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Fibronectin alters the rate of formation and structure of the fibrin matrix

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ABSTRACT

Plasma fibronectin is a vital component of the fibrin clot; however its role on clot structure is not clearly understood. The goal of this study was to examine the influence of fibronectin on the kinetics of formation, structural characteristics and composition of reconstituted fibrin clots or fibrin matrices. Fibrin matrices were formed by adding thrombin to 1, 2 or 4 mg/ml fibrinogen supplemented with 0–0.4 mg/ml fibronectin. The rate of fibrin matrix formation was then monitored by measuring light absorbance properties at different time points. Confocal microscopy of fluorescein conjugated fibrinogen was used to visualize the structural characteristics of fibrin matrices. The amount of fibronectin in fibrin matrices was determined through electrophoresis and immunoblotting of solubilized matrices. Fibronectin concentration positively correlated with the initial rate of fibrin matrix formation and with steady state light absorbance values of fibrin matrices. An increase in fibronectin concentration resulted in thinner and denser fibers in the fibrin matrices. Electrophoresis and immunoblotting showed that fibronectin was covalently and non-covalently bound to fibrin matrices and in the form of high molecular weight multimers. The formation of fibronectin multimers was attributed to cross-linking of fibronectin by trace amounts Factor XIIIa. These findings are novel because they link results from light absorbance studies to microcopy analyses and demonstrate an influence of fibronectin on fibrin matrix structural characteristics. This data is important in developing therapies that destabilize fibrin clots.

1. Introduction

Elevated levels of plasma fibronectin have been associated with an increased risk of blood clot formation in the blood vessels of individuals suffering from thrombotic diseases [1–3]. Thrombosis is the formation of fibrin clots in blood vessels, which can obstruct blood flow and result in stroke [4,5]. Fibrin is formed by proteolytic processing of fibrinogen, an inert plasma protein consisting of two sets of α, β and γ chains linked together by disulfide bonds [6–8]. The conversion of fibrinogen to fibrin is catalyzed by thrombin. A fibrin clot is formed by covalent and non-covalent interactions that cross-link fibrin monomers into a three dimensional extracellular matrix [6–8]. Fibrin clots also contain cross-linked fibronectin, which is a glycoprotein found in blood plasma [3,9].

Fibronectin consists of two nearly identical 230–270 kDa monomers that have binding sites for fibrin. The domain structure of a single monomer of fibronectin is shown in Fig. 1. The structure consists of homologous structural modules classified as type I, II and III repeats. The incorporation of fibronectin into a fibrin clot involves both covalent and non-covalent interactions between the two components. Non-covalent interactions take place between the fourth and fifth type I repeats of fibronectin and the α chain of fibrin [10–13]. Covalent bonding occurs between the amino-terminal fibrin binding site and the α chain of fibrin in the presence of Factor XIIIa [10,14,15]. Non-covalent interactions also occur between the tenth through twelfth type I repeats of fibronectin and the α chain of fibrin [16,17].

The formation of fibrin clots as well as the role of fibronectin on the rate of fibrin clot formation and fibrin clot structure have been studied by light absorbance in reconstituted clots [18,19]. Reconstituted fibrin clots or fibrin matrices formed in the absence of cells are easier to study because they allow for tight control of clot composition [18–20]. A study by Okada and co-workers showed that fibronectin had no impact on the rate of fibrin polymerization but changed the light absorbance properties of the fibrin matrix after gelation [18]. They inferred that the changes in light absorbance correlated to thicker fibrin fibers. Similarly, Carr and co-workers used light absorption studies to demonstrate that fibronectin increased both size and density of fibrin fibers but did not affect the rate of fibrin matrix formation [19]. Both studies seem contrary to the turbidity, microscopy and modeling findings which demonstrate that clot structure is kinetically determined [21]. Other studies have shown that fibronectin increases the rate of fibrin matrix formation, with and without covalent cross-linking

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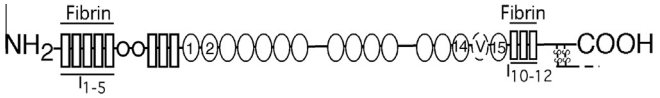

Fig. 1. Domain structure of human plasma fibronectin monomer with fibrin binding sites. Homologous type I, II and III structural repeats are represented as rectangles, circles and ovals respectively. Plasma fibronectin non-covalently binds fibrin at the type I repeats $I_{4\text{-}5}$ and $I_{10\text{-}12}$. Covalent binding also occurs at the amino-terminal binding site.

