FIBRONECTIN INCREASES THE RATE OF FIBRIN CLOT POLYMERIZATION AND ALTERS MATRIX MORPHOLOGY

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Abstract

Plasma fibronectin is a component of the fibrin clot, a provisional matrix that arrests bleeding but that can also obstruct flow in blood vessels. The goal of this study was to examine the influence of fibronectin on the formation, structural characteristics and composition of reconstituted fibrin clots. Fibrin clots were formed by adding thrombin to 1 mg/ml, 2 mg/ml or 4 mg/ml fibrinogen supplemented with 0-0.4 mg/ml fibronectin. The formation of fibrin clots with varying amounts of fibronectin was monitored by measuring light absorbance during polymerization. Confocal microscopy of fluorescein conjugated fibrinogen was used to determine the structural characteristics of fibrin-fibronectin clots. The composition of the fibrin-fibronectin clots was determined through electrophoresis and immunoblotting of solublized clots. Fibronectin concentration positively correlated with the initial rate of polymerization and with the steady state light absorbance values in fibrin clots. The effect of fibronectin on fibrin clots was saturable; there was no significant difference in the steady state light absorbance value of fibrin-fibronectin clots formed from 2 mg/ml and 4 mg/ml fibrinogen. At fibrinogen concentration of 2 mg/ml and above, an increase in fibronectin concentration resulted in thinner and denser fibers in the fibrin-fibronectin clots. Electrophoresis and immunoblotting showed that fibronectin bound to fibrin clots and formed covalently bonded high molecular weight multimers. These findings are novel because they correlate results from light absorbance studies to microscopy images and demonstrate an influence of fibronectin on fibrin clot structural characteristics. This data is important in developing therapies that destabilize fibrin clots.
Introduction

Elevated levels of plasma fibronectin have been correlated to 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 [4]. Fibrin clots cause occlusion of vasculature and obstruction of blood flow and can result in stroke [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 29 disulfide bonds [6-9]. The conversion of fibrinogen to fibrin is catalyzed by thrombin and results in the formation of a fibrin clot. A fibrin clot is formed by covalent and non-covalent interactions between fibrin monomers [7, 10]. Fibrin clots also contain cross-linked fibronectin, which is a soluble glycoprotein that exists in blood plasma [4, 11].

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 Figure 1. The structure consists of homologous structural modules classified as type I, II and III repeats. There is also an additional variable region on a single fibronectin monomer due to alternative splicing [12-14]. Non-covalent interactions take place between the fourth and fifth type I repeats of fibronectin and the α chain of fibrin [15-19]. Covalent bonding occurs between the amino-terminal fibrin binding site and the α chain of fibrin in the presence of Factor XIIIa [14, 15, 18, 20]. Non-covalent interactions also occur between the tenth through twelfth type I repeats of fibronectin and the α chain of fibrin [21, 22].

The formation of fibrin clots as well as the role of fibronectin on the rate of fibrin polymerization and clot structure has been studied by light absorbance [23, 24]. The 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 clot after gelation. They inferred that the changes in the light absorbance correlated to thicker fibrin fibers. Similarly, Carr and co-workers used light absorption studies to demonstrate that fibronectin impacted both size and density of fibrin fibers but did not affect the rate of fibrin clot formation. Microscopy studies on the effect of fibronectin on fibrin clots have yet to be conducted. Such information is important for supporting light absorbance studies and is useful in the design of therapeutics aimed at destabilizing the fibrin clot.

