THROMBOSIS AND HEMOSTASIS

FXIa and platelet polyphosphate as therapeutic targets during human blood clotting on collagen/tissue factor surfaces under flow

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Key Points

- Contribution of FXIa and platelet-derived polyphosphate in thrombin generation varies depending on surface tissue-factor level.
- Platelet-derived polyphosphate potentiates pathways downstream of FXIIa that require low participation of extrinsic pathways.

Factor XIIa (FXIIa) and factor XIa (FXIa) contribute to thrombosis in animal models, whereas platelet-derived polyphosphate (polyP) may potentiate contact or thrombin-feedback pathways. The significance of these mediators in human blood under thrombotic flow conditions on tissue factor (TF) –bearing surfaces remains inadequately resolved. Human blood (corn trypsin inhibitor treated [4 μg/mL]) was tested by microfluidic assay for clotting on collagen/TF at TF surface concentration ($[TF]_{wall}$) from ~0.1 to 2 molecules per μm^2. Anti-FXI antibodies (14E11 and O1A6) or polyP-binding protein (PPXbd) were used to block FXIIa-dependent FXI activation, FXIa-dependent factor IX (FIX) activation, or platelet-derived polyP, respectively. Fibrin formation was sensitive to 14E11 at 0 to 0.1 molecules per μm^2 and sensitive to O1A6 at 0 to 0.2 molecules per μm^2. However, neither antibody reduced fibrin generation at ~2 molecules per μm^2 when the extrinsic pathway became dominant. Interestingly, PPXbd reduced fibrin generation at low $[TF]_{wall}$ (0.1 molecules per μm^2) but not at zero or high $[TF]_{wall}$, suggesting a role for polyP distinct from FXIIa activation and requiring low extrinsic pathway participation. Regardless of $[TF]_{wall}$, PPXbd enhanced fibrin sensitivity to tissue plasminogen activator and promoted clot retraction during fibrinolysis concomitant with an observed PPXbd-mediated reduction of fibrin fiber diameter. This is the first detection of endogenous polyP function in human blood under thrombotic flow conditions. When triggered by low $[TF]_{wall}$, thrombosis may be druggable by contact pathway inhibition, although thrombolytic susceptibility may benefit from polyP antagonism regardless of $[TF]_{wall}$. (*Blood*. 2015;126(12):1494-1502)

Introduction

Many anticoagulants target prothrombinase formation or thrombin, but they can be associated with bleeding risks.[1,2] Reducing thrombotic risk without affecting normal hemostasis may require targeting factors that promote thrombus propagation and stability.[3] The contact pathway is not essential for hemostasis because factor XII (FXII) deficiency is not associated with a bleeding phenotype, and factor XI (FXI) –deficient (hemophilia C) patients display a relatively mild bleeding disorder.[4] The prothrombotic function of FXII and FXI has been demonstrated in several animal vessel injury models.[5-15] Additionally, platelet-derived polyphosphate (polyP; ~60-70mer) has recently been identified as a weak FXII activator[16] that also promotes the feedback activation of FXI by thrombin[17] and factor V (FV) activation by FXIa, factor Xa (FXa), or thrombin[18,19] and enhances fibrin physical structure.[20,21] Cationic inhibitors of polyP also reduce venous and arterial thrombosis in animal injury models.[22] These observations suggest that the contact pathway is a potential source of therapeutic targets for safer antithrombotic therapies.[23] Distinct from testing in animal models, testing inhibitors of FXIa and polyP in human blood under thrombotic flow conditions helps to prioritize and inform inhibitor development against these targets. Importantly, platelet concentrations in wall-attached thrombi that form under flow are 50- to 200-fold greater than those found in whole blood, a complexity that distinguishes microfluidic flow studies from test tube studies.[24-26]

Recently, a phase 2 trial demonstrated that FXI-antisense oligonucleotide (FXI-ASO) reduced FXI levels and decreased the incidence of deep vein thrombosis (DVT) after knee arthroplasty without increasing bleeding, thus providing evidence that FXIa can contribute to thrombosis in humans.[27] However, postoperative bleeding in knee arthroplasty is relatively uncommon, and the rate of bleeding with FXI-ASO was not significantly lower than that seen with enoxaparin.[28,29] The FXI-ASO trial demonstrated a role for FXIa in DVT but did not resolve the role of either platelet polyP or FXIIa in postarthroplasty DVT because FXIa can inactivate tissue factor pathway inhibitor,[30] and FXI can be feedback-activated by thrombin.[31] During knee arthroplasty, FXIIa may (or may not) be activated by polyP, DNA/histones, RNA, sulfatides, or other factors.[32]

We used a microfluidic assay of platelet deposition and coagulation on a type I fibrillar collagen/lipidated tissue factor (TF) surface that could trigger thrombin generation via the contact pathway and/or the extrinsic pathway.[24,33] We used two distinct monoclonal anti-FXI antibodies, 14E11 and O1A6, to explore the role of FXI in contact activation and in promoting thrombin amplification. 14E11 selectively

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inhibits FXI activation by FXIIa but not FXI activation by thrombin, and O1A6 interferes with both FXI activation by FXIIa and factor IX (FIX) and FV activation by FXIa.[34-36] O1A6 does not directly inhibit FXI activation by thrombin but disrupts the FXIa-dependent thrombin amplification mechanism by inhibiting FIX activation by FXIa, which is the downstream reaction of FXI feedback activation in the thrombin feedback loop. By using a low level (4 μg/mL) of corn trypsin inhibitor (CTI), the contact pathway contributions can be studied in whole blood in vitro without the overwhelming dominance of the container. With 4 μg/mL CTI, drawn blood does not clot in the reservoir during the experiment but will generate thrombin via FXIIa in ~300 seconds when perfused over collagen or surface-linked contact activators.[33] We investigated the role of platelet-derived polyP in promoting thrombin generation and enhancing fibrin structure and clot stability by inhibiting polyP with polyP-binding protein (PPXbd), the recombinant polyP-binding domain of *E coli* exopolyphosphatase (concentration that inhibits 50%, 8.5 μg/mL).[17,22] This microfluidic data with human blood demonstrated specific conditions during which FXIa and platelet polyP play a kinetically significant role in clotting under flow conditions that can be targeted with inhibitors.