[22]. Therefore, there is a critical need for microscopy studies on the effect of fibronectin on fibrin matrices that can then be linked to kinetic findings obtained by light absorption studies. Such studies have yet to be conducted but would yield important data that would be useful in the design of therapeutics aimed at destabilizing the fibrin clot.

In this report we present new evidence on an influence of fibronectin on fibrin matrix formation and structure in reconstituted fibrin clots. We found that: (i) the initial rate of change and the steady state values of light absorbance of fibrin matrices increase with increasing fibronectin concentration, (ii) increased fibronectin concentration results in decreased fiber size and increased fiber density, (iii) fibronectin interacts with the fibrin matrix through covalent and non-covalent bonds and (iv) the amount of fibronectin bound to the fibrin matrix increases with increasing solution concentration of fibronectin but is saturable. These results are strong evidence that fibronectin influences both the rate of formation as well the structure of the fibrin matrix.

2. Materials and methods

2.1. Purification of human plasma fibronectin

Fibronectin was isolated from frozen human plasma. Human plasma was obtained from the blood bank at Rush Hospital. Fibronectin was isolated from human plasma through established procedures [23,24]. The optical density of fibronectin at 280 nm was used to determine its concentration. An extinction coefficient of 1.3 $(mg/ml\ cm)^{-1}$ was used for fibronectin. The purity of fibronectin was characterized through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Fibronectin was dialyzed at 4 °C overnight in 50 mM Tris, 50 mM $CaCl_2$, 150 mM NaCl at pH 7.4 (TBS) for subsequent experiments.

2.2. Dynamic light absorbance measurements of fibrin matrices

Lyophilized bovine fibrinogen (MP Biomedicals, Solon, OH) was reconstituted in TBS. Before measurements of fibrin absorbance were conducted, the amount of native fibronectin that may have co-purified with fibrinogen was determined by immunoblotting. The fibronectin concentration in lyophilized fibrinogen was less than 0.2% by mass. Solutions of 1, 2 or 4 mg/ml fibrinogen supplemented with 0–0.4 mg/ml fibronectin were placed in a 96 microwell plate (Fisher Scientific, Pittsburgh, PA). Thrombin (Fisher Scientific) in TBS was added to each well at a concentration of 0.05 U/ml. Time-lapse light absorbance measurements at 405 nm were collected in a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT).

2.3. Confocal microscopy of fibrin matrices

Fibrinogen was conjugated with fluorescein to visualize it by fluorescence microscopy. It was incubated with excess N-Hydroxy-Succinimide (NHS) fluorescein (Fisher Scientific). A molar ratio of fibrinogen to NHS fluorescein of 1:15 was used and conjugation was carried out at room temperature for one hour. The fluorescein labeled fibrinogen solution was dialyzed overnight in TBS at 4 °C. The concentration of fluorescein labeled fibrinogen was determined by measuring absorbance at 280 nm. A 1:10 mixture of fluorescein conjugated and unconjugated fibrinogen at concentrations of 1, 2 or 4 mg/ml of protein was supplemented with 0, 0.2 or 0.4 mg/ml fibronectin. This solution was placed on glass coverslips and enclosed in a mold. Polymerization was initiated by the addition of 0.05 U/ml thrombin. Fibrin matrices were allowed to polymerize for 5 h at room temperature. Thrombin was inactivated by washing the matrices with 2 mM phenylmethylsulfonyl fluoride (PMSF, Fisher Scientific) in TBS. The matrices were imaged using a PASCAL Laser Scanning Microscopy system (LSM, Carl Zeiss Microscopy, LLC, Thornwood, New York) with a 488 nm argon laser, 505 nm low pass filter and a 63x oil objective. Exposure parameters were kept constant when imaging different treatments. The reading frame was 1024×1024 pixels per 143×143 μm. The fibrin matrices were imaged as Z stacks consisting of three images spaced 1 μm apart and compressed using PASCAL software (Carl Zeiss).

