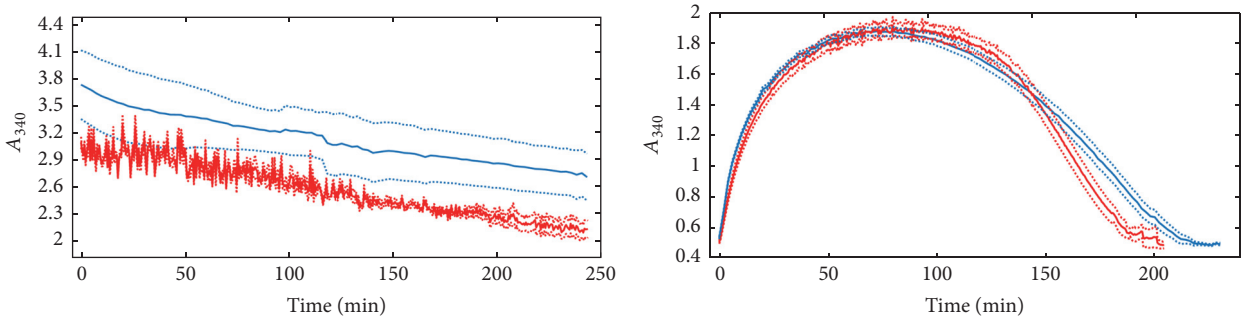

FIGURE 3: Lysis of fibrin by SSL_T layered on the clot surface or homogeneously dispersed in the clots. The liposomal solution was layered over preformed clots (a) or added to fibrinogen before the clotting phase (b) and thereafter formation and/or dissolution of fibrin were followed as changes in absorbance at 340 nm shown as mean (solid lines) ± SEM (dotted lines) of three measurements. Red and blue colours indicate clot lysis at 45°C and 37°C, respectively.

the complete dissolution of fibrin clot was achieved in 4 h. No significant difference was found between lysis times at the two different temperatures applied (Figure 3(a) and Table 1).

In the "intrinsic lysis" setup, SSL_T was incorporated into the clots during the clotting phase. Then, clots were let to dissolve on their own at different temperatures. The time needed for complete dissolution was about 2–2.5 hours at both examined temperatures, although a significant decrease in T_{80} (time needed to reduce the turbidity of the clots by 80% of A_{max}) was observed at 45°C, implying faster trypsin release at the higher temperature again (Figure 3(b) and Table 1).

Under static conditions, there was no change in the absorbance values after the clotting phase in the control fibrin and plasma clots treated with HBS or SSL or in the plasma clots with HBS, SSL, or SSL_T (data not shown).

The failure to dissolve plasma clots under static conditions can be traced back to the effect of α_1-antitrypsin retained in the clot. Furthermore, the SSL_T suspension was diluted to a greater extent in the intrinsic lysis setup compared to the permeation of plasma clots; thus the concentration of trypsin released from liposomes was not sufficiently high to counteract its inhibitor(s). Finally, the permeation pressure in the dynamic assay is an additional factor contributing to the enhanced release of the entrapped enzyme compared to

TABLE 1: Lysis times needed to dissolve fibrin clots by 20, 50, or 80%. Mean values ± SEM are shown ($n = 3$). Asterisk indicates statistical significance between the T_{80} values measured at the two temperatures at $p < 0.05$ according to Kolmogorov-Smirnov test.

	T_{20} (min)	T_{50} (min)	T_{80} (min)
Extrinsic lysis			
37°C	21.23 ± 6.96	89.75 ± 13.50	190.16 ± 9.02
45°C	42.99 ± 6.64	72.77 ± 14.71	167.71 ± 17.80
Intrinsic lysis			
37°C	131.44 ± 4.39	165.80 ± 4.70	192.24 ± 5.06
45°C	133.71 ± 1.15	156.41 ± 2.08	175.78* ± 2.88

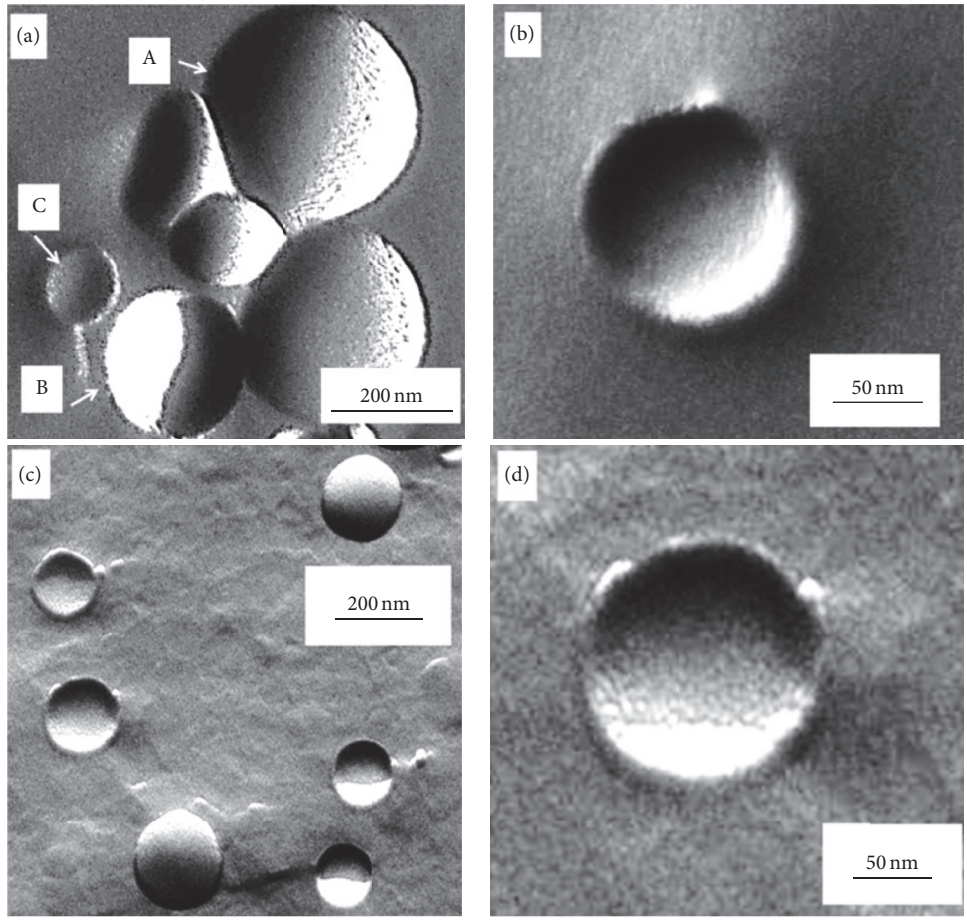

Figure 4: The surface morphology of the trypsin-free ((a) and (b)) and trypsin-loaded ((c) and (d)) vesicles by freeze fracture TEM. (a) Three types of fractures can be observed: (A) convex fractured surfaces of the vesicles protruding from the aqueous medium, (B) concave areas of the imprints of vesicles broken out, and (C) bottom parts of remaining vesicles broken through entirely. (b) The inner surface of a vesicle, broken through entirely, is typically smooth. All three types of fractured surfaces are observed in the trypsin-loaded vesicles (c), but the inner surface contains also closely packed grains (d).

the liposomes stationary incorporated into the clot. The heat-dependence of the trypsin release from SSL_T was much less pronounced and observed only in the late stage (T_{80}, Table 1) of the static lysis assays, probably due to the smaller difference between the two temperatures applied.

The large difference in the time needed for total clot dissolution during surface (about 4 h), and inner (about 2 h) dissolution despite the tenfold liposome concentration in the former case is not unexpected. A similar difference is observed in the fibrinolytic potency of plasmin layered over fibrin and incorporated into fibrin clots; about a 100-fold higher enzyme concentration is needed to achieve a comparable lysis rate from the surface than with clot-entrapped plasmin [35, 36].

3.2. Structural Characterization of SSL_T. The method of TEM combined with freeze fracture provides an excellent tool to visualize the internal structure of vesicle systems of sizes spanning from nanometers up to several micrometers [37]. The typical fractured surfaces of the nearly spherical vesicles are shown on Figure 4(a). Apart from the convex fractured surfaces of the vesicles protruding from their flat neighbourhood ("A") (corresponding to the original, aqueous medium) we can observe the concave imprints of vesicles broken out entirely from the medium ("B"). These two types of fractured creations feature sharp contours. The third type of characteristic fractured surfaces represents vesicles that are broken through ("C"), leaving their bottom parts in the medium. Instead of being sharp, the contours of these are rather wide, corresponding to the wall thickness of the vesicles.

Taking a closer look at the electron micrographs, we can see that the outer surfaces of the vesicles are smooth (Figure 4(a)). The limited roughness of these areas, and also that of the inner surface of the remaining vesicle part, is the consequence of the platinum grains formed during the shadowing procedure, with a mean size of approx. 1-2 nm (Figure 4(b)).

In the trypsin-containing vesicles, a similar surface pattern can be observed in the outer convex and concave surfaces (Figure 4(c)), but a distinct morphological feature emerges in the third type of fracturing. The fractured surface

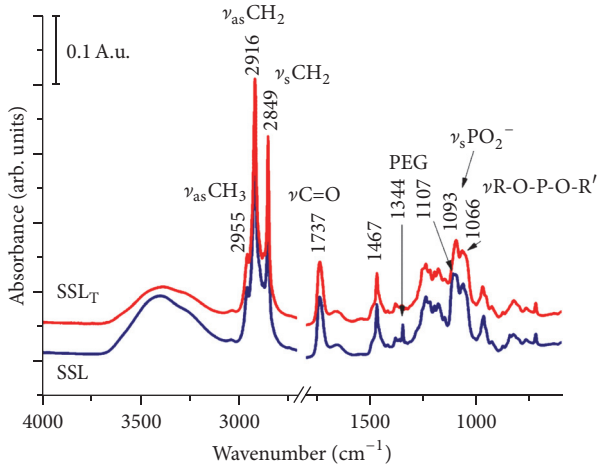

FIGURE 5: Dry film spectra of trypsin-loaded (SSL_T) and empty (SSL) liposomes.

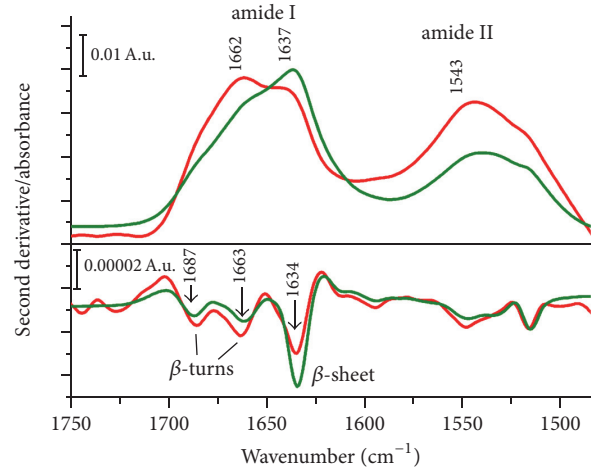

FIGURE 6: Amide I spectral region of trypsin before (green) and after (red) encapsulation in liposomes. The second derivatives of the measured spectra were obtained by the Savitzky-Golay method (3rd grade polynomial, 5 smoothing points).

morphology of the loaded vesicles exhibits a bit higher surface roughness: in the matrix around the vesicles the grain size is about 2 ± 1 nm, while inside it is about 4 ± 1 nm as seen in higher magnification image (Figure 4(d)). This observation indicates that the trypsin molecules are attached to the inner surface of the vesicles and are presumably not dispersed in their inner/aqueous core.

IR spectroscopy is suitable to reveal possible enzyme-lipid interaction by analysis of small spectral changes (band position and intensity) of both lipid- and enzyme-related IR bands. Figure 5 shows the ATR-FTIR spectra of empty and trypsin-loaded liposomes recorded as dry films (after slow evaporation of buffer solvent on the top of the diamond ATR element).

Subtracting the spectra reveals the presence of amide I and amide II bands around 1653 and 1547 cm^{-1}, respectively, corresponding to the presence of trypsin in trypsin-loaded liposomes (Figure 6, SSL_T spectrum). FTIR spectroscopy is widely applied to study protein secondary structure and aggregation. In particular, the amide I band (1700–1600 cm^{-1}), corresponding mainly to the C=O stretching vibration of the peptide bond (~80%), is sensitive to the protein backbone conformational changes. Figure 6 shows the amide I spectral region of trypsin spectra before (in solution) and after encapsulation in liposomes. Trypsin is classified as a "β-protein" with dominant content of β-sheet and β-turn conformations [38]. After encapsulation, the relative amount of β-sheet conformers is slightly decreased, while the band component corresponding to turns and β-turns structure is slightly increased, reflected also in the shape of the amide I band envelope.

To get information about the spectral changes linked to the lipid molecules/bilayer, spectra recorded as suspension were analyzed after buffer subtraction. The C-H stretching region of the IR spectra (3020–2800 cm^{-1}) involved mostly the fatty acyl chains of the lipids (not shown). No differences were observed for trypsin-loaded and empty liposomes, suggesting that the order/disorder of the acyl chains remained intact. Concerning the lipid head group spectral region (1800–900 cm^{-1}), no changes in the phosphate vibrations (νasPO_2^- at 1234 cm^{-1}, νsPO_2^- at 1088 cm^{-1}, and νR-O-P-O-R′ around 1070 cm^{-1}) and in the antisymmetric C-N^+ stretching vibrations (971 cm^{-1}) of the choline groups could be revealed.

The lipid carbonyl ester group is situated in the interfacial region of the lipid bilayer and is a potential H-bond formation site. In highly hydrated phospholipid bilayers, this band splits in two overlapping components: a high wavenumber band around 1742 cm^{-1} of the non-hydrogen-bonded C=O groups and a low wavenumber band around 1728 cm^{-1} due to the hydrogen bonding of the C=O groups [39]. Detailed analysis of the νC=O band around 1737 cm^{-1} (Figure 7) indicated an increase of the relative amount of H-bonded C=O subpopulation: A (νC=O H-bonded)/A (νC=O free) from 2.5 for SSL to 5.2 for SSL_T. These data suggested that trypsin was located close to the lipid hydrophilic/hydrophobic interface driven by weak interaction (H-bonds).

In summary, the IR spectroscopic data evidence that the presence of trypsin molecules does not distort the acyl chain order of the lipid bilayers composing the liposomes despite the weak interaction-driven (H-bonds) location of the encapsulated trypsin close to the lipid hydrophilic/hydrophobic interface.

Scattering techniques, especially small-angle X-ray scattering (SAXS), are a powerful tool for identifying various structural forms on the nanometer scale. To follow the trypsin-induced changes in the layer structure of the liposomes, SAXS measurements were performed. The one-dimensional scattering patterns of the empty and trypsin-loaded vesicles are shown in Figure 8. The scattering in the initial range of the variable is intense and decreases monotonously in both systems. This pattern originates from the scattering of domains spanning a size range of several, up to hundreds of nanometers. The two vesicle systems exhibit

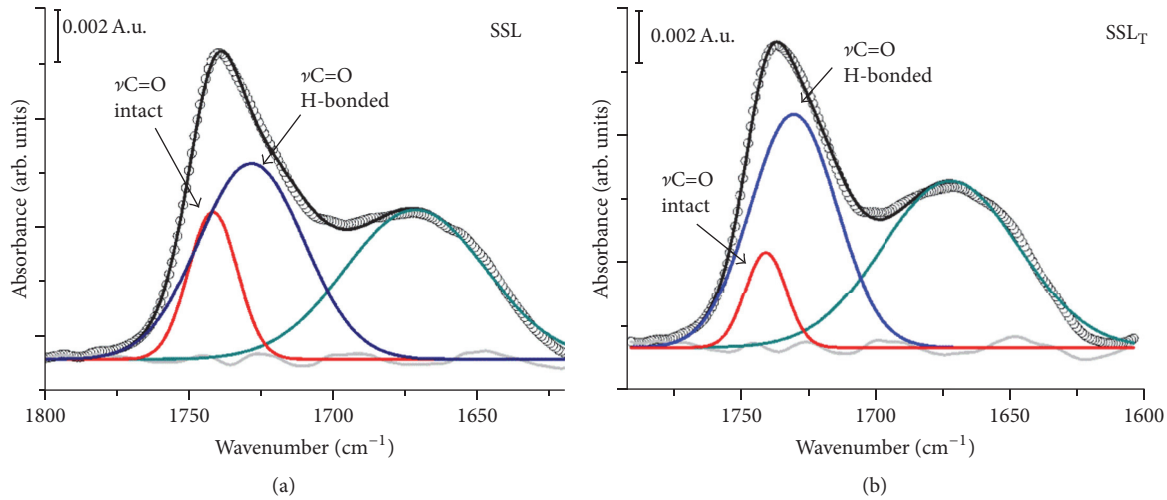

FIGURE 7: Lipid ester carbonyl stretching band region for empty (SSL) and trypsin-loaded (SSL$_T$) liposomes. The solid black lines are the measured spectra; the blue and the red lines are the fitted bands corresponding to intact and H-bonded C=O groups, respectively. The green band component can be attributed to residual water (after subtraction) and to amide I band positions for curve fitting using the second derivative; band shapes were approximated by Lorentzian functions. The intensities and the bandwidth of each component were optimized according to a χ^2 minimization procedure. The relative contribution of each of the particular components was calculated from their integrated areas in the best fit.

a similar scattering pattern with a broad peak in the q-range from 0.4 to 2 nm^{-1}, which can be attributed to the scattering of the lipid bilayers, known as a form factor of the bilayer. However, there is a slight but important difference between the curves: the local minimum of the trypsin-containing vesicle is not as deep as in the case of empty vesicles.

The scattering of trypsin in its solvated form shows a monotonously decreasing SAXS pattern that is typically seen in the scattering of uncorrelated, nearly globular particles and corresponds to the nearly spherical shape of the trypsin molecule with a 3 nm diameter (Figure 8).

The SAXS curve of the trypsin-containing vesicle can be interpreted to the first approximation as the sum of the contributions of the empty vesicle and the trypsin. There is a slight difference, however, between the generated composite and the measured scattering of SSL$_T$ curves (Figure 8). The difference is significant in the regime of the form factor, indicating the change in the bilayer structure and the association between the bilayer and the protein.

The scattering pattern of SSL$_T$ can be fully reconstructed by a spherical shell model, enabling an approximate description of the layer structure (see Supplementary Material available online at https://doi.org/10.1155/2017/5130495). The model for the least-squares fitting of the bilayer scattering involves two identical, symmetrically placed Gaussian functions for the two head group regions, one Gaussian for the carbon chain region and two independent Gaussians for the guest molecules on the inner and outer side of the bilayer. The size distribution of the vesicles is assumed to be Gaussian.

The best fit (Supplementary Material) yields an asymmetric distribution in the head region of vesicle bilayer; therefore we may conclude that the protein molecules localize close to the head region. The thickness of the inner region of guest molecules (rich in trypsin) can be approximated by

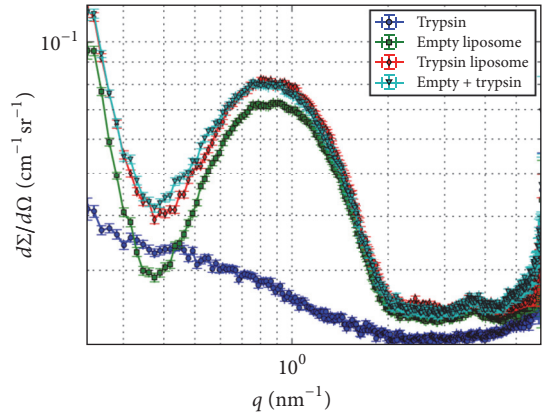

FIGURE 8: The one-dimensional scattering patterns of liposomes with (red) and without (green) trypsin. The scattering of trypsin in free solution (blue) and the curve generated as a sum of the scattering of empty liposomes and solved trypsin (cyan) are also shown.

the sum of the radial displacement of the maximum of the corresponding Gaussian and its half width at half maximum (HWHM) which is 3.73 nm + 1.74 nm ≈ 5.5 nm. The SAXS fit has been executed without any a priori assumptions and the results are in agreement with those obtained by IR, namely, the fact that trypsin is located at the inner leaflet of bilayer in the vicinity of the lipid head group.

4. Conclusions

We have developed a phospholipid-based thermosensitive nanocarrier, in which trypsin is attached to the inner leaflet of the bilayer shell of the liposome. Our findings also

represent a unique physicochemical description of a protein encapsulated in a lipid vesicle and are important in order to appreciate that the enzyme action depends on heat-dependent release and not simply on attachment to the outer surface of the vesicle. The fibrinolytic efficiency of these liposomes is improved in the dynamic fibrinolytic assay under conditions of permeation-driven fibrinolysis. Because intravascular thrombi are exposed to permeation forces [40, 41], these properties of our construct suggest that it could be a successful candidate as a therapeutic tool, the utility of which deserves further investigation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Prothrombotic Fibrin Clot Phenotype in Patients with Deep Vein Thrombosis and Pulmonary Embolism: A New Risk Factor for Recurrence

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Prothrombotic fibrin clot phenotype, involving faster formation of dense meshwork composed of thinner and highly branched fibers that are relatively resistant to plasmin-induced lysis, has been reported in patients with not only myocardial infarction or stroke, but also venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT), and/or pulmonary embolism (PE). Prothrombotic fibrin clot phenotype, in particular prolonged clot lysis time, is considered a novel risk factor for VTE as well as venous thrombosis at unusual location, for example, cerebral sinus venous thrombosis, retinal vein obstruction, and Budd-Chiari syndrome. Growing evidence from observational studies indicates that abnormal fibrin clot properties can predict recurrent DVT and PE and they are involved in serious complications of VTE, for example, thromboembolic pulmonary hypertension and postthrombotic syndrome. The purpose of this article is to review our current understanding of the role of fibrin clot structure and function in venous thrombosis with emphasis on clinical issues ranging from prognosis to therapy.

1. Introduction

Fibrinogen is converted to fibrin by thrombin [1, 2]. Thrombin-mediated release of fibrinopeptide A (FPA) and FPB from the N-termini of the Aα- and Bβ-chains, respectively, results in the formation of fibrin monomer $(\alpha, \beta, \gamma)_2$. At the first stage of polymerization of fibrin monomers half-staggered and double-stranded protofibrils are formed supported by "A:a" interactions and then there is the assembly of protofibrils into fibers that are composed of thousands of protofibrils arranged side-by-side. Lateral aggregation is associated with FPB release and is likely caused by release of the C-termini of the Aα-chains which interact and form bridges between protofibrils. Lateral aggregation is promoted mainly not only by intermolecular αC:αC interactions between protofibrils, but also probably by interactions between both α- and γ-chains [1, 2].

Formation of covalent cross-links formed by activated factor (F)XIII improves elasticity of fibrin clots and resistance to enzymatic degradation [3]. Dissolution of a fibrin clot is mediated by the interaction of tissue plasminogen activator (tPA) and plasminogen on fibrin fibers, which enhances the conversion of plasminogen to plasmin by tPA. Plasmin cleaves Lys-X and Arg-X bonds in the fibrin molecule. Plasminogen and tPA bind to lysine residues exposed by proteolysis of fibrin by plasmin. Plasmin that is bound to fibrin is relatively protected from inhibition by α_2-antiplasmin bound to fibrin by FXIIIa. A key inhibitor of tPA is plasminogen activator inhibitor-1 (PAI-1). Additional fibrinolysis inhibitor, thrombin activatable fibrinolysis inhibitor (TAFI), downregulates plasminogen activation by removing plasmin-binding C-terminal lysine and arginine residues on fibrin [3].

Fibrin structure itself directly affects the rate of fibrinolysis, reflected by faster lysis of looser fiber networks regardless of the fiber thickness, which might affect the thrombotic risk [3, 6]. This effect of fibrin structure on lysis is mediated both by differences in accessibility of the clot to fibrinolytic proteins and differences in the binding of tPA and plasminogen to clots with different structures [7].

2. Evaluation of Fibrin Clot Characteristics

Architecture of a fibrin clot can be characterized by (1) the pore size typically estimated using clot permeability or

permeation (Darcy's constant, or K_s), a measure of clot surface allowing flow through networks under different hydrostatic pressures; (2) the lag phase by turbidimetry that reflects the time to the start of lateral protofibril aggregation; (3) maximum absorbance of a clot that reflects an average fibrin fiber thickness. Clot turbidity is related to the number of fibrin fibers, their thickness, the number of branching points, and the uniformity of fiber distribution [8–10]. The size of the pores in the fiber network or the density of clots can be assessed visually on scanning electron microscopy (SEM) or confocal microscopy images. The stiffness of fibrin clots generated from purified fibrinogen, plasma, or whole blood is evaluated using rheometry and its measure is the maximum elastic storage modulus. Other parameters of the clot including the fractal dimension reflecting its structural complexity can also be calculated using this approach usually in whole blood clots [11].

Since more than 30 years it has been proven that fibrin networks composed of thinner, highly branched fibers usually are less permeable, more rigid, and less susceptible to lysis [10, 12, 13]. The so-called prothrombotic fibrin clot phenotype involves faster formation of dense meshwork (reflected by lower K_s values) composed of thinner and highly branched fibers that are relatively resistant to plasmin-induced lysis. Contrary, fibrin networks composed of thick fibers have larger pores, which results in functional assays to increased permeability and susceptibility to fibrinolysis [10, 12, 13].

Nowadays the most commonly used assay to assess efficiency of fibrinolysis in patients is the measurement of clot lysis time (CLT) developed by Lisman et al. [14] in 2001. In this assay blood clotting is triggered by tissue factor (TF) in the presence of phospholipid vesicles and fibrinolysis is activated by addition of recombinant tPA together with TF [12]. Other assays to test fibrinolytic capacity use varying tPA concentrations with or without addition of thrombin before or after fibrin gel formation.

Fibrin clot properties can be studied when thrombin or other clotting activators are added to purified or recombinant fibrinogens. However, assays in which clots are generated from citrated plasma represent the most commonly used approach in large groups of patients. Structural and functional differences between plasma fibrin clots and those made from purified fibrinogen have been well documented. For example, fibrin network formed from citrated plasma is composed of thicker fibers making looser meshwork compared with that formed from purified fibrinogen [10, 12].

In summary, plasma fibrin clot permeability and clot lysis are valuable measures of clot structure and function, which can be used in evaluation of various diseases. However, these methods are not standardized and large interlaboratory differences are observed.

3. Modifiers of Fibrin Clot Properties

Experimental and clinical studies have identified a number of modifiers of clot structure, both genetically and/or environmentally determined, that in most cases are implicated—in a direct or indirect manner—in the occurrence of the prothrombotic fibrin clot phenotype (Figure 1).

Most of these factors are associated with increased risk of venous thrombosis. A key regulator of fibrin structure is the concentration of fibrinogen and its function. Variation in fibrinogen concentrations explains only up to 18% of the variation in clot permeability [15]. Other recognized modulators of fibrin structure involve acute phase proteins including C-reactive protein which rises together with fibrinogen in several common diseases associated with elevated risk of thrombosis (Figure 1).

It has been established that genetic factors explain 10–50% of variance in fibrin clot measures [16]. About 25% of rare congenital dysfibrinogenemias resulting from mutations in all three fibrinogen genes, despite low levels of functional fibrinogen measured using the Clauss assay, are linked with VTE and at least some of them are known to significantly alter fibrin clot structure [17]. The incomplete penetrance of the thrombotic phenotype in some dysfibrinogenemic subjects with the same genotype is highly suggestive of other genetic or environmental confounders. A stronger impact on fibrin clot characteristics could be related to common genetic polymorphisms largely in genes encoding three fibrinogen chains. A common β-chain polymorphism, due to a substitution of Lys with Arg at residue 448, has been shown to affect the clot structure [18], but not all studies demonstrated it [19]. Aα fibrinogen Thr312Ala polymorphism has been reported to increase FXIIIa-catalyzed reactions and to lead to the formation of thicker fibrin fibers [20]. A functional splice variant of the γ-chain, fibrinogen γ', with 20 new residues that contain binding sites for both thrombin and the FXIII B-subunit is also linked with an unfavorable prothrombotic clot phenotype [21]. Finally, FXIII Val34Leu significantly alters fibrin structure resulting in the formation of a clot with smaller pores and thinner fibers [22]. At high plasma fibrinogen concentrations, the FXIII Leu34 variant showed a protective effect through the formation of more permeable fibrin clots that are more susceptible to lysis, while at low fibrinogen concentration the effect was reversed, suggesting that protection against thrombosis by FXIII 34Leu only occurs in hyperfibrinogenemia [23]. Taken together, a role of common genetic factors in fibrin properties appears to be minor as compared to a broad spectrum of potent acquired factors largely driven by their complex proinflammatory and prothrombotic effects.

Wolberg et al. have shown that fibrin fiber mass-to-length ratio decreases with increasing prothrombin levels (above 100% of plasma levels), in a dose-dependent manner [24]. In both purified fibrinogen and plasma-based systems, clots produced in the presence of high thrombin concentrations are characterized by thin fibers that form a network with small pores [25].

Lipoprotein(a) that contains apolipoprotein(a), apo(a), with domains homologous with the kringle domains IV and V of plasminogen, has been found to be associated with lower clot permeability composed of thinner fibers and impaired susceptibility to fibrinolysis [26]. Small apo(a) isoforms are associated with abnormal clot characteristics and hypofibrinolysis [26].

A number of common diseases, including those known as the risk factors for thrombosis, have been demonstrated

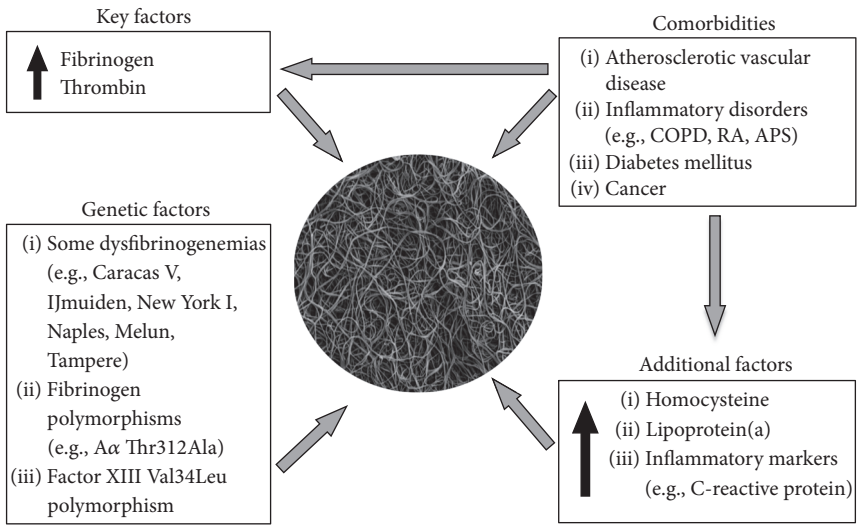

Figure 1: Factors that modify normal plasma fibrin clot phenotype to prothrombotic phenotype. A plasma fibrinogen concentration is a major determinant of clot properties. The larger fibrinogen concentrations, the denser fibrin networks. Alterations to fibrinogen function could be genetically determined and acquired largely associated with posttranslational modifications, for example, glycation or homocysteinylation. Environmental factors have a larger impact on clot phenotype and lysability, largely related to enhanced inflammation and thrombin generation observed in several common chronic diseases, including cancer and rheumatic disorders that represent well-established risk factors for venous thrombosis. COPD, chronic obstructive pulmonary disease; RA, rheumatoid arthritis; APS, antiphospholipid syndrome.

to deteriorate plasma fibrin clot phenotype by rendering them denser and resistant to lysis (Figure 1). Mechanisms underlying these associations are the consequence of post-translational modifications such as oxidation, glycation, and homocysteinylation (often combined like in diabetes) and binding proteins to the fibrin fibers that can confound physiological interactions, for instance, with proteins involved in fibrinolysis [27–29]. The most common diseases related to thrombosis are inflammatory by nature. Reduced clot permeability and susceptibility to lysis have been observed in rheumatoid arthritis [30], chronic obstructive pulmonary disease [31], and inflammatory bowel disease [32]. Binding of C-reactive protein (CRP) with fibrinogen and fibrin [33] is likely to contribute to the prothrombotic clot phenotype as evidenced by correlations of CRP with plasma clot permeability and lysis [30–32].

4. Fibrin Clot Properties Assessed In Vitro in Patients with VTE

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), affects 1 to 3 per 1000 persons per year [34–36], which renders this entity a common medical condition associated with significant morbidity and mortality [37].

Growing evidence from multiple studies using various plasma-based assays indicates that both DVT and PE are associated with altered fibrin clot properties including impaired fibrinolytic capacity [10, 12, 38, 39]. The most compelling evidence for involvement of plasma fibrin clot characteristics in the pathogenesis of VTE is indirect and comes from several studies exploring efficiency of clot fibrinolysis. Impaired clot lysability is in part related to abnormal clot structure, which is commonly reflected by an inverse association between K_s and CLT reported in many disease states [10, 12]. Curnow et al. [40] showed that hypercoagulable patients with arterial thrombosis or VTE, pregnancy complications, or autoimmune diseases have increased fibrin generation and reduced fibrinolysis. Hypofibrinolysis measured in plasma using CLT values introduced by Lisman et al. [14] has been shown in subjects following the first DVT episode [41]. An increase in risk for DVT in patients with hypofibrinolysis and a 2-fold increased risk of DVT in subjects with CLT above the 90th percentile have been observed [41]. 77% of variation in CLT in venous thrombosis patients can be attributed to levels of PAI-1, TAFI, prothrombin, and α_2-antiplasmin, but not to plasma fibrinogen [42].

Analysis of VTE patients demonstrated that three established risk factors for VTE, namely, oral contraceptives, immobilization, and the presence of FV Leiden, markedly increase the risk associated with the longest CLT; hypofibrinolysis with these factors gives 20-, 10.3-, and 8.1-fold increases in the VTE risk, respectively, compared with individuals with the shortest lysis time without the risk factors [43].

In 2009 unfavorably altered plasma fibrin clot properties were documented in patients with prior unprovoked VTE [44]. After excluding known thrombophilia, cancer, trauma, surgery, pregnancy, and other established risk factors, VTE patients have been found to have lower plasma clot permeability, higher maximum clot absorbance, and prolonged clot lysis than controls free of thrombotic events, while similarly unfavorable abnormalities were in relatives of VTE patients, which indicates genetic background of abnormal fibrin clot properties in VTE with no history of thrombotic events [44]. This study might suggest that abnormal plasma fibrin

characteristics represent novel risk factors for idiopathic VTE. However, long-term cohort studies are needed to validate this hypothesis. Higher maximum clot absorbance usually inversely correlated with clot permeability is considered a marker of thicker fibers [8]. However in contrast to many in vitro studies in which clots composed of thinner fibers are more resistant to fibrinolysis, plasma clots from real patients with thrombotic manifestations can be composed of fibers with a similar or larger thickness as compared to the control subjects, but they are less permeable and poorly lysable. Patients following VTE represent such individuals, which suggests that plasma clots possess a far more complex structure in terms of its functional consequences.

Traby et al. [45] have reported that there is a weak association between CLT and risk of VTE recurrence only in women, who experienced a first unprovoked VTE without cancer or thrombophilia and were followed for an average of 46 months after anticoagulation withdrawal.

Using a global assay of fibrinolysis that tested the patient's blood fibrinolytic capacity by application of the euglobulin fraction of plasma to a preformed clot of plasminogen-rich bovine fibrin, Skov et al. [46] demonstrated that fibrinolytic capacity is impaired in VTE patients below 50 years compared with young stroke survivors and this difference remained significant after adjustment for multiple confounders including PAI-1.

A history of provoked and unprovoked DVT episodes has been later shown to be associated with unfavorable fibrin plasma clot properties [47]. It remains to be established whether unfavorable plasma fibrin clot phenotype may predispose to provoked DVT.

We have demonstrated that residual vein obstruction following DVT is linked with faster formation of denser plasma fibrin clots displaying impaired lysability [48]. These patients had 14% lower clot permeability and 11% longer lysis time, with no differences related to thrombophilia and duration or stability of anticoagulant therapy [48].

It remains unclear whether plasma fibrin clot features could predict recurrent VTE episodes. Regarding fibrinolytic parameters, in a two-centre case-control study performed in English and Dutch patients, hypofibrinolysis, defined as CLT values (at 3 months after discontinuation of the anticoagulant therapy) above the 90th percentile calculated in control subjects (>122 min), was associated with a 1.8-fold increased risk of a first VTE, while in the follow-up study the relationship between a recurrent VTE and hypofibrinolysis was estimated as weak (hazard ratio 1.5, $p > 0.05$) [49]. A prognostic value of plasma fibrin clot phenotype following DVT alone has been shown recently [50]. We have observed that subjects with recurrent DVT during follow-up were characterized by slightly lower plasma clot permeability and 15% longer CLT measured at 3 months since the index event compared with the remainder (Figure 2) [50].

