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Differential roles for the coagulation factors XI and XII in regulating the physical biology of fibrin

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Abstract

In the contact activation pathway of the coagulation, zymogen factor XII (FXII) is converted to FXIIa, which triggers activation of FXI leading to the activation of FIX and subsequent thrombin generation and fibrin formation. Feedback activation of FXI by thrombin has been shown to promote thrombin generation in a FXII-independent manner and FXIIa can bypass FXI to directly activate FX and prothrombin in the presence of highly negatively charged molecules, such as long-chain polyphosphates (LC polyP). We sought to determine whether activation of FXII or FXI differentially regulate the physical biology of fibrin formation. Fibrin formation was initiated with tissue factor, ellagic acid (EA), or LC polyP in the presence of inhibitors of FXI and FXII. Our data demonstrated that inhibition of FXI decreased the rate of fibrin formation and fiber network density, and increased the fibrin network strength and rate of fibrinolysis when gelation was initiated via the contact activation pathway with EA. FXII inhibition decreased the fibrin formation and fibrin density, and increased the fibrinolysis rate only when fibrin formation was initiated via the contact activation pathway with LC polyP. Overall, we demonstrate that inhibition of FXI and FXII distinctly alter the biophysical properties of fibrin.

Keywords

fibrin; stability; structure; coagulation; FXI-deficiency; FXII-deficiency

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DISCLOSURE OF CONFLICTS OF INTEREST

A. Gruber, E.I. Tucker, and OHSU have financial interest in Aronora Inc., a company that may have a commercial interest in the result of this research. This potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest in Research Committee.

INTRODUCTION

Upon exposure of blood to a foreign surface or site of vascular injury, activation of coagulation factors via the contact and extrinsic pathways, respectively, leads to thrombin generation and ultimately fibrin formation. In the contact activation pathway, factor XII (FXII) is converted to protease FXIIa, which then catalyzes the activation of FXI, subsequently leading to activation of FIX to lead to thrombin generation. Additionally, both FXI and FXII facilitate clot formation through auxiliary mechanisms besides the well-established contact pathway. For instance, FXIa is able to enhance thrombin generation independently of FXII when the extrinsic pathway of coagulation is initiated with small concentrations of tissue factor.^{18,22,35} More specifically, FXI can be activated by thrombin and can also feedback to activate itself. Moreover, besides activating FIX, FXIa can directly activate FV and FVIII, weakly activate FX, and inactivate tissue factor pathway inhibitor (TFPI)^{18,22,35} to promote thrombin generation.

FXII is activated when blood is exposed to negatively-charged foreign surfaces or molecules such as RNA, DNA, misfolded proteins, and polyphosphates (polyP). Long chain polyP (LC polyP) is a polymer of inorganic phosphate groups that accumulates in certain microorganisms and has been observed to accelerate thrombin generation.²⁷ LC polyP can directly activate FXII and serve as a cofactor of FIX and prothrombin activation bypassing the activation of FXI.²³

Independent roles of FXII and FXI have been observed in patients. Congenital deficiency of FXI is associated with postoperative or posttraumatic bleeding in tissues with elevated fibrinolytic activity,²⁵ while FXII deficiency is not associated with a bleeding phenotype. Deficiencies of either FXI and FXII are associated with a reduction in thrombosis in animal models, making the contact activation of the coagulation pathway a potentially desirable target for pharmacological inhibition, balancing safety with efficacy.¹⁷ Yet the influence of FXI and FXII on clot structure and stability of the fibrin network remain undefined.

The structure of fibrin fiber network is inherently coupled to the kinetics of thrombin generation. Under conditions in which faster coagulation reactions are found, such as with elevated concentrations of thrombin, calcium, or fibrinogen, fibrin fiber diameter decreases and fiber density increases.³⁸ Highly branched networks of thin fibrin fibers exhibit an increase in mechanical stiffness and are increasingly resistant to enzymatic digestion via fibrinolysis.³⁸ *Ex vivo* fibrin gels formed from plasma from patients with premature coronary artery disease are stiffer, form a denser fibrin network and are more difficult to lyse as compared to clots formed from control patients' plasma.³⁴ In contrast, plasma from hemophilia A or B patients, where there is a deficiency of FVIII and FIX, respectively, yields a loose network of thick fibers and increased porosity, making these clots susceptible to mechanical failure and fibrinolysis.³⁶ Taken together, these studies suggest that the pathways leading to thrombin generation play a key role in determining the biophysical properties of fibrin clots.