2.4. SDS–PAGE and immunoblotting

The fibrin matrices were solubilized in 8 M urea, 2% SDS (Fisher Scientific) and 1% β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO) in TBS. Samples were resolved on 5% polyacrylamide gels and stained with silver nitrate or electrophoretically transferred to nitrocellulose for immunoblotting. The nitrocellulose was labeled with monoclonal antibody 7.1 against human fibronectin (Developmental Studies Hybridoma Bank, Iowa City, IA) using procedures previously described elsewhere [23,24]. The blots were treated with Pierce ECL Western Blotting Substrate (Fischer Scientific) and exposed in a ChemiDoc XRS+ imaging system (BioRad, Hercules, CA). Densitometric analysis was carried out with Image Lab software (BioRad). A loading control of 100 ng fibronectin was used.

3. Results

3.1. Light absorption of fibrin clots formed with varying concentrations of fibronectin

The light absorbance of fibrin clots formed in the presence of varying concentrations of fibronectin was monitored to determine the influence of fibronectin concentration on the rate of fibrin clot polymerization. Fig. 2 shows light absorbance of fibrin clots formed from 1, 2 or 4 mg/ml fibrinogen with varying concentrations of fibronectin. At a constant fibrinogen concentration, an increase in fibronectin resulted in an increase in light absorbance during fibrin matrix formation. The initial rates of fibrin matrix formation were determined from Fig. 2A and B. This data is shown in Fig. 2C. Varying the fibronectin concentration between 0 and 0.1 mg/ml had no effect on the initial rate of fibrin polymerization (Fig. 2C). The initial rate of fibrin polymerization increased with increasing fibronectin concentration between 0.1 and 0.4 mg/ml fibronectin. Interestingly, the initial rate of fibrin matrix formation was higher in fibrin matrices formed from 2 mg/ml fibrinogen than those formed from 4 mg/ml fibrinogen with or without fibronectin.

An increase in fibronectin concentration resulted in an increase in the steady state value of fibrin matrix light absorbance (Fig. 2D). Similar results have been reported by Okada and co-workers for the steady state light absorbance of fibrin matrices formed in the presence of comparable amounts of fibronectin [18]. The steady state values for light absorbance of matrices formed from 2 and 4 mg/ml fibrinogen were comparable with those of Okada and