Regarding PE, Martinez et al. have reported that clots made from plasma of patients with acute isolated PE were characterized by faster CLT and lower fiber density when compared with DVT alone [4]. Absent perfusion in some segments of the pulmonary arteries (perfusion defects) following

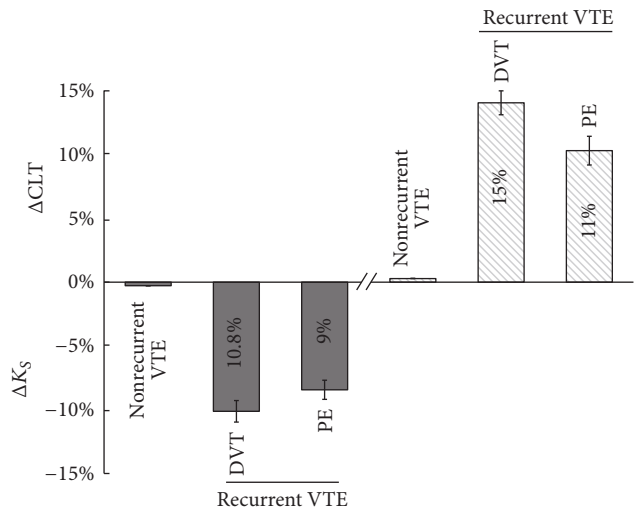

Figure 2: Recurrent VTE episodes during follow-up in relation to plasma fibrin clot properties measured after 3–6 months since the index event (based on [4, 5]). Lower clot permeability and prolonged clot lysis have been reported in patients who experienced VTE recurrences after anticoagulation withdrawal, with a larger impact on deep vein thrombosis. Data are shown as difference between means and confidence interval ranges in %. VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism; K_s, fibrin clot permeability coefficient; CLT, fibrin clot lysis time.

PE detected on angiography has been shown to be associated with impaired fibrinolysis [51].

We have demonstrated recently the association of plasma clot properties and the risk of PE recurrence during a 4-year follow-up [52]. These associations were observed in subjects who discontinued anticoagulant therapy after the first episode of provoked or unprovoked PE (Figure 2). It is possible that screening for the prothrombotic plasma fibrin clot phenotype together with plasma D-dimer and thrombin generation may help identify patients at high risk of recurrent PE.

Analysis of thrombotic material removed from the right atrium and pulmonary (lobar and segmental) arteries of a patient with acute severe PE who underwent surgical embolectomy showed that distally located thrombi contain more densely packed fibrin fibers compared with the proximal thrombi from the lobar pulmonary arteries and the right atrial thrombus [5]. It indicates that abnormal fibrin is present in thrombi retrieved from patients with DVT and the subsequent PE.

In summary, DVT and/or PE are associated with the prothrombotic plasma fibrin clot phenotype especially reduced clot permeability and lysability, and these features might have a prognostic value both in the prediction of first and recurrent episodes.

5. Venous Thrombosis at Unusual Location

Studies on clot properties in patients who developed thrombosis at unusual location suggest that prothrombotic plasma clot phenotype contributes to such thromboembolic events,

indicating that venous thrombosis is associated with certain common fibrin characteristics and the location of the event is largely driven by anatomical peculiarities or abnormalities.

The Budd-Chiari syndrome is a rare disorder caused by obstruction of the hepatic outflow tract, and its classic form involves thrombosis of one of the hepatic veins. This disease has been shown to be linked to less efficient fibrinolysis in part associated with elevated PAI-1 activity, but not with TAFI [53]. Marked differences in clot lysis between Chinese and European patients with this syndrome have been reported [54]. Data on clot architecture in this syndrome have not been published, but based on a significant inverse association of CLT with plasma clot permeability observed in most studies, it might expected that denser fibrin networks assessed in vitro are formed at least in some patients with this disease.

Cerebral sinus venous thrombosis (CSVT) is a rare disorder. Its recurrent episodes have been observed more frequently in patients with unfavorable clot phenotype; CVST was associated with 21% higher baseline fibrinogen, 20% lower plasma clot permeability and 17% greater fibrin mass within a clot [55]. Altered fibrin clot properties similar to those found following CVST have also been reported in patients who experienced retinal vein occlusion [56]. Prolonged lysis time associated with increased endogenous thrombotic potential following retinal vein thrombosis has been confirmed by Italian investigators [57].

In summary, prior thrombosis at unusual location may be linked to the prothrombotic plasma fibrin clot phenotype.

6. Inherited and Acquired Thrombophilia

It is estimated that genetic factors are responsible for up to 25% of unprovoked VTE [58]. Factor V Leiden is the most common inherited thrombophilia that occurs in 5% of Europeans. A heterozygous form of FV Leiden, perceived as a mild thrombophilic defect, has been reported to be associated with impaired efficiency of lysis in apparently healthy women below 50 years, which is independently predicted by the TAFI activity, and it has no significant effect on the fibrin network structure, reflected by clot permeability [59]. The FV Leiden paradox, that is, a relatively lower PE risk compared with high DVT risk in carriers, has not been elucidated [60]. Until now no evident fibrin clot-related mechanisms have been reported as a potential explanation of this paradox.

The G20210A prothrombin mutation is the second most common inherited abnormality that is found in 4.6–8% of patients after VTE and in 0.7–2.6% of the general white population [58]. The G20210A mutation carriers have about 20–25% higher prothrombin levels than noncarriers. Analysis of 32 carriers versus 30 noncarriers showed impaired fibrinolysis reflected by 14.5% longer CLT but this difference disappeared after TAFI inhibition [61]. They also confirmed that addition of prothrombin to normal plasma to raise prothrombin level to 125% and 150% results in longer CLT [61]. Our data indicated that also clot permeability is reduced in the G20210A prothrombin mutation carriers (A. Undas, unpublished data).

Genetically determined deficiencies of natural anticoagulants, that is, antithrombin, protein C, and protein S, increase the risk of a first VTE by at least 10-fold and are observed in <0.5% of the general white population with the incidence up to 5% among subjects with unprovoked VTE [58]. It is unclear whether such abnormalities alter fibrin clot properties. Reduced clot permeability and lysability have been reported in a single patient with antithrombin deficiency with normalization of clot permeability upon addition of antithrombin to plasma [62].

Antiphospholipid syndrome (APS) is a systemic autoimmune disease associated with thrombotic complications, including VTE, in the setting of detectable antiphospholipid antibodies [63, 64]. APS patients were characterized by higher maximum plasma clot absorbency, lower clot permeability, shorter lag phase, prolonged clot lysis time, lower maximum rate, and higher maximum level of D-dimer released from clots, also after adjustment for fibrinogen, body mass index (BMI), and smoking status [65]. There were no differences in plasma fibrin clot characteristics between primary and secondary APS. Patients with "double" or "triple" antibody positivity had less permeable plasma clots compared with those with one positive antibody, with no difference in clot lysis. Patients who experienced PE formed plasma fibrin clots of higher permeability and lysability than those with DVT alone. Clots generated from plasma of APS patients who experienced stroke and/or myocardial infarction were less permeable and were lysed slower compared with those with VTE alone [65]. This study is the first to show that APS is associated with prothrombotic plasma fibrin clot phenotype, with worse characteristics in patients following arterial thrombosis. Low clot permeability in APS was confirmed by Vikerfors et al. [66] and moreover, prolonged fibrinolysis was observed in particular in APS patients with previous arterial thrombosis. A role of microparticles in abnormal clot structure in APS patients has also been suggested [66]. Prothrombotic plasma clot properties in young or middle aged APS subjects following thrombotic events have been shown to be linked to progression of atherosclerosis measured in carotid arteries, which highlights again associations between fibrin-mediated mechanisms observed in both atherosclerosis and thrombosis [67].

In summary, there is evidence that most inherited thrombophilias and APS are associated with unfavorable plasma fibrin clot properties, which might contribute to their thromboembolic clinical manifestations.

7. Complications of VTE

Chronic thromboembolic pulmonary hypertension (CTEPH) is a severe complication that occurs in 1–4% of patients following PE. It has been reported that fibrin clots made from fibrinogen purified from patients with CTEPH are in part resistant to plasmin-mediated lysis as compared to healthy individuals [68]. This observation suggests that structural or functional abnormalities in fibrinogen molecules and the subsequent fibrin properties may be involved in the development of CTEPH by prolonged fibrin clot presence within the pulmonary arteries and stimulation of remodeling of the thrombi into fibrotic intravascular material. Interestingly, analysis of purified fibrinogen and fibrinogen

gene sequencing of patients with CTEPH showed a relatively high incidence of inherited dysfibrinogenemias in which abnormal fibrin clot structure and lysis were described [69]. Moreover, it has been reported that patients with these CTEPH-associated dysfibrinogenemias have low clot turbidity, decreased porosity, and fibrinolytic resistance, along with disorganized fibrin networks composed of thinner fibers and more extensive fiber branching [70]. Since abnormal clot architecture and fibrinolytic resistance may contribute to incomplete clot resolution in this form of CTEPH, clot properties may help identify patients at risk of this complication. Given increased therapeutic options in CTEPH and their benefits [71, 72], efficacy of the therapy can also be in part determined by fibrin clot characteristics.

The most common complication of DVT is the post-thrombotic syndrome (PTS) observed in 20–50% of patients within the first 1-2 years after the index event [73]. Patients who developed PTS suffer from light pain, occasional swelling, and venous ectasia, but with time they complain of chronic pain, tough edema, skin induration, and leg ulcer, which decrease the quality of life. The pathophysiology of PTS is not fully understood, but most investigators highlight a key role of systemic inflammation with the subsequent tissue remodeling and fibrosis [74]. Recently we have demonstrated that prothrombotic plasma clot phenotype, in particular lower clot permeability determined 3 months since the index event, predisposes to PTS [50]. Severe PTS was associated with more unfavorable clot variables, including almost 20% lower plasma clot permeability [50]. The Villalta scale, a measure of the severity of PTS, showed associations with clot permeability and lysability. We provided evidence that denser plasma fibrin clot formation and their impaired lysis may allow identifying patients following DVT who are likely to develop PTS.

8. Antithrombotic Agents and Fibrin Clot Properties

Anticoagulation is the primary approach to therapy of VTE. Heparins are commonly used as the first-line therapy in acute VTE, especially in cancer-associated episodes [75]. The current guidelines recommend lifelong anticoagulation for patients after a second unprovoked VTE and in most subjects after first episodes [36]. Vitamin K antagonists (VKA) have been the standard of care in VTE for 60 years. Nonvitamin K oral antagonists (NOACs) have shown similar efficacy and improved safety profile when compared to warfarin in VTE and they are now used worldwide [36].

It is easy to foresee that all anticoagulant agents that decrease thrombin generation and consequently fibrin formation may improve plasma fibrin clot properties. Unfractionated and low-molecular-weight heparins have been shown to improve clot properties as evidenced by analysis of nanostructure of fibrin clots in the absence of antithrombin [76].

Warfarin administration when International Normalized Ratio is in the range of 2-3 increased plasma fibrin clot permeability by 28–50% compared with the control clots [77]. Similar increases in clot permeability can be observed at therapeutic plasma concentrations of fondaparinux and apixaban (by 58–76% and 36–53%, resp.) [78]. Looser clot structure has also been shown in the presence of argatroban, bivalirudin, lepirudin, and danaparoid [79].

Rivaroxaban can improve clot properties as evidenced by formation of clots composed of thicker fibrin fibers and larger pores with 2-fold increased clot permeability and 3-fold faster lysis compared with the control clots generated in the absence of rivaroxaban [80]. Similar improvement in the presence of rivaroxaban has been reported in whole blood clots [81]. Direct thrombin inhibitors have been demonstrated to increase clot susceptibility to lysis and this effect was in part mediated by TAFI [82]. Reduced thrombin formation and suppressed thrombin-mediated reactions during NOAC treatment appear to account for the formation of less compact and more lysable fibrin clots. Whether the extent of changes in fibrin clots contributes to bleeding risk in VTE remains to be explored.

Low-dose aspirin has also been found to increase clot permeability and susceptibility to lysis, as demonstrated in clots generated from human plasma, purified fibrinogen and in cellular system [83, 84]. Acetylation of fibrinogen molecule is considered a major mechanism underlying this effect [83]. It is unclear whether these actions of aspirin contribute to its clinical outcomes observed also in secondary prevention of VTE.

Statins, a potent cholesterol-lowering class of drugs producing multiple antithrombotic actions which is increasingly used for other indications, have also been reported to increase clot permeability and lysability with no association with reduced cholesterol levels [85, 86]. It has been postulated that modulation of fibrin clot structure and function represents an additional beneficial effect of this widely used medications that have been shown to reduce the risk of VTE [87].

In summary, VKA, NOAC, and other anticoagulant agents favorably alter plasma fibrin clot properties. Less pronounced beneficial fibrin-related effects produce statins and aspirin, and these effects can be detected in vitro. Clinical relevance of these effects is unknown.

9. Concluding Remarks

The so-called prothrombotic characteristics of a fibrin clot, including dense fiber networks displaying reduced plasmin-induced lysability measured in vitro in plasma, are considered a potential novel risk factor for VTE like MI or ischemic stroke. Observational studies have demonstrated that altered plasma clot phenotype, in particular low clot permeability and CLT, characterize patients following VTE and thrombosis at unusual sites as well as those who experienced recurrent VTE or PE during follow-up. The precise molecular mechanisms underlying this association are still poorly understood and involve genetic and acquired factors with a major contribution of increased thrombin generation and inflammation. Of note, plasma fibrin networks are favorably modulated in subjects receiving not only various anticoagulant agents, but also aspirin or statins, which might contribute to beneficial effects of these medications in terms of prevention of VTE.

It remains to be established to what extent unfavorable fibrin clot properties affect the risk of first or recurrent DVT and PE. Further large prospective studies are needed to evaluate a prognostic value of fibrin clot measures in a broad spectrum of venous thromboembolic disorders.

Conflicts of Interest

The author declares that she has no conflicts of interest.

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Research Article

Intracardiac Hemostasis and Fibrinolysis Parameters in Patients with Atrial Fibrillation

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Aims. To identify intracardiac hemostasis or fibrinolysis abnormalities, which are associated with atrial fibrillation (AF) and increase the risk of thromboembolism. *Patients and Methods*. Patient group consisted of 24 patients with AF and control group included 14 individuals with other supraventricular tachycardia undergoing transcatheter radiofrequency ablation. Blood samples were drawn from the femoral vein (FV), left atrium (LA), and left atrial appendage (LAA) before the ablation procedure. Fibrinogen, factor VIII (FVIII) and factor XIII activity, von Willebrand factor (VWF) antigen, thrombin-antithrombin (TAT) complex, quantitative fibrin monomer (FM), plasminogen, α_2-plasmin inhibitor, plasmin-α_2-antiplasmin (PAP) complex, PAI-1 activity, and D-dimer were measured from all samples. *Results*. Levels of FVIII and VWF were significantly elevated in the FV and LA of AF patients as compared to controls. TAT complex, FM, PAP complex, and D-dimer levels were significantly elevated in the LA as compared to FV samples in case of both groups, indicating a temporary thrombotic risk associated with the catheterization procedure. *Conclusions*. None of the investigated hemostasis or fibrinolysis parameters showed significant intracardiac alterations in AF patients as compared to non-AF controls. AF patients have elevated FVIII and VWF levels, most likely due to endothelial damage, presenting at both intracardiac and systemic level.

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia associated with a high risk of mortality and morbidity from stroke and thromboembolism [1]. The precise mechanism by which AF causes thromboembolism and subsequent cerebrovascular events is yet to be fully elucidated. It is widely recognized that thromboembolism in AF is associated with a combination of pathophysiological mechanisms, which fulfill the requirement of Virchow's triad for thrombogenesis: stasis, abnormal change in the vessel wall and pathological imbalance of hemostasis, and fibrinolysis [2]. Although several risk factors (age, hypertension, diabetes mellitus, etc.) predispose to stroke in patients with AF, it has been controversial whether the arrhythmia itself leads to hypercoagulability [3–5]. Nonetheless, it is a well-known fact that anticoagulation therapy reduces the risk of ischemic stroke in AF patients by two-thirds [6]. There are a number of reports available on the activation of coagulation cascade in AF, but fibrinolytic activity in AF patients has been less studied [7–10]. Most studies have focused on the relation of AF-associated thromboembolism with various endothelial damage markers, prothrombotic or inflammatory factors, and plasma markers of platelet activation [4, 8, 11, 12]. It has been shown that AF is associated with increased levels of prothrombin fragments 1 + 2 (F1 + 2) and thrombin-antithrombin (TAT) complex, elevated plasma fibrinogen levels alongside with endothelial markers of soluble thrombomodulin, and von Willebrand factor (VWF) [2, 13–16]. The role of fibrinolysis in the pathomechanism of AF-associated

thromboembolism has been much less studied and controversial findings have been published. AF has been associated with hypofibrinolysis due to increased levels of plasminogen activator inhibitor type 1 (PAI-1) [17], although in other studies hyperfibrinolysis with elevated tissue plasminogen activator (t-PA) [18–22] and plasmin-α_2-antiplasmin (PAP) complex levels were found [23]. Impaired fibrinolysis due to unfavorably altered fibrin clot properties has been also described in AF patients [9]. These reports present data on coagulation and fibrinolytic markers measured in peripheral samples; however, the prothrombotic effects of AF are most likely to develop on a local, cardiac level and may not manifest in the peripheral circulation. Recent studies suggest that there is a difference between the left atrium (LA) and the systemic circulation in AF patients and the thrombogenic pathology is more likely to be restricted to the heart [24, 25]. However, most possibly due to the difficulty of intracardiac blood sampling, in the last decade only few studies, involving a relatively small number of patients, investigated the hemostasis or fibrinolytic system in samples obtained from the LA of patients with AF [11, 26–28]. Unfortunately, these few studies using LA samples published so far examined only a subset of coagulation markers and even less is known about the fibrinolytic system in this respect. As to our knowledge, the levels of blood coagulation factor XIII (FXIII) or α_2-plasmin inhibitor (α_2-PI), although key regulators of fibrinolysis, have not been studied from intracardiac samples as yet. Moreover, in most cases, results obtained from the intracardiac samples of AF patients were not compared to age and sex-matched non-AF intracardiac samples [11, 26]. As the intervention of intracardiac blood sampling is an invasive procedure with a potential effect on the hemostasis system, it is essential to compare results to a population undergoing the same intervention, but not having AF. Furthermore, in some studies patients received unfractionated heparin prior intracardiac blood sampling, which of course has a major impact on the hemostasis system and limits the number of parameters that could be determined from these samples [26]. The investigation of blood samples obtained from the left atrial appendage (LAA) of AF patients is also intriguing, as it has been suggested that the LAA is the most thrombogenic part of the heart in AF, being the most frequent location of embolic thrombi [29]. Nevertheless, available data on coagulation or fibrinolysis markers in LAA samples of AF patients are a rarity.

In this study we aimed to identify local hemostasis and fibrinolysis abnormalities, which are associated with AF and increase the risk of thromboembolism. Intracardiac blood samples taken from the LA and LAA of AF patients and non-AF controls were tested for a comprehensive set of hemostasis and fibrinolytic factors in order to assess AF-associated alterations.

2. Methods

2.1. Study Population. Consecutive patients undergoing radiofrequency ablation for symptomatic paroxysmal or persistent AF (AF group) as well as age- and sex-matched patients with any arrhythmia other than AF requiring left atrial access (non-AF control group) were enrolled in the study. Patients were enrolled between 2013 October and 2015 December. All AF patients were undergoing pulmonary vein isolation (PVI) with phased radiofrequency (RF) or cryoballoon ablation procedure. Non-AF controls were undergoing routine RF ablation of a left atrial substrate (mostly a left-sided accessory atrioventricular pathway).

Inclusion criteria for the AF group were the following: age 18–75 years, documented, symptomatic paroxysmal or persistent AF, failure of at least one antiarrhythmic drug, and patient being willing to sign a written informed consent. Inclusion criteria for the control group were age 18–75 years, documented non-AF arrhythmia including one of the following: left atrial tachycardia, paroxysmal supraventricular tachycardia (orthodromic or antidromic), or FBI (fast, broad, and irregular) tachycardia due to a left-sided accessory pathway, preexcitation on the 12-lead electrocardiogram in an asymptomatic individual in whom the electrophysiology study revealed a left-sided accessory pathway potentially resulting in significant arrhythmia based on its conduction properties, and patient being willing to sign a written informed consent. Exclusion criteria for the patient and control groups were previous heart surgery, valvular heart disease, left ventricular ejection fraction (LVEF) $\leq$ 30%, heart failure of New York Heart Association functional classification (NYHA) class III or IV, documented carotid stenosis, history of ischemic stroke or TIA, prior cardiac surgery, unstable angina or myocardial infarction within the last 3 months, severe chronic obstructive pulmonary disease, known bleeding or thrombotic disorders, acute inflammation, contraindication to oral anticoagulation or to diffusion weighted magnetic resonance imaging (DW MRI), and pregnancy. Additional exclusion criteria for the patient group were long-standing persistent AF, reversible cause of AF (e.g., hyperthyroidism), and presence of AF thrombus.

Risk factors for stroke (hypertension, diabetes mellitus, smoking, BMI, etc.) together with the list of current medications were assessed before the enrollment of patients. The $CHADS_2$ score (congestive heart failure, hypertension, age $\geq$ 75 years, diabetes mellitus, and stroke/transient ischemic attack), CHA_2DS_2-VASC score (congestive heart failure, hypertension, age $\geq$ 75 years, diabetes mellitus, stroke/transient ischemic attack/thromboembolism, vascular disease (prior myocardial infarction, peripheral vascular disease, or aortic atherosclerosis), age (65–74 years), and sex category (female)), and EHRA score (European Heart Rhythm Association score) [30] were recorded for every AF patient.

The study design was in accordance with the guiding principles of the Declaration of Helsinki, and was approved by the Institutional Ethics Committee of the University of Debrecen and the Ethics Committee of the National Medical Research Council (ETT-TUKEB). All patients signed a written informed consent form prior to inclusion.

2.2. Electrophysiology Procedure and Blood Drawing. Patients were hospitalized 1 or 2 days before the procedure. All medications with a potential effect on coagulation or platelet

activity were discontinued for a period of at least three half-lives (or a period needed for reaching complete decay of their action) before the procedure. Transesophageal echocardiography was carried out within 24 h prior to the procedure in order to rule out the presence of a cardiac thrombus in all AF patients. All procedures were carried out under conscious sedation, using midazolam and fentanyl. The ablation procedures were performed as described previously [31, 32]. Blood samples were taken before the ablation procedures from multiple sites: (1) peripheral femoral venous (FV) sheath, (2) left atrial (LA) sheath, and (3) left atrial appendage (LAA) sheath. Intracardiac blood samples were collected before the administration of unfractionated heparin.

Briefly, three punctures of the right femoral vein were performed using the Seldinger technique and introducers with side arms were placed in the vein. Forty-five ml blood sample was drawn through the side arm of a short introducer immediately after access to the vein, from which the first 5 ml of blood was discarded in order to exclude intrasheath hemostasis activation (FV sample). Blood samples were collected into vacutainer tubes (tubes anticoagulated with K_3-EDTA for complete blood count, tubes containing 0.109 M sodium citrate and CTAD (buffered citrate, theophylline, adenosine, and dipyridamole)) for hemostasis and fibrinolysis tests (Becton Dickinson, Franklin Lakes, NJ). After blood drawing, a decapolar catheter and an intracardiac echo (ICE) catheter were advanced from the femoral vein and positioned in the coronary sinus and in the right atrium, respectively. A single ICE-guided transseptal puncture was performed using a Mullins transseptal sheath and a Brockenborough needle (Medtronic, Kirkland, QC, Canada) under fluoroscopic and ICE guidance using standard technique. After crossing the septum, the dilator of the Mullins sheath was removed and 45 ml blood sample was drawn from the LA, from which the first 5 ml of blood was discarded (LA sample). LA blood samples were collected into vacutainer tubes as described above. After the blood drawing of LA samples, the LAA was accessed by using a 5 F pigtail catheter (Medtronic, Kirkland, QC, Canada) under fluoroscopy and ICE control. A blood sample of 45 ml was taken from the LAA, of which, again, the first 5 ml was discarded (LAA sample). LAA blood samples were collected into vacutainer tubes as described above. Immediately after blood samplings, 150 IU/kg body weight i.v. heparin was administered and ablations were performed according to standard protocols.

2.3. Laboratory Investigations. Blood samples anticoagulated with K_3-EDTA were immediately tested for complete blood count. Blood samples anticoagulated with citrate or CTAD were centrifuged twice at 1500 g at room temperature for 20 min and plasma samples were stored at −70°C until further analysis. The measurement of plasminogen activator inhibitor-1 (PAI-1) activity was performed from plasma samples anticoagulated with CTAD; besides this measurement, all hemostasis and fibrinolysis tests were performed using citrated plasma. Hemostasis and fibrinolysis tests were performed from all sample types (FV, LA, and LAA samples). Screening tests of hemostasis (prothrombin time, activated partial thromboplastin time, and thrombin time) were performed using routine methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Fibrinogen concentrations were measured by the Clauss method. Commercially available ELISA tests were used to determine PAI-1 activity (Technozym PAI-1 Actibind, Technoclone, Vienna, Austria), plasmin-α_2-antiplasmin (PAP) complex (Technozym PAP complex ELISA kit, Technoclone, Vienna, Austria), and thrombin-antithrombin (TAT) complex (Enzygnost TAT micro, Siemens Healthcare Diagnostic Products, Marburg, Germany). Factor VIII (FVIII) activity using a chromogenic assay, von Willebrand factor (VWF) antigen level, α_2-plasmin inhibitor (α_2-PI) activity, plasminogen activity and D-dimer levels were measured on a BCS coagulometer by standard methods (Siemens Healthcare Diagnostic Products, Marburg, Germany). Plasma levels of FXIII activity were determined by ammonia release assay [33] using a commercially available reagent kit (REA-chrom FXIII kit, Reanalker, Budapest, Hungary). Soluble fibrin monomer levels (FM) were measured using the Liatest FM assay (Diagnostica Stago, Asnières, France).

High sensitivity C-reactive protein (CRP) and a comprehensive lipid profile including total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were measured from antecubital vein blood samples of all patients upon hospital admission by routine methods (Roche Diagnostics, Mannheim, Germany).

2.4. Statistical Analysis. All data were analyzed using the GraphPad Prism Software version 5.0 (La Jolla, CA) and the Statistical Package for Social Sciences (SPSS, Release 22.0, Chicago, IL). Normality of the data was evaluated by the D'Agostino and Pearson omnibus normality test. A paired t-test or Wilcoxon matched pairs rank-sum test was applied for comparing results obtained from intracardiac and FV samples. In case of two-group analyses between AF patients and controls, unpaired t-test or in case of nonparametric data Mann–Whitney U test was used. ANOVA or Kruskal-Wallis test was applied for multiple comparisons. Pearson's or Spearman's correlation coefficient was used to determine the strength of correlation between variables. Differences between categorical variables were assessed by the Fisher's exact test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Characteristics of AF Patients and Non-AF Controls. Clinical characteristics of the AF patient group and control group are shown in Table 1. In total 32 AF patients and 18 controls were enrolled in the study. Unfortunately, 8 AF patients and 4 controls had to be excluded from the study population due to technical problems arising during the intracardiac blood drawing procedure (clot formation in the sample during the blood drawing procedure, clot formation on the sheath requiring instant heparin administration, etc.). In case of 12 AF patients and 8 non-AF controls an LAA sample was not possible to obtain due to technical/anatomic

Table 1: Characteristics of atrial fibrillation (AF) patients and controls.

Variables	AF group	non-AF control group	p value
Number of patients after exclusions	24	14	
Age (years)	56.45 (47.43–59.80)	50.60 (32.80–56.10)	0.061
Male, n (%)	15 (62.50)	10 (71.43)	0.728
BMI (kg/m^2)	29.43 ± 5.52	26.10 ± 4.75	0.068
Cerebrovascular risk factors, n (%)			
Arterial hypertension	13 (54.17)	8 (57.14)	1.000
Hypercholesterolemia	19 (79.17)	8 (57.14)	0.266
Current smoking	2 (8.33)	6 (42.86)	0.013
Diabetes mellitus	2 (8.33)	0	—
Previous myocardial infarction, n (%)	0	0	—
Previous ischemic stroke, n (%)	0	0	—
Heart failure, n (%)	1 (4.17)	0	—
Left atrium size (mm)	39.71 ± 4.82	36.55 ± 4.80	0.081
AF period during procedure, n (%)	2 (8,33)	—	—
$CHADS_2$ score, n (%)			
0	10 (41.67)	n.a.	—
1	10 (41.67)	n.a.	—
2	4 (16.67)	n.a.	—
CHA_2DS_2-VASC score, n (%)			
0	6 (25.00)	n.a.	—
1	9 (37.50)	n.a.	—
2	5 (20.83)	n.a.	—
3	3 (12.50)	n.a.	—
4	1 (4.17)	n.a.	—
EHRA score, n (%)			
1	3 (12.50)	n.a.	—
2	1 (4.17)	n.a.	—
3	16 (66.67)	n.a.	—
4	4 (16.67)	n.a.	—
Medication, n (%)			
Statin	6 (25.00)	2 (14.29)	0.684
ACEI	8 (33.33)	6 (42.86)	0.729
Beta-blocker	17 (70.83)	9 (64.29)	0.728
Laboratory parameters			
C-reactive protein (mg/L)	1.50 (0.60–2.75)	1.10 (0.13–2.05)	0.281
Total cholesterol (mmol/L)	5.30 ± 1.16	4.69 ± 1.09	0.122
LDL cholesterol (mmol/L)	3.42 ± 0.98	2.79 ± 0.91	0.061
HDL cholesterol (mmol/L)	1.44 (0.32)	1.46 ± 0.42	0.812
Triglyceride (mmol/L)	1.80 (1.23–2.18)	1.4 (0.75–1.95)	0.208
Zero blood group, n (%)	7 (29.17)	2 (14.29)	0.446

Continuous variables are expressed as mean ± SD or median (interquartile range). Categorical variables are indicated as number (percentage), unless otherwise stated. AF: atrial fibrillation; n: number; IQR: interquartile range; SD: standard deviation; n.a.: nonapplicable; BMI: body mass index; $CHADS_2$ score: congestive heart failure, hypertension, age ≥ 75 years, diabetes mellitus, stroke/transient ischemic attack; CHA_2DS_2-VASC score: congestive heart failure, hypertension, age ≥ 75 years, diabetes mellitus, stroke/transient ischemic attack/thromboembolism, vascular disease (prior myocardial infarction, peripheral vascular disease, or aortic atherosclerosis), age (65–74 years), sex category (female); EHRA score: European Heart Rhythm Association score; ACEI: angiotensin converting enzyme inhibitor; LDL cholesterol: low density lipoprotein cholesterol; HDL cholesterol: high density lipoprotein cholesterol.

difficulties. The final numbers of AF patients and non-AF controls included in the study were 24 and 14, respectively (Table 1). No significant differences were observed between the AF patients and non-AF controls regarding BMI and cerebrovascular risk factors except for smoking, which was more frequent in controls. Only two patients experienced paroxysmal AF periods during the procedure. Most AF patients had low or moderate risk for stroke according to the $CHADS_2$ and CHA_2DS_2-VASC score. A similar fraction of AF patients and non-AF controls received statins and

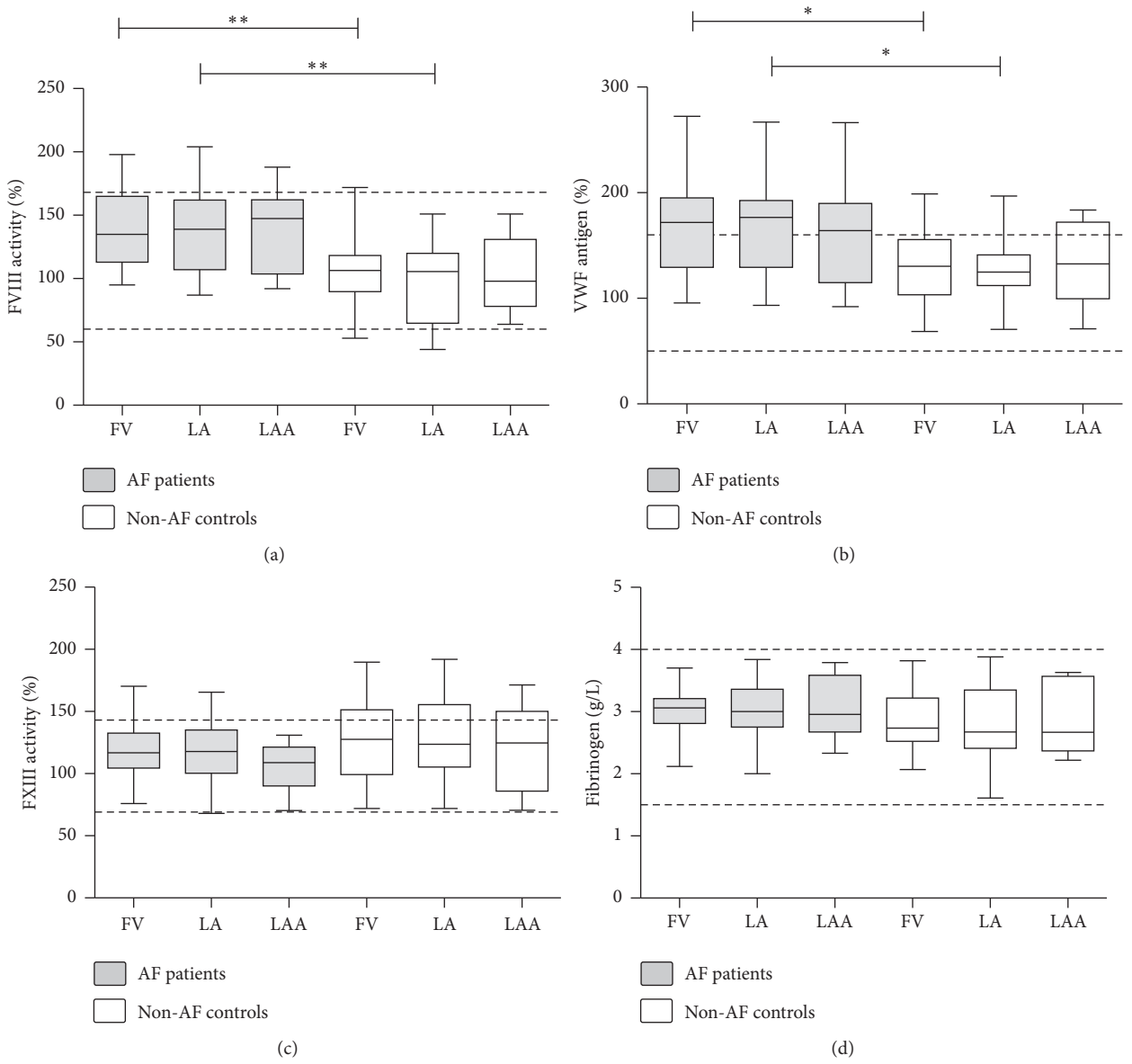

Figure 1: Levels of various coagulation factors in patients with atrial fibrillation (AF) and non-AF controls. Box and whisker plots indicate median, interquartile range, and total range. Dashed lines indicate upper and lower limits of reference interval. FVIII: factor VIII; VWF: von Willebrand factor; FXIII: factor XIII; FV: femoral vein; LA: left atrium; LAA: left atrial appendage. $^{*}P < 0.05$; $^{**}P < 0.01$.

antihypertensive drugs. CRP levels and lipid parameters, measured from peripheral venous blood samples, did not differ significantly between AF patients and non-AF controls.

3.2. Intracardiac Levels of Hemostasis Factors in AF Patients and Non-AF Controls. FVIII activity and VWF antigen levels were significantly higher in the AF patient group as compared to the control group in the samples obtained from the FV and from the LA (Figure 1). LAA levels of both proteins showed a marked elevation in AF patients as well; however, very likely due to the lower number of LAA samples, results were only borderline significance. Elevated levels were not due to acute phase reaction as CRP levels of all individuals were in the normal range. In case of AF patients, median values of VWF antigen levels were above the upper limit of the reference interval in all sample types (171% (IQR: 129.4–195.1%), 176.7% (IQR: 129.3–192.7%), and 164% (IQR: 114.8–189.8%) for FV, LA, and LAA sample types, resp.). The observed differences between patients and controls remained significant after adjustments for AB0 blood type in the statistical model. No local differences were found in the FVIII and VWF levels of intracardiac samples as compared to the FV samples in either group. FVIII and VWF levels showed good correlation in AF patients (Spearman $r = 0.808$, 95% CI: 0.691–0.884, $p < 0.0001$) as well as in non-AF controls (Pearson $r = 0.737$, 95% CI: 0.502–0.871, $p < 0.0001$), suggesting that they are in a complex form. No considerable differences were seen in the correlation of FVIII and VWF levels with respect to sampling

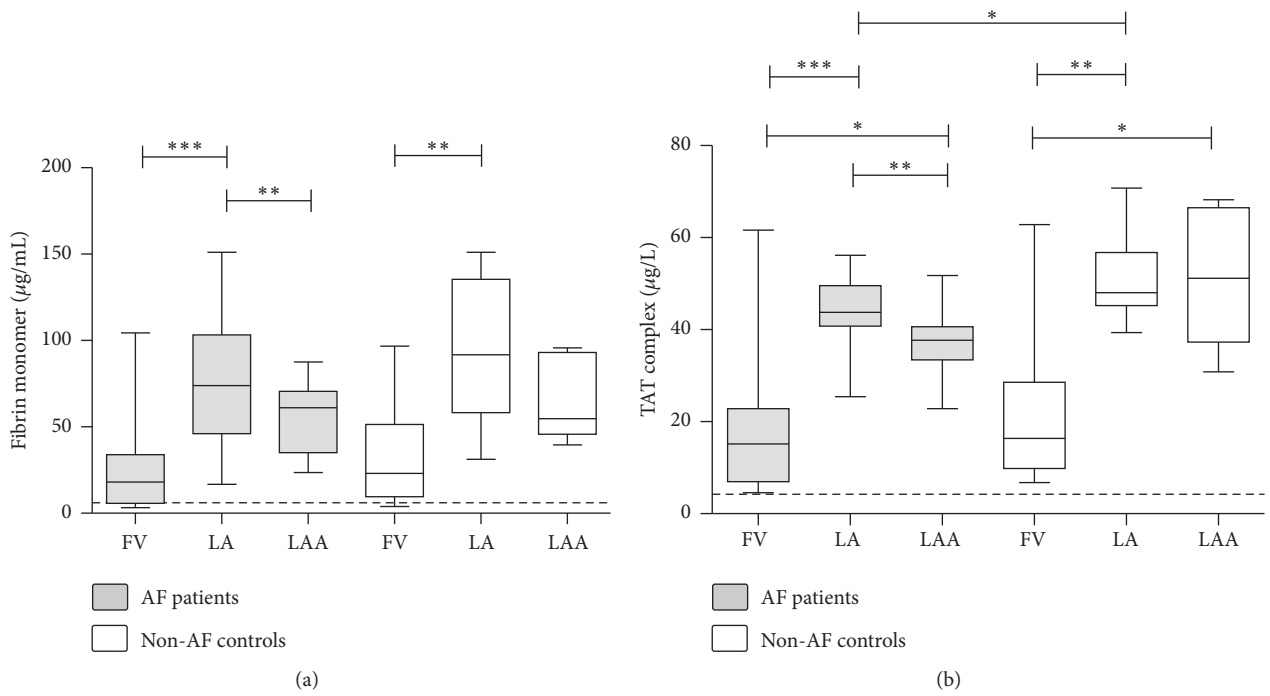

Figure 2: Levels of quantitative fibrin monomer and thrombin-antithrombin (TAT) complex in patients with atrial fibrillation (AF) and non-AF controls. Box and whisker plots indicate median, interquartile range, and total range. Dashed lines indicate upper limit of reference interval. FV: femoral vein; LA: left atrium; LAA: left atrial appendage. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

sites (data not shown). No significant differences were found between sample types and patient groups in case of FXIII activity and fibrinogen levels.