In this report, we aimed to determine whether FXII and FXI serve similar or distinct roles in fibrin formation, structure, and stability. Activation of the coagulation cascade was started by the extrinsic pathway initiator, tissue factor, or contact activation pathway initiators, ellagic acid (EA) and LC polyP (Suppl. Fig. 1). Fibrin clots were interrogated via turbidity, rheological and fibrinolysis measurements and confocal and super resolution microscopy. Our data indicate that inhibition of FXI decreased the rate of fibrin formation and fiber density, and increased gel strength and rate of fibrinolysis when the fibrin formation was initiated with ellagic acid, which acts through the contact activation pathway. FXI inhibition decreased the rate of fibrin formation on surfaces coated with low levels of TF, which acts through the extrinsic pathway. Inhibition of FXII reduced the rate of fibrin formation and fiber density, and increased the rate of fibrinolysis only when coagulation was initiated with LC polyP, which acts through the contact activation pathway.

MATERIALS AND METHODS

Materials

Fibrinogen Alexa Fluor 546 and 647 were from Thermo Fisher Scientific. Corn trypsin inhibitor (CTI) was from Haemotologic Technologies. LC polyP (>1000 phosphate units) and biotinylated polyP (200–1300 phosphate units) were prepared as described previously.^{6,27} Anti-FXI antibodies 1A6, 10C9, and 14E11 were created as described previously.^{5,13} Hirudin was obtained from Hypen Biomed, lipidated recombinant tissue factor (TF; Dade[®]Innovin reagent) was purchased from Siemens, and ellagic acid (EA; aPTT reagent) came from Pacific Hemostasis. HEPES buffered saline (HBS, composed of 25 mM HEPES and 150 mM NaCl, pH 7.4) and polyP-binding buffer (composed of 50 mM Tris HCl, pH 7.4, 1% bovine serum albumin (BSA), 0.05% Tween-20 and 5 mM EDTA) were made in-house. Streptavidin from *Streptomyces avidinii* was obtained from Sigma Aldrich. Platelet-poor plasma (PPP) was prepared as previously described.²³

Turbidity measurements of fibrin formation

Fibrin polymerization of PPP was measured in clear flat bottom polystyrene 96- well plates (Greiner) by monitoring turbidity at 405 nm every 25 seconds for 2 hrs using an Infinite M200 plate reader (TECAN, Switzerland). In select experiments, plasma was incubated with vehicle control, the monoclonal antibodies 1A6 (inhibits FXI activation by FXIIa and FIX activation by FXIa) (50 µg/mL), 10C9 (binds near the active site of FXIa) (50 µg/mL), 14E11 (inhibits FXI activation by FXIIa) (50 µg/mL), the FXIIa inhibitor CTI (40 µg/mL), or the direct thrombin inhibitor hirudin (25 µg/mL) for 10 min. In the last 3 min of the incubation, vehicle control, EA (1:50 dilution) or LC polyP (100 µM) was added to the plasma solution. Plasma solutions (75% plasma) were pipetted into a 96-well polystyrene plate and gelation was initiated with CaCl₂ (13.7 mM) for all the experiments. TF (0.01 or 0.1 nM) was added in combination with CaCl₂ for select experiments.

For surface-initiated fibrin formation, polystyrene plates were incubated with vehicle control, EA (1:50 dilution), or TF (0.01 or 0.1 nM), and then blocked (5 mg/mL BSA in HBS). To surface-immobilize LC polyP, biotinylated LC polyP was covalently bound on the polystyrene plate via biotin-streptavidin linkage and also subsequently blocked (5 mg/mL

BSA in HBS).⁶ Briefly, streptavidin (10 $\mu\text{g/mL}$ in deionized water) was incubated in the wells at room temperature overnight. The concentration of the biotinylated-LC polyP was 150 μM to match the activity of 100 μM LC polyP in solution (Suppl. Fig. 2A,B) and biotinylated-LC polyP produced the similar fibrin formation kinetics observed with the LC polyP with FXI and FXIIa inhibitors (Suppl. Fig. 2C,D). Plasma was incubated with vehicle control, the monoclonal antibodies 1A6 (50 $\mu\text{g/mL}$), 10C9 (50 $\mu\text{g/mL}$), 14E11 (50 $\mu\text{g/mL}$), CTI (40 $\mu\text{g/mL}$), or the direct thrombin inhibitor hirudin (25 $\mu\text{g/mL}$) for 10 min. Plasma solutions (75% plasma) were pipetted into a 96-well polystyrene plate and gelation was initiated with CaCl_2 (13.7 mM). All the concentrations listed are final concentrations.