Methods

Materials

Reagents were obtained as follows: DBCO-Sulfo-NHS Ester (Click Chemistry Tools, Scottsdale, AZ), azide-free anti-human CD61 antibody (BioLegend, San Diego, CA), annexin V- fluorescein isothiocyanate, anti-human CD41a, anti-human CD61 antibody (BD Biosciences, San Jose, CA), Alexa Fluor 647–conjugated human fibrinogen (Life Technologies, Grand Island, NY), CTI (Haematologic Technologies, Essex Junction, VT), Dade Innovin prothrombin time (PT) reagent (Siemens, Malvern, PA), collagen (type I; Chrono-Log, Havertown, PA), recombinant human tissue plasminogen activator (tPA; Abcam, Cambridge, MA), EDTA, grade I glutaraldehyde, sodium cacodylate, hexamethyldisilane (Sigma, St. Louis, MO), and H-Gly-Pro-Arg-Pro-OH (GPRP; EMD Chemicals, San Diego, CA). The murine anti-human FXI monoclonal antibodies O1A6 and 14E11 were gifts from Dr Andras Gruber (Oregon Health & Science University). PPXbd was prepared as described in the supplemental Data available on the *Blood* Web site.[17] The custom-made thrombin-sensitive peptide azidoacetyl-AK(5FAM)-GALVPRGSAGK(CPQ2)-NH2 was obtained from CPC Scientific (Sunnyvale, CA) for click reactions to anti-CD61, as previously described.[37]

Preparation and characterization of collagen/TF surface

Glass slides were first rinsed with ethanol and then with deionized water and dried with filtered air. A volume of 5 μL of collagen was perfused through the patterning channel (250 μm wide × 60 μm high) of a microfluidic device to create a single stripe of fibrillar collagen, as previously described.[38] Lipidated TF was then sorbed to the collagen surface by introduction of 5 μL of Dade Innovin PT reagent (20 nM stock concentration)[39] diluted 300-, 100-, and fivefold with *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffered saline to obtain low, medium, and high TF surface concentrations ($[TF]_{wall}$) of ~0.1, ~0.2, and ~2 molecules per μm^2, respectively, as estimated by imaging of sorbed annexin V-fluorescein isothiocyanate–stained vesicles (supplemental Figure 1). In all experiments, the Dade Innovin PT reagent was incubated with the collagen for 30 minutes without flow and then rinsed and blocked with 20 μL 0.1% bovine serum albumin buffer.

Blood collection and preparation

Blood was obtained via venipuncture into a syringe containing CTI (4 μg/mL) from healthy donors who self-reported as free of alcohol use and medication for at least 72 hours prior to blood collection. In some experiments, blood was collected without the use of CTI. All donors provided informed consent under approval of the University of Pennsylvania Institutional Review Board. Blood was treated with anti-FXI antibodies or PPXbd immediately after blood collection. Platelets were labeled with anti-human CD61 antibody (or anti-human CD41a antibody when thrombin was measured with the platelet targeting sensor). Fluorescent fibrinogen was added (1 mg/mL stock solution, 1:80 v/v [%] in whole blood) for measuring fibrin generation. All experiments were initiated within 5 minutes after phlebotomy.

Microfluidic clotting assay on collagen surfaces with or without TF

An 8-channel polydimethylsiloxane flow device was vacuum-mounted perpendicularly to collagen/TF surfaces forming 8 parallel-spaced prothrombotic patches (250 × 250 μm), as previously described.[25] Treated blood was perfused across the 8 channels by being withdrawn through a single outlet. Initial wall shear rate was controlled by a syringe pump (Harvard PHD 2000; Harvard Apparatus, Holliston, MA) connected to the outlet on the flow device. Thrombi were formed either under constant flow rate (constant Q; CTI-treated blood in all 8 channels) or under constant pressure drop (constant ΔP) conditions.[24] To achieve constant ΔP, EDTA-treated blood was delivered into alternating channels to abolish thrombus formation, thus allowing CTI-treated blood to clot and divert flow into the matched EDTA channels. Experiments with added recombinant tPA were conducted under constant ΔP to avoid clot embolism before acquiring fibrinolysis profiles. Platelet, fibrin, and/or thrombin activity were monitored simultaneously by epifluorescence microscopy (IX81; Olympus America Inc., Center Valley, PA). Images were captured with a charged coupled device camera (Hamamatsu, Bridgewater, NJ) and were analyzed with ImageJ software (National Institutes of Health). To avoid side-wall effects, fluorescence values were taken only from the central 75% of the channel.

Scanning electronic microscopy

In some experiments, thrombi were fixed under flow in situ with 2% grade I glutaraldehyde in N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid buffered saline solution. The glass slides were then removed from the polydimethylsiloxane device, and the fixed thrombi were washed 6 times in 0.2 M sodium cacodylate, incubated in sodium cacodylate overnight at 4°C, dehydrated in graded ethanol in sodium cacodylate buffer, rinsed with hexamethyldisilane, air dried, and sputter coated with gold/palladium (Au/Pd) alloy. Samples were imaged with a Quanta 600 FEG Mark II scanning electron microscope equipped with a Schottky field emission electron gun. The thickness of fibrin fibers was averaged across measurements (ImageJ, NIH) from 40 random selected fibers in images captured at ×3500 magnification.

Statistical analysis

Differences between control and treated groups were analyzed with Student t test. The difference was considered significant at $P < .05$.