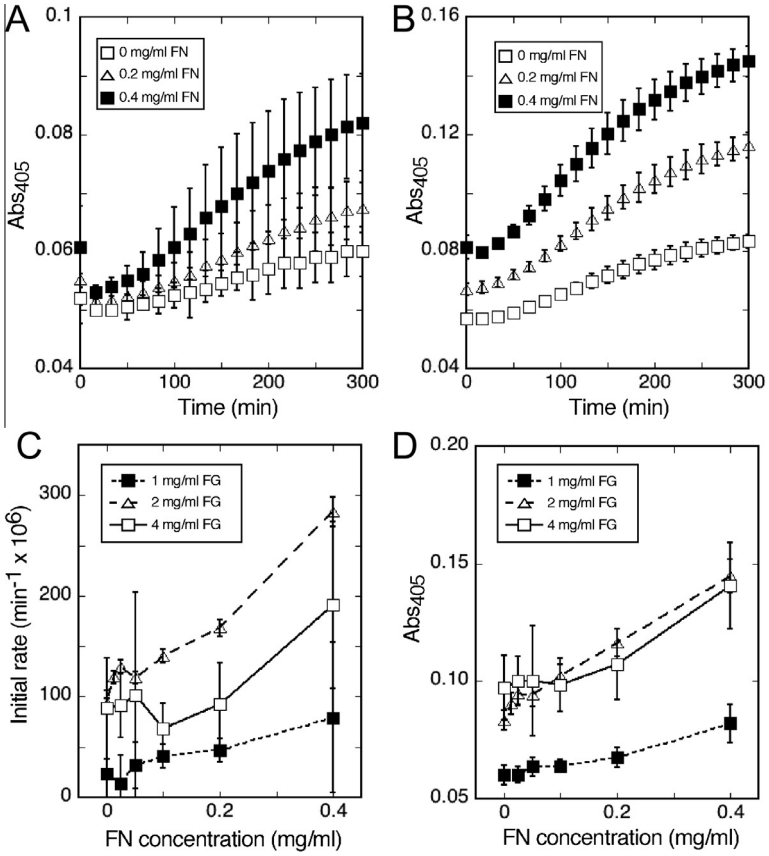

Fig. 2. Fibronectin influences the kinetics of light absorbance during fibrin polymerization. (A and B) Light absorbance at 405 nm for fibrin polymerization from 1 and 2 mg/ml fibrinogen, respectively, with varying fibronectin concentrations. (C) The initial rate of fibrin polymerization versus fibronectin concentration. The initial rate of polymerization was estimated as the slope of the absorbance-time profile from 2000 to 4000 s. (D) The steady state light absorbance versus fibronectin concentration. Error bars represent a 95% confidence interval of the mean for $n = 4–16$ replicates in three different experiments. FN = fibronectin, FG = fibrinogen.

co-workers. Therefore, the influence of fibronectin concentration on fibrin matrix light absorbance properties is at a maximum at 2 mg/ml fibrinogen. The effect of fibronectin concentration on the kinetics of light absorbance in fibrin matrices suggested that it has an influence on fibrin matrix structure.

3.2. Confocal microscopy of fibrin matrices

Microscopy studies have demonstrated that fibrin matrix light absorbance is related to the size and density of fibrin matrix fibers [21,25]. The effect of fibronectin concentration on fibrin matrix structure was investigated by fluorescent confocal microscopy. Fig. 3 shows fluorescently labeled fibrin matrices formed in the presence of different concentrations of fibronectin. The fluorescently labeled fibrin matrices were formed from 1 mg/ml fibrinogen (Fig. 3A–C) and 2 mg/ml fibrinogen (Fig. 3D–F). A higher fibrinogen concentration resulted in denser and finer fibrin fibers (Fig. 3A and D), which was associated to higher absorbance values (Fig. 2A and B). Increasing fibronectin concentration from 0 to 0.4 mg/ml in fibrinogen solutions of 1 mg/ml did not significantly alter the structure of the fibrin matrix but the fiber boundary seemed more indistinct (Fig. 3A–C). This same change in fibronectin concentration corresponded to an increase of approximately 40% in the steady state light absorbance values of fibrin matrices formed from 1 mg/ml fibrinogen (Fig. 2D). On the other hand, increasing fibronectin concentration from 0 to 0.4 mg/ml in fibrinogen solutions of 2 mg/ml resulted in finer and denser fibers (Fig. 3D–F). This change corresponded to an increase of 130% in the steady state light absorbance values for fibrin matrices formed from 2 mg/ml fibrinogen (Fig. 2D). Increasing fibronectin content in fibrinogen solutions results in denser fibrin matrices that absorb more light.

3.3. Quantification of fibronectin in fibrin matrices

Microscopy and spectroscopy findings were linked to the composition of the fibrin matrix through SDS–PAGE and immunoblotting. Reduced, denatured and solubilized fibrin matrices were resolved on 5% polyacrylamide gels and immunoblotted for fibronectin. Fig. 4A and B represent silver stained polyacrylamide gels of solubilized fibrin matrices. In the absence of fibronectin, the solubilized fibrin matrix (Fig. 4A and B, Lane 4) had a prominent band at 180 kDa which was not present in fibrinogen solution, indicating that covalent cross-linking of fibrin had taken place [26]. While Factor XIIIA was not added to the fibrinogen solutions, it is possible that trace amounts of this cross-linker may have co-purified with fibrinogen and resulted in the covalent cross-linking of fibrin. The intensity of the 180 kDa band and lower molecular weight bands did not change with the addition of fibronectin or with increasing amounts of fibronectin (Fig. 4A and B, Lanes 5–10). The presence of fibronectin in fibrin matrices resulted in double bands below and above the molecular weight of monomeric fibronectin. The intensity of the double bands increased with increasing amount of fibronectin and these double bands were positive for fibronectin (Fig. 4C and D). The smaller of the double bands was attributed to cleavage of one of the dimer arms of fibronectin by thrombin [27]. In addition to catalyzing the formation of covalent bonds between fibrin monomers, Factor XIIIa also ligates