Fiber mass-to-length ratio

The fibril mass-to-length ratios was determined by modifying a previously established method³⁷ for characterizing clots with high turbidity.⁴ Fibrin gel maturation proceeded for 2 hrs and an absorbance scan was performed from 400 to 800 nm (20 nm step) on the microplate reader. The data was plotted as the inverse of the absorbance (τ) $\times$ wavelength (λ)³ versus the inverse of (λ).² The y-intercept of the plot and its inverse were determined, which is proportional to the mass-to-length ratio of the fibers.⁴

Confocal Microscopy

Microchannels (μ -slide VI for Live Cell analysis; Ibidi) were incubated with vehicle control, EA (1:50 dilution), or TF (0.01 or 0.1 nM), or LC polyP (150 μM) was covalently bound to the microchannels and then the channels were blocked (5 mg/mL BSA in HBS) for 1 hr. Normal PPP (75% plasma) was incubated with exogenous Alexa Fluor 546-conjugated fibrinogen (1:100 labeled:unlabeled fibrinogen) and was incubated with vehicle control, the mAbs 1A6 (50 $\mu\text{g/mL}$), 10C9 (50 $\mu\text{g/mL}$), 14E11 (50 $\mu\text{g/mL}$), or CTI (40 $\mu\text{g/mL}$) for 10 min. CaCl_2 (13.7 mM) was added to the solution, and the complete solution was quickly withdrawn through the microchannel. Clots were formed in the dark for 2 hr at room temperature, and then rinsed with HBS and fixed with 2% paraformaldehyde for 1 hr. A laser scanning confocal (Inverted Zeiss LSM-880) was used to visualize the clots with a 60 $\times$ /1.4 NA oil objective and an excitation laser with a 561 nm wavelength using Zen 2 software (Zeiss, Germany).

For each condition, images were taken in 5 locations in the 5 mm section of the fibrin gel near the inlet of the channel. Fiji software, with a Diameter J plugin¹¹ was used to sharpen, smooth, and segment the images of the fibers and to determine the average number of intersections, characteristic length, and porosity.

Super Resolution Microscopy

Alexa Fluor 647-labeled fibrin gels were formed in EA-coated microchannels, blocked, and incubated with 50-nm gold particles (1:5 dilution in DPBS) for 1 hr. The gold nanoparticles were used as fiducial markers. Fibrin polymers were rinsed and imaged in a freshly made stochastic optical reconstruction microscopy (STORM) imaging buffer (10 mM mercaptoethylamine, 0.56 mg/mL glucose oxidase, 40 $\mu\text{g/mL}$ catalase, and 10% glucose in a Tris buffer).

STORM experiments were performed on a Nikon Ti-U inverted microscope equipped with an oil immersion objective (APON, 60× OTIRF, NA 1.49, Olympus). Light from two lasers emitting at 647 nm and 405 nm (Coherent), respectively, was collimated and focused onto the target in a total internal reflectance (TIR) illumination configuration. An Andor iXon Ultra 897 EM-CCD camera recorded fluorescence. For 3D imaging, a cylindrical lens ($f=700$ mm) was placed approximately 40 mm in front of the camera chip to produce astigmatism and encode z-information.

Data was collected using the software μ Manager.³⁰ Image reconstruction was performed using custom scripts written in MATLAB (Mathworks, USA). The 3D reconstruction algorithm made use of existing software as well as a home-written MATLAB/C adaptation of the ThunderSTORM Z-recovery routine.²¹

Rheological Measurements

PPP was incubated with a vehicle control, 1A6 or CTI for 10 min as the upper geometry of a rheometer spun at 100 s^{-1} to induce induced shear flow in the solution. In the last 3 min of incubation, LC polyP or EA was added to the solution, and then CaCl_2 (13.7 mM) was added to initiate clotting. The upper geometry was lowered to a 400- μm gap. All rheology tests were completed on a DHR-3 rheometer (TA Instruments, USA) using a 20-mm aluminum plate upper geometry at 25°C . The following oscillation tests were conducted: 1) time sweep (2% strain 5 rad/s for 60 mins); 2) frequency sweep (0.1 – 10 rad/s at 2% strain); 3) amplitude sweep (0.1–200% strain, 2% increments at 5 rad/s). For the frequency and amplitude sweeps, all the results were normalized by the initial value of the vehicle control-incubated and -initiated condition.

Fibrinolysis

PPP was incubated with a vehicle control, 1A6 or CTI for 10 min. In the last 3 min of incubation, TF (0.01), LC polyP (100 μM) or EA (1:50) was added to the solution with tissue plasminogen activator (tPA; 0.2 $\mu\text{g/mL}$) before the addition of CaCl_2 (13.7 mM). A_{405} was recorded over time and each lysis curve was normalized to the maximum turbidity. The 50% lysis time was defined as the time required to achieve a half maximal A_{405} from the time the $A_{405,\text{max}}$ was achieved.

Statistical Methods

Data was analyzed using PRISM 4.0 software (GraphPad, USA). All data are shown as the mean $\pm$ standard error mean. For pairwise comparison, a Mann-Whitney test was performed. Comparisons of grouped data were performed with a two-way ANOVA with a post-hoc Bonferroni's comparison test. Differences were considered significant for a p value less than 0.05.