Results

Contact pathway was indispensable for thrombin generation on collagen surface

Both anti-FXI antibodies (14E11 and O1A6) were tested individually in whole blood (20 μg/mL) perfused at a venous wall shear rate of 100 s^{-1} over a collagen surface (no $[TF]_{wall}$). Perfusion of whole blood (treated with only 4 μg/mL CTI) without antibodies resulted in immediate and rapid platelet accumulation, with thrombin and fibrin production detected after 300 seconds of perfusion. Both antibodies had minimal effect on platelet deposition, which is driven by the collagen and platelet-derived secondary aggregation mediators adenosine diphosphate and thromboxane.[40] Both antibodies caused complete inhibition of thrombin generation and fibrin formation (Figure 1). In the presence of a low level of CTI, the contact pathway was required as the most proximal trigger of thrombin production as demonstrated by the inhibitory activity of 14E11 and O1A6. This result is consistent

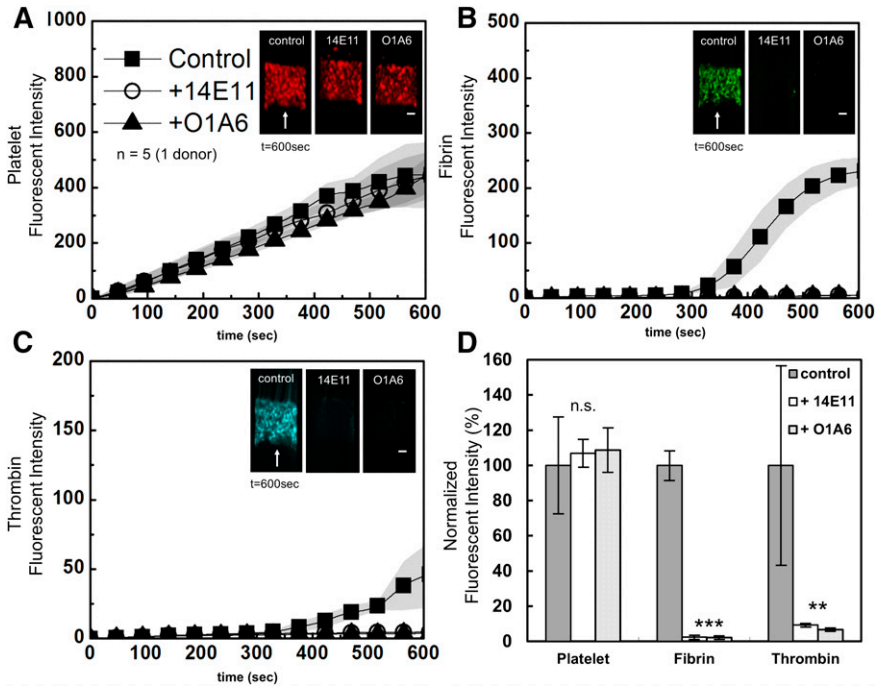

Figure 1. 14E11 and O1A6 individually block thrombin/fibrin generation on collagen. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with FXI antibody 14E11 or O1A6 (20 μg/mL) and was perfused over the collagen surface at 100 s^{-1} under constant flow rate condition (constant *Q*). FXI antibodies efficiently abolished thrombin and fibrin generation on collagen surface without affecting platelet deposition. (A) Dynamics of platelet deposition and (B) generation of fibrin and (C) thrombin based on 5 clotting events (± standard deviation [SD], shaded). End point images are shown for platelet (red; panel A inset), fibrin (green; panel B inset), and thrombin (cyan; panel C inset) on collagen surface (white arrows indicate flow direction; scale bar = 50 μm). (D) Final platelet, fibrin, and thrombin fluorescence (t = 600 s) was normalized to control. Adding FXI antibodies caused more than 90% reduction in final fibrin and thrombin generation. $^{**}P < .01$; $^{***}P < .005$. n.s., not significant.

with prior observations made with whole blood from a patient with a severe FXI deficiency.[41] Furthermore, in an experiment with raw blood (no CTI), which allows for rapid and massive thrombin and fibrin formation through unrestricted FXIIa generation, 14E11 substantially delayed clotting (supplemental Figure 2). Because the addition of CTI and 14E11 together (Figure 1 and supplemental Figure 2C-D) prevented thrombin and fibrin formation, the presence of blood-borne TF can be excluded.

