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 XIIa that requires low participation of extrinsic pathway.

ABSTRACT

Factor XIIa (FXIIa) and Factor XIa (FXIa) contribute to thrombosis in animal models, while 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, albeit highly relevant to drug target validation. Human blood (treated only with low level 4 μg/mL corn trypsin inhibitor) was tested by microfluidic assay for clotting on collagen/TF at \([\text{TF}]_{\text{wall}}\) from ~0.1 to 2 molecule-TF/μm². Anti-FXI antibodies (14E11, O1A6) or polyP binding protein (PPXbd) were used to block FXIIa-dependent FXI activation, FXIa-dependent FIX activation, or platelet-derived polyP, respectively. As expected, both antibodies fully blocked thrombin and fibrin generation on collagen alone. Fibrin formation was 14E11-sensitive at 0 to 0.1 molecule-TF/μm² and O1A6-sensitive at 0 to 0.2 molecule-TF/μm². However, neither antibody reduced fibrin generation at ~2 molecule-TF/μm² when the extrinsic pathway became dominant. Interestingly, PPXbd reduced both thrombin and fibrin generation only at low \([\text{TF}]_{\text{wall}}\) (0.1 molecule-TF/μm²), but not at zero or high \([\text{TF}]_{\text{wall}}\), suggesting a role for polyP distinct from FXIIa activation and requiring low extrinsic pathway participation. Regardless of \([\text{TF}]_{\text{wall}}\), PPXbd enhanced fibrin sensitivity to tPA 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 whole blood under thrombotic flow conditions. When triggered by low \([\text{TF}]_{\text{wall}}\), thrombosis may be druggable by contact pathway inhibition, although thrombolytic susceptibility may benefit from polyP antagonism regardless of \([\text{TF}]_{\text{wall}}\).
INTRODUCTION

Many anticoagulants target prothrombinase formation or thrombin, but can be associated with bleeding risks.\textsuperscript{1,2} Reducing thrombotic risk without effect on normal hemostasis may require targeting factors that promote thrombus propagation and stability.\textsuperscript{3} The contact pathway is not essential for hemostasis since 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.\textsuperscript{4} The prothrombotic function of FXII and FXI has been demonstrated in several animal vessel injury models.\textsuperscript{5-15} Additionally, platelet derived polyphosphate (polyP, ~ 60-70mer) has recently been identified as a weak FXII activator\textsuperscript{16} that also promotes the feedback activation of FXI by thrombin,\textsuperscript{17} FV activation by FXIa, FXa, or thrombin\textsuperscript{18,19} and enhances fibrin physical structure.\textsuperscript{20,21} Also, cationic inhibitors of polyP reduce venous and arterial thrombosis in animal injury models.\textsuperscript{22} These observations suggest that the contact pathway is a potential source of therapeutic targets for safer antithrombotic therapies.\textsuperscript{23} Distinct from 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 forming 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{28,29} The FXI-ASO trial demonstrates a role for FXIa in DVT, but does not resolve the role of either platelet polyP or FXIIa in post-arthroplasty DVT since FXIa can inactivate tissue factor pathway inhibitor (TFPI)\textsuperscript{30} and FXI can be feedback-activated by thrombin.\textsuperscript{31} During knee arthroplasty, FXIIa may (or may not) be activated by polyP, DNA/histones, RNA, sulfatides, or other factors.\textsuperscript{32}