RESULTS

Turbidity Measurements of Surface- and Solution-Initiated Fibrin Formation

Our initial experiments were designed to test the effects of inhibiting the activity of factor XIa or XIIa on fibrin gel formation. Platelet poor plasma (PPP) was treated with function-

blocking FXI antibodies or a FXIIa inhibitor, and gelation was triggered with either TF, EA, or LC polyP; turbidity of the fibrin was tracked over time (Fig. 1). The kinetics of the fibrin formation were described by the lag time required to initiate fibrin formation, rate of fibrin formation or slope, and maximum turbidity, which is related to the overall fiber density. The lag times were unaffected by the presence of 14E11, 10C9, and CTI, yet they nearly doubled from 29 ± 3 min to 57 ± 5 min in the presence of 1A6 in the absence of an initiator (Fig. 1A). The presence of the FXI and FXIIa inhibitors had no influence on the rate of fibrin formation in the absence of an initiator (Fig. 1B). The maximum turbidity of the samples were unaffected by the inhibitors 14E11, 10C9, and CTI, yet decreased in the presence of 1A6, indicative of incomplete gel formation within the 2 hr observation period (Fig. 1C).

Lag times of gel formation initiated by EA in the presence of 1A6, 14E11, 10C9, and CTI were elevated to 13 ± 0.7 min, 9 ± 0.8 min, 7 ± 0.5 min, and 8 ± 0.4 min, respectively, from the baseline of 3 ± 0.3 min (Fig. 1A). The rate of fibrin formation was reduced by 2.7-, 1.9-, 1.2- and 1.6-times in the presence of 1A6, 14E11, 10C9 and CTI, respectively (Fig. 1B). While the presence of 14E11, 10C9, and CTI did not affect the maximum turbidity, the FXI antibody 1A6 caused a significant increase (Fig. 1C).

We subsequently assessed fibrin formation in the presence of LC polyP and found that though FXI inhibitors had no effect on any of the kinetic metrics, the presence of the FXIIa inhibitor, CTI, resulted in a 5-fold decreased rate of fibrin formation and nearly doubled lag times from 6 ± 0.4 min to 11 ± 0.7 min (Fig. 1A,B). The maximum turbidity remained unchanged regardless of the inhibitors used (Fig. 1C). We then measured fibrin formation in the presence of either 0.01 or 0.1 nM TF and found no effect of blocking either FXII or FXI on any of the kinetic measurements (Fig. 1). As a negative control, we treated plasma with a thrombin inhibitor, hirudin, which abrogated gelation regardless of the initiator (data not shown).

Kinetic measurements were made for fibrin gels in which the initiators were immobilized on a surface rather than being added in the bulk solution. In comparison to solution-initiated fibrin gels (Fig. 1A–C), surface initiation of fibrin gels resulted in substantially reduced rates of fibrin formation, prolonged lag times, and lower maximum turbidities (Fig. 1D–F). Lag times were prolonged in the presence of 1A6, from 27 ± 4 min to 69 ± 8 min, whereas other FXI function-blocking antibodies and FXII inhibitor, CTI, had no significant effect in the absence of an initiator (Fig. 1D). Likewise, only 1A6 had an effect on the rate of formation and maximum turbidity, resulting in a 5-fold reduction in the rate of fibrin formation as compared to vehicle and a truncated maximum turbidity (Fig. 1E,F).

We immobilized EA and found that lag times for fibrin gel formation increased from 10 ± 0.2 min for vehicle conditions to 48 ± 4 min, 32 ± 2 min, 21 ± 2 min and 20 ± 1 min in the presence of 1A6, 14E11, 10C9 and CTI, respectively (Fig. 1D). As compared to vehicle, we observed 15-, 4-, 3- and 4-fold decreases in the rate of fibrin formation in the presence of 1A6, 14E11, 10C9, or CTI, respectively (Fig. 1E). Interestingly, only 1A6 decreased the maximum turbidity (Fig. 1F).

We immobilized LC polyP, and found that lag times for fibrin formation increased from 17 ± 1 min for vehicle conditions to 37 ± 4 min and 67 ± 8 min in the presence of 10C9 and CTI, respectively, while the FXI inhibitors 1A6 and 14E11 had no effect on lag times (Fig. 1D). We observed 2-, 1.4-, 2.3-, and 3.6-fold decreases in the rate of fibrin formation in the presence of 1A6, 14E11, 10C9, and CTI, respectively, as compared to vehicle (Fig. 1E). The maximum turbidity observed from surface-immobilized LC polyP was unaffected by the plasma inhibitors (Fig. 1F). We immobilized either 0.01 or 0.1 nM TF, and while none of the FXIIa or FXI antibodies affected the lag time for fibrin formation (Fig. 1D), we observed 1.8- and 1.1-fold decreases in the rate of fibrin deposition and maximum turbidity on surfaces coated with 0.01 nM TF in the presence of 1A6 (Fig. 1E,F). All gels had similar lag times, rates of fibrin formation, and maximum turbidities with surfaces coated with 0.1 nM TF.