We report on a study examining the influence of fibronectin on fibrin clot polymerization and morphology in reconstituted fibrin clots. Reconstituted fibrin clots have been used extensively to study fibrin clot formation [24-26], as they allow for tight control of clot

![Figure 1: Domain structure of human plasma fibronectin monomer with fibrin binding sites.](image)

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_{4-5} and I_{10-12}. Covalent binding also occurs at the amino-terminal binding site.
composition. Fibrin clot formation was monitored in the presence of varying concentrations of fibronectin by measuring light absorbance. The steady state values of light absorbance for fibrin-fibronectin clots were correlated to clot structural characteristics through confocal fluorescence microscopy. The composition of solubilized fibrin-fibronectin clots was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. We found that the initial rate of fibrin clot polymerization and steady state light absorbance increased with increasing fibronectin concentration. Fibronectin influenced fiber size and fiber density in fibrin-fibronectin clots. Fibronectin multimers were present in solubilized fibrin-fibronectin clots. Fibronectin binding to fibrin was saturable. These results are strong evidence that fibronectin influences the rate of fibrin clot formation and the structure of the fibrin clot.

Materials and Methods

1. Purification of Human Plasma Fibronectin
   Human plasma fibronectin was isolated from frozen human plasma obtained from the blood bank at Rush Hospital. Human plasma was thawed at 37ºC and centrifuged to remove residual cells and precipitates. The supernatant was passed through a sepharose 4B column (Sigma-Aldrich, St. Louis, MO) and collected. The sepharose column flow-through was passed through a gelatin sepharose column (GE Healthcare Biosciences, Pittsburgh, PA). The gelatin column was washed with phosphate buffered saline (PBS, Fisher Scientific, Pittsburgh, PA). The bound fibronectin was eluted in 6 M urea (Fisher Scientific) in PBS. The optical density of the 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). The yield of fibronectin was approximately 30 mg of protein for 150 ml of human plasma. Fibronectin was dialyzed at 4ºC overnight in 50 mM Tris, 50 mM CaCl₂, 150 mM NaCl, pH 7.4 (TBS) for subsequent experiments.

2. Dynamic Measurement of Fibrin Clot Absorbance
   Lyophilized bovine fibrinogen (MP Biomedicals, Solon, OH) was reconstituted in TBS. Immunoblotting with polyclonal antibody R184 against fibronectin was carried out to determine the amount of native fibronectin that may have co-purified with fibrinogen. The fibronectin concentration in lyophilized fibrinogen was determined to be less than 0.2% by mass. Solutions of 1, 2 or 4 mg/ml fibrinogen supplemented with 0 to 0.4 mg/ml fibronectin were placed in a 96 microwell plate (Fisher Scientific). Thrombin (Fischer Scientific) in PBS 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).
3. Confocal Microscopy of Fibrin Clots

Fibrinogen was labeled with fluorescein by incubating with NHS fluorescein (Fisher Scientific) at a molar ratio of 1:15, for 1 hour at room temperature. 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. Fluorescein labeled fibrinogen and unlabeled fibrinogen were combined at a 1:10 mass ratio. The mixture of fluorescein conjugated and unconjugated fibrinogen at concentrations of 1 and 2 mg/ml supplemented with 0, 0.2 or 0.4 mg/ml fibronectin was placed on glass coverslips and enclosed in a mold. Polymerization was initiated by the addition of thrombin at a final concentration of 0.05 U/ml. Clots were allowed to polymerize for 5 hours at room temperature. Thrombin was inactivated by washing the clots with 2 mM PMSF in TBS. The clots 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 x 1024 pixels per 143 x 143 µm. The clots were imaged as Z stacks consisting of three images spaced 1 µm apart and compressed using PASCAL software (Carl Zeiss).

4. SDS-PAGE and Immunoblotting

The clots were solubilized in 8M urea, 2% SDS (Fisher Scientific) and 1% β-mercaptoethanol (Sigma-Aldrich) in TBS. Samples were resolved on 5% polyacrylamide gels and stained with silver nitrate or electrophoretically transferred to nitrocellulose for immunoblotting. The nitrocellulose was blotted with monoclonal antibody 7.1.

**Figure 2**: Fibronectin influences the kinetics of light absorbance during fibrin polymerization.

(A, 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 – 4000 seconds. (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. FG=fibrinogen.
against human fibronectin (Developmental Studies Hybridoma Bank, Iowa City, IA), followed by goat anti-mouse IgG (H+L) horseradish peroxidase (Invitrogen, Eugene, OR). The blots were treated with Pierce ECL Western Blotting Substrate (Fischer Scientific) and imaged 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.