We used a microfluidic assay of platelet deposition and coagulation on a type I fibrillar collagen/lipidated tissue factor (TF) surface that can trigger thrombin generation via the contact pathway and/or extrinsic pathway.\textsuperscript{24,33} We utilized 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 inhibits FXI activation by FXIIa but not FXI activation by thrombin, and O1A6 interferes with both FXI activation by FXIIa and FIX and FV activation by FXIa.\textsuperscript{34-36} O1A6 does not directly inhibit FXI activation by thrombin but disrupts 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 use of low level corn trypsin inhibitor (CTI, 4 μg/mL), 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 sec 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 PPXbd, the recombinant polyP-binding domain of E. coli exopolyphosphatase (IC50, 8.5 μg/mL).17,22 This microfluidic data with human blood demonstrated specific conditions when 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, USA), azide free anti-human CD61 antibody (BioLegend, San Diego, CA, USA), annexin V-FITC, anti-human CD41a, antibody anti-human CD61 antibody (BD Biosciences, San Jose, CA, USA), Alexa Fluor 647 conjugated human fibrinogen (Life Technologies, Grand Island, NY, USA), corn trypsin inhibitor (CTI, Haematologic Technologies, Essex Junction, VT, USA), Dade® Innovin® PT reagent (Siemens, Malvern, PA, USA), collagen (type I, Chrono-log, PA, USA), recombinant human tissue plasminogen activator (tPA, abcam, MA, USA), ethylenediaminetetraacetic acid (EDTA), grade I glutaraldehyde, sodium cacodylate, hexamethyldisilane (sigma, St. Louis, MO, USA) and H-Gly-Pro-Arg-Pro-OH (GPRP, EMD Chemicals, San Diego, CA, USA). The murine anti-human FXI monoclonal antibodies O1A6 and 14E11 were gifts from Dr. Andras Gruber (Oregon Health & Science University). Polyphosphate binding protein (PPXbd) was prepared as described in the supplemental material.17 A custom made thrombin sensitive peptide azidoacetyl-AK(5FAM)-GALVPRGSAGK(CPQ2)-NH2 was obtained from CPC scientific (Sunnyvale, CA, USA) for click reactions to anti-CD61 as previously described.37

Preparation and characterization of collagen/TF surface

Glass slides were rinsed with ethanol followed by deionized water and dried with filtered air. A volume of 5 μL collagen was perfused through the patterning channel (250 μm wide x 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 5-fold with HEPES buffered saline to obtain low, medium, and high [TF]wall surface densities of ~0.1, ~0.2, and ~2 molecule-TF/μm², respectively, as estimated by imaging of sorbed FITC-annexin V-stained vesicles (Fig. S1). In all experiments, the PT reagent was
incubated with the collagen for 30 min without flow, followed by rinsing and blocking with 20 μL bovine serum albumin (0.1% BSA) buffer.

**Blood collection and preparation**

Blood was obtained via venipuncture into CTI (4 μg/mL) from healthy donors who self-reported to be 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 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 the measurement of fibrin generation. All experiments were initiated within 5 min after phlebotomy.

**Microfluidic clotting assay on collagen/±TF surface**

An eight channel PDMS (polydimethylsiloxane) flow device was vacuum-mounted perpendicularly to collagen/TF surfaces forming eight parallel spaced prothrombotic patches (250 × 250 μm) as previously described. Treated blood was perfused across the 8 channels by withdraw through a single outlet. Initial wall shear rate was controlled by a syringe pump (Harvard Apparatus PHD 2000, 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) condition. 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, USA). Images were captured with a CCD camera (Hamamatsu, Bridgewater, NJ, USA) and were analyzed with ImageJ (NIH). To avoid side-wall effects, fluorescence values were only taken 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 HBS buffer. The glass slides were then removed from the PDMS 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 (with balance of sodium cacodylate), rinsed with hexamethyldisilane, air dried, and sputter coated with gold/palladium. Samples were imaged with Quanta 600 FEG Mark II scanning
electron microscope equipped with Schottky field emission electron gun. Thickness of fibrin fibers was averaged across measurements (Image J, NIH) from 40 random selected fibers in images captured at 3500x magnification.

**Statistical analysis**

Difference between control and treated groups was analyzed with Student’s t-test. The difference was considered significant when p-value is smaller than 0.05.