Mass-to-Length Ratios

Mass-to-length ratios of the matured fibrin gels were established by quantifying the turbidity of each gel over 400 to 800 nm for solution- and surface-initiated fibrin gels, plotting the inverse of the absorbance (τ) $\times$ wavelength (λ)³ versus the inverse of (λ)², and determining the inverse of the y-intercept, which is proportional to the mass-to-length ratio. A high mass-to-length ratio is indicative of a thick fiber with more lateral aggregation of protofibrils, whereas a low mass-to-length ratio is indicative of a thin fiber. For samples either initiated with TF or in the absence of an initiator, no differences in mass-to-length ratios were found in the presence of FXI antibodies or CTI. When we initiated clotting with EA in solution, ~60%, 20%, 30% and 30% increases in the mass-to-length ratios were found in the presence of 1A6, 14E11, 10C9 and CTI, respectively. When clotting was initiated in solution with LC polyP, the FXI antibodies failed to induce a mass-to-length ratio change, yet we found a 1.6-fold increase in the mass-to-length ratio in the presence of CTI (Fig. 2).

Confocal and Super Resolution Microscopy

We formed fibrin gels in microchambers to analyze the physical parameters of fibrin fibers by confocal microscopy. Fibrin gels formed in the absence of an initiator were characterized by having long fibers, a large porous fraction, fewer fiber intersections, and longer characteristic lengths (total length of fibers/number of intersections) (Fig. 3,4). Gels were found to be unstable, and they could not be properly processed for imaging in the presence of 1A6, while 14E11, 10C9, and CTI did not influence the gel structure in the absence of an initiator. We measured the effect of inhibitors on fibrin gel structure that had been initiated with EA. A decrease in fibrin density was observed for EA-initiated gels in the presence of either 1A6 or 14E11 (Fig. 3). The two FXI inhibitors caused an increase in porous fraction (Fig. 4A), ~50% reduction in the number of intersections (Fig.4B), and an increase in characteristic length from ~3 μ m to 5 μ m (Fig.4C), while neither CTI nor 10C9 affected the structure of gels initiated by EA. We subsequently characterized the physical parameters of gels initiated by LC polyP. We found that treatment of plasma with CTI, but not FXI inhibitors, had a dramatic effect on fibrin density for LC polyP initiated gels, resulting in an increase in porous fraction, ~60% reduction in the number of intersections, and characteristic length increase from ~3 to ~5 for CTI as compared to controls (Fig.4). We used TF as an initiator and found that fibrin gels initiated with TF were characterized by

high fibrin density and composed of slightly aligned thin fibrin fibers (Fig. 3). This alignment may be attributed to rapid coagulation, such as would occur with high TF, as the plasma is slowly entering the flow chamber. Fibrin gels initiated with 0.01 nM TF displaying a slightly larger porous fractions and increased characteristic length as compared to gels initiated the 0.1 nM TF (Figs.3, 4). The physical parameters of TF-initiated gels were insensitive to FXII or FXI inhibitors.

To measure dimensions close to or below the diffraction limit (200–300 nm), super resolution microscopy was performed to quantify fiber diameters of the fibrin gels. As such, we found that fibrin gels initiated with EA had a diameter of 320 ± 10 nm, which in the presence of 1A6 was increased to 420 ± 35 nm (Fig. 5).