3.3. Intracardiac Levels of Coagulation Activation Markers in AF Patients and Non-AF Controls. Median values of soluble FM and TAT complex levels exceeded the upper limit of reference interval in the FV samples of AF patients (18.16 μg/mL (IQR: 5.83–33.91 μg/mL) and 15.17 μg/L (IQR: 6.96–22.83 μg/L) for FM and TAT, resp.) and non-AF controls (23.05 μg/mL (IQR: 9.55–51.41 μg/mL) and 16.36 μg/L (IQR: 9.84–28.59 μg/L) for FM and TAT, resp.) (Figures 2(a) and 2(b)). Moreover, both parameters were significantly elevated in the samples obtained from the LA as compared to the FV samples in case of both groups, suggesting that that the observed differences are not AF-specific and most probably the catheterization procedure itself has a major effect on the results. FM levels showed a decrease in the LAA samples as compared to the LA samples; this decrease was significant in case of the patients ($p < 0.001$, Wilcoxon matched pairs rank-sum test) (Figure 2(a)). TAT complex levels were also significantly lower in the LAA samples versus LA samples of AF patients ($p < 0.01$, Wilcoxon matched pairs rank-sum test), while such significant association was not observed in case of the non-AF control patients (Figure 2(b)). TAT complex levels were significantly increased in the LAA samples of both AF patients and non-AF controls as compared to the FV samples. Surprisingly, a marginal but significant elevation was observed in the TAT complex levels of the LA samples of non-AF controls versus AF patients ($p < 0.05$).

3.4. Intracardiac Parameters of Fibrinolysis in AF Patients and Non-AF Controls. Plasminogen activity, α_2-PI activity, and PAI-1 activity levels showed no difference between AF patients as compared to non-AF controls (Figures 3(a), 3(b), and 3(d)). In general, no difference was observed between the intracardiac and peripheral levels of these parameters, except for a small, but significant reduction of plasminogen level in the LAA versus FV sample of the AF patients (Figure 1(a)). PAP complex and D-dimer levels were significantly increased in the LA samples of both AF patients and non-AF controls as compared to the respective FV samples (Figures 3(c) and 3(d)), suggesting that the activation of the fibrinolytic system took place during the transcatheter procedure in both groups. In fact, approximately half of the AF patients and non-AF controls had D-dimer levels exceeding the cut-off value in the LA sample, while median values of D-dimer were well below the cut-off in the FV samples (0.26 mgFEU/L (IQR: 0.17–0.48 mgFEU/L) and 0.30 mgFEU/L (IQR: 0.18–0.48 mgFEU/L) in AF patients and controls, resp.) (Figure 3(d)).

4. Discussion

Although it is a general belief that in AF the intracardiac milieu is more thrombogenic than the peripheral blood, supporting pieces of evidence derived from measurements using intracardiac blood samples are scarce. In this study, we investigated the levels of a comprehensive list of hemostasis and fibrinolysis markers from intracardiac blood samples of

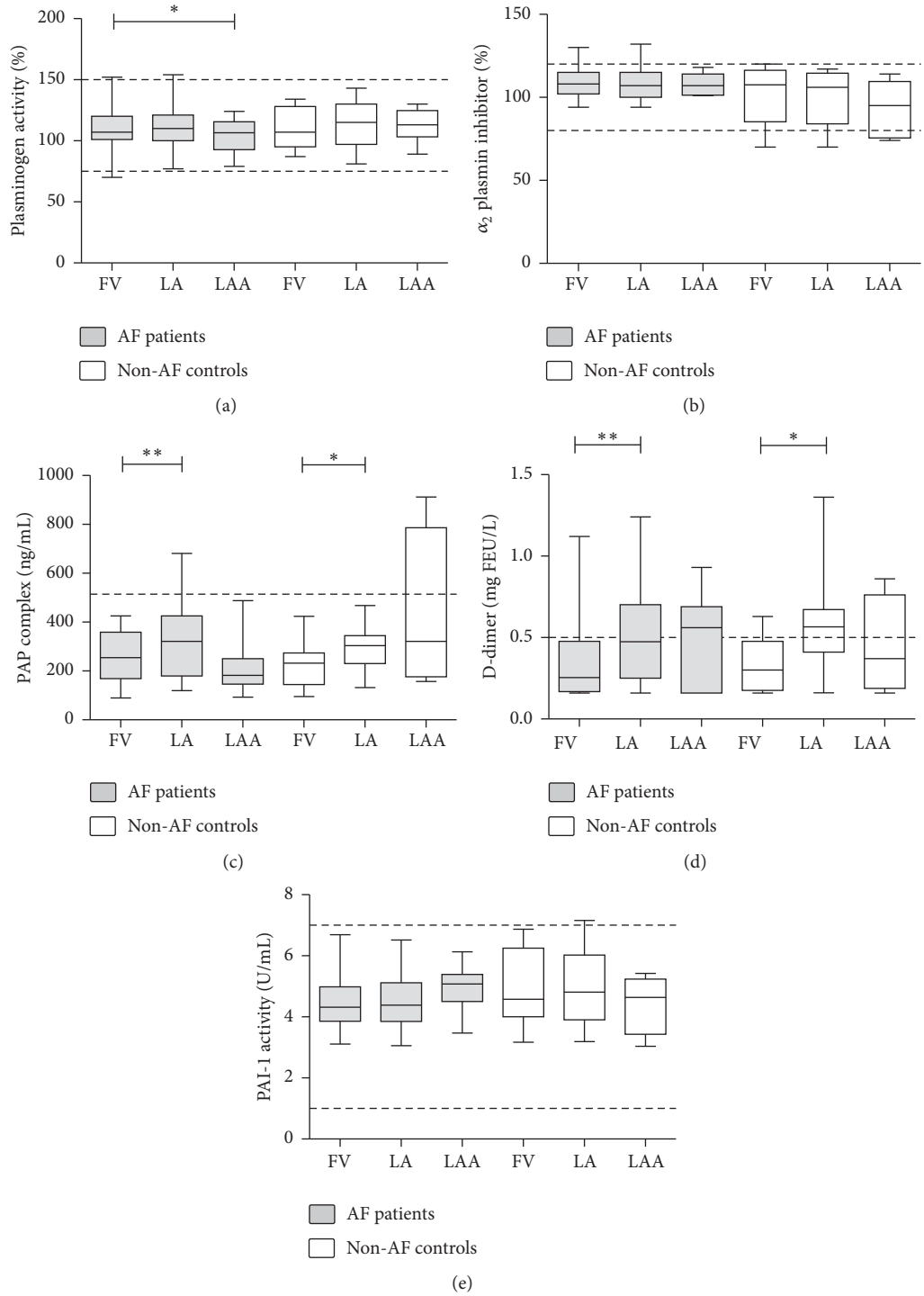

Figure 3: Levels of various fibrinolytic markers in patients with atrial fibrillation (AF) and in non-AF controls. Box and whisker plots indicate median, interquartile range, and total range. Dashed lines indicate upper and lower limits of reference interval or diagnostic cut-off levels. PAP complex: plasmin-α_2-antiplasmin complex; PAI-1: plasminogen activator inhibitor type 1, FV: femoral vein; LA: left atrium; LAA: left atrial appendage; $^{*}p < 0.05$; $^{**}p < 0.01$.

AF patients and non-AF controls and failed to detect significant AF-specific alterations of hemostasis or fibrinolysis in intracardiac blood samples. It is to be noted, however, that only two patients experienced paroxysmal AF periods during the procedure, which means that most patients were on sinus rhythm during blood sampling. Our results suggest that as compared to peripheral samples, paroxysmal/persistent AF patients have no significant alterations in the intracardiac levels of the investigated hemostasis and fibrinolytic parameters, at least when they are not experiencing AF periods.

Although significant local differences were observed for certain coagulation activation and fibrinolytic markers (namely, for FM, TAT complex, PAP complex, and D-dimer levels) in the intracardiac samples as compared to the FV samples, the same differences were found in non-AF control individuals. Moreover, in the LAA sample of both groups, a general tendency of decrease was observed in the level of most investigated markers as compared to LA samples. In earlier studies, in which non-AF control population was not investigated, these differences were attributed to AF pathophysiology [26]. However, our results imply that changes in the level of these markers are not specific for AF and are likely to be attributed to the invasive nature of the catheterization procedure, including transseptal puncture and tissue damage.

Among all investigated hemostasis and fibrinolysis parameters, only the elevation of FVIII and VWF levels was found to be AF-associated in our study. Interestingly, FVIII and VWF levels were significantly elevated in both peripheral and intracardiac blood samples of AF patients as compared to controls. Elevation of VWF levels was particularly considerable in the AF patient group as the medians of VWF levels were at the upper limit of the reference interval in all sample types. Although the levels of VWF in AF patients have been studied earlier using peripheral samples, the relationship between intracardiac and peripheral VWF levels has been obscure. An elevation of FVIII [18, 34] and VWF [35–38] has been described earlier in the peripheral samples AF patients and it has been proposed to be attributed to endothelial damage. Moreover, elevated levels of VWF have been associated with increased stroke risk and poor prognosis [36, 39, 40]. Only few papers enrolling a limited number of patients have investigated the levels of VWF in AF patients from both intracardiac and peripheral blood samples, but in these studies FVIII levels were not determined [27, 41]. In line with our findings, in these earlier reports it was found that VWF levels were similar in the intracardiac samples and in samples obtained from the peripheral sampling site. In our study FVIII and VWF levels showed good correlation in all sample types, suggesting that they were in complexed form. As both proteins are stored in the Weiber-Palade bodies of the endothelium [42], these results imply that the elevation of VWF and FVIII levels are the consequence of endothelial damage not necessarily restricted to the LA. It has to be noted that in the LAA of patients a similar tendency of FVIII and VWF elevation was observed as in case of FV and LA samples, but most likely due to the limited number of LAA samples, differences were not proved to be significant between patients and controls for this sample type.

Despite the important role of fibrinolytic system in preventing intravascular thrombosis, previous studies have paid little attention to the investigation of fibrinolytic abnormalities associated with AF [8]. Moreover, little is known about the levels of important regulators of fibrinolysis in intracardiac samples in AF. Here we assessed a series of fibrinolytic markers from both peripheral and intracardiac blood samples of AF patients and non-AF controls. Besides a small, but significant decrease in the levels of plasminogen in the LAA samples of AF patients as compared to the FV samples, no significant differences were observed between AF patients and non-AF controls and among sample types concerning FXIII activity, α_2-plasmin inhibitor, PAI-1 activity, and plasminogen activity measurements. There was no difference between PAP complex and D-dimer levels in AF patients and non-AF controls as well. These finding suggests that the investigated components of the fibrinolytic system are mostly unaltered in AF.

5. Limitations

Our study has some limitations. First, the number of patients enrolled in the study was limited, which was obviously due to the highly invasive nature of the blood sampling, during which technical difficulties were often encountered. We would like to highlight, however, that the number of patients enrolled in our study is still more than the average number of patients undergoing this kind of blood sampling as published so far. Moreover, in our study a non-AF control patient group was also enrolled, which is often missing from earlier studies. Despite the particularly difficult and potentially risky technique of LAA sampling, a considerable number of patients were sampled from the LAA as well, which is a rarity in the literature as yet. Based on our findings larger studies are warranted to corroborate our observations.

Second, most patients enrolled in the study had low or moderate stroke risk according to the $CHADS_2$ or CHA_2DS_2-VASC score, which limits the extrapolation of our findings to the general AF patient population. It has to be noted, however, that the stroke risk of our patient population reflects the current practice of most ablation centers, which offer ablation for younger patients with mostly paroxysmal AF, structurally normal heart, and no significant comorbidity [43]. In addition, the necessity and safety of the discontinuation of anticoagulation preablation (which was a requirement in our study in order to carry out certain measurements) are only evident in low-risk patients [44].

Third, only 2 patients experienced a paroxysmal AF period during the catheterization and blood drawing procedure. Naturally, more patients having AF period during sampling could have supplemented our results with a further interesting aspect.

6. Conclusion

AF patients have elevated FVIII and VWF levels, most likely due to endothelial damage, which is present in the intracardiac and peripheral environment as well. Intracardiac activation of hemostasis and fibrinolysis was demonstrated in AF patients and in non-AF controls to a similar extent, indicating that this might be a consequence of the catheterization procedure itself rather than a footprint of AF pathophysiology.

Conflicts of Interest

The authors declare that there are no conflicts of interest concerning this study.

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Research Article

Anti-Platelet Factor 4/Heparin Antibody Formation Occurs Endogenously and at Unexpected High Frequency in Polycythemia Vera

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Background. Myeloproliferative neoplasms (MPN) encounter thromboses due to multiple known risk factors. Heparin-induced thrombocytopenia (HIT) is a thrombotic syndrome mediated by anti-platelet factor 4 (PF4)/heparin antibodies with undetermined significance for thrombosis in MPN. We hypothesized that anti-PF4/heparin Ab might occur in MPN and promote thrombosis. *Methods.* Anti-PF4/heparin antibodies were analyzed in 127 MPN patients including 76 PV and 51 ET. Screening, validation testing, and isotype testing of anti-PF4/heparin Ab were correlated with disease characteristics. *Results.* Anti-PF4/heparin antibodies were detected in 21% of PV and 12% of ET versus 0.3–3% in heparin-exposed patients. Validation testing confirmed anti-PF4/heparin immunoglobulins in 15% of PV and 10% of ET. Isotype testing detected 9.2% IgG and 5.3% IgM in PV and exclusively IgM in ET. IgG-positive PV patients encountered thromboses in 57.1% suggesting anti-PF4/heparin IgG may contribute to higher risk for thrombosis in MPN. Overall, 45% of PV patients experienced thromboses with 11.8% positive for anti-PF4/heparin IgG versus 7.1% in PV without thrombosis. *Conclusion.* Anti-PF4/heparin antibodies occur endogenously and more frequently in MPN than upon heparin exposure. Thrombotic risk increases in anti-PF4/heparin IgG-positive PV reflecting potential implications and calling for larger, confirmatory cohorts. Anti-PF4/heparin IgG should be assessed upon thrombosis in PV to facilitate avoidance of heparin in anti-PF4/heparin IgG-positive PV.

1. Introduction

Myeloproliferative neoplasms (MPN) are clonal disorders of hematopoiesis with excessive proliferation of mature myeloid cells. They comprise several related entities [1] including chronic myeloid leukemia (CML) characterized by translocation t(9;22) known as the Philadelphia chromosome and the classical, Philadelphia-negative MPN. These present as essential thrombocythemia (ET) characterized by thrombocytosis, polycythemia vera (PV) with predominant erythrocytosis and concomitant leuko- and thrombocytosis, or myelofibrosis (MF) with expansion of megakaryocytes and reactive bone marrow fibrosis [2]. More rarely, chronic neutrophilic leukemia (CNL) or chronic eosinophilic leukemia (CEL) are also seen [1]. PV, ET, and MF represent the focus of this study and are subsequently referred to as MPN. They are characterized by hyperactive signaling of the JAK2 kinase [3, 4] essentially involved in hematopoiesis [5] which is constitutively activated by acquired gain of function mutations. These driver mutations affect *JAK2* itself, such as *JAK2*V617F in 95% of PV and 50% of ET and MF [6–9] and *JAK2* exon 12 mutations in PV [10]. In addition, mutations in the thrombopoietin receptor *MPL* [11], such as *MPL*W515L, or in the chaperone protein calreticulin *(CALR)* were identified in ET and MF, which converge on activation of JAK2 signaling [12–15].

While MPN potentially transform to acute myeloid leukemia with dismal prognosis [16], the most frequent complications in MPN are thrombohemorrhagic events [17, 18]. They relevantly contribute to the substantial disease burden and their avoidance is a major goal of current therapies. Thrombotic events exceed the hemorrhagic complications in MPN which occur mainly due to depletion of ultralarge von Willebrand factor (VWF) multimers by thrombocytosis leading to acquired von Willebrand syndrome (VWS) and altered platelet function [17, 19]. Thromboses in MPN affect both the arterial and the venous vascular beds comprising stroke or transient ischemic attacks, cardiac events, deep vein thrombosis, and pulmonary embolism as well as peripheral arterial or venous thrombosis [20]. Thromboses in MPN frequently present in atypical locations such as the splanchnic veins including mesenteric, splenic, portal, and hepatic vein thrombosis (Budd-Chiari syndrome) or cerebral venous sinuses. Thrombotic risk is highest in PV with 16–27% of patients affected by arterial and 7.4–11% by venous events [21, 22] and in ET with 10–50% of patients affected by a thrombotic complication within a decade from diagnosis [23]. Several risk factors have been identified and have been implemented in prognostic scores estimating thrombotic risk. While age over 60 years and history of thromboses represent the strongest predictors for thrombotic events [22, 24–30], the implication of leukocytosis, elevated hematocrit, and the *JAK2*V617F driver mutation have also been established. In addition, common cardiovascular risk factors as well as hereditary thrombophilia are considered relevant for overall risk of thromboses in MPN [17]. The pathogenesis of thromboses in MPN is currently perceived as multifactorial since general and disease-specific prothrombotic factors coincide. Thrombocytosis which is a characteristic presenting feature in ET, PV, and prefibrotic forms of MF has not been validated as a thrombotic risk factor in MPN.

The panoply of factors currently known to contribute to thrombotic risk in MPN might not be exhaustive and additional effects should be considered. Heparin-induced thrombocytopenia (HIT) represents a rare thrombotic syndrome mediated by an immune response to platelet factor 4 (PF4) secreted from platelet alpha granules [31]. PF4 complexes with heparin when the latter is administered for treatment or prophylaxis of thrombosis, giving rise to an immunogenic neoantigen. Consecutive antibody formation against PF4/heparin complexes is seen in 0.3–3% of patients on heparin treatment [32] and mediates activation and clearance of platelets leading to thrombocytopenia and potentially thrombosis, the full manifestation of HIT. Anti-PF4/heparin immunoglobulin production ceases upon withdrawal of heparin and anti-PF4/heparin antibodies subsequently become undetectable after heparin treatment is stopped. Even after overt HIT, anti-PF4/heparin antibodies are undetectable by 50–80 days [33]. Differential effects of several types of heparin are known such as increased risk of anti-PF4/heparin antibody formation by unfractionated heparin (UFH) as compared to low molecular weight heparin (LMWH) [34]. In addition, increased levels of PF4 in settings of platelet activation or high turnover have been shown to promote anti-PF4/heparin antibody formation probably via increased abundance of antigen [31]. Inflammatory stimuli by bacterial infection [35, 36] or by tissue damage upon surgery or major trauma are also considered promoting development of HIT [37, 38]. Interestingly, a recent study in orthopedic patients after knee or hip arthroplasty reported anti-PF4/heparin antibody formation in the absence of heparin treatment which was enhanced by dynamic versus static compression therapy for thromboprophylaxis [39, 40].

Insight into the significance of HIT for thrombosis in MPN is limited. The risk for thrombotic complications in MPN patients entails a high probability of heparin exposure during the course of the disease which could thereby put MPN patients at increased risk of developing HIT. However, diagnosis of HIT which includes substantial thrombocytopenia or a 50% fall in platelet counts is impeded by elevated baseline platelet levels in PV and ET. Thrombocytosis in MPN might mask occurrence of thrombocytopenia due to development of HIT, thereby leading to false negative assessments. Importantly, thrombocytosis in PV and ET is reflective of excessive platelet production and turnover, and platelets in MPN are known to circulate in an activated state, thus providing ample PF4 which could promote the formation of anti-PF4/heparin antibodies in PV and ET [17]. A limited number of PV and ET patients with occurrence of HIT have been reported (Table 1) [41–53]. In addition, analysis of a cohort of HIT patients found overrepresentation of ET and PV with 4.7% (2/42) [54]. The true incidence of HIT in PV and ET has not been clearly assessed given the masking of thrombocytopenia by excessive platelet production and the scarcity of the literature on this topic limited to a handful of case reports and series. A comprehensive case series studying 29 MPN patients [41] observed strong clinical evidence for HIT in five patients, but only two were tested for anti-PF4/heparin antibodies. Therefore, we employed a systematic approach to assess PF4/heparin antibody formation and its significance for thrombosis in a large cohort of PV and ET patients given their high thrombotic risk. We hypothesized that anti-PF4/heparin antibodies could be prevalent in these patients with excessive platelet production and turnover and might contribute to thrombotic risk in MPN.

2. Materials and Methods

2.1. Characterization of Patients. A cohort of 127 MPN patients including 76 PV and 51 ET patients from our tertiary care center diagnosed according to the WHO classification were retrospectively analyzed for MPN disease characteristics. Blood counts including hematocrit, hemoglobin concentration, platelet counts, white blood cell (WBC), and neutrophil counts were assessed on an ADVIA hemocytometer. The *JAK2*V617F mutation was assessed by allele-specific PCR in peripheral blood granulocyte DNA. Patient histories were assessed for occurrence, number, and localization of thrombotic events which were diagnosed by duplex sonography for deep vein thrombosis or CT angiography for pulmonary embolism and arterial events. Splenomegaly was assessed by clinical examination, sonography, or CT scan. Informed consent was available from all individuals and approval from the local ethics committee was obtained.

Table 1: Reports of heparin-induced thrombocytopenia in myeloproliferative neoplasms. Three case series with more than one patient and nine single cases of heparin-induced thrombocytopenia (HIT) in polycythemia vera (PV) and essential thrombocythemia (ET) have been reported. Number of patients, manifestation of thrombosis, and diagnostic work-up of anti-PF4/heparin antibodies are indicated. PE: pulmonary embolism, DVT: deep vein thrombosis, SVT: sinusoidal vein thrombosis, TIA: transitory ischemic attack, PF4: platelet factor 4, Ab: antibody, ELISA: enzyme linked immunosorbent assay, PAT: platelet aggregation testing, HIPA: heparin-induced platelet aggregation, SRA: serotonin release assay, PaGIA: platelet factor 4/heparin-particle gel immunoassay, and HemosIL-HIT: automated rapid testing for platelet factor 4/heparin antibodies.

	Report	MPN type	Patients (*n*)	Thrombosis type (*n*)	PF4/heparin Ab testing (*n*)	Test type	Ab isotype testing
Case series	Bovet et al., 2016	ET	2	Stroke (2)	2	ELISA, PAT	−
	Randi et al., 2010	PV	2	PE (2)	1	ELISA, HIPA	+
		ET	3	PE (3)	1		+
	Spectre et al., 2008	PV	2	Catheter-associated (1),	2	PaGIA	−
		ET	1	PE (1), skin necrosis (1)	1		−
Single cases	Biagioni et al., 2013	PV	1	Budd-Chiari (1)	1	HemosIL-HIT	−
	Akoum et al., 2009	PV	1	Budd-Chiari (1)	1	ELISA	−
	Hayashi et al., 2004	PV	1	PE	1	ELISA	−
	Garcia et al., 1991	PV	1	DVT, PE (1)	1	PAT	−
	Kyritsis et al., 1990	PV	1	Thrombophlebitis (1)	1	HIPA, SRA	−
	Murawaki et al., 2012	ET	1	Stroke (1)	1	ELISA	−
	Richard et al., 2011	ET	1	SVT (1)	1	ELISA	+
	Lapecorella et al., 2010	ET	1	DVT, PE (1)	1	ELISA	−
	Houston, 2000	ET	1	Axillary DVT (1)	1	SRA	−
	Risch et al., 2000	ET	1	TIA (1)	1	ELISA	−
	Walther et al., 1996	ET	1	SVT (1)	—	—	−

Table 2: Baseline characteristics of MPN patient cohort. Characteristic parameters of the study population of 127 MPN patients are displayed (mean for age and peripheral blood counts, frequency for all other parameters). Frequencies are indicated in percent of total patients: absolute numbers are given in parentheses.

MPN type	Polycythemia vera	Essential thrombocythemia
Patient cohort	total $n = 127$	
Patients (n)	76	51
Male (n)	42	18
Female (n)	34	33
Parameters at diagnosis		
Age (y)	55.8	53.2
Hematocrit (%)	53.2	41.6
Hemoglobin (g/l)	176.2	139.2
Platelets (G/l)	602.9	984.3
Leukocytes (G/l)	12.4	9.0
Splenomegaly (%)	55.3 (42/76)	41.2 (21/51)
*JAK2*V617F (%)	84.2 (64/76)	52.9 (27/51)
Thrombohemorrhagic events		
Thrombosis	44.7 (34/76)	39.2 (20/51)
Hemorrhage	18.4 (14/76)	3.9 (2/51)
Medications		
Antiaggregation	94.7 (72/76)	88.2 (45/51)
Oral anticoagulation	30.3 (23/76)	11.8 (6/51)
Cytoreduction	75.0 (57/76)	68.6 (35/51)

2.2. Anti-PF4 Antibody Testing. Testing for anti-PF4 immunoglobulins was performed in PV and ET patient serum samples stored at −80°C. We employed a sequential approach including a screening and a subsequent validation assay in positive samples for optimized specificity. Initial screening for anti-PF4 antibodies was performed by a commercial anti-PF4 ELISA globally detecting IgG, IgA, and IgM antibodies (ZYMUTEST HIA IgGAM ELISA, product number RK040D). Positive samples were subsequently subjected to specific isotype testing for validation and for determination of IgG, IgM, and IgA isotypes (ZYMUTEST HIA IgG, IgA, IgM, product number RK040E). IgG subclasses and FcgRIIa H131R polymorphism were not specifically assessed in the study.

2.3. Statistical Analysis. Statistical analysis was performed by SPSS software. Pearson's $X2$ test (two-by-two table) was used to compare categorical variables. Significance level was set at $p < 0.05$ in two-sided tests.

3. Results

3.1. Baseline Characterization of MPN Patient Cohort. The study population of 127 individuals consisted of 76 PV and 51 ET patients. Baseline characteristics are shown in Table 2. Among PV patients, there was a slight male predominance ($n = 42$ male, $n = 34$ female), while ET affected more women ($n = 18$ male, $n = 33$ female). Mean age at diagnosis was 55.8 years in PV and 53.2 years in ET. PV patients were characterized by erythrocytosis reflected by increased red cell parameters (mean hematocrit 53.2%, mean hemoglobin 176.2 g/l) as well as mild leukocytosis with mean white blood cell count (WBC) of 12.4 G/l. Red cell parameters and WBC were within normal range in ET patients, who displayed pronounced thrombocytosis with mean platelet count of 984.3 G/l. Thrombocytosis was also present in PV (mean platelet count 602.9 G/l). *JAK2*V617F was detected in 84.2% of PV and 52.9% of ET patients concordant with previous studies [6–9]. Splenomegaly by clinical assessment or imaging was more prevalent in PV (55.3%) than ET (41.2%). Thrombohemorrhagic complications were frequent with 63.1% in PV and 43.1% in ET. Thereof, 18.4% and 3.9% of patients suffered from bleeding events, respectively, while thromboses clearly outweighed bleeding events both in PV and in ET. Nearly all patients were on antiaggregatory prophylaxis with low dose aspirin (94.7% in PV, 88.2% in ET), while much less individuals had received anticoagulation with vitamin K antagonists during the course of their disease (30.3% in PV, 11.8% in ET). Substantial proportions had received cytoreductive therapy with 75.0% in PV and 68.6% in ET patients (Table 2). These analyses match well with established disease characteristics in PV and ET [2] and demonstrate that this patient population is representative and well suitable for investigations into the role of anti-PF4 immune responses in MPN.

3.2. Thromboses Are Frequent in PV and ET and Relate to Multiple Risk Factors. Thromboses occurred in 34/76 PV patients (44.7%) and 20/51 ET patients (39.2%) during the course of disease highlighting the very substantial contribution of thrombotic events to disease burden in MPN.

Table 3: Site of thrombotic events in MPN. The site of thromboses in 127 MPN patients is displayed highlighting the relevance of thrombotic complications for disease burden in MPN. CNS: central nervous system, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism, and PAD: peripheral arterial disease.

Site of thrombosis	Frequency	
	(%)	*n*
Total	100	54
CNS	37.0	20
VTE		
DVT	20.4	11
PE	11.1	6
Cardiac	14.8	8
Splanchnic	13.0	7
PAD	3.7	2

The slightly higher incidence of thrombotic complications in PV as compared to ET patients is in accordance with previous reports [20, 21] (Table 2). In the presented patient population, cerebrovascular events were the most prevalent manifestation of thrombosis in 37.0% of patients, followed by venous thromboembolism in 31.5% including 20.4% of deep vein thromboses, 11.1% of pulmonary embolism, and 14.8% with cardial ischemic events (Table 3). Splanchnic thromboses which are characteristic for MPN were seen in 7/54 (13.0%) patients which represents a high incidence given their overall rarity. Two patients (3.7%) with peripheral arterial disease and additional vascular risk factors such as previous cigarette smoking, arterial hypertension, and dyslipidemia showed peripheral arterial occlusions. A substantial proportion (19/54 patients) suffered from repeated thrombotic events with 3 patients even showing multiple (3 and more) thromboses during the disease course. Platelet counts >400 G/l did not significantly affect thrombotic risk ($p = 0.106$), whereas patients with hematocrit >0.46 showed a significantly increased incidence of thromboses ($p = 0.010$), as did patients with leukocytosis >10 G/l ($p = 0.033$) reflecting established risk factors [17].

3.3. Anti-PF4/Heparin IgG Antibodies Occur at High Frequency in PV Patients. Qualitative screening for anti-PF4/heparin antibodies was positive in 22 individuals (17.3%) of the entire cohort of 127 patients. Incidence in PV patients was higher than in ET with 21.0% tested positive in PV and 11.8% among ET patients (Table 4). Positive patients underwent subsequent validation testing, which confirmed anti-PF4/heparin antibodies in 14.5% of PV and 9.8% of ET patients (Figure 1). Immunoglobulin isotype testing showed that 9.2% of PV patients had circulating IgG against PF4, for which a functional role as platelet activators in pathogenesis of HIT has been demonstrated [31]. The prevalence of anti-PF4/heparin IgG among PV patients in our cohort is clearly higher than in patients treated with unfractionated heparin, for whom anti-PF4/heparin IgG have been reported in 0.3–3% after a treatment duration of more than four days [32] or at lower frequencies when LMWH is used [34]. Sequential antibody testing and isotype testing in 3/7 IgG-positive PV patients revealed persistent positivity after 847, 674, and 182 days, respectively, which supports the notion that anti-PF4/heparin IgG immunoglobulins can occur endogenously in PV patients and that antibody formation can be maintained despite the absence of concurrent heparin exposure. Immunoglobulin isotype testing also identified anti-PF4/heparin IgM in 5.3% of PV patients. A thrombogenic potential of anti-PF4/heparin IgM has not been clearly established and a causative implication for thrombosis in HIT remains on debate. All anti-PF4/heparin antibodies detected in ET patients were of IgM isotype at a prevalence of 9.8%. No anti-PF4/heparin IgG were observed in ET patients in our cohort. As patients with ET showed more pronounced thrombocytosis than PV patients, the absence of IgG isotypes in ET suggests that excessive platelet production associated with increased circulating levels of PF4 may not be the sole factor facilitating anti-PF4 immune responses in MPN but that additional promoting factors are at play specifically in PV patients (Table 4). However, it is noteworthy that anti-PF4/heparin IgM antibodies persisted in a majority (5/9) of positive patients with 3/4 PV and 2/5 ET patients showing detectable IgM at two sequential assessments with intervals of 1003, 904, 811, 539, and 42 days. As several cases of clinically manifest HIT in ET patients have been reported (Table 1) and exclusively anti-PF4/heparin IgM were detectable in ET patients of our cohort, a potential implication of anti-PF4/heparin IgM for platelet activation in the setting of MPN with thrombocytosis may warrant further evaluation in functional studies.

3.4. Anti-PF4/Heparin IgG Antibodies Confer a Tendency for Increased Thrombotic Risk in PV. We next assessed to what extent anti-PF4/heparin IgG immunoglobulins with the known potential to activate platelets in the pathogenesis of clinical HIT would contribute to thrombotic complications in PV. We observed that 57.1% of PV patients with circulating anti-PF4 IgG suffered from a thrombotic event at least once during the course of disease (Table 5) including both arterial and venous events according to the known spectrum of thromboembolic complications in MPN (Table 6). Anti-PF4/heparin IgG-negative PV patients encountered thromboses in 43.5%, reflecting a 31% increase in relative thrombotic risk for PV patients with anti-PF4/heparin IgG as compared to IgG-negative PV in our cohort (Table 5, $p > 0.05$). Predictors of whether IgG-positive PV patients would actually develop thrombosis were absent suggesting that thrombosis in PV is a strongly multifactorial process, while the FcγRIIa H131R polymorphism was not assessed in this study. When PV patients with a positive history of thrombosis were assessed for anti-PF4 immune responses, we observed that 11.8% had anti-PF4/heparin IgG antibodies, as compared to 7.1% IgG positivity in PV patients who never had a thrombosis. Although the increased risk for thrombotic events in PV patients with anti-PF4/heparin IgG is statistically nonsignificant in our cohort, a subtle but relevant contribution of anti-PF4/heparin IgG to thrombosis in PV cannot be excluded at this point, particularly in view of the multifactorial nature of thrombosis in MPN with hematocrit

Table 4: Characterization of anti-PF4/heparin antibody formation in MPN. A cohort of 127 patients with myeloproliferative neoplasms (MPN) including polycythemia vera (PV, $n = 76$) and essential thrombocythemia (ET, $n = 51$) were characterized for anti-PF4/heparin antibodies. Analysis by a screening ELISA was subsequently validated by follow-up testing and antibody isotype testing determining IgG, IgM, and IgA. PF4: platelet factor 4; Ig: immunoglobulin.

MPN		PF4/heparin antibody characterization				
Subtype	Patients (*n*)	Screening (% positive)	Validation (% positive)	Isotype testing (% positive)		
				IgG	IgM	IgA
PV	76	21.1 (16/76)	14.5 (11/76)	9.2 (7/76)	5.3 (4/76)	0.0 (0/76)
ET	51	11.8 (6/51)	9.8 (5/51)	0.0 (0/51)	9.8 (5/51)	0.0 (0/51)
Total	127					

Table 5: Impact of anti-PF4/heparin antibodies on thrombotic risk in polycythemia vera. Anti-PF4/heparin antibodies of IgG isotypes, which have a known implication in pathogenesis of HIT, were assessed for a potential impact on thrombotic risk in polycythemia vera. IgG positivity conferred a 31% increase of relative risk for thrombosis as compared to IgG-negative PV. Statistical significance was not reached due to sample number. PF4: platelet factor 4; Ig: immunoglobulin.

	Anti-PF4/heparin IgG isotype		Relative thrombotic risk	*p* value
	Positive	Negative		
Thrombotic complications (%)	57.1	43.5	1.31	>0.05
No thrombotic complications (%)	42.9	56.5		

Table 6: Thromboembolic complications in anti-PF4/heparin IgG-positive PV patients. Thromboembolic complications are indicated in 7 PV patients positive for anti-PF4/heparin antibodies of IgG isotype. Thromboembolic events occurred in patients 1–4 and not in patients 5–7. Both arterial and venous complications as well as repeated events were seen. Splenic infarcts as in patient 5 are not considered classic thromboembolic complications in MPN. Grade IV PAD induced critical ischemia in patient 6 without an acute occlusive event. Pat: patient, Dgn: diagnosis, DVT: deep vein thrombosis, TIA: transitory ischemic attack, CVI: cerebrovascular infarction, NIHSS: National Institute of Health Stroke Scale, PE: pulmonary embolism, CHD: coronary heart disease, and PAD: peripheral arterial disease.