**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 sec\(^{-1}\) over a collagen surface (no [TF]\_wall). Perfusion of whole blood (treated only with 4 μg/mL CTI) without antibodies resulted in immediate and rapid platelet accumulation with thrombin and fibrin production detected after 300 sec of perfusion. Both antibodies had minimal effect on platelet deposition, which is driven by the collagen and platelet-derived secondary aggregation mediators ADP and thromboxane. Both antibodies caused complete inhibition of thrombin generation and fibrin formation (Figure 1). In the presence of 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 with prior observations made with whole blood from a patient with a severe FXI deficiency. 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 (Figure S2). Since addition of CTI and 14E11 together (Figure 1 and Figure S2C-D) prevented thrombin and fibrin formation, the presence of bloodborne tissue factor can be excluded.

*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 over the first 420 sec (Figure 2A-C). By 500 sec, 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 sec 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 min also increased. At low [TF]\_wall ~0.1 molecule-TF/μm\(^2\), fibrin formation was detectable at ~240 sec and was significantly reduced by both 14E11 or O1A6 at times between 300 and 400 sec (Figure 2D). O1A6 maintained its ability to inhibit fibrin generation up to medium [TF]\_wall ~0.2 molecule-TF/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) 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 sec (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 (Figure S3).

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

Since 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 where 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 condition, 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 = 0.012) and 70% (p = 0.037) on collagen/low [TF]wall ~0.1 molecule-TF/µm² (Figure 4D, F). The reduction in thrombin signal became significant after 300 sec (Figure 4E, F). Under this flow condition, occlusive thrombi were observed at around 400 sec into 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 only observed when polyP was inhibited at no [TF]wall (Figure S4).

PPXbd blocked mechanisms downstream of FXIIa on collagen/low-medium [TF]wall

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

**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 due to 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 (Video 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 two extreme conditions: (i) preserving all formed fibrin by not adding lytic reagent, and (ii) blocking fibrin polymerization with GPRP. PPXbd had no significant effect on retraction under these two conditions (Figure 7B-C).

We also examined the impact of PPXbd on fibrin physical structure by measuring the fibrin fiber diameter in SEM micrographs of whole blood clots formed under flow condition (100 s⁻¹, Q = constant) 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 activation of FIX and FV using FXI antibodies 14E11 and O1A6, respectively. The extrinsic pathway was left intact but its relative contribution was tuned by varying wall TF concentration. Based on our observations, we propose a model showing relative contributions of the three major mechanisms of thrombin generation: FXIIa dependent thrombin generation, FXIa mediated thrombin amplification, and TF induced thrombin generation (Figure S5). The contact pathway was required for thrombin generation on collagen surfaces since 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. Thrombin feedback mechanism became more detectable since O1A6 but not 14E11 caused significant reduction in fibrin generation. Finally, when [TF]wall exceeded 2 molec-TF/µm², neither of the FXI antibodies reduced fibrin formation, indicating thrombin was generated primarily via the extrinsic pathway. The activity of the two antibodies, especially at low
[TF]wall, in reducing fibrin formation was essentially due to the inhibition of contact activation, as was observed 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 endogenous activator of FXII\textsuperscript{16,43-45}, which raises the question whether polyP is physiologically important, as TF is usually present at injury sites. Our microfluidic data supports 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.\textsuperscript{27} The fact that adding PPXbd to FXIIa inhibited blood caused further reduction in fibrin at low-medium [TF]wall suggests that polyP potentiating pathway(s) downstream of FXIIa activation of FXI that requires low participation of the extrinsic pathway. Insignificant thrombin feedback caused by insufficient thrombin generation on collagen or overwhelmed thrombin generation 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 (Figure S4). 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 only detectable 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 condition at low (but not no) [TF]wall suggests a similar promoting role of polyP in arterial thrombosis. However, we found the role of polyP as FXII activator in this microfluidic model was less important as surface-immobilized long chain polyP (700mer), which was shown in well plate as a much more potent FXII activator than platelet-derived short chain polyP \textsuperscript{42}, failed to promote fibrin generation on a collagen (no [TF]wall) surface (Figure S6). 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 only occurred when TF and polyP were both absent even though total platelet fluorescence was not affected, indicating factor(s) other than total deposited platelet mass (i.e. 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\textsuperscript{20,42} and attenuating binding of fibrinolytic proteins to fibrin.\textsuperscript{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 polyP attenuated retraction of occlusive thrombi during fibrinolysis. We speculate that polyP modulated contraction by enhancing fibrin structure based on 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 only significant upon fibrinolysis. Platelets are known to generate heterogeneous contractile force based on the stiffness of surrounding environment.\(^{46}\) It is possible that polyP incorporated into fibrin fibers caused the fibers to exhibit better retention of 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\(^{20}\) since similar attenuating effect was observed at both low and high [TF]\(_{\text{wall}}\). 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 upon [TF]\(_{\text{wall}}\). To 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 with the presence of surface immobilized TF.