Rheology

We then studied the mechanical properties of fibrin gels that had been initiated in solution with either EA or LC polyP. The viscoelastic properties and mechanical stability of fibrin gels were quantified in the presence of either the FXI antibody 1A6 or the FXIIa inhibitor CTI. Gels were monitored over time to determine the lag times for gel formation (Suppl. Fig. 3). The gels had reduced lag times prior to fibrin polymerization in the rheometer compared to fibrin solutions formed in the plate reader for turbidity measurements potentially due to differences in the length scales of the geometries in which the gels formed, but the kinetics of fibrin formation were maintained in the presence of the inhibitors were maintained between both setups. We then measured the G' and G'' , indicative of storage and loss moduli respectively, through a frequency sweep to quantify frequency-dependent viscoelastic properties. The G' was independent of the frequency and was 60% higher in the presence of 1A6 either in the absence of an initiator or when gels were initiated with EA (Fig. 6A). No measureable differences were found between G' and G'' with 1A6 and CTI in the presence of LC polyP as the initiator (Fig. 6B). Overall, all of the fibrin networks showed gel-like behavior, with the storage modulus dominating and the moduli remaining constant across the measured frequencies. EA-initiated fibrin gels were then monitored during an amplitude sweep, and we quantified strain-hardening start (%), the maximum G' attained and its corresponding strain (%) before clot rupture (Fig. 6C–F). When plasma gelation was initiated with EA, the start of the strain hardening occurred at ~26% with 1A6 compared to ~33% for vehicle, while CTI did not have a significant effect compared to the control (Fig. 6D). No changes in the maximum G' could be discriminated between all the conditions of EA-initiated fibrin gels (Fig. 6E). The maximum strain the gel could withstand prior to rupture was only affected by 1A6, as 1A6-treated clots had a higher max strain of $125 \pm 5\%$ compared to the control max strain of $100 \pm 6\%$, while addition of CTI made no significant difference compared to the control (Fig. 6F).

Fibrinolysis

We studied the role of the contact activation pathway in fibrinolysis induced by tPA. Our data show that inhibitors of either FXIIa or FXI had no effect on the lysis dynamics for clots initiated with 0.01 nM TF (Fig. 7A). The presence of the FXI antibody 1A6 significantly increased the rate of lysis for clots initiated with EA compared to the control (Fig. 7B). In contrast, the presence of the FXIIa inhibitor CTI significantly increased the rate of lysis for

clots initiated with LC polyP (Fig. 7C). We quantified the 50% lysis time (time required for clot to reach its half maximum during the lysis phase). In EA-initiated gels, the presence of 1A6 or CTI caused a 0.7 or 1.2-fold change in the 50% lysis time relative to vehicle, respectively. For gels initiated with LC polyP, the presence of 1A6 or CTI resulted in a 1.1 or 0.8-fold change in the 50% lysis time relative to vehicle, respectively (Fig. 7D).

DISCUSSION

Recent studies have supported the notion that FXI and FXIIa inhibitors may represent a therapeutic strategy to prevent thrombosis. Congenital FXI deficiency protects patients from stroke and venous thromboembolism, and is only associated with minor bleeding episodes.^{9,10} Additionally, blockade of FXII might be protective in microorganism-driven thrombotic complications accompanying sepsis, as inhibition of FXII activation of FXI reduced thrombotic complications in animal models of sepsis.^{5,9} Here, we sought to better understand whether FXII and FXI differentially regulate the physical biology of fibrin networks. We quantified fibrin formation, structure and stability with turbidity measurements, confocal and super resolution microscopy, rheological measurements, and fibrinolysis studies. The main conclusions drawn from these studies are that: 1) FXI inhibition by 1A6 decreased the rate of fibrin formation and fibrin density, and increased fibrinolysis kinetics and gel strength when the contact activation pathway was initiated with ellagic acid. Additionally, inhibition of FXI by 1A6 decreased the kinetics of fibrin formation by the extrinsic pathway at a low surface TF concentration. 2) Inhibition of FXII lead to a decreased fibrin formation rate and fiber network density, and increased the rate of fibrinolysis when the contact activation pathway was initiated by LC polyP.

Our kinetic measurements are consistent with other studies showing the hemostatic role of FXI can promote thrombin generation independent of FXII with initiation with EA or low levels of TF.^{18,22,35} While an effect of the FXI function-blocking antibodies was detected on a surface coated with 0.01 nM TF, no effect of FXI function-blocking antibodies was found at 0.1 nM TF, which would suggest that at some threshold concentration of TF, enough thrombin is produced that the role of FXI becomes insignificant for fibrin formation. However, assays under flow have demonstrated an effect in fibrin formation of FXI deficiency on surfaces coated with high TF concentration (~1 mM),²⁰ as enhanced FXI-mediated thrombin production may compensate for thrombin and reagent removal by convection. The rate of fibrin formation and fiber density were dramatically reduced by combined inhibition of FXIIa activation of FXI and FXIa activation of FIX with the inhibitor 1A6 in the absence of an initiator. When only activation of FXI by FXIIa was blocked with the inhibitor 14E11, changes in the rate of fibrin formation and fiber density were insignificant compared to the vehicle-treated condition. This result would suggest that FXIa activation FIX is a critical step of the coagulation cascade compared to FXII activation of FXIa, which is consistent with clinical studies demonstrating that FXII-deficient patients have no bleeding symptoms, while a FIX deficiency (hemophilia B) results in bleeding episodes into joints and muscles.²

The polyanionic molecule, LC polyP, has been shown to accumulate in a variety of microorganisms, inducing a FXII-dependent pathological response in mice.²⁶ We found that