Pat	Age at Dgn	Sex	MPN type	PF4/heparin Ab isotype	Thromboembolic complications (*n*)	1st event	2nd event
1	71	m	PV	IgG	2	2-level DVT with postthrombotic syndrome	TIA
2	49	m	PV	IgG	2	CVI (NIHSS 5 pt)	CVI (NIHSS 16 pt)
3	57	m	PV	IgG	1	Bilateral PE with pulmonal-arterial hypertension	—
4	33	m	PV	IgG	1	In-stent thrombosis in early onset CHD	—
5	41	m	PV	IgG	0	Splenic infarcts, no splenic vein thrombosis	—
6	61	m	PV	IgG	0	Critical ischemia of lower extremity in grade IV PAD	—
7	69	f	PV	IgG	0	—	—

and leukocyte levels as major risk factors. Further studies of more extensive patient populations are warranted to confirm the tendency for increased thrombotic risk in PV patients with anti-PF4/heparin IgG antibodies which we observe in our study. However, as PV is a rare hematological disorder, this will eventually require multicenter collaborative efforts.

Overall, we find a high frequency of circulating anti-PF4/heparin IgG antibodies in patients with PV which are detectable in the absence of concurrent exposure to heparin. There is a tendency for increased thrombotic complications in PV patients positive for anti-PF4/heparin IgG. Due to the retrospective nature of this study, data on antibody positivity at the time of thrombosis is not available for our cohort. However, our data suggest that PV patients should undergo anti-PF4/heparin IgG testing as soon as a thrombotic event occurs to prevent potential boosting of the anti-PF4/heparin

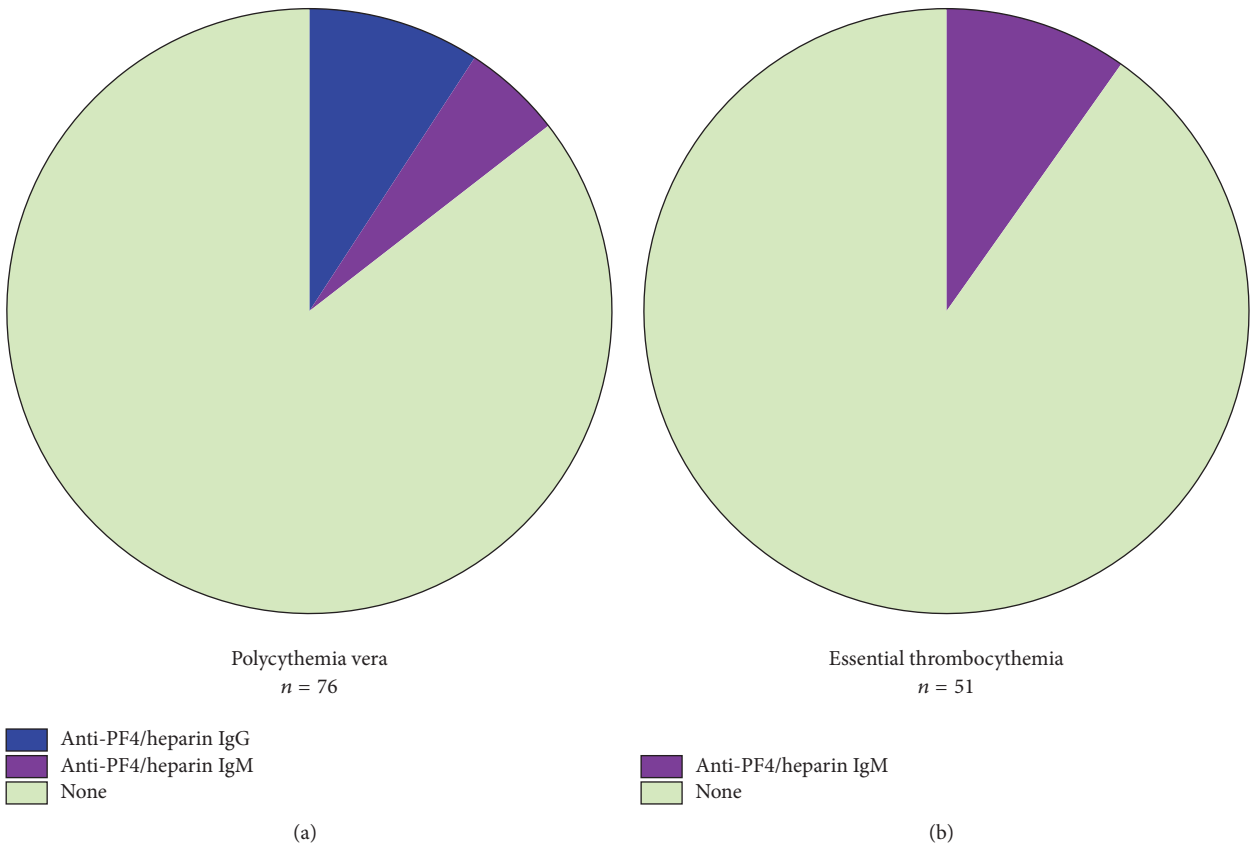

Figure 1: Endogenous anti-PF4/heparin IgG and IgM antibodies occur in polycythemia vera and essential thrombocythemia. (a) Anti-PF4/heparin antibodies of IgG isotype are detected at considerable frequency in polycythemia vera (PV), while IgM isotype antibodies are also found. (b) Anti-PF4/heparin antibodies in essential thrombocythemia are of IgM isotype in our cohort. No IgA isotypes were detected. PF4: platelet factor 4; Ig: immunoglobulin.

immune response by initiation of heparin treatment as seen in rare reported cases [55] and to facilitate alternative anticoagulation in IgG-positive PV patients.

4. Discussion

We report on the prevalence of anti-PF4/heparin antibodies in a large cohort of PV and ET patients and evaluate to what extent anti-PF4/heparin IgG known to mediate platelet activation and consequent thrombosis in HIT would contribute to thrombosis in MPN. MPN are characterized by an acquired prothrombotic condition with multifaceted pathogenesis of interplaying proaggregatory and procoagulatory factors [17]. As a consequence, thrombotic events are frequent in MPN, particularly in PV. Clinically relevant thrombosis is found at time of diagnosis in 11–39% of PV and in 8–29% of ET [56, 57]. Thromboses often also complicate the further course of MPN as seen in 8–19% of PV and 8–31% of ET patients and outweigh the risk of bleeding complications, which also typically occur in MPN patients, but at lower frequencies than thromboses [19]. Thus, thromboses are a substantial contributor to symptom burden of MPN patients and impact on their life expectancies [58] as demonstrated by the largest epidemiologic study in PV (European Collaboration on Low dose Aspirin in Polycythemia vera, ECLAP), which showed cardiovascular events to account for 41% of all deaths [59]. The arterial circulation is more prone to thrombosis in MPN accounting for 60–70% of events mostly as cerebro- or cardiovascular complications and less frequently as peripheral arterial occlusions. Venous thromboembolism accounts for the remainder with a high incidence of thrombosis in atypical locations such as cerebral venous sinuses and splanchnic veins including mesenteric, portal, and hepatic vein thrombosis (Budd-Chiari syndrome). As MPN represent the most common cause of splanchnic vein thromboses, such events should always raise a high suspicion of underlying MPN [17].

As thrombosis related to HIT has been reported in patients with PV or ET (Table 1), we hypothesized that an immune response to PF4 may be relevant in MPN with a potential implication in thrombotic events. Strong predictors of thrombotic complications in MPN have long been known as a history of previous thrombosis and advanced age >60 years [22, 24, 26–30]. General risk factors for arterial or venous thrombosis such as cardiovascular parameters and hereditary thrombophilia, respectively, are prevalent also in MPN patients and appear to mediate additive prothrombotic

propensity [17]. In addition, multiple MPN-specific factors promoting thrombosis have been identified recently. They comprise both quantitative and qualitative alterations of cellular blood components including erythrocytes, leukocytes, and platelets and extend to functional changes of the endothelium and the hemostatic cascade, thus mediating an overall hypercoagulable state. It has also been established that the presence of the *JAK2*V617F mutation associates with increased thrombotic potential, whereas the role of the actual *JAK2*V617F allele burden remains controversial. The recently developed international prognostic score for risk of thrombosis in ET (IPSET-thrombosis) is implementing advanced age, previous thrombosis, *JAK2*V617F, and cardiovascular risk factors [25]. Although not considered by the IPSET scoring, leukocytosis and increased hematocrit represent recognized and relevant promoting factors of thrombosis acting mainly via increased blood viscosity, platelet activation through leukocyte-platelet interactions, and secretion of microparticles of activated leukocytes [60]. Elevated platelet count has not been validated as an independent contributing factor, but functional changes have been observed. MPN platelets show increased biosynthesis of thromboxane A2 [61] and the circulating platelet pool in PV and ET is enriched in immature platelets which circulate in an activated state with increased expression of P-selectin and tissue factor [61, 62]. It has been found that platelets in PV and ET, particularly if carrying the *JAK2*V617F mutation, show increased potential of thrombin generation [63, 64]. Of note, platelet activation products such as PF4 are increased in plasma of MPN patients [63], which could facilitate an anti-PF4 immune response based on increased abundance of antigen. This process could be even more pronounced in patients not on low dose aspirin, which very efficiently reduces thromboxane A2 biosynthesis already at low doses and counteracts platelet activation [61]. Other factors like platelet-leukocyte aggregates, increased production of inflammatory cytokines and reactive oxygen species (ROS), and increased circulating endothelial cells as well as lower levels of protein C illustrate the broad variety of factors implicated in thrombosis in MPN [17]. Functional studies will be needed to determine the significance of their contribution to overt thrombosis in MPN patients. In addition, the broad range of proaggregatory and procoagulatory factors promoting thrombosis formation in MPN is probably not exclusive and additional, yet unidentified factors may further contribute to the complex pathogenesis of multifactorial thromboses in MPN.

Anti-PF4/heparin antibodies as seen in HIT could represent an additional prothrombotic factor in MPN. Several case reports of HIT occurring upon heparin treatment in PV and ET have been published suggesting that patients with MPN might be at risk for HIT-related immune responses and potentially thrombotic events [41, 42, 54]. HIT is a severe, prothrombotic condition facilitating arterial or venous thromboses triggered by antibody formation against PF4/heparin complexes upon treatment with heparin [31]. Diagnostic criteria of HIT include onset of thrombocytopenia within 5–14 days of heparin exposure along with detection of platelet-activating anti-PF4/heparin antibodies, while overt thrombosis is seen in up to 50% of cases [65]. However, these diagnostic criteria are hampered in PV and ET by baseline thrombocytosis which may mask thrombocytopenia and by the fact that thrombosis in MPN is obviously multifactorial [41, 42]. Insight into the pathogenesis of HIT has been gained which could provide links to a role of anti-PF4/heparin antibodies in PV and ET. Crystallization studies have shown that negatively charged heparin is complexing with tetramers of the cationic platelet factor 4 (PF4) after release from activated platelets. Linearization of heparin molecules allows multiple PF4 tetramers to bind, building substantial amounts of PF4/heparin antigen with neoepitopes on PF4 emerging under specific conditions [66]. Formation of anti-PF4/heparin antibodies is induced probably via recognition of PF4/heparin complexes by toll-like receptors [67], and PF4/heparin antibody complexes mediate activation of platelets via binding to the surface FcγRIIA receptor. Activation of monocytes via binding of PF4/heparin complexes to FcγRIIA and FcγRI enhances thrombin formation by increased tissue factor expression, which further promotes antibody formation by increasing platelet activation and PF4 levels [68, 69]. FcγRIIA and FcγRIIIA are involved in clearance of activated platelets mediating platelet depletion in HIT. Polymorphism in exon 4 of the FcγRIIA, H131R (c519G>A; rs1801274) associated with a histidine to arginine substitution, is known to infer a higher risk for overt thrombosis in patients with HIT homozygous for the 131RR allele due to increased platelet activation and tissue factor expression [69]. Also, FcγRIIA 131RR homozygous patients show reduced inhibitory effects by normal immunoglobulins of IgG2 subtype on FcγRIIA due to decreased affinity of FcγRIIA 131RR for IgG2 [69]. In addition to platelet activation via binding of anti-PF4/heparin antibodies to FcγRIIA via their Fc part, anti-PF4/heparin antibodies have also been found to bind PF4 complexed with endogenous GAGs on platelet, monocyte, and endothelial cell surfaces via their Fab domains [67].

From studies so far, it is evident that heparin represents the major trigger of the anti-PF4 immune response in HIT and shows differential immunogenicity for distinct types of heparin. Exposure to unfractionated heparin (UFH) provokes HIT in 0.3–3% [32], while frequencies are lower at 0.2–0.8% on low molecular weight heparin [70]. A study on minimized heparin use showed reductions in anti-PF4/heparin antibody positivity and clinical HIT in hospitalized patients, which highlights heparin exposure as the central inducing factor [71]. Additional factors supporting the development of anti-PF4/heparin antibodies and HIT have been described. Upon heparin exposure, trauma and surgery were associated with increased risk for HIT as compared to general internal medicine patients. This higher propensity for anti-PF4/heparin immune responses in surgical patients was related to tissue damage which could mediate release of glycosaminoglycans (GAG) as endogenous heparin-like polysaccharides and promote platelet activation with higher levels of PF4 in plasma [31] facilitating anti-PF4/heparin antibody formation due to increased availability of antigen. Inflammatory stimuli related to surgical tissue damage could further enhance the immune response to PF4/heparin complexes [37, 38]. This concept was supported by increased IL6

levels in patients after cardiovascular surgery with high-titer anti-PF4/heparin antibodies [72]. Also, the inflammatory milieu in the setting of bacterial infections has been shown to enhance anti-PF4/heparin antibody formation and HIT [35, 36]. PF4/heparin antibody formation may also be facilitated in other conditions characterized by elevated plasmatic PF4 levels such as diabetes [73], atherosclerosis [74], and cardiovascular [75] and renal disease [76]. A study on diabetic patients with increased vascular risk found anti-PF4/heparin antibodies more frequently than in nondiabetic patients [77]. Similar findings have been reported for patients with acute coronary syndrome (ACS) [78, 79] or cardiac surgery. Of note, positivity for anti-PF4/heparin antibodies has been associated with increased complication rate and mortality in cardiovascular patients [80].

Interestingly, one study suggested that inflammatory stimuli and platelet activation due to tissue injury upon joint replacement surgery would suffice to evoke anti-PF4/heparin antibodies in the absence of heparin treatment [39, 40]. Anti-PF4/heparin antibodies were detected in 6.5% of patients with arthroplasty in the absence of heparin and in 15% when dynamic compression as thromboprophylaxis was applied. We hypothesized that a similar constellation could be at play in a nonsurgical setting in patients with PV and ET. Platelet production and turnover is increased in PV and ET patients and it is known that MPN platelets are circulating in an activated state and show prolonged activation as compared to normal platelets. As a consequence, plasma levels of PF4 are increased [17]. Higher abundance of PF4 antigen and platelet activation could increase the susceptibility for anti-PF4/heparin antibody formation in MPN in the absence of heparin treatment. In addition, it is established that MPN create an inflammatory milieu with increased plasma levels of multiple cytokines including IL6 [81]. Detailed in vivo studies have revealed that excessive inflammatory cytokines in MPN originate from both the malignant clone and nonmutant cells [82]. Substantial disease burden in MPN is attributed to inflammatory symptoms including fatigue, pruritus, and bone and muscle pain. Treatment with the JAK1/2 inhibitor ruxolitinib reduces cytokine levels and inflammatory symptoms in most MPN patients highlighting a relevance of the inflammatory milieu for disease burden [83]. The inflammatory condition in MPN could further increase the probability for anti-PF4/heparin antibody formation in the absence of heparin exposure similarly to the findings in nonheparinized surgical patients [40], thereby increasing the risk of overt HIT in PV and ET.

Evidence of anti-PF4/heparin immune responses in MPN is scarce so far. A limited number of case series and reports of single cases describe the occurrence of HIT in PV and ET, while cases in MF have not been reported to date (Table 1) [41, 42, 54]. It is challenging to determine the significance of HIT for thrombosis in PV and ET based on this limited data. Furthermore, diagnostic work-ups vary among different reports. The presence of anti-PF4/heparin antibodies has not been assessed in all patients and antibody isotype testing was performed in a minority. Therefore, we investigated a large cohort of 127 MPN patients for anti-PF4/heparin antibodies using a systematic diagnostic approach. As no cases of HIT have been reported so far in MF, we focused our analysis on 76 patients with PV and 51 with ET. We applied a 2-step algorithm using first an anti-PF4/heparin antibody screening ELISA for analysis of patients' plasma followed by confirmatory testing. As differential effects of immunoglobulin isotypes for platelet activation and thrombin generation in HIT are known, positive patients were subjected to immunoglobulin isotype testing with a specific anti-PF4/heparin antibody ELISA determining positivity for IgG, IgM, and IgA, respectively. This uniform work-up in a large cohort of MPN patients for the first time provides consistent data on the frequency of anti-PF4/heparin antibody formation in PV and ET.

Antibody screening detected anti-PF4/heparin immunoglobulins in 17.3% of all MPN patients in the absence of heparin exposure (Table 4). Antibody formation was more frequent in PV than in ET with 21% and 11.8% of patients, respectively. These findings are notable, as anti-PF4/heparin antibodies in PV and ET patients without direct heparin exposure occur at a clearly higher frequency than in patients on heparin treatment known to develop antibodies in 0.3–3% [32]. Anti-PF4/heparin antibodies were also substantially more prevalent in PV and ET than in healthy individuals, in whom detection of anti-PF4/heparin antibodies is rare [84]. Subsequent validation testing confirmed anti-PF4/heparin antibodies in 14.5% of PV and 9.8% of ET patients which consolidates that anti-PF4/heparin antibody formation in MPN exceeds the risk in heparin-treated patients (Table 4, Figure 1). Also, anti-PF4/heparin antibody production was sustained as documented in a subset of PV and ET patients with serial testing at average intervals of 568 days for anti-PF4/heparin IgG and 660 days for IgM isotypes. A similar susceptibility for anti-PF4/heparin immune responses has been reported in nonheparinized surgical patients with dynamic compression therapy which lends strong support to our findings of prevalent anti-PF4/heparin antibodies independent of direct heparin exposure in nonsurgical, but hematological patients. Although there is no direct tissue damage in PV and ET, increased platelet activation and PF4 plasma levels are characteristic [17] and an inflammatory milieu is prominently present, as shown by clinical trials and experimental animal models [82, 83].

For further characterization of the anti-PF4/heparin immune response in MPN, we performed immunoglobulin isotype testing in PV and ET to delineate the proportion of anti-PF4/heparin IgG with an established role in platelet activation via FcγRIIA [31]. Anti-PF4/heparin IgG were detected in 9.2% of PV patients which exceeds the frequencies seen in heparin-exposed patients [32] or healthy individuals [84] (Table 4, Figure 1). Interestingly, anti-PF4/heparin antibodies in ET patients were of IgM isotype for which a role in pathogenesis of HIT is controversial. As case reports of overt HIT in ET have been published [41, 42, 46–51], but exclusively IgM are detected in our ET cohort, functional effects of IgM should be evaluated in future studies. Isotype specification of anti-PF4/heparin antibodies in our cohort did not identify any IgA-positive patients and none of the patients tested positive for multiple isotypes, whereas studies on the dynamics of anti-PF4/heparin immune responses

have reported simultaneous detection of IgG, IgM, and IgA [33]. These differences in antibody isotype pattern may relate to differential immune mechanisms involved in anti-PF4/heparin immune responses in patients on heparin treatment versus patients with PV and ET.

Correlation with clinical characteristics revealed a tendency for more thrombotic events in PV patients with anti-PF4/heparin IgG immunoglobulins. We observed a 31% increase of relative risk for thrombotic complications in IgG-positive PV patients as compared to IgG-negative PV suggesting that anti-PF4/heparin IgG antibodies could potentially contribute to the multifactorial thromboses occurring in PV. Higher patient numbers would have been required to reach statistical significance of this finding, but the rarity of MPN is impeding the formation of more extensive patient cohorts (Tables 5 and 6). The fact that anti-PF4/heparin antibodies in PV patients with thrombosis were of IgG isotype may support a functional relevance of anti-PF4/heparin immune responses for increased thrombotic risk. Collaborative multicenter efforts will be required to further extend patient numbers even in rare disorders like MPN, to specifically explore the effect of anti-PF4/heparin IgG for overt thrombosis in PV and ET.

Special forms of HIT including spontaneous or delayed onset HIT are being discussed in rare cases. State-of-the-art diagnostic criteria for spontaneous HIT have been proposed and include thrombocytopenia and thrombosis without previous heparin exposure along with detectable anti-PF4/heparin antibodies with platelet-activating potential in the absence of heparin [85]. Delayed onset HIT may correspondingly occur days to weeks after heparin exposure. However, diagnostic criteria for these particular HIT phenotypes do not correspond well to the situation in PV and ET. Thrombocytopenia is often masked by excessive platelet production and pathogenesis of thromboses is strongly multifactorial. In MPN, it is the finding of surprisingly frequent, endogenous anti-PF4/heparin antibody formation including particularly IgG isotypes in PV in the current study which deserves attention. It should increase our awareness for platelet activation and inflammatory stimuli as contributors to the anti-PF4/heparin immune response in nonsurgical patients even in the absence of heparin exposure. Future collaborative multicenter studies will hopefully allow for analyses of more extensive patient cohorts also in these rare disorders to unequivocally determine the significance of anti-PF4/heparin antibodies for thrombotic risk in MPN. Functional studies will be required to characterize the mechanisms of the anti-PF4/heparin immune response in the absence of heparin exposure. Further insight into the functional basis of this phenomenon could inform measures to reduce anti-PF4/heparin antibody formation and potential thromboses. Our data demonstrate that PV patients need to be tested for anti-PF4 IgG at the latest when an actual thrombotic event occurs. As anti-PF4 IgG are endogenously prevalent at a substantial frequency in PV, their presence needs to be excluded before treatment with heparin, which could enhance anti-PF4/heparin immune response and thrombotic risk [55], is initiated to facilitate alternative anticoagulation in anti-PF4 IgG-positive PV patients. Prospective studies of anti-PF4/heparin antibody formation in MPN should follow and will lead to more general recommendations for diagnostic and clinical management of PV and ET patients with anti-PF4/heparin antibodies.

Conflicts of Interest

There are no conflicts of interest to declare.

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Research Article

The Phosphatase Inhibitor Calyculin-A Impairs Clot Retraction, Platelet Activation, and Thrombin Generation

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The aim of this study was to investigate the effect of the serine/threonine protein phosphatase inhibitor, calyculin-A (CLA), on clot formation and on the procoagulant activity of human platelets. Platelet-rich plasma (PRP) samples were preincubated with buffer or CLA and subsequently platelets were activated by the protease-activated receptor 1 (PAR-1) activator, thrombin receptor activating peptide (TRAP). Clot retraction was detected by observing clot morphology up to 1 hour, phosphatidylserine- (PS-) expression was studied by flow cytometry, and thrombin generation was measured by a fluorimetric assay. For the intracellular Ca^{2+} assay, platelets were loaded with calcium-indicator dyes and the measurements were carried out using a ratiometric method with real-time confocal microscopy. CLA preincubation inhibited clot retraction, PS-expression, and thrombin formation. TRAP activation elicited Ca^{2+} response and PS-expression in a subset of platelets. The activated PRP displayed significantly faster and enhanced thrombin generation compared to nonactivated samples. CLA pretreatment abrogated PS-exposure and clot retraction also in TRAP-activated samples. As a consequence of the inhibitory effect on calcium elevation and PS-expression, CLA significantly downregulated thrombin generation in PRP. Our results show that CLA pretreatment may be a useful tool to investigate platelet activation mechanisms that contribute to clot formation and thrombin generation.

1. Introduction

Platelets play a crucial role in the pathogenesis of atherosclerotic diseases including acute coronary syndrome or ischemic stroke that are leading causes of death and disability worldwide. These events are triggered by disruption of the endothelium and plaque rupture or during interventions on coronaries, when platelets are tethered to surface-bound von Willebrand Factor (vWF), which initiates platelet activation and allows them to adhere to subendothelial components.

Protease-activated receptor 1 (PAR-1), the primary platelet thrombin receptor, is G-protein-coupled. The activator of this receptor is the strongest platelet agonist and an important contributor to atherothrombotic processes. Modulation of the PAR-1 receptor is the target for novel and promising antiplatelet drugs [1, 2]. PAR-1 receptor activation via thrombin or relevant thrombin receptor activating peptides (TRAPs) results in a series of signaling events terminating in platelet shape change and granule secretion via the $G_{12/13}$ proteins and intracellular calcium release via the G_q mediated inositol triphosphate (IP_3) pathway. Intracellular IP3 receptors can be directly activated by pharmacological agents like thiomersal that has been used previously as a calcium mobilizer and cell function-modulating agent [3].

Serine/threonine protein phosphatases (PP) are present in platelets predominantly as type 1 protein phosphatase (PP1) and type 2A protein phosphatase (PP2A) subtypes. Calyculin-A (CLA), a naturally occurring phosphatase inhibitor, present in marine sponges, in nanomolar concentration, primarily inhibits PP2A and indirectly PP1 [4, 5].

Among the other frequently used PP inhibitors tautomycetin selectively inhibits PP1, while okadaic acid at nanomolar concentrations preferentially suppresses the activity of PP2A [6–8]. Both PP1 and PP2A can be found in the membrane and cytosolic fractions of resting platelets [9].

A previous study from our group has described the effects of calyculin-A on TRAP-stimulated human platelets [8] and it was concluded that phosphatase inhibition prevents platelet-derived microparticle (MP) formation and degranulation in TRAP-activated platelets.

Surface exposure of phosphatidylserine (PS) is increased during platelet activation or apoptosis [10] and PS-expression is a key event in the control of blood coagulation, localizing prothrombin activation to the platelet plug, and regulating thrombin generation [11]. The initiation of early platelet activation events such as Ca^{2+}-influx can also be affected by CLA, which blocks any further propagation of platelet reactivity [12].

The aim of this study was to explore the effects of CLA on resting and activated platelets and to simultaneously investigate clot formation, platelet activation, and thrombin generation and their modulation in platelet-rich plasma. PS-expression, intracellular calcium responses using a novel cytosolic Ca^{2+} level measurement, clot retraction, and thrombin generation were studied with or without TRAP activation. We show here, for the first time, that in platelet-rich plasma CLA blocked clot retraction and inhibited cytosolic Ca^{2+} elevation upstream of the IP_3 receptor, abolished PS-expression, and subsequently inhibited thrombin generation. Thus, we suggest that this phosphatase inhibitor can be utilized in a wide variety of platelet functional assays for exploring biochemical pathways during thrombus formation.

2. Materials and Methods

2.1. Antibodies and Reagents. For flow cytometric analysis of PS-exposure we used FITC-labeled Annexin-V, Annexin-V binding buffer (10x concentrate), from Becton Dickinson (San Jose, CA), and a monoclonal anti-human CD41-PE antibody from Dako (Glostrup, Denmark). For platelet preparation either for flow cytometry or for the thrombin generation assay or intracellular Ca^{2+} measurements we used the following materials: paraformaldehyde (PFA), dimethylsulfoxide (DMSO), bovine serum albumin (BSA), HEPES, apyrase from potato, and Arg-Gly-Asp-Ser (RGDS) which were obtained from Sigma-Aldrich (St Louis, MO).

We used thrombin receptor activating peptide (TRAP) and thiomersal from Sigma-Aldrich (St Louis, MO) as platelet agonists. The protein phosphatase inhibitor, CLA, was from Calbiochem (San Diego, CA). HEPES buffer for flow cytometry and thrombin generation methods contained 150 mM NaCl and 25 mM HEPES, pH 7.4. HEPES-buffered saline for intracellular Ca^{2+} level measurements contained 145 mM NaCl, 10 mM HEPES, 10 mM D-glucose, and 5 mM KCl, pH 7.4, supplemented with 0.1% (w/v) BSA, 100 μM RGDS, 200 μM $CaCl_2$, and 0.1 U/mL apyrase. For some experiments during the analysis of free calcium levels instead of $CaCl_2$ the previously described HEPES buffer was supplemented with 500 μM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) from Sigma-Aldrich (St Louis, MO). We shall refer to this buffer hereafter as Ca^{2+}-free buffer.

2.2. Preparation of Platelet-Rich Plasma. Whole blood was drawn from healthy volunteers with no medications for at least 2 weeks prior to the experiments. Blood sample collection from volunteers was approved by the Ethical Committee of the University of Debrecen. The DEOEC RKEB/IKEB 4318-2015 protocol grants permission to draw citrated blood samples from patients and controls both for analysing plasma and platelets. Blood was anticoagulated with 0.105 M sodium citrate. Platelet-rich plasma (PRP) was prepared from venous whole blood by centrifugation at 170 ×g for 15 minutes at room temperature (RT). Platelet count of PRP was adjusted to 250 G/L by adding platelet poor plasma (PPP). PPP was obtained by centrifugation of the citrated blood sample at 1500 ×g for 15 minutes at RT. In subsequent experiments the following PRP samples were analysed: (i) nonactivated (NA), (ii) CLA-pretreated nonactivated (NA + CLA), (iii) TRAP-activated (TRAP), and (iv) CLA-pretreated TRAP-activated (TRAP + CLA). In preliminary experiments 50 nM CLA was found to inhibit degranulation of platelets as well as PAC-1 binding without being toxic to cells so this concentration of CLA was applied in the experiments. TRAP was used at a final concentration of 20 μM.

2.3. Clot Retraction Analysis. PRP (720 μL) was preincubated with buffer control or CLA for 30 minutes at 37°C in a water bath and then activated by TRAP. In a glass tube, 800 μL of every four samples was incubated with $CaCl_2$ (at a final concentration of 25 mM) for 60 minutes at 37°C in a water bath. At time points 0, 20, 40, and 60 minutes, photos were taken to document clot formation. At the end of the experiment, the volume of the extruded serum was determined by an analytical scale. The amount of fibrin monomer in the extruded serum was measured by a latex enhanced quantitative immunoassay (Stago, Asniére, France) on the ACL-TOP coagulation analyser (Instrumentation Laboratory, Bedford, MA).

2.4. Flow Cytometric Assays. PRP (110 μL) was preincubated with either HEPES buffer containing 0.5% DMSO as control or the protein phosphatase inhibitor CLA, for 30 minutes at 37°C in a water bath. CLA was used at a final concentration of 50 nM. After preincubation, platelets were activated either by TRAP at a final concentration of 20 μM or by thiomersal at a final concentration of 200 μM for 15 minutes at 37°C in a water bath. Then PRP (5 μL) was stained with 5 μL monoclonal CD41-PE antibody and 5 μL Annexin-V-FITC and Annexin-V binding buffer (1x concentrate) was added to examine the PS-expression of the platelets. In each experiment 10,000 events were collected in the platelet gate, measured by an FC500 flow cytometer, and results were analysed with the Kaluza software (Beckman Coulter, CA).

2.5. Thrombin Generation Assay. Eighty microliters of pretreated PRP was incubated with 20 μL of standard preparations of 1 pM recombinant tissue factor (rTF, PRP-Reagent,

Thrombinoscope BV, Maastricht, The Netherlands) for 10 minutes in round-bottomed 96-well black microplates. For each PRP sample a simultaneously run calibrator, a stable complex of α_2-macroglobulin (α_2M) and thrombin (Calibrator-Reagent, Thrombinoscope BV, Maastricht, The Netherlands), was used to eliminate the differences between samples [13]. Thrombin generation was initiated by the addition of 20 μL of a mixture of fluorogenic substrate and Fluo-Buffer that contained $CaCl_2$ (Thrombinoscope BV, Maastricht, The Netherlands). Fluorescence was detected by a Fluoroskan Ascent® fluorimeter (Helsinki, Finland) and the thrombin generation curves were analysed by the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). Thrombin generation curves were characterised by the following parameters (calculated and presented by the Thrombinoscope software): Lagtime is the delay in minutes until thrombin formation starts. Peak thrombin expressed in nanomoles is the highest thrombin level and the time required to reach the peak thrombin level is designated as time to peak (in minutes). Velocity index (in nanomoles per minute) is the slope between lagtime and time to peak.

2.6. High-Speed Confocal Measurement of Platelet Cytosolic Ca^{2+} Levels. For measurements of intracellular Ca^{2+} levels, PRP was prepared as described above and RGDS peptide was added to prevent aggregation [14]. PRP (100 μL) was loaded with 1.5 μL of 2 mM Fluo-4-AM for 10 minutes at 37°C in a water bath. The cells were coloaded with Fura-Red by addition of 2 μL of 1 mM Fura-Red-AM for 30 minutes at 37°C in a water bath. Before centrifugation, 2 mL of HEPES-buffered saline was added and after centrifugation at 350 ×g for 20 minutes at RT, the coloaded platelets were collected and resuspended in 100 μL of the same buffer.

Platelets loaded with the calcium-indicator dyes were imaged using a Zeiss LSM 5 LIVE (Carl Zeiss AG, Jena, Germany) high-speed confocal scanning unit with a 40x oil immersion objective (NA: 1.3). The final 100 μL solution was pipetted onto a glass coverslip secured above the objective within a temperature-controlled chamber and was allowed to rest for 2 minutes before the measurement. Both the chamber and the objective had been preheated to 37°C and this temperature was kept constant during the measurement. Frames (x-y images) were recorded at a rate of 5 Hz for 5 minutes using 488 nm excitation wavelength and two detection channels: a band pass filtered channel between 500 and 525 nm for the Fluo-4 signal and a long pass filtered channel above 635 nm for the Fura-Red signal. The wide gap between the cutoff wavelengths ensured that there was minimal crosstalk between the channels.

Details of the ratiometric method are demonstrated in Supplementary Figure 1 in Supplementary Material available online at https://doi.org/10.1155/2017/9795271. The green (Figures S1A, S1D, and S1G) and red (Figures S1B, S1E, and S1H) fluorescence channels were measured separately; then composite images were formed where color changes from red to green indicated increased intracellular Ca^{2+} concentrations (Figures S1C, S1F, and S1I). All platelets that could be observed for at least 30 s before and 90 s after the addition of TRAP were manually marked as regions of interest (ROIs) on the recorded image series. Time series curves of fluorescence values were analysed using a custom-built computer program. The average fluorescence level within a ROI on each channel was determined for all frames ($F_{\text{Fluo-4}}$, green, and $F_{\text{Fura-Red}}$, red; Figures S1J and S1M). Exponential fitting was used to determine the baseline fluorescence intensity on each channel ($F_{0(\text{Fluo-4})}$ and $F_{0(\text{Fura-Red})}$) taking into account the different bleaching levels for the two dyes during the experiment. Relative fluorescence values for the Fluo-4 ($F'_{\text{Fluo-4}}$, green) and Fura-Red ($F'_{\text{Fura-Red}}$, red) channels were calculated from the original fluorescence values by normalization to the respective baseline fluorescence curve (Figures S1K and S1N). Fluorescence ratio ($F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$, blue) values corresponding to the intracellular Ca^{2+} level in the platelet were calculated by dividing the relative fluorescence value for the Fluo-4 channel by that of the Fura-Red channel at every time point (Figures S1L and S1O). Calcium transients, but not nonspecific changes of fluorescence intensity, are characterised by a simultaneous negative deflection of the $F'_{\text{Fura-Red}}$ and positive deflection of the $F'_{\text{Fluo-4}}$ curves and thus the $F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$ trace. The magnitude of the transients can be characterised by the amplitude measured on the fluorescence ratio curve.

TRAP was added by pipetting 4 μL of 500 μM TRAP solution to the platelet suspension on the coverslip. Measurement was continuous during the addition of TRAP.

2.7. Statistical Analysis. Normality of the data was evaluated by the Kolmogorov-Smirnov test. Data are expressed as mean ± SD or mean ± SEM. Predetermined pairwise differences were analysed by paired Student's t-test and p values less than 0.05 were considered as statistically significant. In case the data showed a nonnormal distribution, the Wilcoxon signed rank-sum test was used for statistical evaluation. 95% confidence intervals for proportions were calculated using the modified Wald method.

3. Results

3.1. The Effect of CLA on Clot Retraction. It could be observed that clot formation and retraction commenced already by 20 minutes and by 60 minutes both the nonactivated and the TRAP-activated samples displayed an intense clot retraction. The CLA-pretreated samples however were much less retracted (Figure 1(a)). In accordance with this morphology the CLA-pretreated samples extruded significantly less serum (Figure 1(b)). The fibrinogen concentration was unmeasurably low in the samples by 60 minutes; nevertheless the fibrin monomers (FM) were determined by a quantitative assay and it was found that in the extruded sera of CLA-pretreated samples there was a tendency to higher FM values compared to nonpretreated samples (Figure 1(c)).