**AUTHORSHIPS**

Contributions: S.Z. and S.L.D. designed research and wrote the manuscript; S.Z. and R.J.T. performed the research; 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.

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REFERENCES


FIGURE LEGENDS

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 collagen surface at 100 s⁻¹ under constant flow rate condition (Q = constant). FXI antibodies efficiently abolished thrombin and fibrin generation on collagen surface without affecting platelet deposition. Dynamics of platelet deposition (A), and generation of fibrin (B) and thrombin (C) based on 5 clotting events (± SD, shaded). Endpoint images (t = 600 sec) of platelet (red), fibrin (green) and thrombin (cyan) on collagen surface are embedded in corresponded subgraphs (white arrows indicate flow direction, scale bar = 50 μm). Final platelet, fibrin and thrombin fluorescence (t = 600 sec) was normalized to control (D). Adding FXI antibodies caused over 90% reduction in final fibrin and thrombin generation. (**, p < 0.01; ***, p < 0.005)

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 (Q = constant). Platelet deposition on collagen was unaffected by FXI antibodies despite the difference in [TF]wall (A-C). O1A6 showed inhibitory effect on fibrin generation at low and medium [TF]wall (D, E). 14E11 only reduced fibrin generation at low [TF]wall (D). Neither of the antibodies caused reduction in fibrin at high [TF]wall (F). (*, p < 0.05; **, p < 0.01)

Figure 3. PPXbd inhibits fibrin generation at low [TF]wall under venous condition. 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 100 s⁻¹ (Q = constant). Platelet deposition on collagen was unaffected by PPXbd despite the difference in [TF]wall (A-C). Fibrin generation was only inhibited by PPXbd on collagen/low [TF]wall surface but not on collagen or collagen/high [TF]wall surface (D-E). (*, p < 0.05; **, p < 0.01)

Figure 4. PPXbd inhibits fibrin and thrombin generation on collagen/low [TF]wall at arterial shear rate. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with PPXbd (250 μg/mL) and was perfused over or collagen or collagen/low [TF]wall surface at 1000 s⁻¹ under constant pressure drop condition (∆P = constant). Platelet deposition on collagen was unaffected by PPXbd (A, B). Fibrin generation was inhibited by PPXbd on collagen/low [TF]wall surface (D) but not on the collagen-alone surface (C). Consistent with the reduction in fibrin on collagen/low [TF]wall, thrombin generation at low [TF]wall was also lowered by PPXbd after 300 sec (E, F). (*, p < 0.05; **, p < 0.01)

Figure 5. PPXbd inhibits pathways downstream of FXIIa. Anticoagulated whole blood (CTI, 4 μg/mL) was treated with 14E11 (20 μg/mL) to inhibit FXIIa activation of FXI (A) or with O1A6 to block FIX activation by FXIa (B) and was perfused over collagen/low-medium [TF]wall surface at 100 s⁻¹ under constant flow rate mode (Q = constant). Adding PPXbd to 14E11 treated blood caused significant reduction in fibrin signal after the first 200 sec. PPXbd showed no effect on fibrin generation when it was added to O1A6 treated blood. (*, p < 0.05; **, p < 0.01)