**Results**

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 fibrin clot polymerization and structure. Figure 2 shows light absorbance of fibrin clots formed from 1, 2 or 4 mg/ml fibrinogen with varying concentrations of fibronectin. Figure 2A and 2B show that at a constant fibrinogen concentration, an increase fibronectin results in an increase in light absorbance. Light absorbance positively correlates with fibrinogen concentration. The light absorbance measurements between 2000 – 4000 seconds were fit to a straight line and the slopes of the lines were used to estimate the initial rates of fibrin polymerization (Figure 2C). The initial rate of fibrin polymerization increased with increasing fibronectin concentration. Varying the fibronectin concentration between 0 to 0.1 mg/ml in Figure 2C had no significant effect on the initial rate of fibrin polymerization compared to varying the concentration between 0.1 to 0.4 mg/ml fibronectin. The influence of fibronectin concentration on the initial rate of fibrin polymerization was significantly higher in fibrin clots formed from 2 mg/ml fibrinogen compared to those formed from 1 mg/ml or 4 mg/ml fibrinogen. Steady state values in fibrin clot light absorbance were reached five hours after the addition of thrombin. The steady state values are plotted against fibronectin concentration in Figure 2D. An increase in fibronectin concentration results in an increase in the final value of fibrin clot light absorbance. Similar results were observed by Okada et al. when measuring the light absorbance of fibrin-fibronectin clots [23]. The steady state values for light absorbance of clots formed from 2 and 4 mg/ml fibrinogen are comparable, suggesting that the influence of fibronectin concentration on fibrin clot light absorbance properties saturates at 2 mg/ml fibrinogen. The effect of fibronectin concentration on the absorbance of light in fibrin clots suggested that it had an influence on fibrin clot structure.

2. **Confocal Microscopy of Fibrin-Fibronectin Clots**

   Microscopy studies have demonstrated that fibrin clot light absorbance is correlated to the size and density of fibrin matrix fibers [27, 28]. The effect of fibronectin concentration on fibrin clot structure was investigated by fluorescent confocal microscopy. Figure 3 shows fluorescently labeled fibrin clots with different concentrations of fibronectin. The fluorescently labeled fibrin clots were formed from 1 mg/ml fibrinogen (Figures 3A, 3B and 3C) and 2 mg/ml fibrinogen (Figures 3D, 3E and 3F). The higher fibrinogen concentrations result in more dense and finer fibrin fibers (Figures 3A and 3D) which correlates to higher absorbance values
(Figures 2A and 2B). Increasing fibronectin concentration in fibrinogen solutions of 1 mg/ml does not significantly alter the structure of the clot (Figures 3A, 3B and 3C). The change in steady state values of fibrin clot steady state light absorbance corresponding to this increase is approximately 40% (Figure 2D). However, increasing fibronectin concentration in fibrinogen solutions of 2 mg/ml results in finer and denser fibers (Figures 3D, 3E and 3F). The change in the steady state light absorbance for fibrin clots formed with 2 mg/ml fibrinogen is an increase of 130% (Figure 2C). Confocal microscopy demonstrates that fibronectin concentration influences the structure of the fibrin clot.

3. Quantification of composition of fibrin-fibronectin clots

Microscopy and spectroscopy findings were correlated to composition through SDS-PAGE analysis of the fibrin-fibronectin clots. Reduced, denatured and solubilized fibrin-fibronectin clots were resolved on 5% polyacrylamide gels in order to quantify the amount of fibronectin bound in the fibrin clots. Figures 4A and 4B represent silver stained polyacrylamide gels of solubilized fibrin-fibronectin clots. In the absence of fibronectin, the fibrin clot in Figures 4A and 4B has several bands exceeding 100 kDa. Factor XIIIa catalyzes the formation of covalent bonds between fibrin monomers [14, 15, 18, 20]; which may result in multimers exceeding 100 kDa in reduced samples. Figures 4A and 4B also show an increase in the

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**Figure 3**: Confocal microscopy of fluorescein labeled fibrin clots formed with varying concentrations of fibronectin.