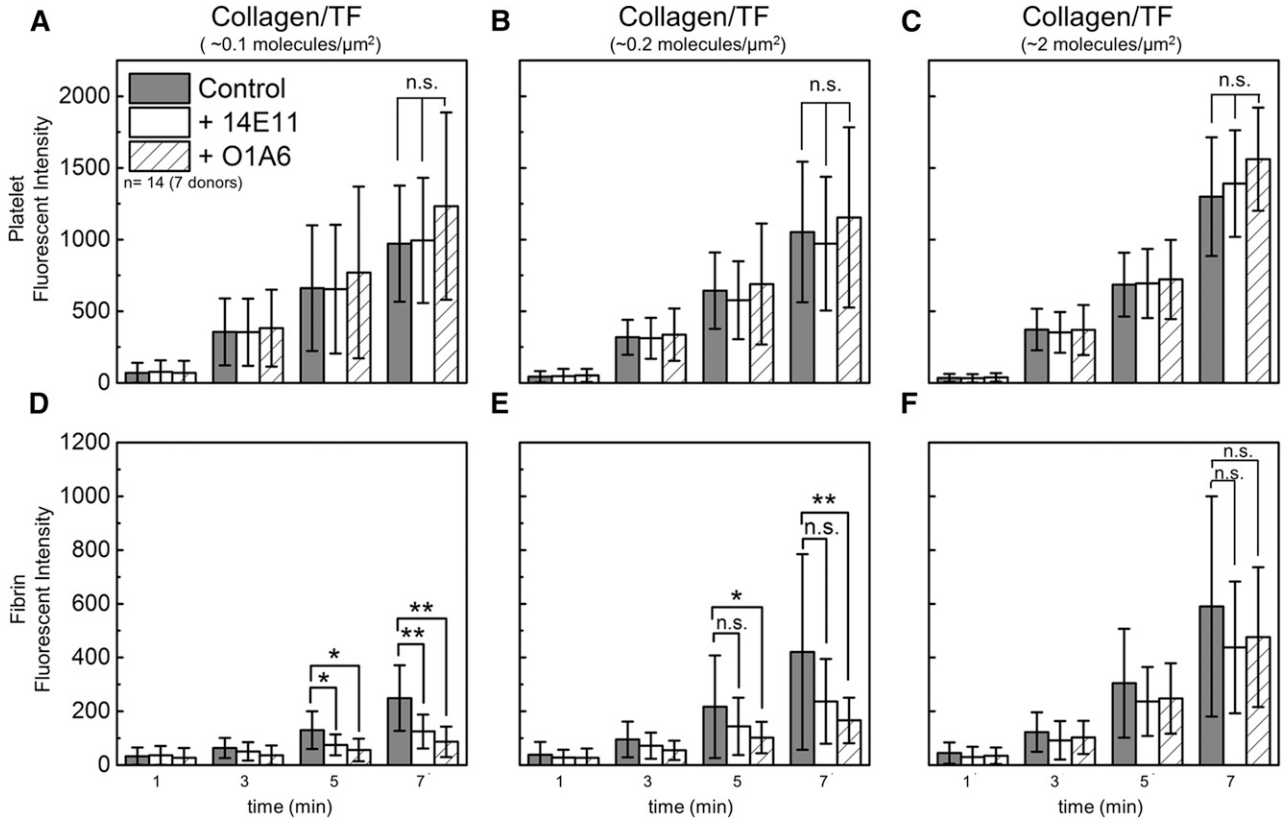

Figure 2. Potency of 14E11 and O1A6 varies depending on $[TF]_{wall}$. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with FXI antibody 14E11 or O1A6 (20 μg/mL) and was perfused over collagen/TF surfaces at 100 s^{-1} (constant *Q*). (A-C) Platelet deposition on collagen was unaffected by FXI antibodies despite the difference in $[TF]_{wall}$. (D-E) O1A6 showed inhibitory effect on fibrin generation at low and medium $[TF]_{wall}$. (D) 14E11 only reduced fibrin generation at low $[TF]_{wall}$. (F) Neither of the antibodies caused reduction in fibrin at high $[TF]_{wall}$. $^{*}P < .05$; $^{**}P < .01$.

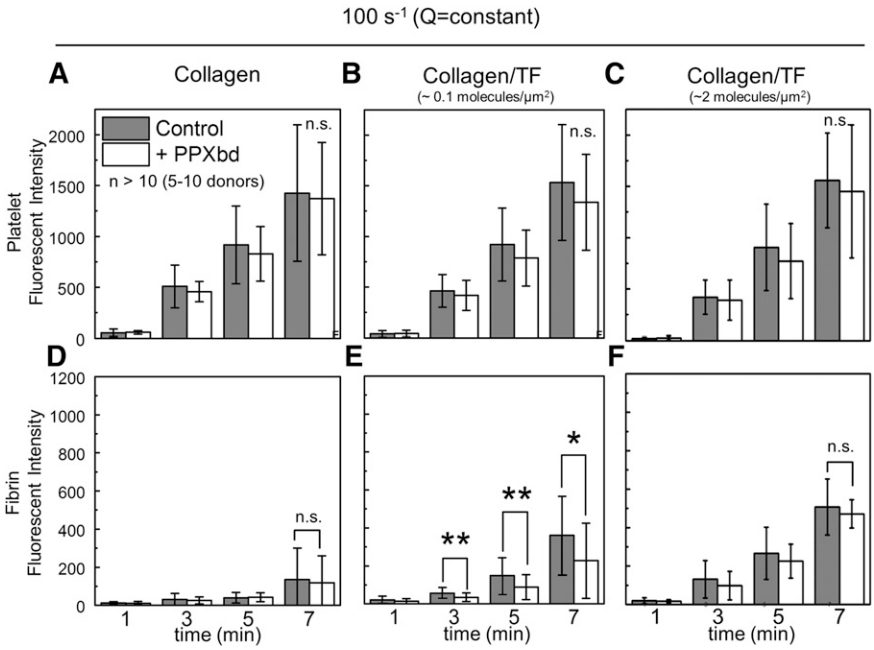

Figure 3. PPXbd inhibits fibrin generation at low $[TF]_{wall}$ under venous conditions. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with PPXbd (250 μg/mL) and was perfused over collagen or collagen/TF surface at 100 s^{-1} (constant *Q*). (A-C) Platelet deposition on collagen was unaffected by PPXbd despite the difference in $[TF]_{wall}$. (D-F) Fibrin generation was inhibited by PPXbd only on collagen/low $[TF]_{wall}$ surface but not on collagen or collagen/high $[TF]_{wall}$ surface. $^*P < .05$; $^{**}P < .01$.

14E11 and O1A6 inhibited thrombin generation at low $[TF]_{wall}$

Regardless of $[TF]_{wall}$, platelet deposition on collagen/TF surfaces was not affected by 14E11 or O1A6 during the first 420 seconds (Figure 2A-C). By 500 seconds, most of the formed thrombi were partially or fully occlusive and subject to large hemodynamic forces that drove embolization. The lag phase before detectable fibrin formation was <100 seconds on collagen/high $[TF]_{wall}$, and this lag time was prolonged as $[TF]_{wall}$ was decreased (Figure 2D-F). As $[TF]_{wall}$ increased, the amount of fibrin produced by 7 minutes also increased. At low $[TF]_{wall}$ of ~0.1 molecules per μm^2, fibrin formation was detectable at ~240 seconds and was significantly reduced by both 14E11 or O1A6 at times between 300 and 400 seconds (Figure 2D). O1A6 maintained its ability to inhibit fibrin generation up to medium $[TF]_{wall}$ of ~0.2 molecules per μm^2. However, at high $[TF]_{wall}$, fibrin formation was not affected by 14E11 or O1A6.

PPXbd inhibited fibrin generation on collagen/low $[TF]_{wall}$ surface at venous shear rate

Under conditions of constant flow rate, PPXbd (250 μg/mL) was tested on collagen/TF at the inlet venous shear rate (100 s^{-1}) by using whole blood with CTI (4 μg/mL). Platelet aggregation remained unaffected when platelet-derived polyP was inhibited with PPXbd (Figure 3A-C), consistent with platelet deposition being largely driven by collagen and released mediators or secondary aggregation. Measurable fibrin accumulation on collagen surface alone (no TF) did not appear until 400 seconds (Figure 3D) and was less than that observed on collagen/TF surfaces. PPXbd did not reduce fibrin formation on a pure collagen surface, indicating that other triggers were more prominent activators of the contact pathway than platelet-derived polyP. This is consistent with the relatively low activity of small forms of platelet-derived polyP to activate FXII.[42] With TF on the surface, PPXbd reduced fibrin formation at low $[TF]_{wall}$, but this inhibition was not detectable at high $[TF]_{wall}$ (Figure 3E-F). This experiment defines a specific condition in which endogenous platelet polyP leads to enhanced fibrin production under a condition of low extrinsic pathway activation. Similarly, for a condition where FXII activation could proceed unimpeded, perfusion of raw blood (no CTI) resulted in rapid and massive platelet and fibrin deposition that was unaffected by PPXbd (supplemental Figure 3).