LC polyP-initiated gel lag times and rates of fibrin formation were increased and reduced, respectively, in the presence of CTI. The prominent role of FXII is consistent with findings from Puy *et al.*, who showed LC polyP with FXIIa can promote FIX and prothrombin activation independently of FXI.²³ Though FXII is activated *in vitro* by anionic surfaces such as diatomaceous earth, dextran sulfate, glass or kaolin, FXII mechanisms independent of FXI have only been identified with LC polyP initiation.²⁹ We also initiated coagulation with LC polyP from a surface, as LC polyP can also be expressed on the surface of microorganisms.³¹ We began to see a contribution of FXI, as addition of FXI antibodies caused an increase in lag time and decrease in rate of fibrin deposition. Interestingly, 10C9, which inhibits the active site of FXIa, had a more potent effect than 1A6 and 14E11, which respectively inhibit FXIIa activation of FXI and FXIa activation of FIX, and FXIIa activation of FXI. This result might suggest that propagation of fibrin formation from a LC polyP surface relies on FXI-mediated mechanisms independent of FIX and FXII, which is supported by studies showing LC does not have any effect on the activation of FIX by FXIa.^{7,8}

Mechanical measurements of fibrin gels formed in the presence of 1A6 indicated an increased stiffness as compared to the control and CTI-treated conditions, despite being categorized as having a lower fibrin density. Though counterintuitive, nonlinear trends with respect to fiber diameter and density have previously been demonstrated. For instance, Konings, *et al.* found that α FXIIa causes a dose-dependent increase in fiber density, but nonlinear elastic moduli, suggesting that an optimal degree of fiber branching and thickness is required for maximum elastic storage modulus.¹² We observed strain hardening of the fibrin fibers when stretched, a well-documented property of fibrin.³⁹ Interestingly, fibrin gels formed in the presence of 1A6 had enhanced deformability, as they were able to sustain a higher strain before tearing. Given the low occurrence of stroke episodes found in FXI-deficient patients,²⁵ it is plausible that the mechanical properties of clots formed in the absence of FXI favor deformation and provide protection from embolization.

Fibrinolysis was found to be more rapid in the presence of 1A6 or CTI with EA or LC polyP initiators, respectively, suggesting that activation of FXI and FXII increase a clot's resistance to digestion. We expect that the increased rate of lysis in the aforementioned conditions is not attributed to differences in tPA permeation throughout the fibrin matrix, because tPA was already well-mixed in the fibrin network. Rather, it is plausible that FXIa and FXIIa might directly modify the fibrin network. For example, studies have demonstrated a co-dependence of FXI and thrombin-activatable fibrinolysis inhibitor (TAFI) in regulating thrombolysis.³² TAFI is a plasma procarboxypeptidase originating from the liver which, upon thrombin activation, downregulates fibrinolysis. In an *in vivo* model, inhibition of FXI or TAFI resulted in a twofold increase in thrombolysis within a rabbit jugular vein as compared to a control.¹⁹ TAFI and FXIa were also found to equally inhibit fibrinolysis in a co-dependent manner in a TEG analysis study.³ These results would suggest that FXIa activity is necessary to promote TAFI-mediated fibrinolysis resistance.

Our experiments were performed in a static purified system without platelets or other blood cells or flow. Platelets, upon activation, contribute to thrombin production and subsequent fibrin formation via exposure of phosphatidylserine (platelet membrane constituent).^{1,14,24}

Platelet phosphatidylserine supports the assembly of tenase and prothrombinase complexes, which lead to the activation of FX and prothrombin, respectively.¹⁵ Platelets also secrete short polyP (60–100mer), which triggers the contact activation pathway via FV and attenuates the anticoagulant function of tissue factor pathway inhibitor.²⁷ As such, we would expect the incorporation of platelets would lead to an increased rate of thrombin generation and fibrin formation and cause the fibrin networks to have higher density, though activated and aggregated platelets might introduce more heterogeneity into the overall clot structure. Moreover, under flow conditions, thrombin is diluted due to convection, even in the presence of potent coagulation cofactors and initiators, such as platelets and TF. Given the dynamic regulation of thrombin production with these added complexities, future work will entail deciphering the role of FXI and FXII in fibrin clot structure as a function of shear rate, platelet count, and TF concentration.