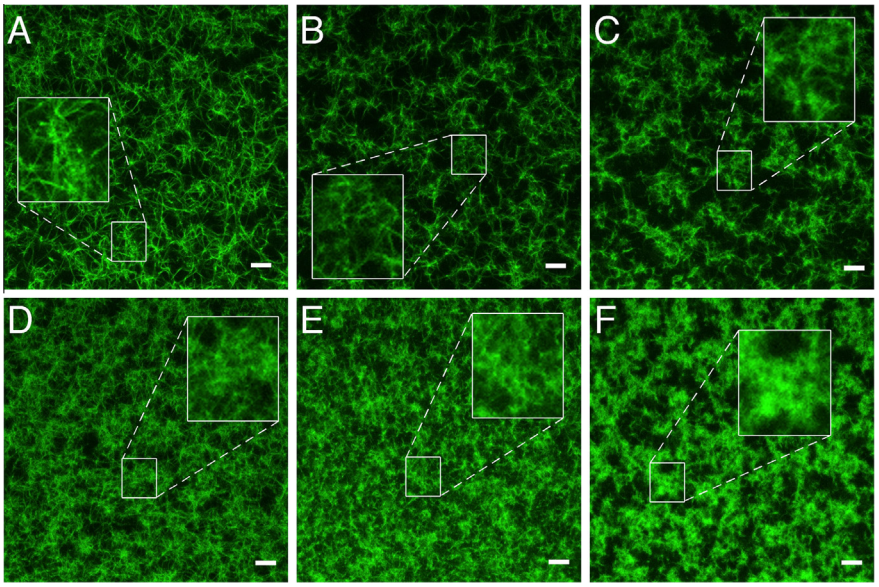

Fig. 3. Confocal microscopy of fluorescein labeled fibrin matrices formed with varying concentrations of fibronectin. Fibrin matrices formed from fibrinogen labeled with NHS fluorescein and different concentrations of fibronectin. Matrices formed from: (A–C) 1 mg/ml fibrinogen and 0, 0.2 and 0.4 mg/ml fibronectin respectively; (D–F) 2 mg/ml fibrinogen and 0, 0.2 and 0.4 mg/ml fibronectin, respectively. The images were acquired using similar exposure parameters. Scale bar = 10 μm.

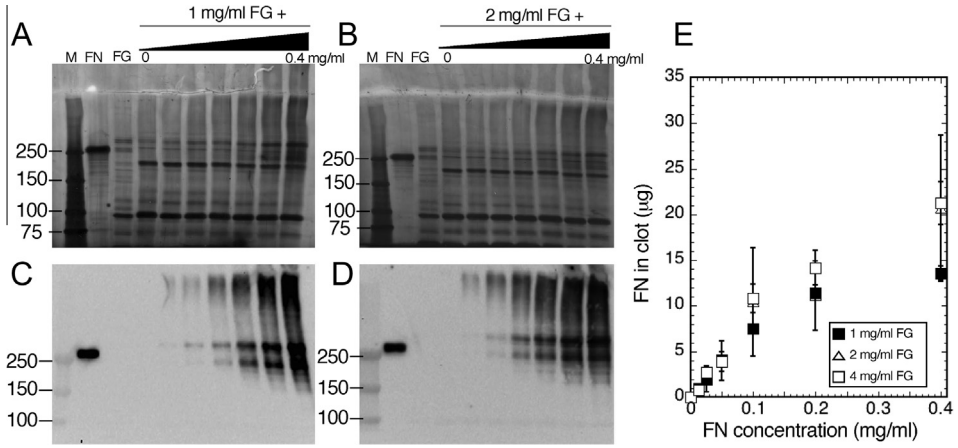

Fig. 4. Quantitative analysis of fibronectin in the fibrin matrix. (A and B) 5% polyacrylamide gels of solubilized fibrin matrices made from solutions of 1 and 2 mg/ml fibrinogen, respectively, and varying fibronectin concentrations. Fibrin matrices were solubilized in 8 M urea, 2% SDS and 1% β-mercaptoethanol in TBS. (C and D) Immunoblots of A and B, respectively with monoclonal antibody 7.1 against fibronectin. M represents molecular weight standards, FN = 100 ng fibronectin loading control, FG = 100 ng of fibrinogen. (E) Densitometric analysis of the immunoblots was used to estimate the fibronectin retained in the matrices versus initial solution concentration of fibronectin. Error bars represent a 95% confidence interval of the mean of two replicates. FN = fibronectin, FG = fibrinogen.