PPXbd inhibited thrombin and fibrin generation on collagen/low $[TF]_{wall}$ surface at arterial shear rate

Because animal studies have supported a role for the contact pathway during arterial thrombosis, we tested PPXbd at arterial shear rate (1000 s^{-1}) under constant pressure drop conditions in which occlusive clots can stop flow.[24] Thrombi were formed on collagen or collagen/low $[TF]_{wall}$ in the presence and absence of PPXbd. Consistent with the observation under venous conditions, platelet deposition was not significantly altered by polyP inhibition (Figure 4A-B). As was seen for venous conditions, PPXbd had no significant effect on platelet, thrombin, or fibrin accumulation for clotting of blood on pure collagen (no TF) at an arterial shear rate (Figure 4A,C,E). PPXbd inhibited thrombin and fibrin by 54% ($P = .012$) and 70% ($P = .037$) on collagen/low $[TF]_{wall}$ of ~0.1 molecules per μm^2 (Figure 4D,F). The reduction in thrombin signal became significant after 300 seconds (Figure 4E-F). Under this flow condition, occlusive thrombi were observed at around 400 seconds into the experiment on collagen/low $[TF]_{wall}$. Simply decreasing $[TF]_{wall}$ to zero did not cause reduction in the time to full channel occlusion. Delayed occlusive thrombi were observed only when polyP was inhibited at no $[TF]_{wall}$ (supplemental Figure 4).

PPXbd blocked mechanisms downstream of FXIIa on collagen/low to medium $[TF]_{wall}$

At venous shear, we found that the addition of PPXbd to blood in which FXIIa activation of FXI is blocked by 14E11 caused significant reduction in fibrin formation on collagen/low to medium $[TF]_{wall}$ after the first 200 seconds (Figure 5A). In contrast, adding PPXbd to O1A6-treated blood in which FIX activation by FXIa is blocked did not cause further reduction in fibrin formation at the same $[TF]_{wall}$ (Figure 5B). This suggests that the inhibited thrombin/fibrin generation at low to medium but not high or no $[TF]_{wall}$ by PPXbd was associated with its inhibition on mechanisms downstream of FXIIa.

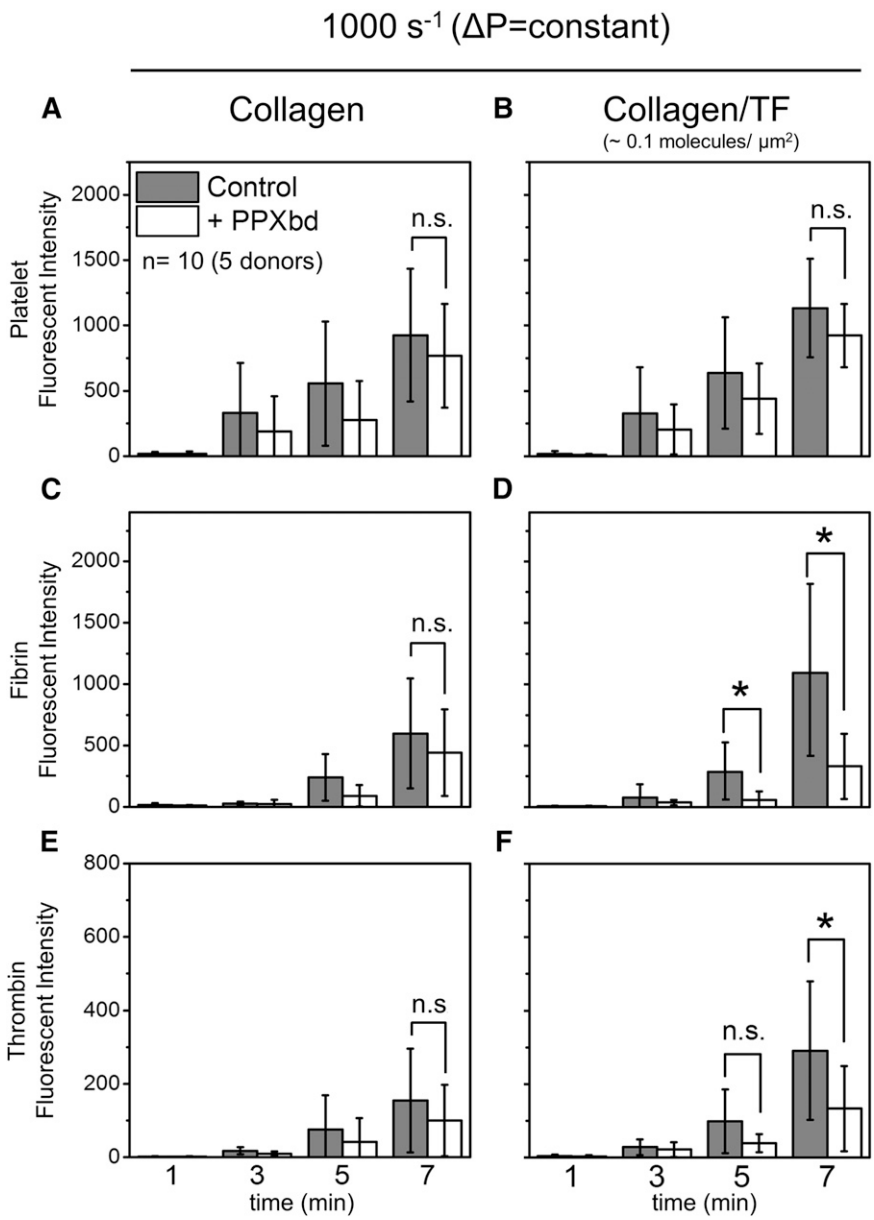

Figure 4. PPXbd inhibits fibrin and thrombin generation on collagen/low $[TF]_{wall}$ at arterial shear rate. (A-B) Anticoagulated whole blood (CTI, 4 μg/mL) was treated with PPXbd (250 μg/mL) and was perfused over collagen or collagen/low $[TF]_{wall}$ surface at 1000 s^{-1} under constant pressure drop conditions (constant ΔP). Platelet deposition on collagen was unaffected by PPXbd. Fibrin generation was inhibited by PPXbd on (D) collagen/low $[TF]_{wall}$ surface but not on (C) the collagen-alone surface. (E-F) Consistent with the reduction in fibrin on collagen/low $[TF]_{wall}$, thrombin generation at low $[TF]_{wall}$ was also lowered by PPXbd after 300 seconds. $^*P < .05$.