3.2. The Effect of CLA on the Level of Platelet PS-Expression. In a series of experiments we determined the level of PS-exposure to analyse the effects of CLA on resting and activated platelets. PRP samples were preincubated with HEPES buffer (control) containing 0.5% DMSO or 50 nM CLA in DMSO for 30 minutes. Platelets were identified according to

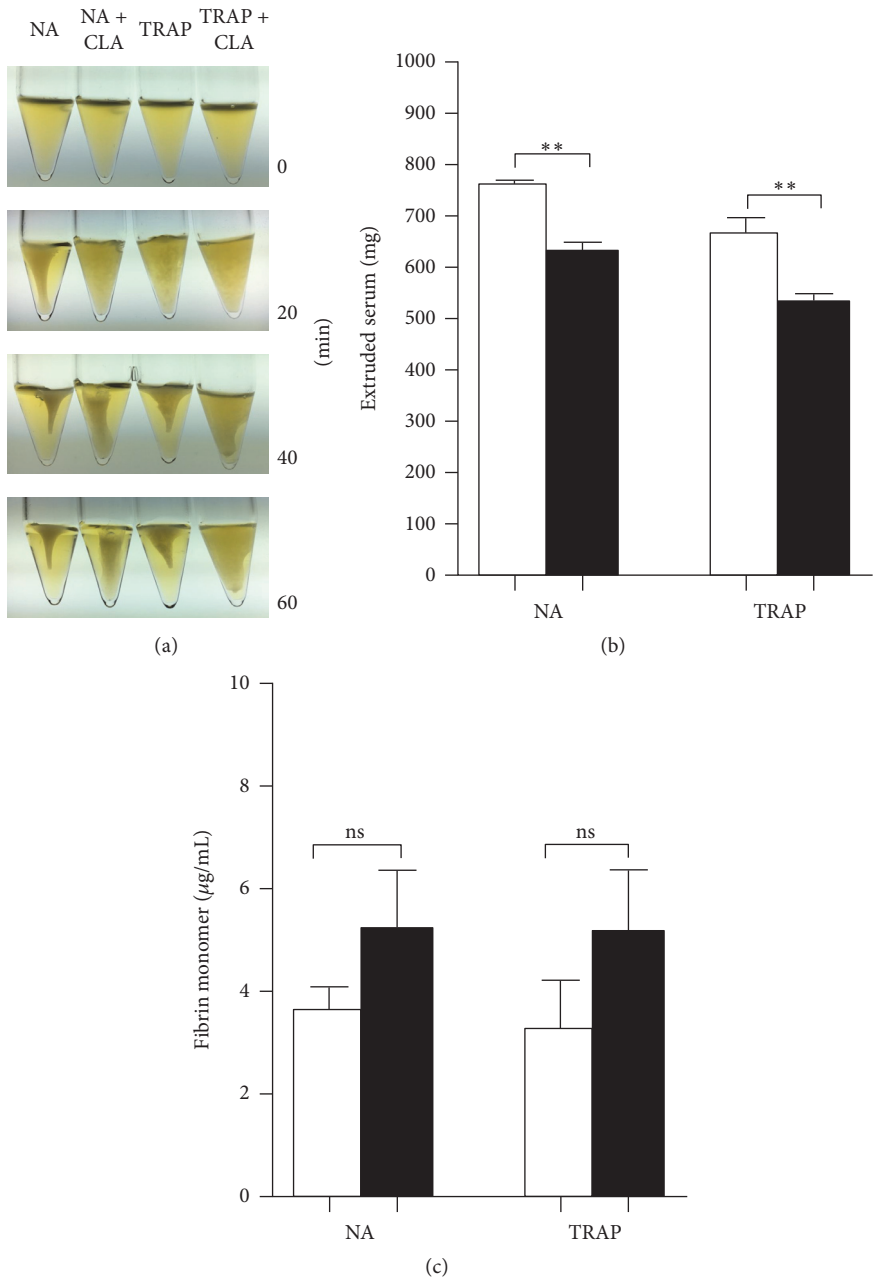

Figure 1: Inhibitory effect of CLA on clot retraction. Nonactivated (NA) and TRAP-activated samples displayed an intense clot retraction by 60 minutes, while CLA-pretreated samples were much less retracted (a). This significant difference could be observed by the lower quantity of extruded serum in CLA-pretreated samples (b) and a tendency could be observed to display higher fibrin monomer concentrations in CLA-pretreated samples (c). Nonactivated and activated samples are indicated with open bars and CLA-preincubated samples are represented by the black bars. The results are the mean and standard error of the mean (SEM) of 5 different experiments. Statistical significance was assessed by paired t-test. $^{**}p < 0.01$. ns = nonsignificant.

their CD41-PE staining; then PS-expression was determined by Annexin-V positivity. The PS-expression of nonactivated (NA) samples (Figure 2) was low (mean ± SD = 3.03 ± 0.84) and it was further decreased by CLA pretreatment (mean ± SD = 1.75 ± 0.67, $p = 0.004$). The PAR-1 receptor agonist, TRAP, significantly increased the number of platelets expressing PS (mean ± SD = 18.7 ± 3.23) compared to the NA sample ($p < 0.0001$) while CLA pretreatment completely prevented PS upregulation in TRAP-stimulated platelets ($p < 0.0001$) (Figure 2).

3.3. The Effect of CLA on Various Thrombin Generation Parameters. Representative thrombin generation curves demonstrate the effect of TRAP and/or CLA on thrombin formation by platelets (Figure 3(a)). The thrombin formation was faster, and the lagtime and time to peak were shorter

Table 1: Proportion of platelets responding to activation by TRAP.

Sample	Total number of investigated platelets	Ca^{2+} transient in response to activation by TRAP		No transient change	
		n	%	*n*	%
TRAP-activated PRP	117	26	22.2% (15.59–30.62%)	91	77.8% (69.38–84.41%)
CLA-preincubated TRAP-activated PRP	122	0	(0.0–3.67%)	122	100% (96.33–100.0%)

Numbers in brackets indicate the 95% confidence interval; total number of samples = 13 for each dataset.

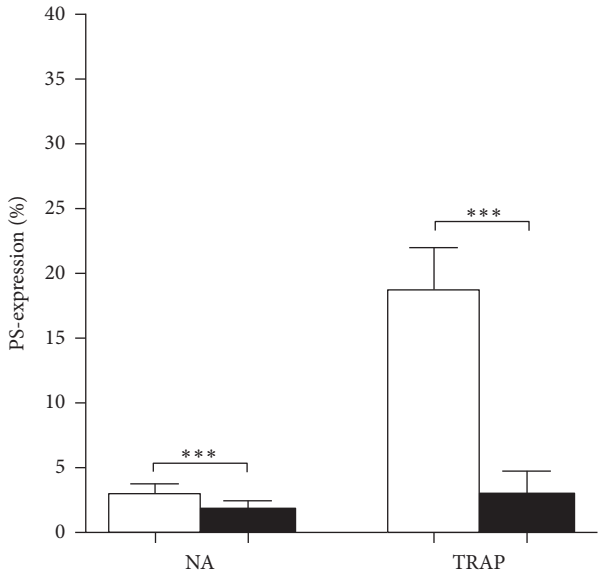

Figure 2: Inhibitory effect of CLA on PS-expression. Nonactivated (NA) and activated samples are indicated with open bars and CLA-pretreated samples with black bars. The results are the mean and standard deviation (SD) of 8 different experiments. Statistical significance was assessed by paired *t*-test. $^{***}p < 0.001$.

in case of TRAP activation compared to the nonactivated sample (Figures 3(b) and 3(c)). TRAP activation increased the peak thrombin and also the velocity index (Figures 3(d) and 3(e)). CLA preincubation significantly prolonged the time of thrombin generation in TRAP-activated samples but did not have significant effect on the NA sample. CLA attenuated the peak thrombin and velocity index already in NA samples and completely blocked the TRAP-elicited augmentation in these parameters.

3.4. Ratiometric Measurement of Platelet Cytosolic Ca^{2+} Levels. Platelets marked on confocal time series images were divided into groups based on the time course and magnitude of calcium transients during the experiment.

Platelet intracellular Ca^{2+} levels were measured after activation of nonactivated or CLA-pretreated sample. Two typical patterns of intracellular calcium concentration changes of platelets were observed on TRAP-activated samples without CLA pretreatment: no transient change after the addition of TRAP (Figure 4(a)) or a transient increase in the Ca^{2+} level (Figure 4(b)). When platelets were preincubated with CLA all calcium transients were abolished (Figure 4(c)). All curves depict $F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$ values where an increase indicates higher cytosolic calcium levels. First, we investigated nonactivated platelets for 60 seconds and TRAP was added to the PRP during the microscopic measurement. Dotted vertical lines mark the time of TRAP addition. One hundred and seventeen platelets were investigated in TRAP-activated samples. In the case of samples that were not pretreated, 22.2% of platelets showed transient cytosolic Ca^{2+} increase upon TRAP addition. In case of platelets preincubated with calyculin-A, none of the investigated 122 cells showed calcium transients after TRAP administration during the time course of the measurement providing evidence for CLA blockage of Ca^{2+} elevation (Table 1). It is important to emphasize that CLA pretreatment did not affect resting cytosolic calcium levels (data not shown).

3.5. The Effect of Thiomersal on Platelet Activation. Both nonactivated and CLA-preincubated samples were treated with thiomersal at a final concentration of 200 μM and PS-expression was determined by flow cytometry. The PS-expression of nonactivated samples was 2.3% (Figure 5 (NA)) that was further decreased by CLA preincubation (Figure 5 (NA + CLA)). The ratio of PS-expressing cells was increased to 16.4% by TRAP activation (Figure 5 (TRAP)) that could be completely blocked by CLA. Contrary to the TRAP activation, thiomersal activation induced 96% PS-expression (Figure 5 (thiomersal)) that could not be prevented by CLA preincubation (Figure 5 (thiomersal + CLA)).

Thiomersal evoked a prolonged elevation of cytosolic calcium in all the platelets followed by sustained store-operated calcium entry (Figure 6(a)). Nonactivated platelets kept in calcium-free buffer show a minimal calcium entry in response to the resupplement of extracellular calcium by calcium chloride, probably due to platelets that were activated by physical contact with the glass coverslip (Figure 6(b)).

In CLA-pretreated platelets thiomersal also evoked a prolonged elevation of cytosolic calcium followed by sustained store-operated calcium entry, similarly to the response seen in NA platelets (Figure 6(c)). All preparations responded to calcium ionophore A23187 with increased calcium levels. All curves are based on $F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$ values where an increase indicates higher cytosolic calcium levels.

4. Discussion

It is widely accepted that cell surface expressed, negatively charged phospholipids are required for the formation of

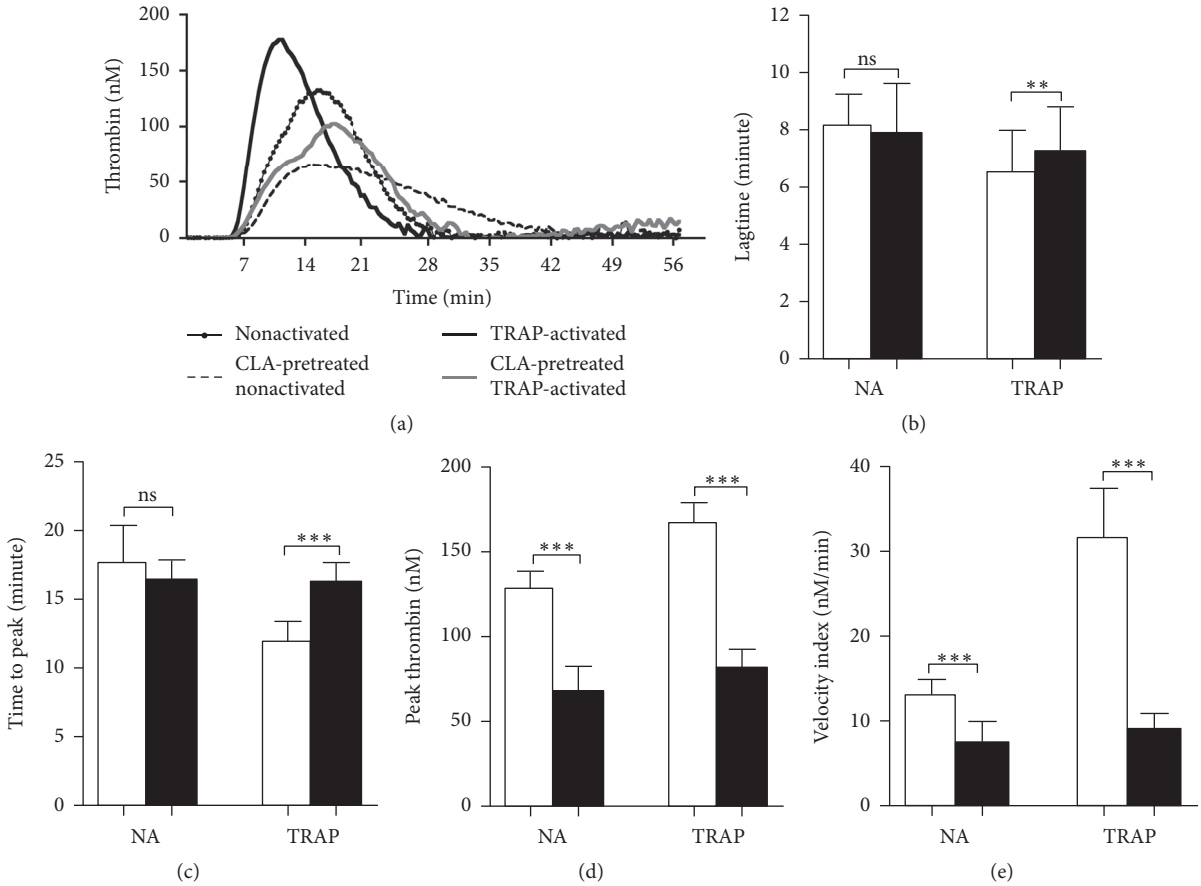

Figure 3: Thrombin generation assay and the effect of CLA on various parameters during thrombin generation. Thrombin generation in PRP was investigated as a procoagulant function of the intracellular Ca^{2+} level changes. The dotted line corresponds to nonactivated sample, the dashed line represents the CLA-pretreated nonactivated sample, black line marks the TRAP-activated sample, and CLA pretreatment on TRAP-activated sample is represented by the grey line (a). Lagtime (b) and time to peak (c) values were observed during TRAP-elicited platelet activation peak thrombin (d) and velocity index (e) values are informative about the amount and speed of generated thrombin. Nonactivated and activated samples are indicated with open bars and CLA-preincubated samples with black bars. The results are the mean and standard deviation (SD) of 5 different experiments. Statistical significance was assessed by paired t-test. $^{**}p < 0.01$; $^{***}p < 0.001$. ns = nonsignificant.

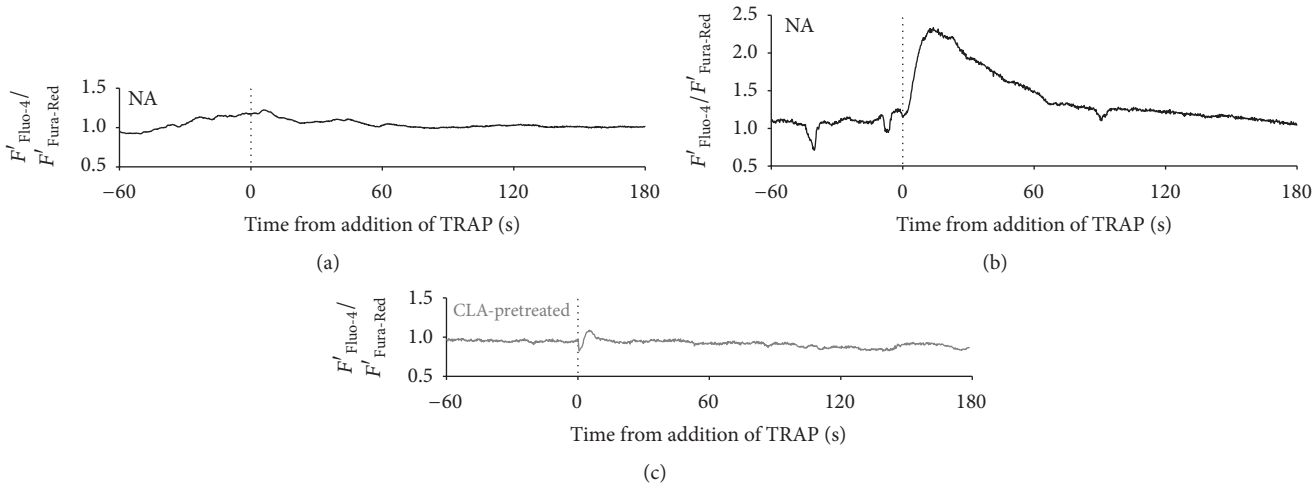

Figure 4: Characteristic changes of the cytosolic Ca^{2+} level of single platelets in relation to TRAP activation. Three typical time courses of the intracellular calcium concentration of platelets: no response to TRAP (a), transient increase in the Ca^{2+} level after the addition of TRAP (b), and no transient change upon CLA pretreatment (c). All curves are $F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$ values where an increase indicates higher cytosolic calcium levels. Dotted lines mark the time of TRAP addition.

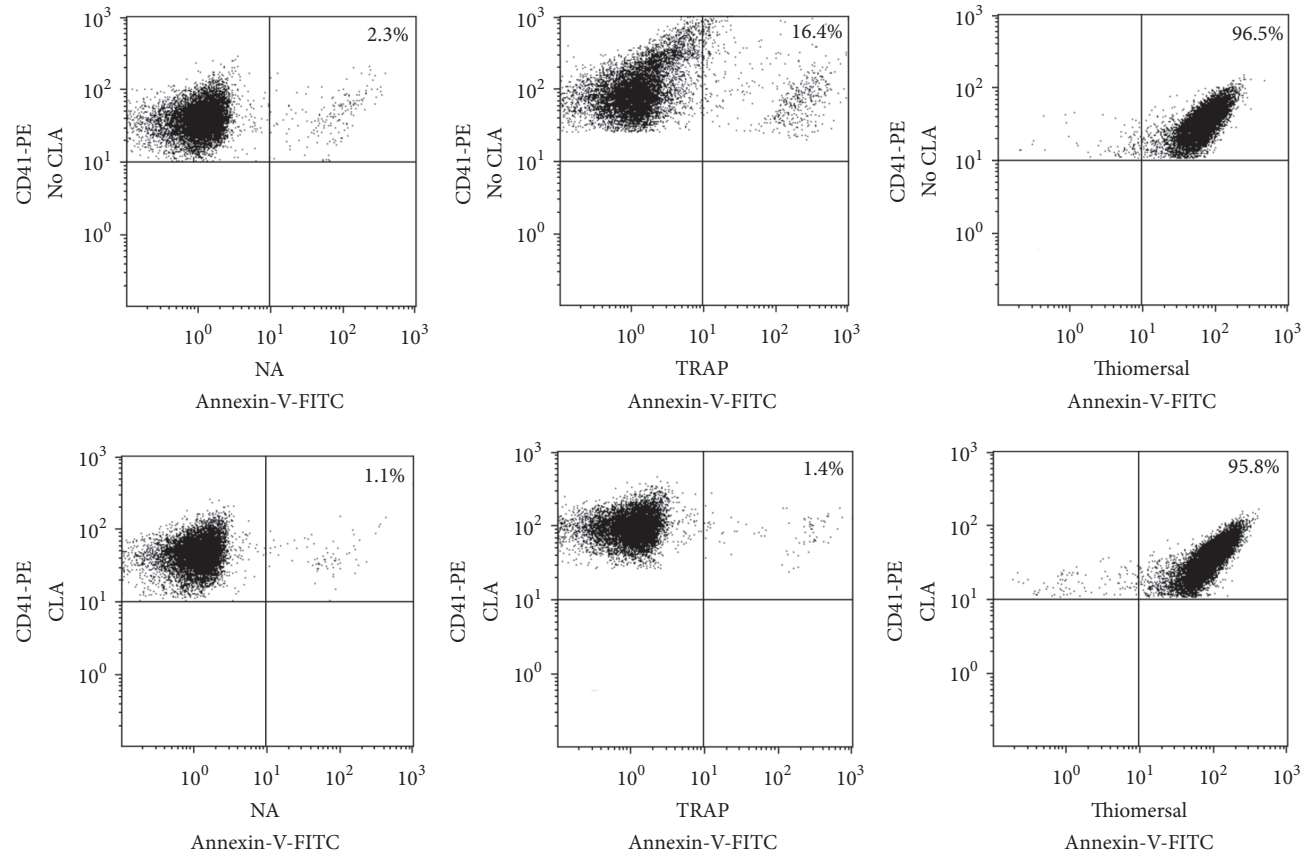

Figure 5: The effects of TRAP, thiomersal activation, and CLA preincubation on phosphatidylserine-expression (PS). The positivity of platelet PS-expression according to the Annexin-V labeling is in the upper right quadrants of the dot plots. The PS-expression of nonactivated sample (NA) was very low. When platelets were preincubated with CLA the PS-expression further decreased. Representative dot plots show that TRAP activation resulted in elevated PS-expression, which was completely blocked by preincubation with CLA. Thiomersal activated almost all of the platelets and this activation could not be inhibited by CLA preincubation.

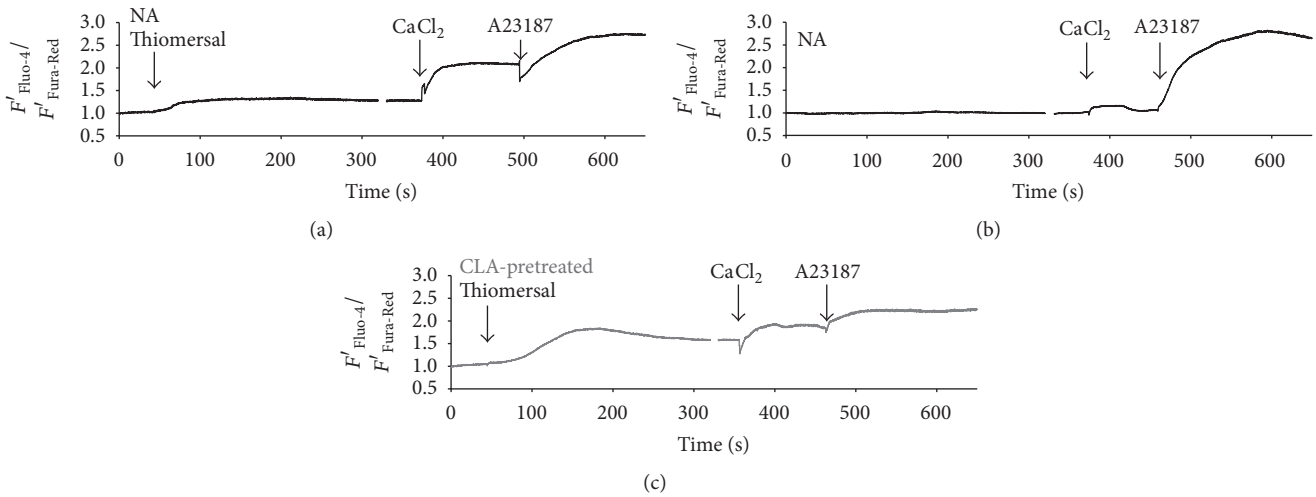

Figure 6: Changes of the cytosolic Ca^{2+} level of platelet groups in relation to activation by thiomersal in Ca^{2+}-free buffer and the consequent store-operated calcium entry. Thiomersal evoked a prolonged elevation of cytosolic calcium in all the platelets followed by sustained store-operated calcium entry (a). Nonactivated platelets kept in calcium-free buffer show a minimal calcium entry in response to the resupplement of extracellular calcium by calcium chloride, probably due to platelets that got activated on physical contact to the glass coverslip (b). Thiomersal evoked a prolonged elevation of cytosolic calcium in CLA-pretreated platelets followed by sustained store-operated calcium entry, similarly to the response seen in NA platelets (c). All preparations responded to calcium ionophore A23187 with an increased calcium level. All curves are $F'_{\text{Fluo-4}}/F'_{\text{Fura-Red}}$ values where an increase indicates higher cytosolic calcium levels. Arrows mark the addition of thiomersal.

multiprotein complexes of the coagulation cascade. Thus, this membrane dependency identified as PS-exposure can be regarded as a cellular control of coagulation processes during thrombus formation.

In our experiments we have investigated calcium signaling and PS-expression of platelets and investigated the role of phosphatase inhibition in these processes and subsequent thrombin formation in nonactivated and PAR-1 activated samples. Instead of thrombin we utilized TRAP, a potent activator of PAR-1 on platelets, to avoid early clotting and any potential pleiotropic effects [15].

Serine/threonine protein phosphatases play an essential role in cellular signaling, metabolism, and cell cycle control [7]. The activity of these phosphatases is needed for the initiation of platelet secretion and aggregation that is evident from the study which described that CLA suppressed aggregation, adhesion, secretion, and platelet spreading on fibrinogen [16].

Under experimental conditions in phosphatase-inhibitor-treated platelets, actin polymerization was inhibited, microtubules were reorganized in sustained pseudopods, and the phosphorylation of myosin light chain (MLC) was not enhanced upon thrombin stimulation [17]. The MLC phosphorylation is crucial in actomyosin contraction for platelet secretion, which is induced by MLC kinase and counterbalanced by myosin phosphatase including associated protein phosphatase-1 catalytic subunit and myosin binding (MBS/MYPT1) regulatory subunit [18].

When platelets are stimulated with thrombin, among several intracellular proteins, Rho-kinase, PKC, and integrin-linked kinase become activated resulting in the phosphorylation of MBS/MYPT1 (at Thr695/Thr696) causing a decreased myosin phosphatase activity accompanied with an elevated MLC phosphorylation [19, 20]. This leads to actomyosin contraction and subsequent secretion.

When CLA was added to platelets, PP was also blocked in this complex and this intervention further increased the phosphorylation of MYPT1 decreasing myosin phosphatase activity [8]. Already 10 nM CLA decreased phosphatase activity in both resting and TRAP-activated samples and with 50 nM the control phosphatase activity was decreased by more than 50%. Therefore, in the present study we used a final concentration of CLA of 50 nM.

Activation of platelets by various agonists is accompanied by a transient intracellular Ca^{2+} elevation, which is obligatory for the initiation and propagation of platelet responses such as adhesion, aggregation, and degranulation. The rise in intracellular calcium is a result of the calcium release from the intracellular stores mediated by the IP_3 receptor. The depletion of the intracellular calcium stores is sensed by stromal interaction molecule (STIM1). Consequently, a calcium entry channel molecule (Orai1) is activated by STIM1 and elicits store-operated calcium entry (SOCE). This STIM-Orai regulated mechanism has been described in several cell types including platelets [21].

In earlier studies, it was found that CLA treatment of activated platelets suppressed the activation induced rise in Ca^{2+} levels measured by fluorescence spectrophotometric techniques [22, 23]. Experimental techniques have considerably improved in the past decades. More recently, a ratiometric flow cytometric method has been described that is suitable for measuring calcium fluxes in platelets [24]. We have investigated platelet cytosolic Ca^{2+} levels by a real-time ratiometric measurement with confocal microscopy, to more appropriately characterise changes in intracellular calcium signals. It was found that activating platelets by TRAP via the PAR-1 receptor causes a clear intracellular Ca^{2+} signal in a proportion of platelets that was abolished by CLA pretreatment and this effect was unrelated to resting calcium levels. In control samples, upon TRAP activation, a heterogeneous response was predictable [25, 26]. Indeed, we have observed two clear patterns: fast and robust Ca^{2+}-transients in 22% of platelets and no Ca^{2+}-response in the rest. Similar to the heterogeneity in calcium signals, not all platelets express PS upon activation. After platelet activation, these distinct platelet subpopulations may have different roles in the coagulation process, depending on their activation state and surface properties [27]. Similarly to calcium signals, not all platelets express PS upon activation; there is current evidence for platelet subpopulations, heterogeneity of platelet responses, and functions in the thrombus-forming process [28–30]. We attribute the two distinct Ca^{2+}-response patterns to the existence of the above-mentioned platelet subpopulations. Response heterogeneity can be due to intrinsic differences between platelets in age and in receptor and signaling proteins. As a result, at least three subpopulations of platelets have been suggested in a thrombus: aggregating platelets with (reversible) integrin activation, procoagulant (coated) platelets exposing phosphatidylserine and binding coagulation factors, and contracting platelets with cell-cell contacts [28].

By using thiomersal, a direct activator of the intracellular IP3 receptors, we could verify both by flow cytometric PS-expression studies and by the ratiometric calcium measurement that PP inhibition by CLA does not impair direct IP3 receptor stimulation, so its effect is exerted upstream of the IP3 receptor.

The thrombin generation test was originally described for plasma [31] and has later been extended to PRP [32] and it was found that this test may reveal important interactions between platelets and the clotting system [33]. In our experiments the speed of thrombin generation as measured by the lagtime and time to peak parameters and the activity of generated thrombin as exemplified in the peak thrombin and velocity index parameters were shown to be the most useful parameters to evaluate the effect of CLA on the thrombin generation of PRP.

Raised Ca^{2+} levels lead to the exposure of PS on the platelet surface that serves as a site for the assembly of intrinsic and extrinsic tenase and prothrombinase complexes; thus it is critical for thrombin generation in PRP [34]. We have found that CLA on TRAP-activated platelets influenced platelet responses by eliminating calcium transients. It also abolished agonist-induced PS-exposure and consequently downregulated thrombin generation in PRP as demonstrated both by the speed of thrombin formation and by the amount of generated thrombin.

PS exposing platelets are procoagulant; however, they are nonadhesive and possess closed (or blocked) integrins;

thus they are unlikely to participate in clot retraction. Nevertheless, in our experiments CLA also inhibited the platelet subpopulation that participates in clot retraction. Fibrinogen was clotted to insoluble fibrin so very little fibrin monomer remained in the extruded serum particularly in the TRAP-activated PRP. We have found that CLA attenuated thrombin formation that may have contributed to less crosslinked fibrin in the clot and thus more FM was detectable in the extruded serum.

Taken together, these findings indicate that inhibition of PP by CLA pretreatment can be regarded as a useful tool to investigate the platelet subsets that contribute to enhanced thrombin formation and clot retraction.

5. Conclusions

Calyculin-A effectively inhibits phosphatases in resting and activated platelets and can be used in a wide variety of platelet functional assays ranging from clot retraction, via calcium measurements, to thrombin generation. With the simultaneous use of selective platelet activators it can be utilized to dissect biochemical pathways during thrombus formation.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Renáta Hudák performed the clot retraction and thrombin generation experiments and wrote most of the manuscript, Ildikó Beke Debreceni did flow cytometric measurements, János Vincze and Tamás Oláh carried out the calcium measurements, László Csernoch worked out the calcium measurement methodology, and Kenneth J. Clemetson and Ferenc Erdődi reviewed the data and helped in writing the manuscript. János Kappelmayer designed the study, supervised the experiments, and finalized the manuscript. Renáta Hudák and János Vincze contributed equally to this work.

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Review Article

The Interaction of Selectins and PSGL-1 as a Key Component in Thrombus Formation and Cancer Progression

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Cellular interaction is inevitable in the pathomechanism of human disease. Formation of heterotypic cellular aggregates, between distinct cells of hematopoietic and nonhematopoietic origin, may be involved in events leading to inflammation and the complex process of cancer progression. Among adhesion receptors, the family of selectins with their ligands have been considered as one of the major contributors to cell-cell interactions. Consequently, the inhibition of the interplay between selectins and their ligands may have potential therapeutic benefits. In this review, we focus on the current evidence on the selectins as crucial modulators of inflammatory, thrombotic, and malignant disorders. Knowing that there is promiscuity in selectin binding, we outline the importance of a key protein that serves as a ligand for all selectins. This dimeric mucin, the P-selectin glycoprotein ligand 1 (PSGL-1), has emerged as a major player in inflammation, thrombus, and cancer development. We discuss the interaction of PSGL-1 with various selectins in physiological and pathological processes with particular emphasis on mechanisms that lead to severe disease.

1. Introduction

In the last three decades, our knowledge on the function of the receptor family of selectins and their ligands has been substantially extended in terms of the development and progression of several diseases, particularly inflammation, atherosclerosis, thrombosis, and malignancy. Furthermore, several mucins expressed on cancer cells and neutrophil extracellular traps (NETs) have been recently implicated to be involved in thrombosis and cancer development via selectin-mediated interaction. Due to substantial involvement of selectins and their counter-receptors in these serious conditions, they have become therapeutic targets in the prevention or at least alleviation of these disorders.

1.1. The Players and Their Nomenclature. Selectins obtained their names because of their ability to selectively bind carbohydrate moieties. Their ligands had long remained unidentified; nevertheless work in the 1980s led to the discovery of a dimeric mucin that is now uniformly designated as P-selectin glycoprotein ligand 1 (PSGL-1). The name PSGL-1 surmises that further molecules also exist as selectin ligands. Thus, the term PSGL-1 is misleading because of two reasons: (i) the mucin is not a glycoprotein ligand for P-selectin only but the major ligand for all three selectins and (ii) although it is true that there are several selectin binding proteins, the numbering of PSGL became obsolete as other selectin ligands are structurally different proteins. In the early years of selectin and PSGL-1 discovery, it was common that independent enthusiastic research groups investigated these phenomena in parallel using different approaches and the discoveries resulted in various nomenclatures on the very same proteins.

1.2. Selectin Structure and Function. There are three types of selectins that are all composed of the same domains and are distinguished from one another by their variable number of consensus repeats [1, 2]. The longest selectin molecule with 9 such motifs is P-selectin and it was named based on its first recognized source, the platelets. Subsequent studies also revealed that P-selectin is also detectable in endothelial cells; additionally these cells also possess a unique selectin naturally designated as E-selectin that is shorter in length compared to P-selectin as it contains 6 consensus repeats.

Table 1: Characteristics of various selectins.

	Cellular expression	Protein ligands	Rolling velocity of leukocytes
P-selectin (GMP-140, PADGEM, CD62P)	Activated endothelium and platelets	PSGL-1 CD24	Slow
E-Selectin (ELAM-1, CD62E)	Activated endothelium	PSGL-1 ESL-1 L-Selectin Podocalyxin	Slow
L-Selectin (MEL-14, CD62L)	Constitutive expression on leukocytes	PSGL-1 GlyCAM-1 MAdCAM-1 CD-34 Podocalyxin	Fast

The shortest selectin molecule, present in leukocytes, is L-selectin and contains only 2 consensus repeats [2]. There is considerable difference in the cellular appearance of these selectins not only in the sense that P-selectin is not cell-specific, as it is present on both platelets and endothelial cells, but also regarding its appearance in normal or activated cells [1, 2].

Research on selectins started with the identification of different monoclonal antibodies. Out of these antibodies one investigated in detail was the S12 clone [3]. It was found that nonactivated platelets do not react with S12, but following platelet activation by thrombin an intense labelling was obtained [3]. Studies revealed that this clone identifies a 140 kDa protein that is present in the alpha-granules of resting platelets and upon stimulation is expressed on the cell surface [4]. Thus, one of its designations was based on molecular mass; as such it was named granule membrane protein, GMP-140. Another group identified the same protein as a platelet activation dependent granule-external membrane protein [5]; as such it was designated as PADGEM [6]. Later it became evident that PADGEM, GMP-140, or P-selectin identifies the same cell surface molecule and subsequently obtained a number in the cluster of differentiation nomenclature as CD62P [2].

A major breakthrough was the discovery that, similarly to platelets, endothelial cells also contain a considerable amount of P-selectin. After its synthesis, this protein is transported to the Golgi apparatus where it is decorated with carbohydrates and finally transported to and stored in the Weibel-Palade bodies [7]. In addition to P-selectin, endothelial cells were also described to express a unique adhesion molecule, subsequently designated as E-selectin [8], but, unlike P-selectin, preformed E-selectin is not present in the endothelium. Thus, there is considerable difference in the kinetics of selectin expression in endothelial cells when stimulated, as P-selectin can be expressed on the endothelial surface within minutes, while E-selectin is de novo synthesized and a minimum of 2-3 hours are required for its surface expression.

The third member of the selectin family is L-selectin that was actually discovered earlier than the other two selectins [9] and is expressed on basically all types of leukocytes. It is different from the other two selectins not only in its size, but also in its expression as it is constitutively present on leukocytes [10]. Upon cell stimulation, their surface expression is usually downregulated. A short summary about the characteristics of selectins is shown in Table 1.

The discovery of the surface expression of selectins immediately implicated two important further areas of investigation. One that was plausible to be studied immediately upon their discovery was the identification of their soluble forms. The molecular mass of the soluble selectins is somewhat smaller than the membrane expressed forms, since they do not contain the transmembrane and intracellular domains. Shedding is the natural fate of surface-expressed receptors, for example, platelet CD40L, that is facilitated by proteolytic cleavage by (metallo)proteinases; nonetheless P-selectin shedding by this mechanism remains elusive [11]. Furthermore, PSGL-1 also regulates the rapid shedding and, in the absence of PSGL-1, P-selectin can be downregulated on activated platelets mostly by internalization [12]. In addition, a soluble form can also be released via the direct expression of its splice variant lacking the cytoplasmic domain [13]. It was shown earlier that, in addition to cell surface expression, the distribution and the intracellular trafficking of P-selectin are also important in leukocyte recruitment [14–16]. Several signaling molecules were described as regulators, and the internalization into clathrin-coated pits is also regulated by an endocytic receptor [17]. Similarly, the distribution of E-selectin in raft domains is also important for its adhesive capacity [18].