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

**Figure 7. PPXbd enhances clot retraction after flow cessation and alters fibrin fiber thickness.** Clot retraction was quantified by the reduction in area at the downstream edge, as measured from just prior to occlusion (pink outline) to endpoint of experiment (white area) (A). Scale bars represent 50 μm. Anticoagulated WB (4 μg/mL CTI) was either untreated (B) or treated with 5 mM GPRP (C) or 30 nM recombinant tPA (D) 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, Q = constant) on collagen/high [TF]_{wall} surface with (E) or without (F) PPXbd or on collagen/low [TF]_{wall} surface with (H) or without (I) PPXbd. Left subgraphs (scale bar = 100 μm) of E,F,H,I show the structure of whole thrombi while right subgraphs (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 high (G) and low [TF]_{wall} (J). (*, p < 0.05; **, p < 0.01)
Figure 1

(A) Platelet Fluorescent Intensity

(B) Fibrin Fluorescent Intensity

(C) Thrombin Fluorescent Intensity

(D) Normalized Fluorescent Intensity (%)

- Control
- +14E11
- +O1A6

n = 5 (1 donor)
t = 600 sec

n.s.

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Platelet
Fibrin
Thrombin
Figure 2

(A) Collagen/TF (≈0.1 molecules/μm²)

(B) Collagen/TF (≈0.2 molecules/μm²)

(C) Collagen/TF (≈2 molecules/μm²)

(D) Fibrin

(E) Platelet intensity

(F) Platelet intensity

- Control
- + 14E11
- + O1A6

n= 14 (7 donors)

* p < 0.05
** p < 0.01
n.s. not significant
Figure 3

100 s⁻¹ (Q=constant)

A) Collagen

- Control
- + PPXbd

n > 10 (5-10 donors)

B) Collagen/TF (~0.1 molecules/μm²)

C) Collagen/TF (~2 molecules/μm²)

D) Fibrin

E) Fibrin

F) Fibrin

- n.s.
- *
- **
Figure 4

1000 s⁻¹ (ΔP=constant)

A. Collagen

- Platelet Fluorescent Intensity
  - Control
  - + PPXbd
  - n=10 (5 donors)

B. Collagen/TF (~0.1 molecules/µm²)

- Platelet Fluorescent Intensity

C. Fibrin Fluorescent Intensity

- Fibrin Fluorescent Intensity

D. Fibrin Fluorescent Intensity

E. Thrombin Fluorescent Intensity

- Thrombin Fluorescent Intensity

F. Thrombin Fluorescent Intensity

- Thrombin Fluorescent Intensity

Figure 4
Figure 5

A. Collagen/TF (~ 0.1 - 0.2 molecules/μm²)

- + 14E11
- + PPXbd and 14E11

n = 16 (4 donors)

B. Collagen/TF (~ 0.1 - 0.2 molecules/μm²)

- + O1A6
- + PPXbd and O1A6

n = 12 (4 donors)

Fibrin Fluorescent Intensity vs. Time (min)

* p = 0.036
* p = 0.039
n.s.
Figure 7

(A) Diagram showing area reduction with a constant ΔP and 100 s⁻¹. (B) Graph showing area reduction in control conditions with high and low TF, n = 9, 3 donors, p = 0.13, n.s., and p = 0.17, n.s., respectively. (C) Graph showing area reduction with +GPRP in high and low TF conditions, n = 12, 4 donors, p = 0.03, * and p = 0.002, **. (D) Graph showing area reduction with +tPA in high and low TF conditions, n = 12, 4 donors, p = 0.002, **.

(F) Images showing fiber thickness with control and PPXbd conditions. (G) Bar graph showing fiber thickness comparison between control and PPXbd conditions, with ** indicating statistical significance.

(H) Images showing fiber thickness with control and PPXbd conditions. (I) Images showing fiber thickness with control and PPXbd conditions. (J) Bar graph showing fiber thickness comparison between control and PPXbd conditions, with * indicating statistical significance.
FXIa and platelet polyphosphate as therapeutic targets during human blood clotting on collagen/tissue factor surfaces under flow

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