PPXbd altered fibrin structure and promoted clot retraction upon fibrinolysis

Recombinant tPA (30 nM) was added to blood prior to perfusion to initiate fibrinolysis. Degradation of fibrin was observed after occlusion when the platelet mass stopped growing and the fibrin signal started to decline as a result of lysis. Fibrinolysis initiated earlier and proceeded faster in the presence of PPXbd (Figure 6C-D), indicating a role for platelet-derived polyP in protecting the fibrin clot from lysis regardless of the $[TF]_{wall}$ (supplemental Videos 1-2). The platelet plateau level was significantly higher in PPXbd-treated clots, which was likely caused by retraction of occlusive thrombi, considering that platelet propagation was unaffected by PPXbd at early time points (Figure 6A-B). We quantified clot retraction by analyzing the platelet area reduction at the downstream edge, which proceeded against the direction of flow (Figure 7A). The presence of PPXbd caused a larger area reduction at both high and low $[TF]_{wall}$ (Figure 7D). We further analyzed the retraction under 2 extreme conditions: (1) preserving all formed fibrin by not adding lytic reagent, and (2) blocking fibrin polymerization with GPRP. PPXbd had no significant effect on retraction under these 2 conditions (Figure 7B-C). We also examined the impact of PPXbd on fibrin physical structure by measuring the fibrin fiber diameter in scanning electron micrographs of whole blood clots formed under flow condition (100 s^{-1}; constant Q) on collagen/TF surfaces (Figure 7E-F,H-I). PPXbd significantly reduced the fiber diameters at both high and low $[TF]_{wall}$ (Figure 7G,J).

Discussion

In this study, we investigated the role of FXI in contact activation and in promoting thrombin amplification by selectively targeting FXIIa-dependent FXI activation and FXIa-dependent FIX and FV activation by using FXI antibodies 14E11 and O1A6, respectively. The extrinsic pathway was left intact, but its relative contribution was tuned by varying wall TF concentration. On the basis of our observations, we

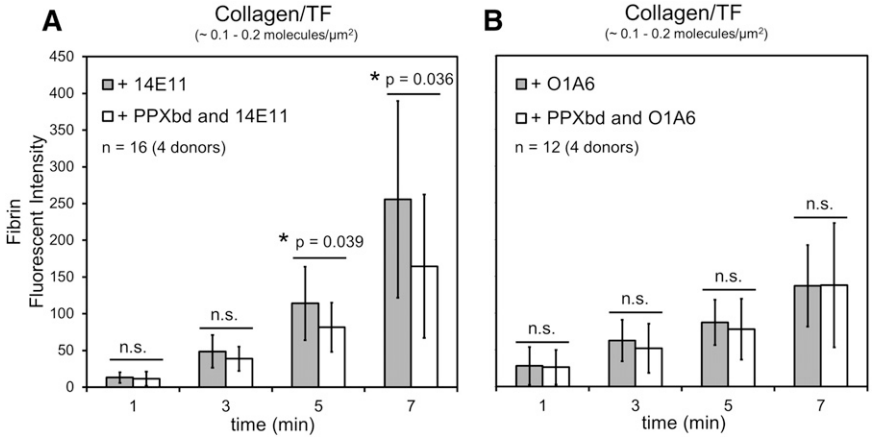

Figure 5. PPXbd inhibits pathways downstream of FXIIa. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with (A) 14E11 (20 μg/mL) to inhibit FXIIa activation of FXI or (B) O1A6 to block FIX activation by FXIa and was perfused over collagen/low to medium $[TF]_{wall}$ surface at 100 s^{-1} under constant flow rate mode (constant *Q*). Adding PPXbd to 14E11-treated blood caused significant reduction in fibrin signal after the first 200 seconds. PPXbd showed no effect on fibrin generation when it was added to O1A6-treated blood.

propose a model showing relative contributions of the 3 major mechanisms of thrombin generation: FXIIa-dependent thrombin generation, FXIa-mediated thrombin amplification, and TF-induced thrombin generation (supplemental Figure 5). The contact pathway was required for thrombin generation on collagen surfaces because 14E11 and O1A6 robustly blocked thrombin and fibrin generation. At low $[TF]_{wall}$, contributions of the contact pathway and the extrinsic pathway were comparable. 14E11 and O1A6 individually caused partial inhibition of fibrin generation. As $[TF]_{wall}$ was increased, FXIIa-dependent contact activation became less significant. The thrombin feedback mechanism became more detectable because O1A6, but not 14E11, caused significant reduction in fibrin generation. Finally, when $[TF]_{wall}$ exceeded 2 molecules per μm^2, neither of the FXI antibodies reduced fibrin formation, indicating that thrombin was generated primarily via the extrinsic pathway. The activity of the 2 antibodies, especially at low $[TF]_{wall}$, in reducing fibrin formation was essentially the result of the inhibition of contact activation, as shown in Figure 1, although the identity of the most proximal activators of FXII and FXI was not resolved.