1.3. PSGL-1 Structure and Function. Another area of research has emerged from the arduous quest for the possible selectin ligands. Selectins interact weakly with small sialylated, fucosylated oligosaccharides, such as the tetrasaccharide sialyl-Lewis^x, and they bind with higher affinity to glycans displayed on glycoproteins or proteoglycans. Thus, characterization of these high affinity selectin ligands was important, as they are key components in selectin-mediated leukocyte adhesion during inflammation. Although there are several glycosylated proteins that are capable of binding one particular selectin type, only one protein has emerged as the best characterized ligand for all three selectins. In the early 1990s, work from the laboratory of Dr. McEver described that endothelial cell P-selectin attaches to neutrophil glycoprotein via a lectin-like interaction [19]. This glycoprotein that was later proved to

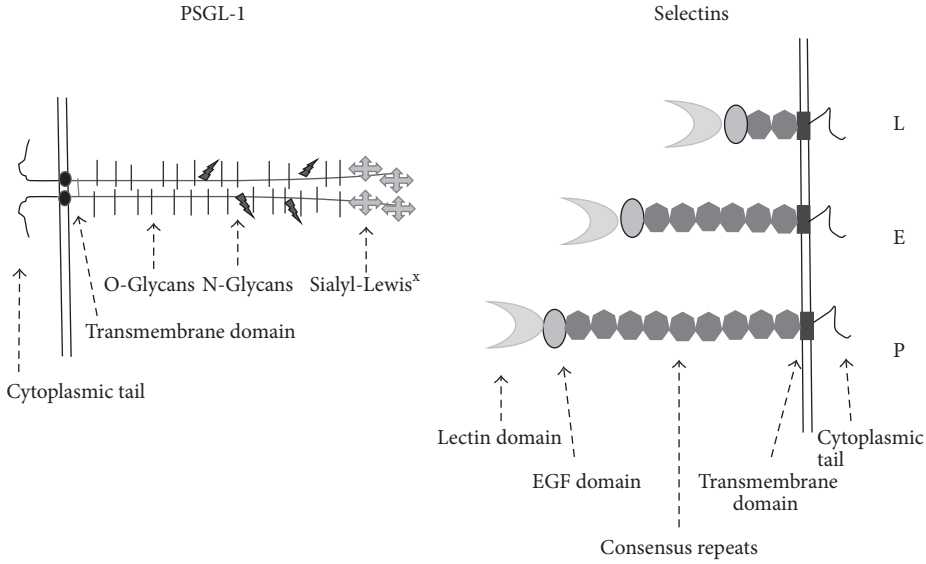


Figure 1: Structural features of selectins and PSGL-1.

be the main counter receptor for selectins is a heavily glycosylated protein, where sialyl-Lewis^x is a necessary partner for the three selectins that bind the platelets, leukocytes, and endothelial cells. It is a 120 kDa homodimeric mucin that is a type I membrane protein consisting of 402 amino acids. The expression of the native protein would be insufficient for selectin binding, as PSGL-1 needs to be posttranslationally modified by sialic acid and fucose exclusively on O-glycans and to be sulfated on tyrosine residues to become functional. The importance of these posttranslational modifications is exemplified by the inability of lymphocyte PSGL-1 to bind to P-selectin. Although most lymphocytes express PSGL-1, only 10–20% actually bind P-selectin [20]. The necessity of PSGL-1 in selectin binding is interesting as it only represents a small fraction of the total sialyl-Lewis^x containing residues on the leukocyte surface and the copy number of this molecule is also relatively low [21].

Unlike P- and E-selectin but similarly to L-selectin, PSGL-1 is a constitutively expressed molecule on the surface of several hematopoietic and in some nonhematopoietic cells [22]. In circulating blood under flow conditions, selectins mediate the first adhesive step that is characterized by tethering and rolling of leukocytes on endothelial cells, platelets, or other leukocytes. L-selectin expressed on most leukocytes binds to ligands on endothelial cells and on other leukocytes; however, these molecules like the peripheral node addressins and the CD34 family of transmembrane sialomucins, such as podocalyxin, are not a part of this review. E-selectin expressed on activated endothelial cells also binds to ligands on most leukocytes and binds to activated platelets. There is a cellular network during inflammation that becomes complete by the participation of P-selectin on activated platelets and endothelial cells. The regulated expression of the selectins and their ligands initiates the inflammatory response and prepares the stage for a firm integrin-mediated leukocyte binding of the slowed leukocytes. It has been found that the inappropriate expression of these molecules contributes to leukocyte-mediated tissue damage in a variety of inflammatory and thrombotic disorders recently reviewed by Nagy Jr. et al. [23]. The structure of selectins and PSGL-1 is depicted in Figure 1.

1.4. PSGL-1 and Selectin Gene Polymorphisms. The functional effects of different individual or complex polymorphisms of selectins and PSGL-1 have been established in the development of vascular and metabolic diseases. PSGL-1 resembles the adhesive platelet receptor glycoprotein Ib alpha (GPIbα), as both proteins mediate the attachment of blood cells from blood. GPIbα is known to be polymorphic, so it was anticipated that PSGL-1 may also be polymorphic. Indeed, there is a relatively common genetic variation in PSGL-1 with variable number of tandem repeats (VNTR) affecting the length of the extracellular domain of PSGL-1 molecule via the distance from the P-selectin binding site to the cell surface [24]. Three allelic variants were identified in the human population. The 3 alleles, A, B, and C, from largest to smallest, contained 16, 15, and 14 decameric repeats, respectively, with the B variant lacking repeat 2 and the C variant retaining repeat 2 but lacking repeats 9 and 10. Allele frequencies were highest for the A variant and lowest for the C variant; the frequencies were described as 0.81, 0.17, and 0.02 in the white population. Homozygous carriers for the shorter B and C short alleles had lower risk for premature myocardial infarction due to lower adhesive capacity [25]. Further studies may clarify whether other haplotypes of P-selectin and/or PSGL-1 gene substantially influence the risk and outcomes of adverse vascular events with or without medication.

P-selectin is highly polymorphic having several genetic variants; the Thr715Pro variant located in the last consensus repeat region of P-selectin is probably the most intensively studied [26–30]. The substitution of threonine for proline induces a conformational change in the precursor protein, which may influence its intracellular transportation and secretion leading to reduced expression and/or shedding of

P-selectin and thus fewer cellular interactions are developed. The Pro715 allele alters the kinetics of P-selectin release in patients with recurrent deep vein thrombosis [31]. The lectin domain of each selectin possesses the carbohydrate binding site and P-, E-, and L-selectin share 70% sequence identity over each of their three individual lectin domains. Changes in the amino acid sequence within the P- and E-selectin EGF domains have been shown to modulate the adherence of the proteins to sialyl-Lewis^x and heparin [32]. Another possibility by which polymorphisms may contribute to atherothrombotic processes is via the modulation of the release of soluble selectins. This has been shown for E-selectin as the Leu554Phe mutation results in a diminished soluble E-selectin release. Soluble E-selectin may be a protective factor in the progression of atherosclerosis as this may have a direct pathological consequence [33]. L-selectin is also polymorphic, but a recent large multiethnic analysis found that although the variants account for a significant level of soluble L-selectin variance, none of these variants were associated with clinical or subclinical cardiovascular disease [34].

2. Role of Selectins and PSGL-1 in Physiological Leukocyte Recruitment and during Inflammation

2.1. Classic Knowledge on Leukocyte Recruitment. The inflammatory process is accompanied by numerous molecular changes and several of these influence cell-cell interactions. Cellular interactions are extremely important also for the normal function of blood cells and the disturbance of this axis may lead to pathological states. As mentioned earlier the mere expression of adhesive proteins is not sufficient, the posttranslational modification of PSGL-1 is crucial for its function, and it is evidenced by the development of a human disease, the type II leukocyte adhesion deficiency (LAD-II). The affected patients have a mutation in the gene encoding a fucose transporter and thus cannot effectively incorporate fucose into selectin ligands [36]. As a result, leukocytes cannot bind any selectins and the patient suffers from bacterial infections of the mucosal membrane and the skin. A phenotype similar to that observed in LAD-II patients is detectable in mice lacking fucosyltransferase-7 [37].

If both P-selectin and PSGL-1 molecules are functional, they are the primary players in slowing down the leukocytes on the surface of activated endothelium resulting in the tethering and rolling of myeloid cells on inflamed endothelium. Endothelial P-selectin on the surface of activated endothelial cells and the constitutively expressed PSGL-1 are ideal molecules for capturing myeloid cells from the circulation as they are very long molecules extending far from the leukocyte surface and the endothelial surface layer. This process is delicately regulated by flow rate, P-selectin density, and receptor dimerization [38]. Each domain of the PSGL-1 molecule plays multiple roles in leukocyte rolling and extravasation, while the short cytoplasmic domain is dispensable for leukocyte rolling on P-selectin but is essential to activate β2 integrin to slow rolling on ICAM-1 [39].

Selectins interact with glycosaminoglycans and one practical consequence of this phenomenon was that unfractionated heparin is an inhibitor of selectin-PSGL-1 interactions. This anti-inflammatory effect of heparin was achieved at concentrations 10–50-fold lower than recommended for anticoagulation. It has also been suggested that low molecular weight heparins are much poorer inhibitors [40]. The inhibition occurs via blockade of P- and L-selectins and requires glucosamine 6-O-sulfation [41].

2.2. New Discoveries on How Leukocyte Recruitment Is Modulated. According to recent results, the leukocyte-endothelial bond strength is considerably influenced by the vessel diameter. If this diameter is comparable or smaller than that of the cell itself, the cells are not rolling as observed in venules but due to the small diameter they are travelling with a bullet motion. Under these circumstances the P-selectin-PSGL-1 interaction is not a weak interaction anymore but can provide a firm adherence to the wall of the capillary [42].

Another factor that may considerably influence physiological and pathological leukocyte recruitment is the variability of the endothelial surface layer in diverse anatomical locations. The majority of the experimental studies use the cremaster vasculature and observe neutrophil movement with microscopic techniques. Here the cell rolling and arrest occurs primarily in the postcapillary venules. Contrarily, in the lungs leukocyte extravasation occurs primarily through the capillaries and similarly in the kidneys the site of neutrophil extravasation is the glomerular capillaries and also in the liver the postcapillary venules have an inferior role in leukocyte diapedesis as it primarily happens in the liver sinusoids. These different anatomical locations may also display a large variability in the thickness of the endothelial surface layer that alters the pro- and antiadhesive properties of the endothelium [43].

But it is not only the endothelial cell that may exert variable contribution to the inflammatory process. A highly cited recent publication describes how neutrophils scan for activated platelets in the circulation to initiate inflammation [44]. By using intravital microscopy the authors elegantly demonstrated that the neutrophils recruited to inflamed vessels extend a PSGL-1 bearing microdomain into the vessel lumen that scans for activated platelets present in the bloodstream through P-selectin. The capacity of neutrophils to switch to a polarized morphology is essential as neutrophils that are unable to polarize or transduce signals through PSGL-1 display an aberrant crawling. Very recently studies have revealed that platelets and neutrophils have a mutual relationship. Platelets, in addition to their role in hemostatic processes, were found to have a considerable role in navigating leukocyte to their exit points in the inflamed microvasculature, as upon inflammation platelets were shown to immediately adhere at endothelial junctions in the smallest venular microvessels and capture neutrophils via CD40/CD40L dependent interactions. In this crosstalk, P-selectin-PSGL-1 ligation is crucial as it induces a conformational change of the surface-expressed leukocyte integrins. The blockade of this cellular partnership leads to misguided inefficient leukocyte responses leading to an ineffective leukocyte trafficking at

the site of inflammation [45]. There are numerous further aspects in the modulation of leukocyte-platelet interactions that may not require the entire neutrophil. When activated platelets attach to neutrophils via P-selectin-PSGL-1 mediated binding, neutrophils extracellular vesicles (microvesicles or microparticles) are released that are involved in a multistep reciprocal crosstalk between platelets and neutrophils. These vesicles can be specifically internalized into platelets and subsequently relocated into intracellular platelet compartments enriched in cyclooxygenase that can process arachidonic acid into the vasoconstrictor and platelet aggregation promoter thromboxane A_2 [46].

Finally there are several ways about how microorganisms may interfere with platelet-leukocyte interactions, thereby modulating the inflammatory reactions. One newer aspect of these effects was the recognition that extracellular fibrinogen binding protein (Efb) from *Staphylococcus aureus* inhibits the formation of platelet-leukocyte complexes via binding to P-selectin. Efb was shown to inhibit P-selectin-PSGL-1 interaction in both cell lysates and cell-free assays [47].

3. The Role of Selectins and PSGL-1 in Thrombus Development

3.1. Platelet and Soluble P-Selectin in Thrombosis. Clinically, P-selectin has been demonstrated to be a risk factor for recurrent venous thromboembolism. We have previously summarized several clinical studies where both platelet and soluble P-selectin were found to be elevated in patients with cardiovascular disorders [48]. Soluble and platelet P-selectin may not always alter their values in parallel. Activated platelets may be sequestered during thrombus formation and thus platelet P-selectin may underestimate the actual platelet activation. Activated platelets may also be attached to leukocytes; thus in a pioneering experimental work by Michelson, it was found that platelet-monocyte complexes are better markers for thrombotic tendency than platelet P-selectin that was formerly regarded as gold standard [49]. Furthermore, soluble P-selectin may be elevated disproportionately to platelet P-selectin because of the presence of selectin polymorphism that can influence P-selectin shedding [29]. On the other hand, although the major source of soluble P-selectin has undoubtedly been shown to be of platelet origin [50, 51], in certain situations endothelial cells as an alternative source have also been suggested [52].

3.2. PSGL-1 Mediated Cellular Interactions during Thrombus Formation. Abnormal neutrophil accumulation has been implicated in several inflammatory disorders like rheumatoid arthritis [53]. The persistent accumulation of neutrophils may lead to the release of elastase and toxic oxygen compounds that both potentiate tissue damage. The important role of the P-selectin-PSGL-1 axis in thrombus development has been demonstrated mostly in animal experiments. By using intravital microscopy, seminal discoveries were made mostly in the laboratory of Dr. Bruce Furie. Their experiments with real time intravital imaging revealed that the absence of P-selectin or PSGL-1 is accompanied by a deficiency of tissue factor accumulation and fibrin generation. On the microscopic images in mice they found that tissue factor antigen and fibrin accumulated within the first minute of vessel injury [54, 55]; however, leukocyte incorporation was not detectable in the developing thrombus in the first minutes. It was verified that circulating cellular microparticles include tissue factor associated with PSGL-1 and they observed that microparticles were captured by thrombus associated platelets through the interaction of microparticle PSGL-1 and P-selectin expressed by activated platelets [56, 57]. The importance of this axis was underlined in further murine studies that were carried out by the in vivo use of blocking antibodies to either adhesion molecule [58]. It was found that both antibodies abrogated lipopolysaccharide stimulation elicited platelet and leukocyte rolling and adhesion. Since platelets were also described to express functional PSGL-1 [59], it remained elusive whether this phenomenon occurs via platelet PSGL-1 or via the platelet-leukocyte binding.

The real time imaging methodology in the study of thrombus formation in mice has become a powerful technique that such studies were worth presented in a real time visualized form [60]. Based on the above results, the P-selectin-PSGL-1 axis is important for tissue factor induction and cell aggregate formation between platelets and leukocytes. The binding of these two adhesive proteins also leads to other alterations like the $\beta 2$ integrin Mac-1 conformational change on the monocyte surface. In addition to these data, biologically relevant concentrations of soluble P-selectin were found to stimulate phosphatidylserine (PS) expression in a time and concentration dependent manner. The effect was already observed slightly above the reference range and was reaching its peak value when soluble P-selectin became 6-fold elevated [61]. The PS-inducing capability was observed with both soluble and platelet membrane-bound form of P-selectin and resulted in a surface-dependent thrombin generation on monocytes. As described in the previous chapter, microvesicles are very important in inflammation and thrombus initiation. These cellular fragments are a result of selective membrane shedding. It has been shown that monocyte/macrophage derived microvesicles are deficient in CD45 but are enriched in PSGL-1 and tissue factor. It was also found that lipid rafts are also rich in tissue factor and PSGL-1 but not CD45 and consistent with the raft origin of these tissue factor-bearing microvesicles their shedding was significantly reduced with depletion of the membrane cholesterol. The microvesicles may fuse with platelets transferring both proteins and lipids to the platelet membrane [62].

Several other groups have addressed the question of thrombus formation in relation to P-selectin ligation. The role in thrombus promotion has been verified and it was also observed by flow cytometry that platelet P-selectin initiates platelet aggregation by inducing microaggregate formation [63]. This process is somewhat similar to the role of L-selectin via its interaction with PSGL-1, where it initiates the aggregation process and increases the $\beta 2$ integrin affinity and avidity for its ligands in neutrophils [64]. If appropriate posttranslational modifications occur and PSGL-1 becomes functional, the expression rate of the protein may become a pathogenic factor. By investigating monocyte subsets in patients suffering from various cardiovascular disorders, it

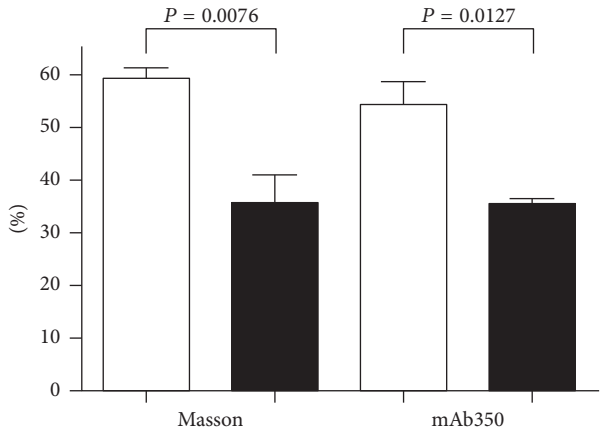


Figure 2: Ratio of vessels occluded with thrombi after collagen-epinephrine challenge in PSGL-1 knockout (filled bars) and wild-type (open bars) mice. A significantly reduced rate of thrombus formation was observed in knockout animals both with Masson trichrome staining and with an immunohistochemical staining to mouse fibrin *(reprinted from Thrombosis Research with permission by Elsevier).*

was found that the expression of PSGL-1 on $CD14^{++}/CD16^{+}$ monocytes is significantly increased in patients with unstable angina and acute myocardial infarction (AMI) compared to controls. Moreover the dramatic increase at the onset of AMI was decreased during the chronic phase and the intensity of PSGL-1 staining was significantly higher in patients with plaque rupture [65].

Since PSGL-1-selectin interactions are crucial to thrombus formation, it was plausible to hypothesize that the lack of PSGL-1 would be protective against thrombosis. Several studies were launched with this hypothesis and they unequivocally proved that the lack of this adhesive protein is indeed protective against induced arterial and venous thrombosis in murine models [66–68].

If thrombotic stimuli are carefully chosen in these models, a difference in the number of vessels occluded with thrombi was evident whether determined by conventional or immunochemical staining (Figure 2); also less fibrin was deposited in the lungs of PSGL-1 knockout animals (Figure 3) that can partly be explained by the PS-inducing capacity of the P-selectin-PSGL-1 axis. All these effects contribute to a better survival rate in PSGL-1 knockout mice upon thrombotic challenge (Figure 4). The above data about the selectin-PSGL-1 connection served as examples for physiological function and as a pathogenic player in vascular disorders.

Nevertheless, there are few examples on the beneficial effects of the selectin-PSGL-1 interactions. As pointed out previously, the formation of microvesicles and their interaction with blood cells have been described as a pathognomic characteristic in thrombotic disorders. However, the presence of microvesicles may become beneficial and the vesicles that were generated by the P-selectin-PSGL-1 interaction corrected the hemorrhagic disorders as was described in an animal model of haemophilia [69]. More recently, it was also shown that, after laser-induced vascular injury in mice, neutrophil granulocytes recruited endothelial colony-forming cells at the site of vascular injury via a PSGL-1-L-selectin interaction and via this effect contributed to angiogenesis and the regeneration of the injured vessel [70].

4. The Role of the Selectin-PSGL-1 Axis in Malignancies

Similarly to inflammation and thrombus formation as discussed above, tumor growth and the development of metastasis comprise of a cascade of various cellular events regulated by a large number of adhesion molecules including selectins from the initial step up to the advanced stages of malignancy [71]. Basically, selectins and their ligands may be involved in cancer progression in two ways: (i) selectin ligands (mucins) are expressed on cancer cells to bind to selectins on the surface of (activated) normal blood cells or endothelial cells that facilitates the arrest and extravasation of tumor cells and (ii) in turn the tumor itself rarely expresses selectin to exploit these interactions above for aggregating with leukocytes and endothelial cells to seed distant metastases. Based on former investigations, there are three main approaches to analyze the role and the mechanism of selectin/selectin ligand pairing in cancer propagation: (i) the investigation of knockout mice lacking selectin or with deficiency of endogenous enzyme(s) involved in ligand expression in comparison to wild-type counterparts, (ii) the application of neutralizing antibodies against selectin(s) in animals to block these interactions in vivo to study altered tumorgenesis, and (iii) the utilization of these agents in tumor cell cultures among in vitro (flow) conditions to influence cellular interactions.

In respect to abnormal selectin ligand expression, malignant cells are characterized by mucins with abnormal glycosylation [72]. Selectins predominantly bind sialyl-$Lewis^{x}$ or sialyl-$Lewis^{a}$ fucosylated carbohydrate ligands on tumor cells, which are synthesized by different glycosyltransferases [73]. The increased function of fucosyltransferase-7 resulted in a higher sialyl-$Lewis^{x}$ expression causing enhanced lung cancer progression [74]. These data have recently been supported by others when fucosyltransferase-7-deficient mice displayed a reduced recruitment of monocytes to metastasizing tumor cells correlated with attenuated metastasis [75]. Hence, the degree of abnormal glycosylation with altered expression of carbohydrate selectin ligands by cancer cells correlates with metastasis formation and poor prognosis for cancer patients [73]. The altered expression of sialyl-$Lewis^{x/a}$ containing mucins allows tumor cells to interact with their microenvironment via binding to selectins on blood cells and endothelium that influences metastatic spread [76]. For example, mucin 16 overexpressed on pancreatic cancer cells bound to E- and L-selectin under flow [77]. Thus, mucin removal from tumor cells could effectively attenuate metastasis [78].

Hematogenous metastasis of cancer occurs in the later stage of tumor progression that is responsible for death in most cancer patients. After tumor cells entered the blood stream, they circulate and bind to (i) platelets, which support their extravasation and protect them from innate immune system and mechanical stress, (ii) leukocytes, which may

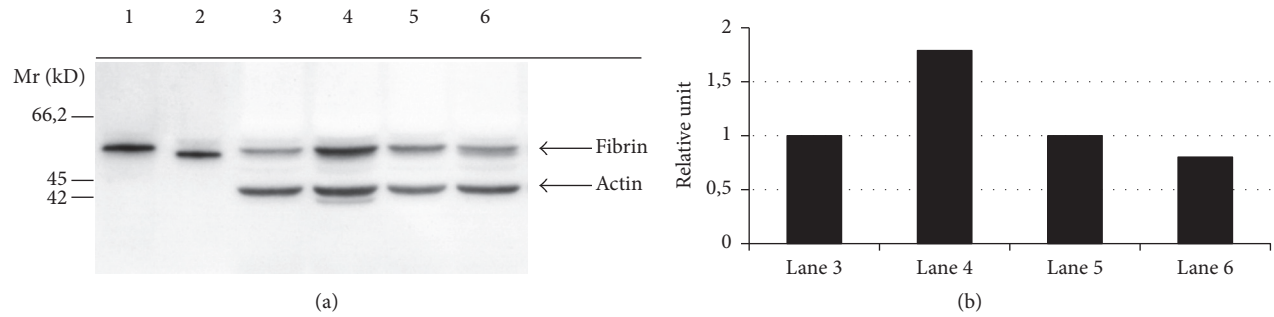


Figure 3: Quantity of fibrin deposition in the lungs. The western blot analysis of lung extracts shows remarkable difference. (a) Lane 1: fibrinogen standard, lane 2: fibrin standard, lane 3: wild-type mice treated with saline, lane 4: wild-type mice treated with collagen and epinephrine, lane 5: knockout mice treated with saline, and lane 6: knockout mice treated with collagen and epinephrine. (b) After thrombotic challenge, twice as much fibrin deposits were found in the wild-type mice compared to knockout animals *(reprinted from Thrombosis Research with permission by Elsevier).*

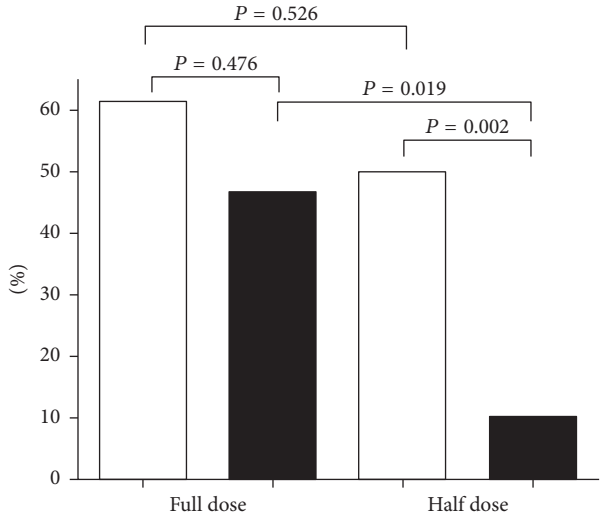


Figure 4: Survival rates of wild-type and knockout mice within 30 minutes after thrombotic challenge. The percentage of perished wild-type mice (open bars) and knockout mice (solid bars) after administration of full dose and half dose collagen + epinephrine (full dose: 15 μg collagen + 3 μg epinephrine, half dose: 7.5 μg collagen + 1.5 μg epinephrine) *(reprinted from Thrombosis Research with permission by Elsevier).*

support the adhesion of cancer cells to vessel wall, and (iii) endothelial cells to adhere and then migrate from the vasculature. First, after binding to activated platelets via P-selectin, tumor cells tether and then roll on the endothelial cells to be finally arrested in the microvasculature of distant organs. Due to the formation of these heterotypic aggregates, endothelial cell activation is induced resulting in enhanced E- and P-selectin expression. The recruitment of reactive neutrophils and monocytes to cancer cells is regulated via L-selectin as well as endothelial-mediated interactions [79, 80]. Subsequently, platelets secrete a number of bioactive mediators, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibrinogen, and thrombospondin to provide mitogenic triggers for cancer [81]. This environment establishes first a "premetastatic niche" where the primary tumor cells can survive, proliferate, and later metastasize. Before the arrival of tumor cells, myeloid precursor cells are mobilized that express VEGF receptor 1 or tumor necrosis factor alpha to provide a permissive niche for migrating cancer [82, 83]. Developing niche is shaped by additional cellular interactions, especially through E-selectin between circulating tumor cells and endothelial cells during extravasation process [84]. Consequently, an E-selectin targeted aptamer could reduce hematogenous metastases of breast cancer in a mouse model [85]. Very recently, myeloid-derived suppressor cells have been shown to promote the arrest of cancer cells via IL-1β-mediated E-selectin expression on endothelial cells [86]. In turn, activated endothelium produces several chemokines (CCL5) to support the extravasation of cancer cells [71, 79, 87]. Attachment of tumor cells to endothelium is also promoted by innate immune cells; for example, neutrophils increased melanoma cell extravasation by IL-8 production [88]. Subsequently, PSGL-1 mediated recruitment of monocytes facilitates tumor extravasation [75]. These cellular events are summarized and depicted in Figure 5.

4.1. Role of P-Selectin during Tumor Progression. P-selectin was started to be widely investigated in relation to cancer biology after its role had been analyzed under inflammatory and thrombotic conditions [54, 89]. During the 1990s, the first reports were published demonstrating that P-selectin can bind to several human cancers [90–92]. As a result, P-selectin deficiency in mice caused attenuated human colon carcinoma growth and metastasis in vivo [93] and impaired murine adenocarcinoma progression [94]. The formation of distant metastases of small cell lung cancer (SCLC) was also significantly reduced when this cell line was xenografted into P-selectin knockout mice [95]. Substantial evidence demonstrated that platelets interact with circulating malignant cells to form tumor microemboli via P-selectin, and these events may help cancer cell arrest in distant organs where they adhere to vessels [81, 96]. Actually, the involvement of platelets in tumor progression was far earlier suggested based on those animal models, when pulmonary metastasis was inhibited in different types of cancers in the presence of

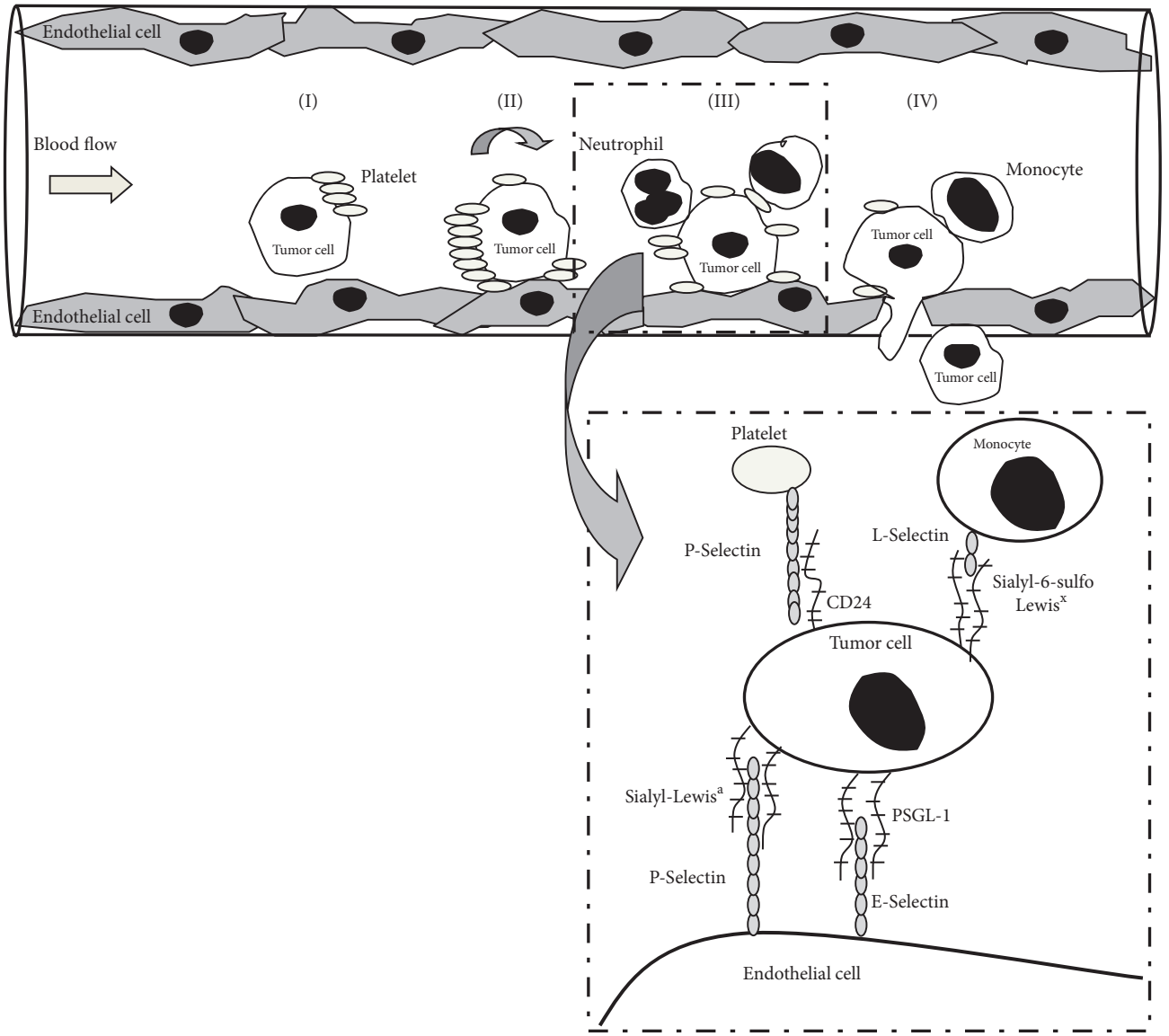


Figure 5: A schematic figure on the development of selectin-mediated heterotypic interactions among tumor cells, leukocytes, platelets, and endothelial cells during metastasis formation. Tumor cells circulate in the blood stream until they tether (I), roll (II), and then are arrested on endothelial cells (III) and finally migrate from the vasculature (IV). Activated platelets aggregate with cancer cells via P-selectin protecting them from innate immune system and permitting further leukocyte binding. Induced endothelial cell activation by tumor cells results in E- and P-selectin expression with additional recruitment of reactive neutrophils and monocytes to cancer cells regulated via L-selectin. As being magnified, selectin ligands (e.g., PSGL-1, sialyl-Lewis^x) are expressed on malignant cells to bind selectins that are expressed on normal blood cells and endothelial cells. See details in the text. Of note, there are several other receptors and integrins involved in these interactions but that cannot be depicted here.

induced thrombocytopenia or by the inhibition of fibronectin and von Willebrand factor with monoclonal antibodies [97]. The expression of sialyl-Lewis^a, sialyl-Lewis^x, or PSGL-1 was demonstrated on the surface of human melanoma [98, 99], breast cancer [100], and different SCLC cell lines [95, 101] to interact with P-selectin on platelets. PSGL-1 negative breast and SCLC cells may express O-glycosylated glycoprotein, CD24, which was found to be important in the dissemination of tumor cells [102]. The "platelet cloak" around tumor cells provides further interactions of monocytes to malignant cells via platelets [78] but also protects against natural killer- (NK-) mediated clearance of tumor cells [103]. Of note, P-selectin on endothelial cells additionally contributes to metastasis based on attenuated melanoma lung metastasis after the transplantation of bone marrow from P-selectin-deficient into wild-type mice [104]. Malignancies are often associated with inflammation, enhanced cytokine expression, and lymphocyte infiltration of tumor tissue that are more likely to facilitate growth and spread of cancer than being effective in a host antitumor response [105]. In breast cancer, the loss of P-selectin inhibited the infiltration of regulatory T-cells and reduced levels of proinflammatory cytokines, such as IL-4,

IL-10, and TGF-β, were measured in the tumors resulting in a better survival ratio [106].

Selective inhibition of selectins may be potential therapeutic targets for preventing hematogenous metastasis. Interaction of cancer cells with platelets and endothelial cells via P-selectin can be blocked by unfractionated heparin in a clinically tolerable concentration range mimicking its ligands that reduce the organ colonization of cancer [104]. Recently, low molecular weight heparin, Tinzaparin, effectively blocked P-selectin in vivo reducing metastasis formation in a B16F10 melanoma mice model [107]. On the other hand, treatment of the tumor cells with O-sialoglycoproteinase prevented endothelial cell activation and chemokine (CCL5) production leading to decreased metastatic microenvironment [87]. Finally, enzymatic removal of sulfation of SM4 from the surface of MC-38 colon carcinoma cells resulted in decreased P-selectin binding on platelets with attenuated metastasis [108]. Overall, platelets with P-selectin are considered as the key enhancers of hematogenous dissemination, tumor survival, and tissue colonization [78], as they initiate the early phase of metastatic tissue colonization via P-selectin [71], while activated endothelium with increased P-selectin exposure supports these events [104].

4.2. Involvement of E-Selectin in Cancer Progression. E-selectin is an important adhesion receptor on activated endothelial cells for leukocytes, but cancer cell migration is also mediated by E-selectin to be arrested on microvasculature as one of the initiating events during metastasis [79]. Subsequently, endothelial cells become activated by the accumulation of malignant cells and P-selectin-dependent platelet-tumor cell interactions. Hence, they show increased expression of E-selectin with induced production of different chemokines and facilitate the subsequent recruitment of monocytes and myeloid cells [66]. The blocking of E-selectin function attenuated liver [109] and lung metastases by colon carcinoma cells [110]; however, others found E-selectin dispensable in lung metastasis indicating its role merely in local activation of lung microvascular endothelial cells [80]. E-selectin binding to colon cancer cells can alter gene expression to promote further metastases [111]. Moreover, signal transductions in endothelial cells are also triggered to regulate the integrity of the endothelial layer for transendothelial migration of colon tumor cells [112].

E-selectin ligands are mostly mucins. Increased expression of CD44 known as hematopoietic cell E-/L-selectin ligand (HCELL) on colon carcinoma resulted in an enhanced adherence to activated endothelium [113]. Additionally, human colon carcinoma cells can express other E-selectin ligands, such as death receptor-3, LAMP-1, and LAMP-2 that provide survival advantages for this malignancy [114, 115]. The recently discovered Mac-2 binding protein can be expressed by breast cancer cells to bind this selectin upon metastasis [116].

Heparin is an excellent inhibitor of P- and L-selectin binding to the sialyl-Lewis^x, but no effect on E-selectin was observed [117]. In contrast, a bile acid acylated-heparin derivative showed an inhibitory effect on E- and P-selectin-mediated interactions reducing adhesion and invasion of B16F10 cells into the lung in mice [118]. When a soluble form of E-selectin, the recombinant fusion protein E-selectin-immunoglobulin, was used against E-selectin, this treatment impaired lung metastasis by colon carcinoma [110]. On the other hand, blocking its sialyl-Lewis^a ligand with a specific antibody [119] or a mimetic peptide (DLWDWVVGKPAG) [120] inhibited the dissemination of pancreatic cancer in the peritoneal cavity of nude mice and the metastasis formation of B16F10 melanoma cells to the lung, respectively.