fibronectin to fibrin [10,14,15] and to itself [28]. Therefore, the larger of the double bands may represent fibronectin cross-linked to fibrin monomers. Other high molecular weight fibronectin positive components in the fibrin matrices (near the stacker in Fig. 4C and D) increased with increasing fibronectin levels. These may have been cross-linked fibronectin or fibronectin cross-linked to fibrin. These results suggest that as fibronectin concentration is increased more fibronectin is bound to the fibrin matrix through both covalent and non covalent interactions.

A densitometric analysis of the solubilized matrices was carried out so as to determine the amount of fibronectin in solubilized fibrin matrices. Fig. 4E shows that the amount of fibronectin bound to the matrix is dependent on fibronectin concentration in fibrinogen solutions and approaches a saturation value (Fig. 4E). Fibrin matrices formed from 2 and 4 mg/ml fibrinogen had comparable binding to fibronectin, which was higher than fibrin matrices formed from 1 mg/ml fibrinogen. This is in line with steady state light absorbance values of fibrin matrices (Fig. 2D). The 2 mg/ml fibrinogen concentration may represent the upper limit concentration for fibrin matrix formation. The saturation level of fibronectin binding to fibrin matrices suggests that the fibrin–fibronectin interactions are specific.

4. Discussion

The goal of this study was to determine the influence of fibronectin on fibrin matrix polymerization and structure. Reconstituted fibrin matrices formed with varying concentrations of fibronectin had differences in the kinetics of light absorbance, structure and composition. The initial rate of change of light absorbance and steady state light absorbance values in fibrin matrices increased with increasing fibronectin concentration. Confocal microscopy showed an altered fibrin matrix structure due to increased fibronectin concentration. An increase in fibronectin

resulted in a decrease in fiber thickness and an increase in fiber density in fibrin matrices. Immunoblotting with monoclonal antibody 7.1 against human fibronectin demonstrated the formation of high molecular weight multimers containing fibronectin from covalent bonding of fibronectin. Covalent binding between fibrin and fibronectin is catalyzed by factor XIIIa [10,14,15]. Factor XIIIa was not added to the fibrin matrices, but trace amounts may have co-purified with the fibronectin, fibrinogen or thrombin used in the studies. A densitometric analysis of immunoblots showed that the amount of fibronectin bound to the fibrin matrix increased with increasing fibronectin concentration and was saturable. Collectively, the data demonstrates that bound fibronectin influences the kinetics of fibrin matrix formation as well as its structure.

Fibrin matrix polymerization and structure have been examined extensively by monitoring light absorbance. We present new microscopy evidence that links fibronectin concentration and fibrin matrix structure. Increasing fibronectin concentration in fibrin matrices formed from low concentrations of fibrinogen did not significantly change fibrin matrix structural characteristics. On the other hand, increasing fibronectin concentrations in high concentrations of fibrinogen, led to a decrease in fibrin fiber size, an increase in density of fibers. This change is linked to an increase in fibrin matrix light absorbance. Our results are in line with published literature that shows that fibrin fiber size is influenced by the rate of matrix formation [21]. Results from light absorbance studies conclusively show that the initial rate of fibrin matrix formation increases with increasing fibronectin concentration. This is in agreement with the rheological findings of Kamykowski and coworkers that the rate of matrix formation increases in the presence of fibronectin, with or without factor XIIIa [22]. Thus the microscopy findings herein are also important in reconciling data from different analytical methods on the influence of fibronectin on the physical and chemical properties of the fibrin matrix.

This study addresses a critical deficiency related to the effect of fibronectin on fibrin matrix morphology by providing both quantitative and qualitative evidence of the effect of fibronectin on fibrin matrix structure. Our study contributes to current knowledge by showing that fibronectin concentration influences fibrin matrix structure in a concentration dependent manner and presents new microscopy evidence on the influence of fibronectin on structural characteristics of the fibrin matrix. Our studies reconcile a number of studies of fibrin matrices using different experimental methods. Our study presents important information that may be useful in the design of therapeutics for targeting fibrin clot formation.

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