Platelet-derived polyP has been proposed as a mediator for coagulation and clot structure. However, the masking effect of TF has caused discrepancy in reported data regarding platelet-derived polyP as an endogenous activator of FXII,[16,43-45] which raises the question of whether polyP is physiologically important because TF is usually present at injury sites. Our microfluidic data support a role for platelet polyP as an enhancer of clotting under specific venous flow conditions with low (but not high) levels of wall-derived TF, consistent with the role of thrombin-feedback activation of FXI implicated in the FXI-ASO study of DVT prevention.[27] The fact that adding PPXbd to FXIIa-inhibited blood caused further reduction in fibrin at low to medium $[TF]_{wall}$ suggests that polyP potentiates pathways downstream of FXIIa activation of FXI that require low participation of the extrinsic pathway. Insignificant thrombin feedback caused by insufficient thrombin generation on collagen or overwhelmed thrombin generation

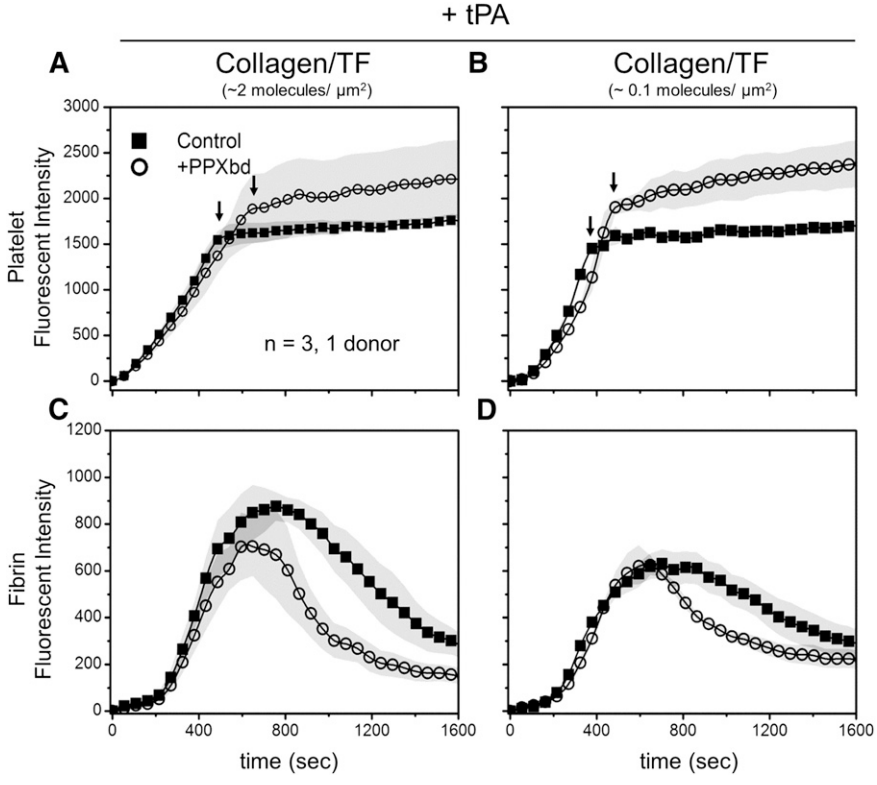

Figure 6. PPXbd reduces thrombus resistance to fibrinolysis induced by recombinant tPA. (A-B) Recombinant tPA (30 nM) was added to PPXbd-treated whole blood (250 μg/mL) right before flow initiation (100 s^{-1}; constant ΔP). Platelet deposition was identical with or without PPXbd during the first 400 seconds. However, the platelet signal reached a higher plateau level after occlusion (black arrows) when treated with PPXbd. Fibrinolysis was initiated after occlusion and proceeded faster in the presence of PPXbd at both (C) high and (D) low $[TF]_{wall}$. Dynamics of platelet and fibrin accumulation were based on 3 clotting events (± SD, shaded area).