4.3. Function of L-Selectin in Cancer Progression. L-selectin expression is restricted to neutrophils, monocytes, and natural killer (NK) cells that also display a role in regulating metastasis. First, it was shown that L-selectin facilitated the lymph node metastasis [121]. In contrast to the contribution of P-selectin positive platelets, leukocyte L-selectin can facilitate tumor metastasis at later stage, as L-selectin mediates the leukocyte recruitment to tumor emboli after P-selectin-mediated platelet-cancer cell aggregates are formed [71]. Thus, L-selectin deficiency does not affect initial tumor cell embolization, since the association of CD11b positive myeloid cells with tumor cells was reduced and tumor cell survival was diminished 24 hours later [122]. Consequently, the enhanced expression of L-selectin ligands in the endothelium and in the tumor emboli also correlates with leukocyte infiltration. In summary, L-selectin facilitates metastasis formation via leukocyte-endothelium interactions, which is supported by L-selectin ligand induction by fucosyltransferase-7 [122]. When the host response of inflammatory cell infiltration in malignant melanoma was investigated to see how this selectin regulated these events as an antitumor reaction, pulmonary metastasis was enhanced by the loss of L-selectin due to impaired migration of NK cells, CD4+ and CD8+ T-cells into the lung tissue; however, cytotoxic response was unaffected [123].

L-selectin has several ligands expressed on various tumor cells. Beside conventional sialyl-Lewis^x, the expression of its main ligand sialyl-6-sulfo Lewis^x is also present in human colorectal cancer; however, it is preferentially expressed in the nonmalignant colonic epithelia rather than cancer cells [124]. Sialofucosylated podocalyxin, which can bind L- and E-selectin on host cells, is upregulated in a number of cancers including breast, colon, and pancreas malignancies. Hence, the specific depletion of this molecule from the cell surface significantly interferes with selectin-dependent cancer cell-host cell interactions [125]. Similarly to P-selectin, heparin effectively blocked L-selectin preventing leukocyte-endothelial interactions at sites of intravascular arrest [122].

4.4. Expression of PSGL-1 on Solid Tumor Cells. PSGL-1, the main counter receptor of selectins, is highly involved not only in inflammation and thrombosis [126, 127], but also in solid tumor progression. Apart from its constitutive presence on normal leukocytes, it is functionally expressed on the surface of tumor cells, such as human prostate carcinoma [128]. The knockdown of PSGL-1 from malignant cells resulted in a significantly reduced aggregate formation between activated platelets and lung cancer cells [129]. Others also claimed that PSGL-1 with CD44 mediated metastasis formation in SCLC

cells [95]. Tumor cells produce microparticles at higher quantity that express active tissue factor and PSGL-1. Based on an animal model by Thomas et al., pancreatic and lung cancer cell-derived microparticles bearing PSGL-1 accumulated at the site of injury and played a role in thrombus formation by binding to P-selectin in mice developing a tumor [130].

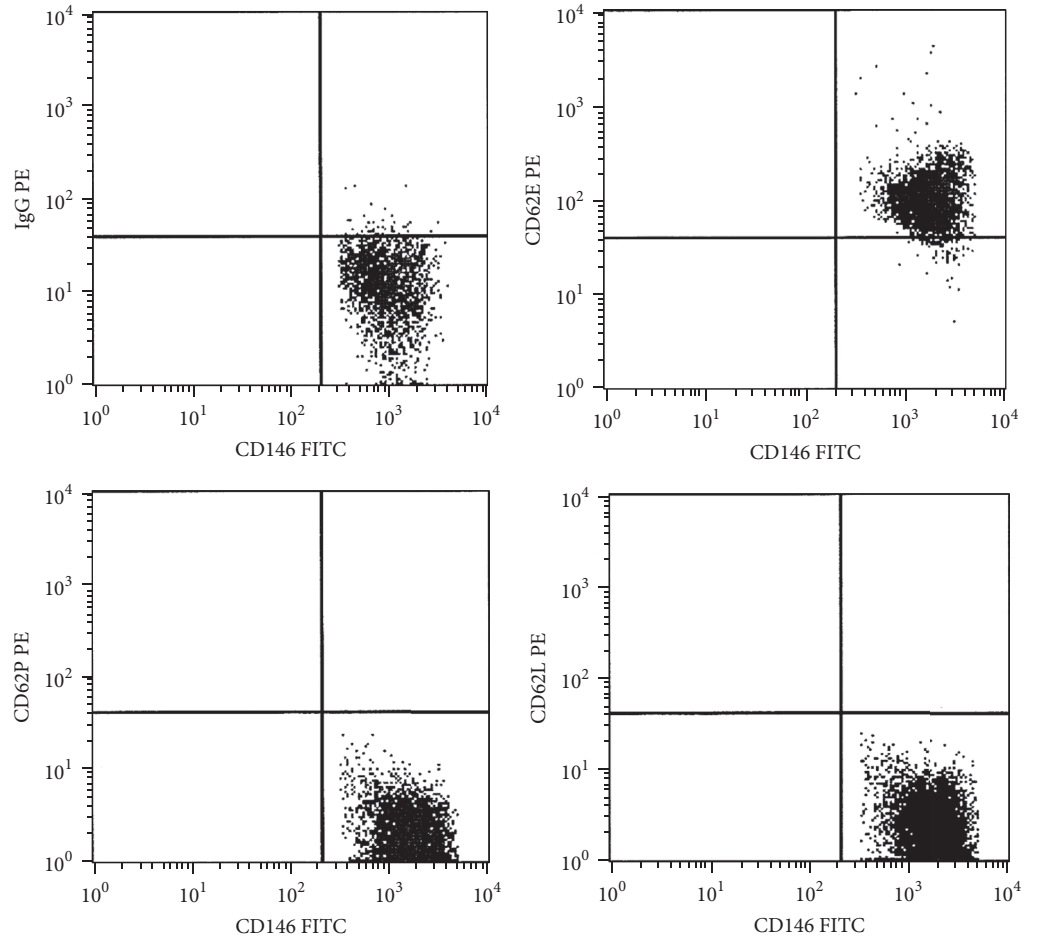
4.5. Selectin Expression on Cancer Cells. Multiple gene defects in tumor cells are generated resulting in phenotypic changes. These "mimicries" are characterized with the reactivation of endothelial or platelet specific genes leading to the expression of E-selectin [131], αIIbβ3 integrin, and thrombin receptor [132–134]. Along this line, selectins are rarely expressed on certain cancer cells to exploit cellular interactions for seeding metastasis. P-selectin was described on metastatic pancreatic tumor cell line, and its expression could be further induced by thrombin stimulation [135].

Our group has also investigated the expression of selectin(s) on a previously characterized aggressive human melanoma cell line (M35/01) [136]. Based on its endothelial mimicry phenotype, selectin expression was analyzed by flow cytometry and confocal laser scanning microscopy (CLSM). We found that M35/01 melanoma cells showed a substantial E-selectin expression but were negative for surface L- and P-selectin (Figure 6). We then studied if this receptor was functional for the interaction with isolated normal peripheral blood mononuclear cells (PBMCs). For this purpose, PBMCs were added to the tumor cell culture in the absence and presence of blocking antibodies against E-selectin on cancer cells and/or PSGL-1 expressed on PBMCs. The alteration in the attachment of these cells was followed by flow cytometry via measuring CD45 positivity of PBMCs in the gate of melanoma cell population. By CLSM, we visualized the ratio of cellular interactions between the melanoma cells and leukocytes. A significantly decreased binding of PBMCs to cancer cells was seen by both blocking antibodies suggesting the binding of cancer cells to normal leukocytes via E-selectin/PSGL-1 pairing (Figure 7). These data are in accordance with former results on the role of PSGL-1 in tethering leukocytes to E-selectin under flow conditions [137].

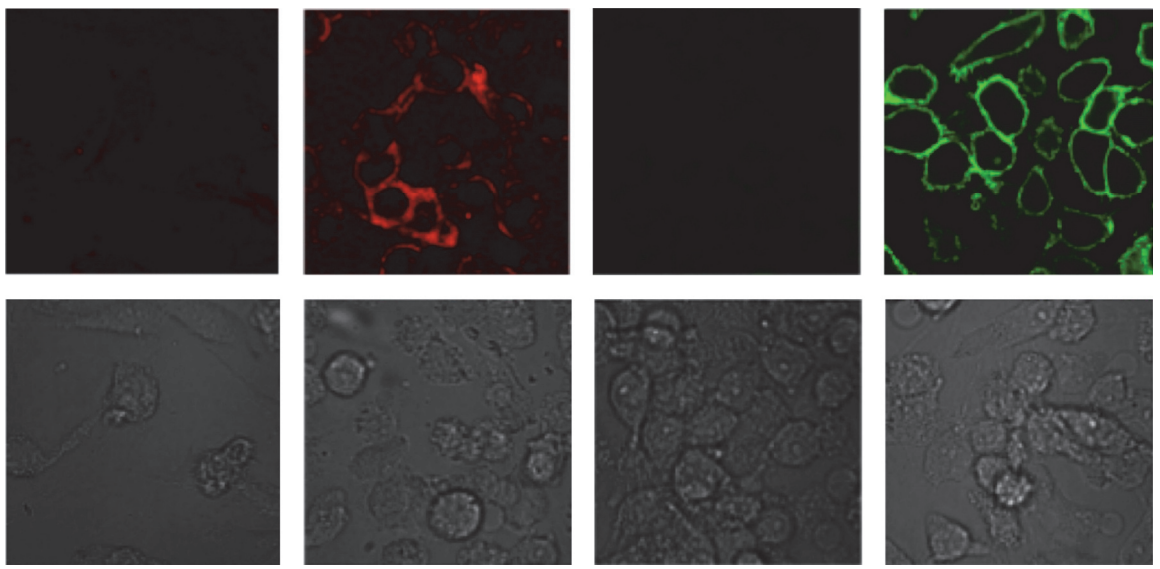
4.6. The Selectin-PSGL-1 Axis in Hematological Malignancies. According to the literature, selectins and their ligands may also participate in the progression of hematological malignancies. In chronic myeloid leukemia (CML), these receptors are required for homing and engraftment of BCR-ABL1+ leukemic stem cells in the bone marrow niche, since deficiency of E-selectin and L-selectin in the bone marrow endothelium of mice significantly reduced the engraftment of BCR-ABL1-expressing stem cells, while P-selectin was not required [138]. These results establish that BCR-ABL1+ leukemic stem cells rely to a greater extent on selectins and their ligands for homing and engraftment than do normal stem cells and may be beneficial in autologous transplantation for CML [138]. The most frequently observed karyotypic abnormality in acute myeloid leukemia (AML) is the 8 : 21 translocation resulting in the formation of the RUNX1/ETO oncoprotein that suppresses the expression of PSGL-1 on hematopoietic progenitor cells and deregulates other genes involved in differentiation and proliferation. These alterations contribute to impaired adhesive behavior of t(8,21)+ AML cells and may partially explain a favorable response to chemotherapy with a better prognosis [139]. Similarly, PSGL-1 copy number was considered as a biomarker to differentiate different types of AML [140]. In multiple myeloma, PSGL-1 is highly expressed on the myeloma cells and regulates their homing into bone marrow microenvironment [141]. However, the mobilization of mature myeloid cells and their precursors from the bone marrow is also mediated by PSGL-1 via the interaction of leukocytes with endothelial or stromal cells [142]. Finally, function of PSGL-1 is associated with hematogenous metastasis of lymphomas as the downregulation of its expression in metastatic lymphoid cells resulted in a significant reduction of liver and spleen colonization in a dose-dependent manner [143].

4.7. Function of Neutrophil Extracellular Traps in Thrombosis and Cancer via P-Selectin. NETs are released from activated neutrophils comprising DNA fibers with histones and various granular proteases [144]. NETs were first identified as a host defense mechanism against bacteria. In activation of neutrophils by pathogens or cytokines, histone H3 becomes hypercitrullinated that leads to chromatin decondensation [145]. Increased NET formation is typical not only in severe infections, but also in thrombotic complications as platelets become activated by NETs [146], and induces thrombin generation and tissue factor expression [147]. In turn, platelets can trigger NETosis via thromboxane A2 release [148]. Solid tumors and leukemias may produce G-CSF that primes more neutrophils for further NET generation [149]. NETs accumulate at the site of neutrophil accumulation and influence cancer environment causing necrotic areas within the tumor being advantageous for tumor growth [150]. Notably, NETs also promote cancer-associated venous thrombosis and arterial microthrombosis as in ischemic stroke [151, 152]. To date, one report is available about the direct association of selectin function and NET formation [153]. Accordingly, surface P-selectin on thrombin activated platelets as well as its soluble form and neutrophil PSGL-1 interaction promotes NETosis [153]. The release of histone was found to induce the neutrophil-endothelium interactions in the muscle microcirculation through P-selectin/PSGL-1 pairing; hence this histone-dependent inflammatory process may be involved after NET generation as well [154]. However, the role of other types of selectin with their ligands in NET formation is still being defined. Several other questions may also arise in terms of NETs, for example, whether neutrophils participating in circulating heterotypic aggregates with platelets may produce more NETs via P-selectin.

4.8. Mucin Associated Abnormal Coagulation at Cancer Progression. Selectins also contribute to the development of coagulation disorders that are often detected in cancer subjects [155]. Particularly mucinous carcinomas expressing high level of mucins trigger platelet-rich microthrombi formation that is accompanied with cancer progression [155]. Furthermore, carcinoma mucins promote reciprocal activation of platelets and neutrophils requiring P- and L-selectin but not



(a)



(b)

Figure 6: To determine whether any type of selectins may be expressed on cell surface on M35/01 human melanoma cancer cells, flow cytometry (FACSCalibur, Becton Dickinson) was first assessed: (a) Cells were gated based on their CD146-FITC positivity (CD146/MUC18 is an adhesion molecule on melanoma cells [35]) and SSC characteristics (data not shown). These malignant cells showed a substantial E-selectin expression with monoclonal anti-E-selectin antibody but were negative for surface L- and P-selectin. To confirm these results, CLSM (Olympus IX 81 with Fluoview FV 1000) was also used to observe E-selectin (red) and CD146 (green) positivities. (b) IgG-PE and IgG-FITC antibodies were applied to exclude the background staining. Transmission photos (grey) were used to visualize the cells.

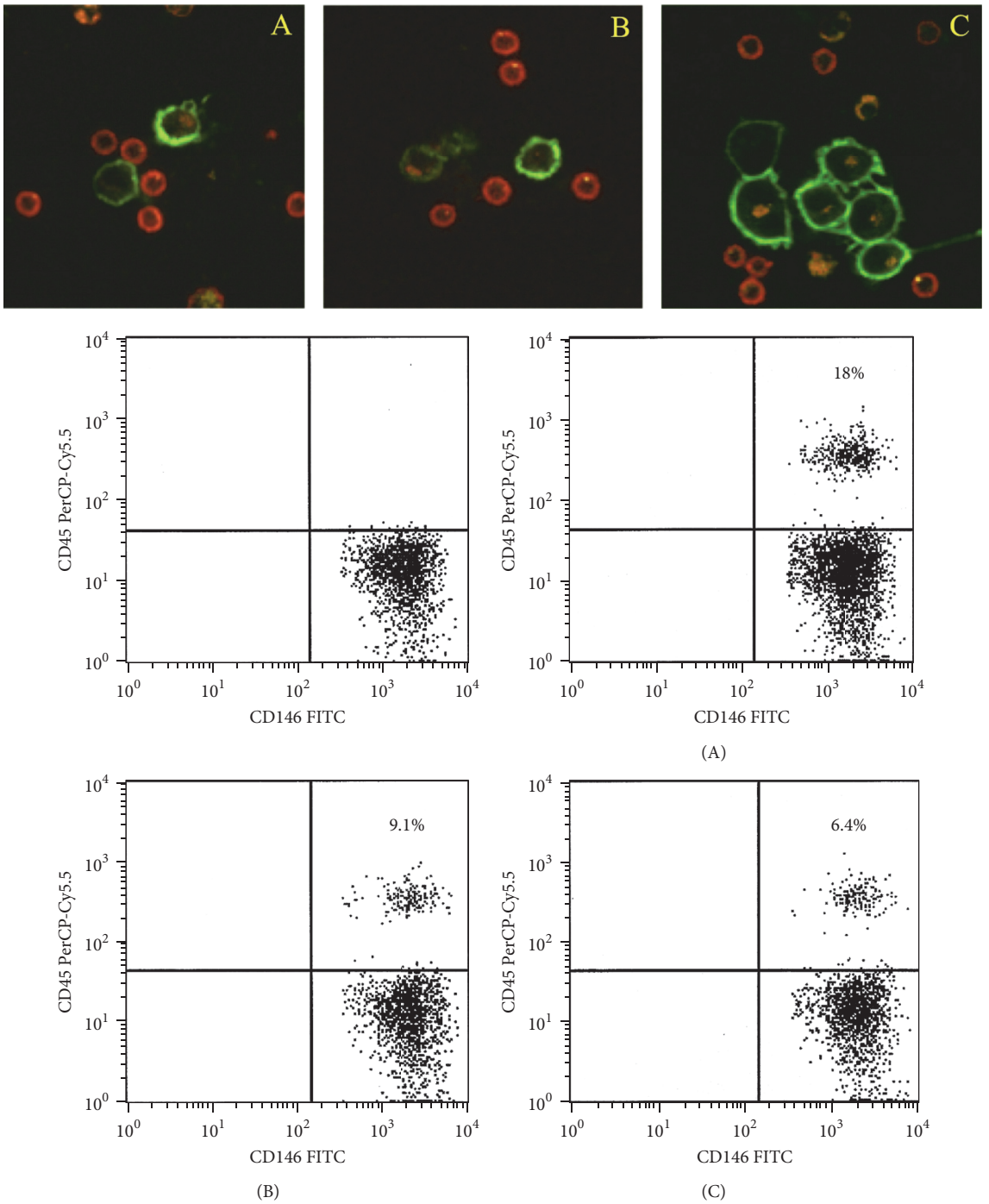


Figure 7: During functional testing of E-selectin on M35/01 melanoma cells by CLSM, these cells were incubated with normal PBMCs for at least 4 hours in the absence (A) and presence of anti-PSGL-1 (B) (clone KPL-1) or anti-E-selectin (C) (clone P2H3) blocking antibodies (BD). Tumor cells were stained with anti-CD146-FITC (green), while anti-CD45-PE (red) (BD) was used for leukocytes to detect heterotypic cellular aggregates. A significantly decreased binding of PBMCs to cancer cells was seen with both blocking antibodies (B, C) suggesting the development of cell-cell interactions via E-selectin and PSGL-1 (A). Representative photos of all conditions are demonstrated. Flow cytometry analysis showed a similar tendency in the ratio of bound PBMCs to cancer cells with (B, C) or without blocking antibodies (A). Double positive events are expressed in % in the dot plots.

thrombin in a murine model of Trousseau syndrome [156]. Overall, mucin-selectin interaction has been implicated as one of the potential mechanisms in the frequent development of venous thrombosis in pancreatic cancer [157].

5. Conclusion

In this review, we have summarized the central function of selectins and their ligands as key mediators in a number of cellular events during development of thrombotic and malignant conditions. Since these receptors are major contributors to the pathological processes, they represent diagnostic biomarkers and ideal targets for intervention of thrombosis and cancer. As only a limited number of safe specific drugs against selectin ligands are available in humans, future studies are required to investigate more details of cellular selectin-mediated interactions discussed above.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Biophysical Mechanisms Mediating Fibrin Fiber Lysis

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The formation and dissolution of blood clots is both a biochemical and a biomechanical process. While much of the chemistry has been worked out for both processes, the influence of biophysical properties is less well understood. This review considers the impact of several structural and mechanical parameters on lytic rates of fibrin fibers. The influences of fiber and network architecture, fiber strain, FXIIIa cross-linking, and particle transport phenomena will be assessed. The importance of the mechanical aspects of fibrinolysis is emphasized, and future research avenues are discussed.

1. Introduction

Coagulation and fibrinolysis serve as complementary but competing mechanisms during the process of wound healing. Activation of the coagulation cascade due to vascular injury results in the formation of a fibrin network, which serves to seal the injury. Formation of fibrin activates the fibrinolytic system, a set of enzymes, and inhibitors whose function is to regulate the breakdown of the fibrin network. These systems have been studied for over sixty years, and many of the main pathways have been studied and identified.

In the past decade the mechanical properties of fibrin have received renewed interest with the revelation that fibrin is among the most elastic and extensible biomaterials [1, 2], and recent studies have begun to explore the direct correlation between fibrin extension and fibrinolytic rates [3]. This review will focus on the intersection of fibrinolysis and fibrin's biophysical properties, with an emphasis on basic scientific discoveries and not clinical treatment strategies. However, it is expected that a deeper understanding of how the mechanical properties of fibrin mediate fibrinolysis could have clinical relevance. Lytic strategies for treating acute myocardial infarctions often see recanalization rates of only 80%–90%, while the mechanical breakdown of blood clots often achieves higher patency [4]. This suggests the need for a further examination of the fibrinolytic determinants and highlights the importance of understanding fibrinolysis in light of fibrin's biophysical characteristics. This review is not exhaustive for all aspects of fibrinolysis but emphasizes major events, and as with any review there are many papers that could have been cited that were not and many topics that could have been covered in greater detail that only receive a surface treatment; the author apologizes for any oversites in these cases.

2. Fibrinogen and Fibrin

2.1. Structure and Polymerization. Human fibrinogen is a soluble, 46 nm long, 340 kDa glycoprotein and is the third most prevalent protein found in blood plasma, circulating at 6–12 μM [5]. It is assembled as a homodimer, with each subunit consisting of three polypeptide chains (called Aα, Bβ, and γ), having 610, 461, and 411 amino acid residues, respectively [6]. Within the fibrinogen molecule, all six chains are oriented so that their N-termini are located in the central region and held together by five disulfide bonds [6, 7]. From both sides of the central nodule, the three chains extend into α-helices that form a triple coiled coil structure, terminating with a series of disulfide bonds, linking the three chains again at the C-terminus of the coiled coil [7, 8]. Beyond this disulfide linkage, the C-terminal segments of the Bβ and γ chains fold independently to form the compact, globular β- and γ-nodules (see Figures 1(a) and 1(b)) [8]. The C-terminal segment of the Aα chain (called the αC region) is different, briefly folding back to form a fourth α-helix, before extending into a primarily unstructured region, which has

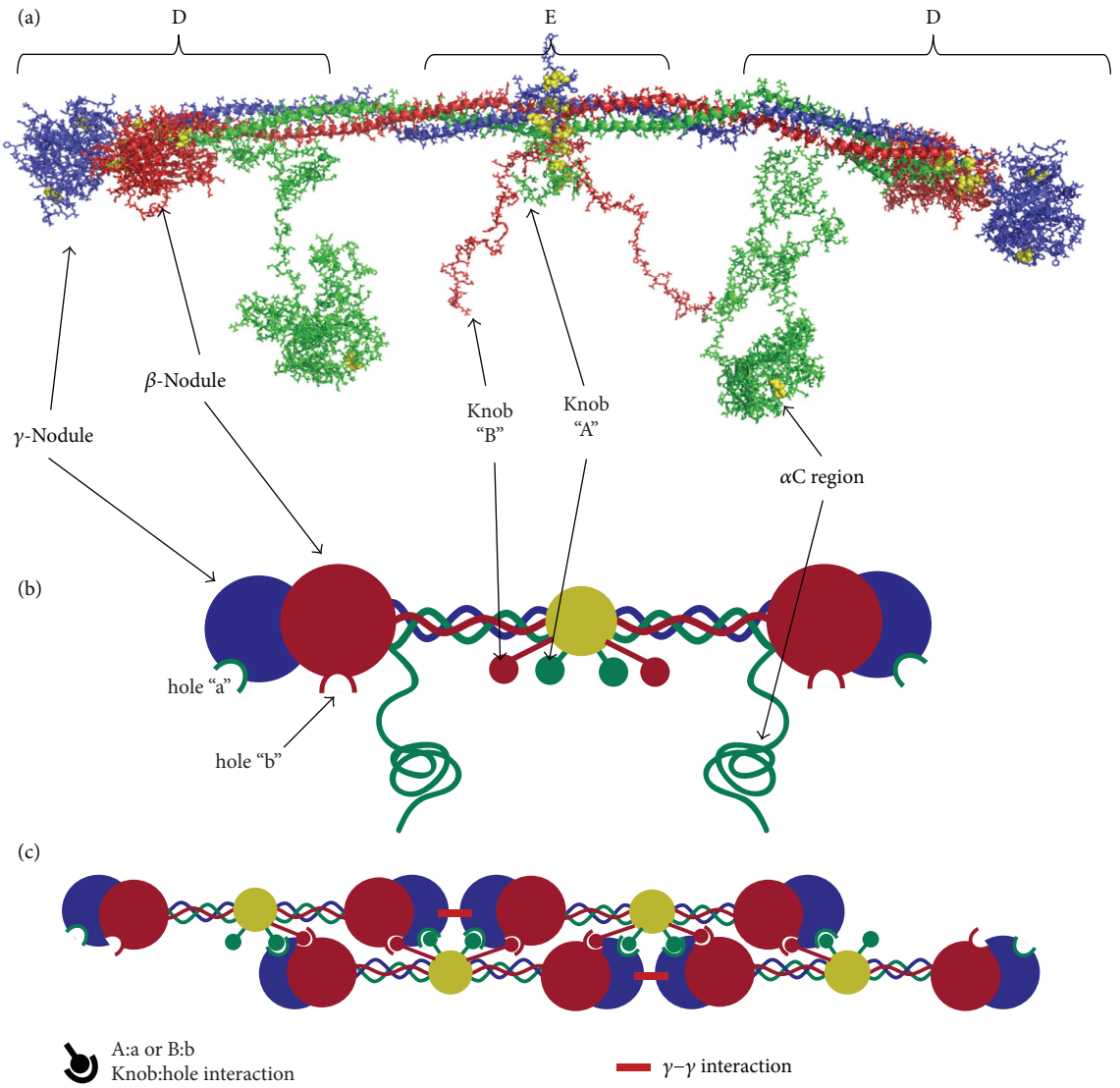

Figure 1: The fibrin molecule and polymerization into fibers. (a) Crystallography-based fibrin molecule: the fibrin molecule structure shown was created using crystal structure 3GHG [9], combined with discrete molecular dynamics methods to fill in amino acids α17–26, α201–610, and β15–57 [15], which were missing in the crystal structure. The α chain is shown in green, β chain in red, and γ chain in blue; disulfide bonds are emphasized as yellow spheres. The αC region was built from homology modeling and molecular dynamics methods as described in [15]. Fibrin degradation fragments D and E are highlighted. Fragment X is formed from plasmin cleavage of the αC region. (b) Cartoon fibrin molecule: upon thrombin cleavage of FpA and FpB, knob A and knob B are exposed to bind the respective hole a and hole b. Cartoon model highlights these interactions and draws structural correlations between the crystal structure and the cartoon (c) Polymerization model for a protofibril: during polymerization, a half-staggered protofibril is formed as the knobs in the central region of one molecule bind to the holes in the distal region of two opposite molecules. Knob B has been implicated in the lateral aggregation of protofibrils and could potentially bind to holes in adjacent protofibrils (not shown).

been uncrystallizable [9]. The αC region is often grouped into two subregions: the αC connector (221–391) and the αC domain (392–610) [10, 11]. The αC connector region is thought to be unstructured and consists of 10, 13-amino acid repeats [10] in humans, while electron microscopy and circular dichroism (CD) studies indicate that the αC domain contains a folded structure [12, 13]. CD and NMR studies have further clarified the αC domain structure, finding that the lone disulfide bond in the αC domain stabilizes a double β hairpin structure in residues α392–503, while a second, uncharacterized, structured region also exists in α504–610 [13, 14]. A similar, but not identical, β-sheet structure was found for the αC domain using homology modeling and molecular dynamics simulations [15]. In fibrinogen, it is thought that the αC domains interact with each other and the FpB in the central region [16].

Fibrinogen is converted into insoluble fibrin when the enzyme thrombin cleaves the R16-G17 bond in each Aα chain and the R14-G15 bond in each Bβ chain. Release of these peptides (fibrinopeptides A and B, or FpA and FpB)

exposes the "A" and "B" knobs, which bind to corresponding "a" and "b" holes in the β- and γ-nodules, allowing fibrin fiber polymerization (see Figures 1(a) and 1(b)). FpA is cleaved more rapidly than FpB, and the "A:a" knob:hole interaction is the primary mediator of polymerization [11]. Cleavage of FpB may release the αC domains from the fibrin molecule, allowing them to interact intermolecularly [16], and also seems to induce a conformational change in the fibrin molecule [5, 17]. Polymerization proceeds using a half-staggered molecular arrangement in which the knobs in the central region of one fibrin molecule bind to holes in two abutting, nearby molecules [18]. The central region of each of those two molecules also contains knobs, which can bind to two other molecules, and so on. Polymerization propagates in this manner, forming a double-stranded protofibril in the process (see Figure 1(c)). Finally, to form fibers, protofibrils bundle together laterally through interactions between the αC regions in adjacent protofibrils (see Figure 2) [5]. Fibers with truncated αC regions (Aα251) display thinner fibers, lower stiffness, and enhanced fibrinolysis, emphasizing the importance of this region of the fibrin molecule [19]. αC domain interactions are thought to be mediated by intermolecular β-sheet swapping of the β-hairpin region [14] and produce high molecular weight digestion products commonly called α-polymers that suggest many αC regions link together in this manner. α-Polymers and the γ-nodules in protofibrils are further reinforced by Factor XIIIa (FXIIIa) cross-linking, as discussed later in this report. The resulting product is fibers ranging from eighty to several hundred nanometers thick and 100's of nanometers to 10's of micrometers long [20, 21].

The nanostructure of fibrin fibers has been of longstanding interest. Multiple experimental methods have determined that fibers themselves also contain ~80% water [22, 23], leading to estimates of pore sizes within fibers ranging from 1 to 30 nm [24, 25] and suggesting that fibrinolytic molecules can diffuse even within a fiber [25–27]. Early EM studies on fibrin showed a distinct 23 nm banding pattern across the diameter of fibers, exactly half the length of the fibrin molecule [28]. Later studies showed that protofibrils twist around the exterior of fibers [20]. The banding patterns indicated a lateral registry between protofibrils in the fiber, although the interactions that cause the lateral registry were and still are unclear. A model based on the crystal structures of fibrin(ogen) suggested a quasicrystalline packing of protofibrils inside the fiber with unit cell of dimensions 19 nm x 19 nm × 45 nm [29]. AFM and Small Angle X-ray Scattering (SAXS) data suggested a modified model with a fractal fiber structure still retaining quasicrystalline banding, but having larger distances between protofibrils [23, 30]. Still other studies suggest that the density of protofibrils is not uniform across a fiber diameter and that the protofibril spacing increases as a function of radial distance [20, 31]. Finally, other studies emphasize the flexibility of protofibrils within fibers, which is hard to reconcile with a quasicrystalline packing [32]. None of the models currently include α-polymer networks into the packing architecture. More work in this area could shed light on fibrin mechanical properties and the mechanisms of molecular diffusion during fibrinolysis.

Finally, it should be mentioned that while fibrinogen is soluble, polymerized fibrin is insoluble. Thus, although commonly used, Michaelis-Menten kinetics are not quite correct in describing the action of an enzyme on an insoluble substrate. Recent work on fractal kinetics has begun to explore this issue [33].

2.2. Plasmin and tPA Binding Sites on Fibrin. Fibrinolytic enzymes including plasminogen and tissue plasminogen activator (tPA) bind to fibrin, and their binding sites will be discussed here, although a detailed description of the fibrinolytic molecules occurs in later sections of this review. Of importance is the fact that fibrin, but not fibrinogen enhances the activation of plasminogen by tPA [34], even though sequence-wise fibrinogen and fibrin only differ by the presence and absence fibrinopeptides A and B. Thus, it has been hypothesized that the conversion of fibrinogen to fibrin causes a conformational change, exposing binding sites for plasminogen and tPA [18].

Intact fibrin has ~100 lysine residues, but no C-terminal lysines. Binding sites have been identified at the periphery of the fibrin molecule for both tPA and plasminogen. The α chain residues 148–160 bind both tPA and plasminogen with equal affinity ($K_D \sim 1\,\mu$M) [35], and a monoclonal antibody raised against the sequence was able to bind fibrin, but not fibrinogen [36]. Electron microscopy studies of plasminogen bound to fibrin also show that it binds to the peripheral "D" region, in agreement with the antibody epitope mapping (see Figure 2(a)) [37]. The binding is lysine dependent, suggesting Kringle domain involvement [38]. A lysine-independent tPA binding site has been localized to γ chain residues 312–324 that is also inaccessible to antibodies in fibrinogen, but accessible in fibrin (see Figure 2(a)) [39]. The spatial localization of these sites is in agreement with the observation that a ternary complex between fibrin, tPA, and plasminogen is required to increase tPA's catalytic efficiency [37, 40].

Dysfibrinogenemias with abnormalities in the fibrin αC region indicate further binding sites for plasminogen and tPA [41, 42]. To test this, αC regions were recombinantly expressed and binding to plasminogen and tPA was measured. Both enzymes bound with high affinity (16–33 nM) [43] to lysine residues in the αC domain (α392–610), and binding was noncompetitive, suggesting different binding sites for plasminogen and tPA. Other work indicates that the tPA finger domain can bind to cross-beta structures in the fibrin αC domain [44]. Thus, there may be multiple bindings sites in the αC domain, but not the αC connector regions (α 221–391) for plasminogen and tPA. The αC region also contains binding sites for FXIIIa (α389–403) [45] and the cross-linking site for α2-antiplasmin (α2AP, L303) [46].

2.3. Degradation Products and C-Terminal Lysine Binding Sites. At least 34 different plasmin cleavage sites have been identified on fibrin(ogen), but different attack points are cleaved at diverse rates. Because of this, plasmin leaves a series of well-defined fibrin degradation products during lysis. The lytic series was worked out in detail several decades ago

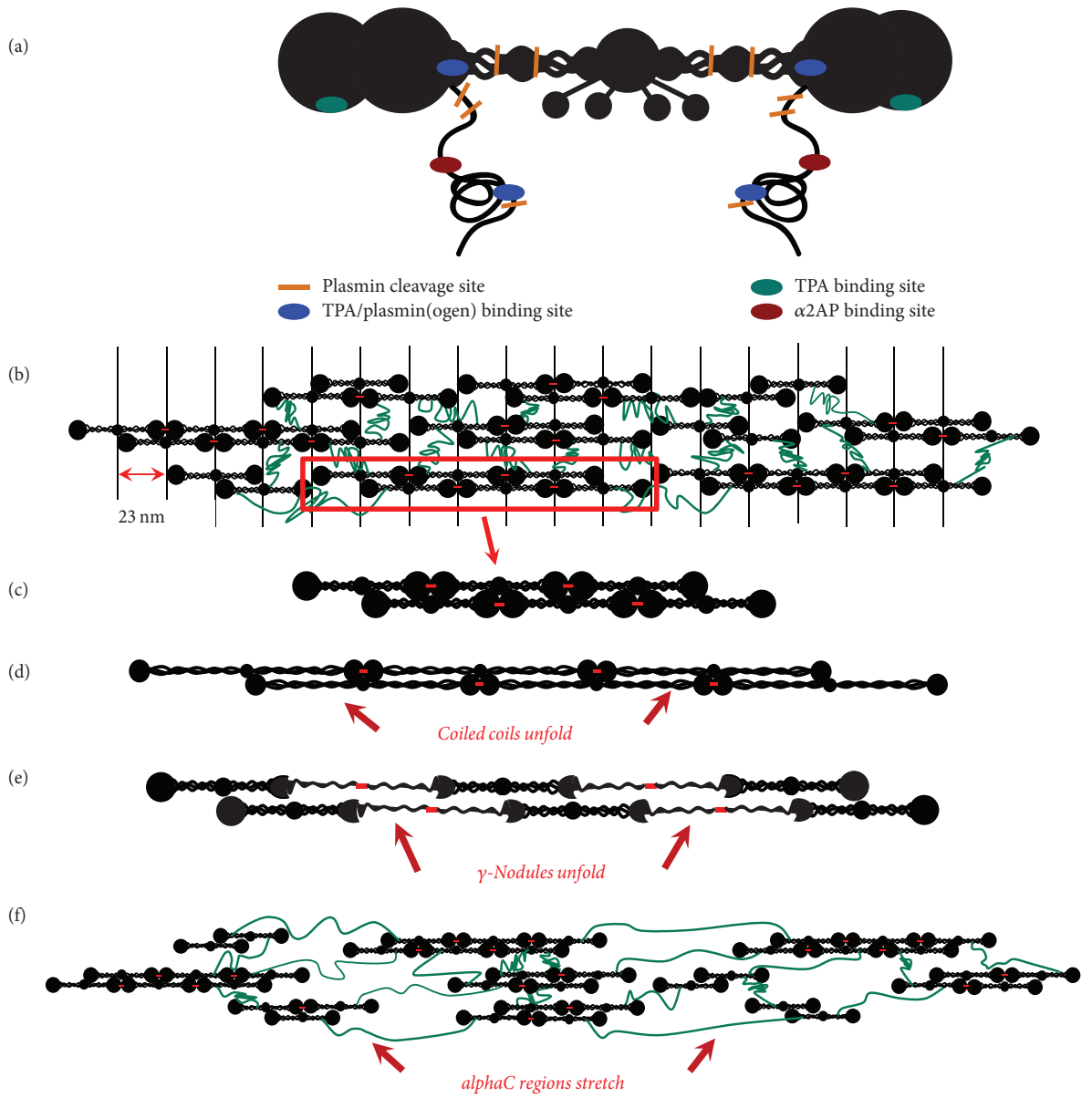

FIGURE 2: Fibrin fiber structure and mechanical stretching. (a) Cartoon model of the fibrin molecule showing fibrinolytic binding and cleavage sites. Relative positions of plasmin cleavage sites, and tPA, plasmin(ogen), and α2AP binding sites are color coded. Mechanical stretching alters each site, as seen below. (b) A structural model for the fibrin fiber, consisting of protofibrils (c) linked together by unstructured αC regions. Knob-hole interactions are not shown and the β- and γ-nodules have been simplified to one structure in (b–f) for clarity. The protofibrils align to give a 23 nm banding pattern as seen in electron microscopy images, although the interactions mediating this alignment are unclear. The red dashes between adjacent molecules indicate the site of γ-γ FXIII cross-linking. (d–f) Cartoon models depicting extension of the fiber arising from stretching of the coiled coil region (d), γ-nodule (e), and the αC regions between protofibrils (f), respectively.

and has been reviewed elsewhere [47], so it will only be mentioned briefly here.