Fibrin clots formed from fibrinogen labeled with NHS fluorescein and different concentrations of fibronectin. Clots 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 = 10µm.
intensity of bands greater than 250 kDa with increasing fibronectin. Immunoblotting of the gels with fibronectin antibodies (Figures 4C and 4D) showed fibronectin positive bands corresponding to the high molecular weight staining in Figures 4A and 4B. These results suggest that as fibronectin concentration is increased more fibronectin is cross-linked to fibrin or other fibronectin molecules.

The total fibronectin binding in fibrin-fibronectin clots was quantified by densitometric analyses of immunoblots of fibronectin in fibrin-fibronectin clots. Figure 5 shows the total fibronectin bound to solubilized fibrin-fibronectin clots. The amount of fibronectin bound to the clot is dependent on fibronectin concentration and approaches a saturation value. Fibrin clots formed from 2 mg/ml and 4 mg/ml fibrinogen have comparable binding to fibronectin and higher binding than fibrin clots formed from 1 mg/ml. This is in line with light absorbance values of fibrin-fibronectin clots and indicates that binding of fibronectin to fibrin is specific.

**Discussion**

The goal of this study was to determine the influence of fibronectin on fibrin clot polymerization and morphology. Reconstituted fibrin clots formed with varying concentrations of fibronectin had differences in light absorbance, structure and composition. Light absorbance varied with time during fibrin clot formation. The initial rate of change of light absorbance and steady state light absorbance values in fibrin clots increased with increasing fibronectin concentration. Confocal microscopy showed an altered fibrin clot structure due to increasing fibronectin concentration. An increase in fibronectin resulted in a decrease in fiber thickness and an increase in fiber density in fibrin clots. Immunoblotting with monoclonal antibody 7.1 against human fibronectin demonstrated the formation of high molecular weight polymers.
containing fibronectin that may be due to covalent bonding between fibronectin and fibrin. Covalent binding between fibrin and fibronectin is catalyzed by factor XIIIa [29]. Factor XIIIa was not added to the fibrin clots, but trace amounts may have co-purified with fibronectin, fibrinogen or thrombin used in the studies. Densitometric analyses of immunoblots showed that the amount of fibronectin bound to the fibrin clot increased with increasing fibronectin concentration and was saturable. Collectively the data demonstrates that bound fibronectin influences the structural features of the fibrin clot.

Fibrin clot polymerization and structure have been examined extensively by monitoring light absorbance. We present new microscopy evidence that links fibronectin concentration and fibrin-fibronectin clot structure. Increasing fibronectin concentration in fibrin clots formed from low concentrations of fibrinogen did not significantly change fibrin clot 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 and correlates with an increase in fibrin clot light absorbance. Our results are in line with published literature that shows that fibrin fiber size is influenced by the rate of matrix formation [28]. Our results from light absorbance studies showed that the initial rate of fibrin clot formation, at the higher levels of fibrinogen sampled, increased with increasing fibronectin concentration. Higher light absorbance values, due to the presence of fibronectin, correlated to a decrease in fiber size. This is in agreement with the rheological findings Kamykowski et al. that the rate of clot formation is increased in the presence of fibronectin, with and without factor XIIIa [30]. Thus the microscopy findings herein are also important in reconciling fibrin-fibronectin data from different analytical methods.

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

**Figure 5: Quantification of fibronectin retention in fibrin clots.**

Densitometric analysis of the immunoblots was used to estimate the fibronectin retained in the clots versus initial solution concentration of fibronectin. Error bars represent a 95% confidence interval of the mean of two replicates. FG = fibrinogen.
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References