Our experiments were limited to determining the effects of FXI and FXIIa inhibitors during thrombin generation and fibrin polymerization. However, FXIa, FXIIa and LC polyP could modulate the structure of fibrin independently of thrombin generation.^{12,33} For example, a concentration-dependent increase in fibrin fiber density and clot stiffness occurred with FXIIa, through the direct binding of the N-terminus of FXIIa to fibrin.¹² Moreover, Smith *et al.* demonstrated polyP enhances fibrin clot structure in a calcium-dependent manner and attenuates fibrinolysis, through incorporation into polymerizing fibrin clots, without affecting thrombin clotting times.²⁸ FXIII is a transglutaminase that circulates in the plasma, which upon activation by thrombin supports a stable fiber network by crosslinking fibrin monomers.¹⁶ Future work is required to delineating fibrin structural and stability changes in normal and FXI- and FXII-inhibited fibrin networks in the presence of an FXIIIa inhibitor. In summary, we demonstrate that inhibition of FXI and FXII distinctly alter the physical biology of fibrin formation depending on how the coagulation cascade is initiated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Solution- and surface-initiated kinetic parameters of fibrin formation. PPP preincubated with a vehicle control (white), FXIa antibodies 1A6 (light gray), 14E11 (dark gray), 10C9 (charcoal), or the FXIIa inhibitor CTI (black) was mixed with vehicle control (HBS), ellagic acid (EA), or long chain polyP (PolyP), and then the reaction was initiated with Innovin (TF) at 0.01 or 0.1 nM and CaCl_2 . Fibrin gel turbidity was monitored at A_{405} for 2 h during gel formation. The curves were analyzed to determine the (A) lag time, (B) rate of fibrin deposition, and (C) maximum turbidity for clot initiated in solution and (D) lag time, (E)

rate of fibrin deposition, and (F) maximum turbidity for clots initiated from the surface. Error bars indicate standard error mean across experiments ($n = 4-5$). Significant differences ($p < 0.05$) are denoted by asterisk.

Figure 2.

Mass-to-length ratios of mature fibrin gels. Following the formation of the fibrin gel, the A_{405} was scanned from 400 to 800 nm at 10 nm intervals. A plot of $1/(\text{turbidity} \times \text{wavelength}^3)$ versus $1/\text{wavelength}^2$ was invoked to determine the y-intercept and its inverse, which is proportional to the mass-length ratio of the fibers. Citrated plasma was initiated in solution with control (HBS), EA, long chain polyP (PolyP), or tissue factor (TF) at 0.01 and 0.1 nM TF. The reaction was initiated with Ca^{2+} and fibrin gel turbidity was tracked at A_{405} for 2 h during gel formation. The curves were analyzed to determine the mass-to-length ratios of the fibers that were initiated in solution. Data are mean plus the standard error across all the experiments ($n = 4-5$). Significant differences ($p < 0.05$) are denoted by asterisk.

Figure 3.

Confocal images of fibrin fibers. Ibidi microchannels (width: 100 μm , height: 1 mm) were coated with vehicle, EA, polyP, or TF (0.01 and 0.1 nm). Citrated plasma with Alexa 546 conjugated fibrinogen was mixed with Ca^{2+} and quickly drawn through the channel. Gels were rinsed and fixed and then imaged by confocal microscopy. Scale bar = 15 μm .

Figure 4.

Quantification of (A) porous fraction, (B) number of intersections, and (C) characteristic length in confocal images (length of all fibers/number of intersections) was determined using an ImageJ plugin Diameter J. Shown are the mean and standard error between the acquired images (n=5). Significant differences ($p < 0.05$) are denoted by asterisk.

Figure 5. SRM images and diameters of vehicle control and 1A6 treated fibrin fibers derived from EA-initiated coagulation. (A) Alexa Fluor 647 labeled fibrin gels were imaged by 3-D super resolution microscopy for 1A6 and vehicle control conditions. Color bar indicates the depth of the fibers. Scale bar = 2 μm. (B) The mean and standard error of the fiber diameters for vehicle and 1A6 plasma treatment conditions with EA initiation. Significant differences ($p < 0.05$) are denoted by asterisk.

Figure 6.

Rheology of fibrin gels. A frequency sweep of fibrin gels initiated with (A) EA, or (B) long chain polyP treated with 1A6 and CTI. (C) An amplitude sweep was performed with an EA initiator and 1A6- and CTI-treated plasma. The (D) start of the strain hardening, (E) the maximum G' obtain prior to clot rupture, and (F) the corresponding strain at the maximum G' are reported. Error bars represent the standard error across experiments. Significant differences ($p < 0.05$) are denoted by asterisk.

Figure 7.

Normalized fibrinolysis curves for fibrin gels that had been initiated with (A) tissue factor (0.01 nM), (B) EA, or (C) long chain polyP with vehicle, 1A6 or CTI treatments. Each data set is normalized by the largest value. (D) The normalized lysis time, which was obtained by determining the amount of time for the clot to reach half its turbidity, is shown for the three initiator conditions with 1A6 and CTI treatments. Error bars represent the mean plus the standard error (n = 4). Significant differences (p < 0.05) compared to the vehicle control for each initiator are denoted by asterisk.