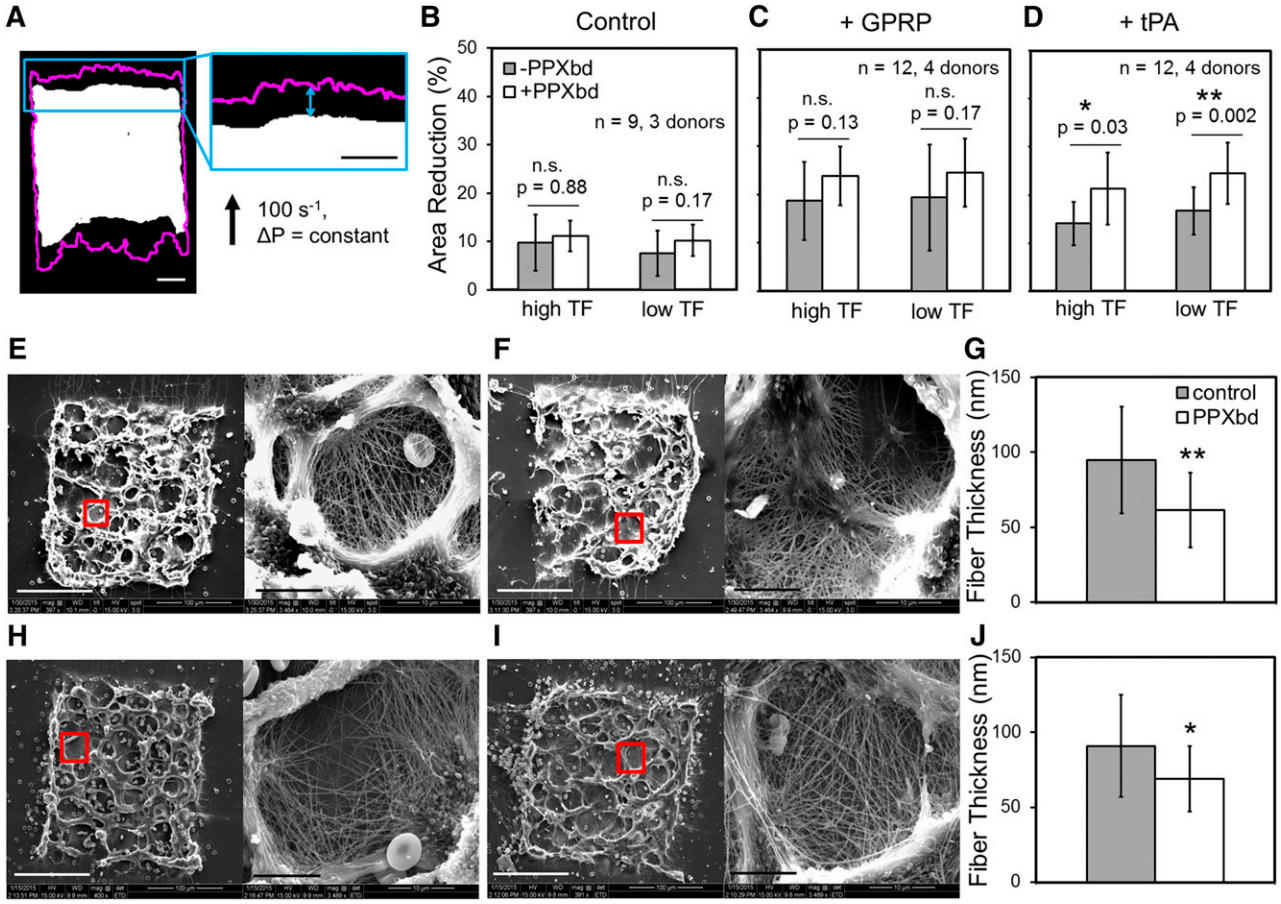

Figure 7. PPXbd enhances clot retraction after flow cessation and alters fibrin fiber thickness. (A) Clot retraction was quantified by the reduction in area at the downstream edge, as measured from just prior to occlusion (pink outline) to the end point of the experiment (white area). Scale bars = 50 μm. Anticoagulated whole blood (CTI, 4 μg/mL) was either (B) untreated or (C) treated with 5 mM GPRP or (D) 30 nM recombinant tPA and was perfused over collagen surface with high or low $[TF]_{wall}$. Area change was averaged across multiple donors. Representative scanning electron micrographs of thrombi formed under flow (100 s^{-1}; constant Q) on collagen/high $[TF]_{wall}$ surface (E) with or (F) without PPXbd or on collagen/low $[TF]_{wall}$ surface (H) with or (I) without PPXbd. Left panels (scale bar = 100 μm) of E, F, H, and I show the structure of whole thrombi; right panels (scale bar = 10 μm) are zoomed-in images of the areas outlined by red boxes. Flow direction was from right to left. Average fiber thickness was smaller in PPXbd-treated thrombi at both (G) high and (J) low $[TF]_{wall}$. *$P < .05$; **$P < .01$.

by high $[TF]_{wall}$ made the potentiating effect of polyP on thrombin feedback mechanism negligible. Interestingly, reduction in thrombin was seen with PPXbd on collagen (no $[TF]_{wall}$) when 14E11 was added to raw blood (no CTI) shortly after blood collection (supplemental Figure 4). In this case, 14E11 only partially blocked thrombin generation, indicating thrombin leakage from surface-induced contact activation during blood collection, which was probably sufficient for initiating the thrombin feedback loop. As expected, the effect of PPXbd was completely masked by massive FXIIa-dependent thrombin generation in raw blood when 14E11 was excluded. Thus, we hypothesize that the contribution of polyP is detectable only when the thrombin feedback loop is primed with adequate but not excessive amounts of thrombin generated via either the contact or extrinsic pathway. The sensitivity of thrombin and fibrin production to the inhibitory effect of PPXbd under arterial shear conditions at low (but not no) $[TF]_{wall}$ suggests a similar promoting role of polyP in arterial thrombosis. However, we found that the role of polyP as FXII activator in this microfluidic model was less important because surface-immobilized long-chain polyP (700mer), which was shown in well plates as a much more potent FXII activator than platelet-derived short-chain polyP,[42] failed to promote fibrin generation on a collagen (no $[TF]_{wall}$) surface (supplemental Figure 6). In this assay, platelet aggregation is primarily mediated by collagen signaling and endogenous secondary aggregation agonists, and thus was not sensitive to the reduction in thrombin generation caused by PPXbd. Delayed full channel occlusion occurred only when TF and polyP were both absent even though total platelet fluorescence was not affected, indicating that factor(s) other than total deposited platelet mass (ie, spatial distribution of platelet mass) could be affected by polyP and cause the change in occlusion time.

PolyP also exerts effects on clot structure by enhancing fibrin polymerization[20,42] and attenuating binding of fibrinolytic proteins to fibrin.[21] But these effects have not been validated in human whole blood under flow conditions in the presence of TF. We were able to show the reduced diameter of fibrin fibers formed in polyP-deficient thrombi regardless of the wall TF concentration. As a result, polyP-deficient thrombi were more prone to tPA-induced lysis. We also noticed that polyP attenuated retraction of occlusive thrombi during fibrinolysis. We speculate that polyP modulated contraction by enhancing fibrin structure on the basis of the observation that thrombi contracted to the same degree with or without polyP when fibrin polymerization was abolished. Furthermore, the attenuating effect of polyP on clot retraction was significant only upon fibrinolysis. Platelets are known to generate heterogeneous contractile force based on the stiffness of the surrounding environment.[46] It is possible that polyP incorporated into fibrin fibers caused the fibers to better retain their stiffness upon fibrinolysis, thus limiting clot retraction. The effect of polyP on clot stiffness is probably thrombin-independent and directly

caused by the incorporation of polyP into fibrin fibers[20] because a similar attenuating effect was observed at both low and high $[TF]_{wall}$. When fibrinolysis was excluded, the stall force generated by the dense and stiff fibrin network prevented platelets from contracting despite the difference in fibrin structure caused by polyP.

In this study, we demonstrated that the role of FXIIa, FXIa, and platelet-derived polyP in thrombus formation on collagen may vary depending on $[TF]_{wall}$. To the best of our knowledge, this is the first study to show the effect of polyP on thrombin generation and fibrin structure with human whole blood under controlled flow condition in the presence of surface immobilized TF.

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Authorship

Contribution: S.Z. and S.L.D. designed the research and wrote the manuscript; S.Z. and R.J.T. performed the research; and S.Z., R.J.T., S.L.D., and J.H.M. analyzed and interpreted the data.

Conflict-of-interest disclosure: J.H.M. and R.J.T. are co-inventors on pending patent applications of medical uses of polyP inhibitors. The remaining authors declare no competing financial interests.

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