The first attack point is AαK583, which is partially degraded even in circulating fibrinogen, and the cleavage of which leaves a C-terminal lysine. Subsequent attack points are αK206 and αK230, which remove nearly the entire αC region of fibrin. This αC-less fibrin structure was historically called fragment "X" (see Figure 1(a)); the detached αC region is further degraded after removal. The next attack region is in the coiled coil, where the α (K78, K81, R95, R104, and R110), β (K122, K133), and γ (K53, K58, K62, K85, and K88) chains all contain 2–5 lysine and arginine residues known to be plasmin cleavage sites. Transection of the coiled coil releases "the D region" containing a portion of the coiled coil and the β- and γ-nodules (see Figure 1(a)). Upon the cleavage of the other coiled coil, "the E region" is produced, which includes the central region of the molecule containing the N-terminal disulfide knot and a portion of the coiled coil. In FXIIIa ligated fibrin, the D region is covalently cross-linked to an adjacent molecule, and "D-D" and "D-E-D" (where a D-D is

noncovalently bound to region E via knob-hole interactions) fragments are released.

The creation of C-terminal lysines in partially degraded fibrin serves as a feedback mechanism for more plasminogen and tPA binding. tPA binding to degraded fibrin is 2–4 orders of magnitude tighter than binding to intact fibrin [48]. Also, removal of the αC region eliminates the protection of α2-antiplasmin (α2AP) from the immediate vicinity of the fibrin molecule, potentially increasing fibrinolysis further.

3. Fibrinolytic Agents: Activation and Inhibition

3.1. Plasminogen and Plasmin Structure, Conformation, and Function. The primary fibrinolytic agent is the serine protease plasmin. It is a trypsin-like enzyme with broad specificity that cleaves peptide bonds at the C-terminal side of lysine and arginine residues. Plasmin is the activated form of the zymogen plasminogen, which circulates as a single 791 amino acid chain at concentrations of ~2 μM [49]. Several structural features regulate the binding and activity of plasmin(ogen) [the nomenclature plasmin(ogen) will refer to features common to both plasmin and plasminogen].

Plasmin(ogen) has historically been subcategorized, based upon its glycosylation state, into Type I and Type II. Type I has an N-linked glycan at N289 and an O-linked glycan at T346 and comprises 40% of circulating material [50, 51]. Type II only has the O-linked T346 glycan and comprises 60% of circulating material. Subsequent studies suggested that some Type II plasminogen could contain a second O-linked glycan at S248 [52] or S339 [53]. Glycosylation plays a dramatic role in regulating the binding of plasmin(ogen) to particular cell types [54] and seems to play a minor role in the activation of plasminogen on fibrin [55].

The crystal structure of plasminogen was recently determined shedding further light on the structural determinants of plasmin(ogen) function [56, 57]. It can be structurally segregated into an N-terminal Pan-apple domain (PAp; ~1–77), 5-Kringle domains (Kr-1, Kr-2, Kr-3, Kr-4, Kr-5; ~78–542), and a serine protease domain (562–791) [56, 58]. Kringle domains 1, 2, 4, and 5 contain a DXD/E motif for binding C-terminal lysine residues, and lysine binding seems to be the primary mechanism for plasmin(ogen) binding to fibrin and/or cell-surface ligands [58, 59]. In Kringle 3, the motif has been mutated to DXK, and lysine binding is abolished [60]. In the closed conformation (discussed below), only Kr-1 is exposed for binding, suggesting that this domain mediates the initial recruitment of plasmin(ogen) to its binding partners [56, 57].

The PAp domain (sometimes called the N-terminal peptide [57] or activation peptide [58]) plays an important role in regulating plasmin activity and activation. With the PAp attached, plasmin(ogen) (referred to as Glu-plasmin(ogen), in this case) is predominantly found in a compact conformation mediated by an interaction between the PAp and Kr-4/Kr-5 [56, 57]. The compact conformation (typically called the closed conformation) has rough dimensions of 9 nm × 6 nm [37, 57] and a radius of gyration of 3.1 [61] to 3.9 nm [62]. However, plasmin(ogen) can also adopt a much larger U-shaped conformation (the open conformation) with rough dimensions of 14 nm × 7 nm [37] and a radius of gyration of 5.0 [61] to 5.6 nm [62]. Natively there is some thermal equilibrium between the two conformations [63], but the open conformation can be stabilized either through the cleavage of the PAp by plasmin at residue Lys77 or by the binding of lysine or lysine-analogs to the Kr-4/Kr-5 domains. Upon cleavage of the PAp domain, plasmin(ogen) is referred to as Lys-plasmin(ogen). Interestingly, Lys77 is buried in the closed conformation and inaccessible to plasmin, so some conformational rearrangement must occur prior to PAp cleavage [56].

Plasminogen activators cleave the bond between R560 and V561 in the C-terminal region of plasminogen [64], exposing the catalytic triad H603, Asp646, and Ser741 in the serine protease domain. This is the crucial step in the conversion of plasminogen to plasmin and results in a double-stranded plasmin molecule whose two chains are held together by disulfide bonds [65]. The plasmin light chain (~25 kDa) contains the catalytic site, while the heavy chain (~60 kDa) contains the Kringle domains. The R560-V561 bond is shielded from proteolysis by both the Kr-3/Kr-4 loop and the T346 O-linked glycan in the closed conformation of plasminogen [56]. However, the open conformation has a 3–50 fold increased rate of activation [63], suggesting that R560-V561 shielding is greatly reduced in this conformation.

Thus, there is a direct connection between the conformation of the plasminogen molecule and its ability to be activated to plasmin. Because the plasminogen conformation is governed by the interaction between PAp and Kr-4/Kr-5, cleaving the PAp domain or plasminogen binding to C-terminal lysines, as is the case when binding to fibrin or cell receptors, will greatly enhance the conversion to plasmin. Additionally, because Glu-plasminogen can bind to ligands prior to PAp cleavage, and binding promotes activation, it is possible to have Glu-plasmin, in addition to Glu-plasminogen. However, higher local plasmin concentrations lead to higher catalytic rates of PAp cleavage, so the predominant activation pathway is Glu-plasminogen → Lys-plasminogen → Lys-plasmin.

3.2. Plasminogen Activators (PA's). The two primary physiological plasminogen activators are the serine proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (for more detailed reviews see [65–68]). While having similar catalytic function, differences in the binding domains of uPA and tPA result in differentiation in localization and different biological roles for the two PA's [66]. Other potential physiological plasminogen activation pathways, such as the contact activation pathway will not be covered in this review [68, 69].

3.2.1. uPA. uPA is secreted as a single-chain, 411-amino acid, protein (sc-uPA) that has very little intrinsic catalytic activity in plasma [70, 71]. uPA activation transpires from the proteolysis of L158-I159 bond, converting sc-uPA to two-chain uPA (tc-uPA) and exposing the serine protease site [72]. tc-uPA also has a low molecular weight form, created via plasmin cleavage of the L135-L136 bond [69, 73], which

circulates in plasma, but most circulating sc-uPA and tc-uPA are cleared from plasma within minutes [74].

uPA consists of three structural regions, a C-terminal serine protease domain (159–411), a Kringle domain (50–132), and a growth factor domain (GFD; 10–43) [75]. Unlike tPA, uPA's Kringle domain does not have a fibrin binding site, and uPA has low affinity for fibrin [69]. uPA binds tightly (K_D < 1 nm) [76] to cell-surface receptor urokinase-type plasminogen activator receptor (uPAR) through its GFD [77, 78], although it also binds and activates plasminogen on platelets, which do not express uPAR [79]. Interestingly, sc-uPA shows ~100 fold increase in activity when bound to cell surfaces, while tc-uPA's activity is not increased further by cell binding [79, 80].

uPA primarily activates cell-surface bound plasminogen, although it can also activate solution-phase plasminogen, in contrast to tPA [68]. Surface-activated plasmin plays an important role in extracellular matrix degradation and growth factor activation [81]. The precise role uPA plays in fibrinolysis is still controversial; however, mouse models show an active role for uPA in fibrinolysis [82, 83] and tc-uPA activates Glu-plasminogen at a 10-fold higher rate in the presence of fibrin in spite of not binding to fibrin [69], so uPA's role in fibrinolysis should not be minimized.

3.2.2. tPA. tPA is synthesized and secreted by endothelial cells as a single-chain, 527-amino acid, glycoprotein. The plasma concentration of tPA is 70 pM, and it has a half-life of 4 minutes, so it is tightly regulated [68, 84]. Unlike other serine proteases like uPA and plasmin, the single chain of tPA (sc-tPA) has inherent catalytic activity and can activate plasminogen [85]; however, cleavage of the R275-I276 bond by plasmin and conversion to two-chain tPA (tc-tPA) increases plasminogen activation rates from 3- to 10-fold in the absence of fibrin [34, 40]. In the presence of fibrin, the activity of tPA is increased from 100- to 1000-fold and sc-tPA and tc-tPA have comparable catalytic rates [34, 40, 86]; the presence of fibrinogen does not increase tPA activity [18, 40]. There is strong evidence that the rate enhancement occurs due to the formation of a ternary complex between fibrin, plasminogen, and tPA [37, 40].

Fibrin stimulation of plasminogen activation by tPA occurs in two phases [87–89]. The first phase is mediated by the conversion of fibrinogen to fibrin and the exposure of cryptic tPA and plasminogen binding sites on fibrin. During this phase the typical tPA K_m value is ~1 μM plasminogen and the catalytic rate constant is ~0.2 s^{-1} [40, 89]. Upon plasmin formation, COOH-terminal lysines become exposed as fibrin is digested; this provides more binding sites for plasminogen and tPA and creates a positive feedback mechanism, resulting in K_m values for tPA ~ 100 nM plasminogen while retaining the same catalytic rate constant [89]. For these reasons, tPA is thought to be the predominant plasminogen activator during fibrinolysis.

Structurally tPA consists of 5 distinct domains: a finger domain, an epidermal growth-factor-like domain, two Kringle domains, and the catalytic domain [90]. The finger domain and the Kringle-2 domain serve as the primary fibrin binding sites [86]. The Kringle-2 domain plays a role in C-terminal lysine binding, while the finger domain can bind to a region in the fibrin γ-nodule in a lysine-independent mechanism [91] or to amyloid-like cross-beta structures [44], which have been hypothesized to form in fibrin α-polymers [14, 92]. Recent work has shown that the tPA finger domain plays the predominant role in binding to fibrin during fibrinolysis [93, 94], even in proteolytically degraded fibrin.

Fibrin thus acts as both a cofactor and substrate for tPA and plasminogen and in so doing provides the mechanism for its own disintegration. These dual roles highlight the intended temporariness of the fibrin mesh network; it is not designed to stick around for longer than is necessary for wound healing in most physiological circumstances.

3.3. Fibrinolytic Inhibitors. The proteases of the fibrinolytic system are all tightly controlled by inhibitors. For recent, thorough reviews, the reader is directed to [68, 95]. For the purposes of this review, they will only be covered briefly.

3.3.1. α2-Antiplasmin (α2AP). α2AP serves as the fast-acting ($4 \times 10^7\ M^{-1}s^{-1}$) [96] primary inhibitor of plasmin and circulates in plasma at concentrations of ~1 μM, usually in excess of plasmin, with a half-life of 3 days [68, 97]. α2AP is also cross-linked to fibrin by FXIIIa, so there is a high local concentration in clots (this is described in more detail later in this review) [98], and there are additional noncovalent α2AP binding sites in fibrin, but not fibrinogen [99]. Like other serpin inhibitors, α2AP inhibits plasmin by inserting a "reactive center loop (RCL)" into plasmin's catalytic site, which then attacks the R364-M365 peptide bond of the loop [100]. This releases the N-terminal portion of α2AP, while forming a covalent ester-bond between α2AP and the catalytic site, inhibiting plasmin. The lysine-rich C-terminus of α2AP is ~55 residues longer than most serpins and contains a binding site for plasmin(ogen) Kringle domains [97, 101]. Plasminogen-fibrin and plasminogen-α2AP binding are competitive; plasmin activated while bound to fibrin is therefore relatively protected from α2AP, although the unbound α2AP can still inhibit plasmin albeit at a 100-fold slower rate [100].

3.3.2. Plasminogen Activator Inhibitors 1, 2 (PAI-1 and PAI-2). PAI-1 is the physiological inhibitor of both uPA and tPA. Like α2AP, PAI-1 is a serpin inhibitor, with its reactive site at R346-M347 [95]. PAI-1 inhibits both tPA and tc-uPA with second-order rate constants of roughly $1–4 \times 10^7\ M^{-1}s^{-1}$ [102, 103]. Activated platelets can release PAI-1 and increase its local concentration 10-fold [104], helping to reduce fibrinolysis at the onset of clotting.

PAI-2 also is a serine protease that inhibits tPA ($1 \times 10^4\ M^{-1}s^{-1}$) and uPA ($2 \times 10^6\ M^{-1}s^{-1}$), but with slower rate constants than PAI-2 [105]. Its primary function may be related to placental maintenance rather than fibrinolysis, as it is only present in plasma during pregnancy [68, 95].

3.3.3. Thrombin-Activatable Fibrinolysis Inhibitor (TAFIa). TAFIa is not a serine protease and has an entirely different mechanism than other fibrinolytic inhibitors [106]. TAFIa

is produced from its zymogen TAFI by cleavage at R92-A93 by the thrombin/thrombomodulin complex [107]. TAFIa removes C-terminal lysine and arginine residues from fibrin as it is degraded, preventing plasminogen binding and the positive feedback mechanisms that stimulate lysis [108]. It also prevents the conversion of Glu- to Lys-plasminogen, reducing the rate of plasminogen activation [109]. Thus, TAFIa does not directly inhibit lysis, but rather slows down several crucial steps in fibrinolytic upregulation.

4. Biophysical Determinants of Fibrinolysis

4.1. Clot Structure and Architecture. Blood clot content and architecture help to determine their lytic susceptibility. Fibrin network structure is determined by the local concentrations of fibrinogen, thrombin, and ions, such as Ca^{2+} [110, 111]. High fibrinogen concentrations, such as those experienced in hyperfibrinogenemia, high local thrombin concentrations, and high plasma ionic strength give rise to clots with thinner fibers at a higher density (less space between fibers) [110–113]. Conversely, increasing the Ca^{2+} concentration or decreasing the thrombin concentration leads to clots composed of thicker fibers and a lower packing density [110]. A long history of rheological studies suggest that clots composed of thin, densely packed fibers are stiffer than those of thick fibers with larger pore sizes [110, 114, 115].

The mechanism by which individual fibers are lysed has been a subject of debate. Several studies, primarily using turbidity as a readout of fibrinolysis, and models suggested that the diameter of fibrin fibers decreases uniformly during lysis due to many plasmin molecules binding and digesting the fiber along its entire length [116–118]. It should be noted that using turbidity to measure lytic rates has been a subject of controversy with respect to whether the data should be normalized by the highest turbidity value [119]; doing the normalization lowers the apparent lytic rates after polymerization reaches its maximum but ignores digestion prior to that point. Other studies, primarily using fluorescent and electron microscopy reported transverse cleavage of fibers at one point [120–123]. Because C-terminal lysines are exposed during lysis, plasminogen binding to partially degraded fibrin will be amplified at points where fibrin has already been cleaved [124]. It has been hypothesized that this serves as a feedback mechanism to promote further lysis at those points, leading to transverse cleavage directly across the diameter of the fiber [26]. Studies of individual fiber lysis showed that cleavage does occur at a specific point but also that the lytic rate for further fiber degradation slows after the initial transverse cleavage event [121]. It is often observed that cleaved fibers bundle together to form thicker fibers [120, 123] prior to being degraded further. It is likely that both transverse cleavage and digestion along the fiber length play a role during fibrinolysis, perhaps with transverse cleavage mediating the initial digestive event.

The conventional wisdom has been that fiber density (the number of fibers per unit volume) and fiber thickness have competing effects on fibrinolysis. Numerous in vitro studies have reported that clots composed of thinner, more closely spaced fibers, are more resistant to fibrinolysis [111, 112, 120, 125]; however, several studies have shown an opposite effect, so this is not necessarily always the case [116, 126]. Hypofibrinolysis has been reported for patients with thin, dense fibers, supporting the idea that clots composed of thinner fibers are more resistant to lysis [127, 128]. Conversely, thin fibers were more rapidly cleaved than thick fibers in a variety of fibrinolysis models [120, 121, 123, 126]. These contrasting results likely come from the interplay between the movement of fibrinolytic agents (plasminogen, plasmin, tPA, etc.) within a clot, and the activity of those agents on fibers upon binding. It is important to note that, in most in vitro studies of fibrinolysis, plasmin or plasminogen activators such as tPA or uPA are added from outside an already formed fibrin network. Therefore, the permeation of fibrinolytic agents into a clot plays a predominant role in determining clot lysis rates. These studies are important for the development of therapeutics that must be administered from outside the clot but may not mimic in vivo fibrinolysis which happens concurrently with polymerization [123].

In the case where lytic agents are released from outside the clot, recent 3D stochastic modeling suggests that the determining factor in fibrinolytic rates is the number of tPA molecules per clot surface area [27]. Bannish et al. found that, for low tPA concentrations, clots of thick fibers lyse more rapidly, but for higher concentrations (high enough for at least one tPA molecule to bind to every fiber on the surface of the clot), clots composed of thinner fibers will actually lyse faster than those of thick fibers [27]. These results may help to explain the discrepancies between previous experiments and suggest avenues for therapeutic development.

The observation that thicker fibers lyse more slowly has been explained, in part, by several phenomena. First, thin fibers are composed of fewer protofibrils within a cross-section, so fewer molecules need to be cleaved in order to transect a fiber [26, 27]. Also, within thin fibers the molecules are more densely packed, so plasmin and tPA binding sites are closer together [31], and it has been observed that thin fibers are better for plasminogen activation by tPA [93]. Therefore, all other things being equal, thin fibers should lyse more rapidly than thick fibers. Secondly, thicker fibers are likely under more tension than thinner fibers due to protofibril packing [20]. Modeling and experiments show that, as fibers are lysed, they lose this inherent tension leading to elongation, and elongation hinders fiber lysis [121]. Elongation is more prominent in thicker fibers than thin fibers providing additional reasons that thin fibers lyse more rapidly. Finally, models predict that the amount of time tPA remains bound to fibers can have a noticeable influence on lytic rates of individual fibers, and tPA remains bound longer to thicker fibers than thin ones independent of the tPA off-rate, if the off-rate is sufficiently slow [27].

4.2. FXIIIa Cross-Linking. The transglutaminase FXIIIa likely regulates fibrinolysis through at least three distinct mechanisms: (1) cross-linking fibrinolytic inhibitors, particularly α2-antiplasmin, to fibrin, (2) cross-linking fibrin fibers, and (3) altering the mechanical properties of fibers and fibrin networks.

FXIII is a protransglutaminase consisting of two A and B subunits (A_2B_2) in plasma and as a homodimer of A subunits (A_2) in cells [129]. Plasma FXIII is activated in the final step of the clotting cascade when thrombin hydrolyzes the R37-G38 bond, releasing an activation peptide, and Ca^{2+} causes the dissociation of the B subunit, resulting in a catalytically active A_2 dimer usually referred to as FXIIIa. The rate of plasma FXIIIa activation is accelerated 6-fold in the presence of polymerized fibrin [130]. Cellular FXIII, such as that released by platelets and monocytes, is activated in a thrombin- and fibrin-independent mechanism involving Ca^{2+}, where the activation peptide is not removed [131].

It now seems clear that cross-linking α2-antiplasmin (α2AP) to fibrin is the primary antifibrinolytic function of FXIIIa [98, 132]. α2AP plays several inhibitory roles in fibrinolysis including rapidly inhibiting plasmin and interfering with the binding of plasminogen to fibrin lysine sites [46, 133]. During fibrin polymerization, FXIIIa covalently cross-links α2AP via its Q2 residue to L303 in the fibrin αC linker region [46, 134]. α2AP cross-linking precedes α chain cross-linking by FXIIIa (see next paragraph) and may inhibit this process [129, 133]. Uncross-linked α2AP has similar plasmin inhibitor activity to cross-linked α2AP, but cross-linked α2AP has a much greater effect on the inhibition of lysis [135]. This inhibitory effect is increased during platelet retraction, when the fibers of the clot are closer together and fluid is expelled from the clot [132, 135]. FXIIIa can also cross-link other fibrinolytic inhibitors to fibrin(ogen), including PAI-2 [136] and TAFI [137]. These results strongly support the hypothesis that FXIIIa inhibits fibrinolysis by the covalent incorporation of fibrinolytic inhibitors into the fibrin network. This may be particularly important during the early stages of clot formation, protecting against immediate elimination of nascent clots [129]. However, other studies have shown that α2-antiplasmin works in concert with α chain cross-linking in fibrinolytic inhibition [138], so FXIIIa cross-linking of fibrin itself likely also has inhibitory effects.

During fibrin polymerization, FXIIIa forms γ-glutamyl-ε-lysyl cross-links between residues in the γ and α chains of fibrin monomers. FXIIIa first catalyzes the formation of isopeptide bonds between γ1L406 and γ2Q398 or γ2Q399 at the C-terminal γ-nodules of adjacent molecules, forming longitudinal γ–γ dimers within protofibrils [139]. Later during polymerization, FXIIIa targets lysine and glutamine residues in the αC region, resulting in the formation of high molecular weight fibrin species including α-polymers and α-γ hybrids [140–142]. Although there is no set order in which α chain glutamine residues are cross-linked, generally Q237 is targeted first, followed by Q366, Q328, and Q221 [143]. The α chain lysine donors are more heterogeneous but involve at least L418, L508, L539, L556, L580, and L601, most of which are located at the C-terminal periphery of the αC region [144–146]. FXIIIa cross-linking causes slight, but not dramatic changes in network morphology, with 10% thinner fibers, and a 2-fold reduction in pore size [110, 147].

Whether FXIIIa cross-linking effects fibrinolytic rates was a subject of historical [148–150] and even recent [98, 147] debate, complicated by sample preparation and the presence of fibrinolytic inhibitors. A recent study, where α2-antiplasmin was inhibited, established that FXIIIa cross-linked fibers have delayed fibrinolysis, even in the absence of external mechanical force [147]. These results agreed with previous studies showing decreased lysis by plasmin on FXIIIa ligated clots [151]. This may be due, in part, to the decreased binding affinity of plasmin or plasminogen to cross-linked fibers [126, 129]. Some studies show that specifically α chain cross-linking plays an important role in reducing the fibrinolytic susceptibility of clots [140, 149], although there is not universal agreement [147, 148]; it seems feasible because α chain cross-linking likely reduces the number of lysines available for plasminogen and tPA binding, decreases the mobility of molecules between protofibrils [129], and makes protofibril packing more dense [152]. Other studies have shown a predominant role for γ-cross-linking and even γ-multimers in regulating fibrinolytic rates [153]. In summary, it seems that cross-linking of fibers plays a measurable, but potentially minor role in fibrinolysis.

FXIIIa cross-linking has a dramatic effect on fibrin mechanical properties. Uncross-linked or partially cross-linked (some low molecular weight species) fibrin fibers are among the most extensible biomaterials found in nature, able to be stretched to triple or quadruple their original length before failing and also able to relax back to their original length within milliseconds [1, 15, 154, 155]. Fully cross-linked fibers (>90% γ and α chains cross-linked) are 2–10 times stiffer, 50% less elastic, and have 40–50% lower extensibility than partially cross-linked fibers [21, 155]. Studies using recombinant fibrin with γ chain cross-linking sites mutated out (γQ398N/Q399N/K406R) reveal that loss of fiber elasticity and extensibility is primarily due to α chain cross-linking [156]. Loss of fiber extensibility may explain the recent observation that during clot retraction erythrocytes are trapped in FXIIIa cross-linked fibrin networks without being covalently bound to the fibers [157, 158], while erythrocytes in uncross-linked fibers were extruded.

Fibrin networks also have remarkable extensibility, due in large part to the mechanical properties of their individual fibers [2, 32]. In rheological studies, where network mechanical properties typically depend more on fiber structural rigidity and network rearrangement than fiber stretching, networks composed of cross-linked fibers also exhibited a 2- to 5-fold higher elastic modulus (stiffness) and a 2-fold higher loss modulus [21, 141, 152, 159]. Rheological measurements on (γQ398N/Q399N/K406R) fibrin suggest that FXIIIa-mediated stiffening comes from contributions of α- and γ-cross-linking, with α chain cross-linking playing the largest role [141]; other studies demonstrate that the mechanism of network stiffening comes from FXIIIa-mediated structural rigidity increases of individual fibers [152].

The effect of different mechanical properties in FXIIIa-cross-linked fibrin on fibrinolytic rates has not been directly explored, but several mechanisms can be proposed. First, the reduced extensibility of FXIIIa cross-linked fibers limits the extension of fibers during platelet retraction. Platelets carry endogenous FXIIIa, so most fibers are highly cross-linked

during retraction, and because retraction plays multiple roles in regulating fibrinolysis (discussed below), it is likely that the increased stiffness of FXIIIa fibers has a mechanism in this regulation. Second, Varjú et al. showed that fiber stretching decreased plasminogen activation and lysis, suggesting that stretching of fibrin alone regulates fibrinolysis [3], and thus reduced extensibility of fibrin by FXIIIa should affect fibrinolytic rates via this mechanism as well. Third, under certain conditions, thick fibers elongate during lysis and reach a lysis resistant state [121]. FXIIIa cross-linking could alter the elongation and lytic resistance of these fibers. While only hypotheses at this point, these ideas highlight the need for further studies to measure the direct effect of altered fibrin mechanical properties on the fibrinolytic susceptibilities of fibrin clots.

Recently it was shown that plasmin can inactivate FXIIIa, by cleaving the enzyme at a variety of sites, predominantly the K468-Q469 bond [160]; contrastingly, FXIII (A_2B_2) was not degraded in the same manner. FXIIIa inactivation by plasmin occurs primarily during fibrinolysis rather than polymerization suggesting it serves as a feedback mechanism to prevent further FXIIIa activity after the cessation of clotting. This could also avert the further incorporation of fibrinolytic inhibitors such as α2AP into the clot, thus promoting fibrinolysis.

4.3. Movement of Fibrinolytics into and within Fibrin Networks. The transport of fibrinolytic agents into a clot and their movement within a clot depend on diffusion (the random movement of molecules due to thermal fluctuations), advection (the conveyance of particles within flowing fluid; sometimes referred to as convective transport, permeation, or perfusion), and binding (to fibrin or other clot constituents like platelets or erythrocytes). Fibrinolytic transport has been covered in other reviews [24, 161]; the physical aspects of this process will be reviewed here.

Penetration of fibrinolytics into blood clots depends on the network architecture and contents. Networks formed of purified fibrin have a fibrin content usually <1% of the total network volume at physiological fibrinogen concentrations [161]. Under these conditions, the average pore size (space between fibers) ranges from 100 nm in gels made of thin fibers to 10 μm in gels made of thick fibers [24]. The diffusion of a molecule such as plasmin(ogen), with a stokes radius of ~5 nm, within the pores between fibers and cells can roughly be thought of as free diffusion [24, 161].

Cells, such as erythrocytes and platelets, modulate network structure through direct fibrin-cell receptor binding and the release of pro- and anticoagulation factors [162, 163]. Moreover, tissue-factor bearing cells promote fibrin production and can lead to high local fibrin concentrations during polymerization [125]. High local fibrin concentration (up to 400 μM) could decrease the fiber pore size to as low as 4 nm [164], but this is likely not the case under most physiological situations. Even for platelet retracted clots, where 99% of the fluid volume has been expelled, the porosity is still >90% [24]. Taken together, these data suggest that under most conditions, the diffusion of fibrinolytic molecules into and within a clot should roughly mimic free diffusion [165]; however, a recent report on fibrinolysis of stretched fibrin clots reported hindered diffusion into the clot based on Fluorescence Recovery after Photobleaching (FRAP), so this might not always be the case [166].

Although network structure usually does not hinder diffusion, the binding of fibrinolytic molecules to fibrin or cellular constituents plays a dramatic role in reducing their mobility [24, 27, 161]. Fluorescent microscopy studies on the lysis of clots initiated by adding lytic agents outside the clot often show a "lysis front" where plasmin, plasminogen, or tPA bind to the first few microns of a clot without penetrating much further [120, 124]. The network is dissolved progressively from outside to inside as the lysis progresses. Streptokinase, which does not bind tightly to fibrin, penetrates clots more rapidly than tPA [167], and studies using a tPA variant defective in fibrin binding also observed more rapid penetration into clots [93]. Networks composed of thin, densely packed fibers, have more binding sites per unit volume for plasminogen and tPA, which helps to explain the hindered fibrinolysis for these types of clots [27, 120].

Molecular penetration into clots and fibrinolysis rates can be enhanced from 10- to 100-fold by the presence of flow and molecular advection [118, 168, 169]. Flow allows fibrinolytics to travel further into a clot prior to binding, enhancing the inner-clot fibrinolytic rate. The direction of flow matters as tangential flow with respect to the clot can lead to a "plasmin steal" effect where flow depletes plasminogen from the clot boundary [124]. However, a study on retracted blood clot dissolution under tangential flow still showed a 10-fold increase in clot degradation [170]. In the case of flow directly into a thrombus, the fluid will flow through the least-permeation-resistant path, so structural heterogeneity can have dramatic effects on the delivery of fibrinolytics [118, 169]. As the fibrin network is digested, channels will be carved out, and further digestion will emanate outward from the channel [124, 171]. In the case of a completely occluded blood vessel, once a channel is carved through a thrombus, reperfusion of the channel is achieved. The accompanying drop in pressure can reduce the transport of further fibrinolytics into the clot due to flow, and further lysis must proceed through diffusion and binding, as described above. The difference in transport in arterial and venous flow rates can have a dramatic difference in the impact of advection on fibrinolytic rates [161], and therapeutic strategies should be designed accordingly.

4.4. Fiber Stress and Extension. Fibrin is among the most extensible biomaterials [1, 172]. Fibrin's elasticity and extensibility may play prominent roles during blood clot formation under shear stress [3, 173–175] and during platelet retraction [176, 177]. The molecular mechanisms underlying fibrin extensibility have recently been debated (see Figures 2(d)–2(f)) [178]. Fiber extension measurements, simulations, and a comparison of human, mouse, and chicken fibrin extensibilities all suggest that the αC connector region plays a large role in fibrin elasticity [32, 154, 156, 179, 180]. Other measurements and simulations suggest that unfolding of either the coiled coil region [2, 181–183] or the γ-nodule could play roles [15, 182, 184]. Because plasmin(ogen) has potential

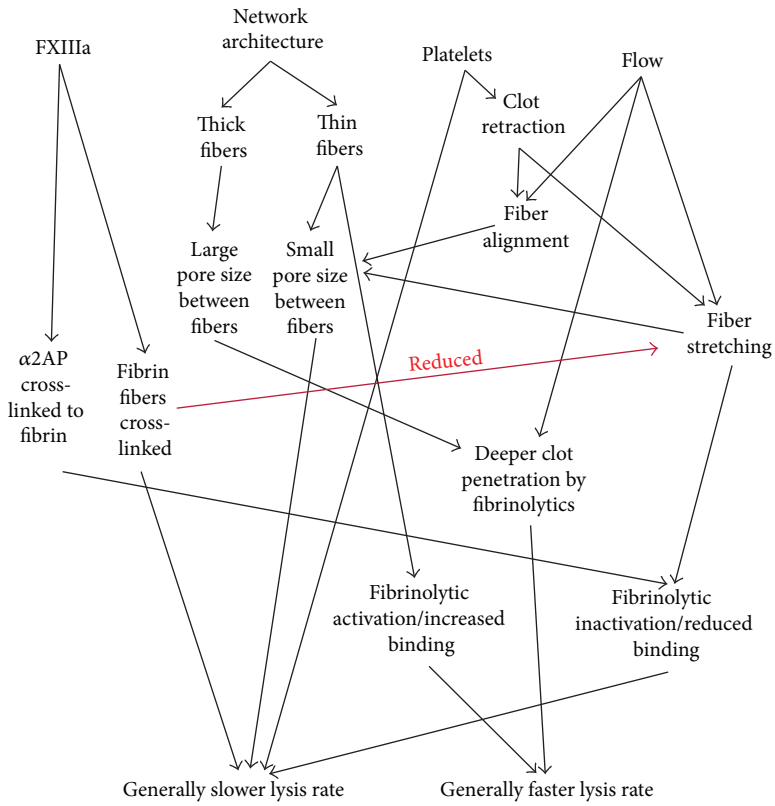

Figure 3: Interaction network of the biophysical determinants of fibrinolysis rates. A diagram highlighting the influence of FXIIIa, network architecture, platelets, and fluid flow on lytic rates. The diagram is simplified and does not include many of the interactions discussed in the paper but is meant to emphasize some of the major impacts. Black arrows show an influence of one property on a downstream property. The end result is either faster or slower network fibrinolytic rates. The red arrow indicates that cross-linked fibrin fibers have reduced extensibility and thus reduced fiber stretching. "Fibrinolytic activation/increased binding" and similarly worded effects are meant to indicate that a fibrinolytic enzyme such as plasmin is activated and/or has increased binding affinity or avidity.

binding and cleavage sites in each of these regions, fibrin stretching could act as a modulator for fibrinolysis.

During clot retraction (contraction) fibrin fibers are stretched by platelets. This reduces the interstitial spacing between fibers, reduces clot volume, and expels up to 99% of the liquid from within the clot [185]. Retraction may also help to segregate red blood cells (erythrocytes) and form a more effective wound seal [186]. For many years, it has been observed that lysis is altered by clot retraction, and the consensus of most in vitro studies is that platelet retraction inhibits fibrinolysis [185, 187–191]; however, the effects and mechanisms have been debated. One proposal is that the expulsion of unbound plasminogen during retraction reduces lytic rates [185, 192, 193]. tPA binding to fibrin also is inhibited by retraction and may play a larger role than plasminogen depletion in hindering fibrinolysis [189, 191]. Other studies suggested that the increased concentration of FXIIIa-cross-linked α2-antiplasmin in retracted clots resulted in higher plasmin deactivation and slower lysis [132, 194, 195]. Studies on the effects clot retraction on fibrinolysis are complicated by the fact that activated platelets contain and/or release a number of hemostatic and fibrinolytic factors including fibrinogen, FXIIIa, plasminogen, plasminogen activators, α2AP, and PAI-1, so it is likely that platelets and retraction have multiple roles in regulating fibrinolysis [190, 194, 196, 197].

Several studies have looked at the direct impact of fibrin strain on lysis rates. A study by Varjú et al. attempted to directly measure the fibrinolysis of stretched networks of fibers in the absence of platelets and found that the digestion of fibers formed under mechanical stress was delayed [3]. Plasminogen activation by tPA decreased by 2- to 3-fold on stretched fibers as compared to unstretched. The digestion of stretched surface fibers by both tPA activated plasminogen, and by the direct addition of plasmin, showed a greater than 50% reduction in lysis at comparable time points when compared to unstretched fibers. Another study by Adhikari et al. found a 10-fold reduction in plasmin degradation of strained clots and correlated this with a reduction of diffusive transport into the network [166]. These results suggest that fiber stretching impairs fibrinolysis by delaying plasminogen activation, reducing the fibrinolytic ability of plasmin, and hindering the entrance of fibrinolytics into the network.

In contrast, a study on the lysis of individual, isolated, unstretched fibrin fibers by plasmin showed that as fibers are lysed, they lose their inherent tension and elongate [121]. Elongated fibers reached a state where further fibrinolysis was impaired and often were not further digested. The effect

was dependent on fiber diameter, with thicker fibers more likely to elongate, but independent of plasmin concentration. The results suggested that a minimum fiber tension may promote plasmin activity [121]. Because fibrin fibers form under tension [20, 198], one resolution is that polymerization tension is required for fibrinolysis, but the addition of external tension, such as in the case of retraction, hinders fibrinolysis.

Taken together, these results suggest that fiber tension and stretching play an important role in the regulation of fibrinolysis, altering the binding of plasminogen activators, the availability of fibrinolytic enzymes, and the activity of plasmin. Models of fibrin extension often rely on protein unfolding to correlate extensibility with molecular structure [2, 184]. Unfolding of the coiled coil or γ-nodule or stretching of αC domains [154] could alter or partially block enzyme binding and cleavage sites. Further studies could help to decouple the roles between these different effects.

5. Conclusions

Coagulation and fibrinolysis are very physical processes, performed amid fluid flow, cellular adhesion, and platelet contraction. This review has highlighted several biophysical mechanisms that regulate fibrinolytic rates (see Figure 3). Additional work in this area is needed to understand the mechanisms undergirding the delayed lytic rates of strained fibrin, given that platelet retracted clots contain almost exclusively stretched fibrin fibers. Improved understanding of the connection between the biophysical aspects of fibrin and fibrinolytic rates could lead to new strategies in the development of future fibrinolytic therapies [199].

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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