

Factor VIIa-mediated tenase function on activated platelets under flow

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Summary. *Background:* Tissue factor (TF) and/or active factor (F)VIIa may be stored inside resting platelets. *Objectives:* The objective of this study was to examine if platelets, following activation of GPVI, could support tenase and prothrombinase activity without any exogenously added tissue factor. *Methods:* Thrombin (IIa) formation on gel-filtered platelets with added factors or the clotting of platelet-free plasma (PFP) or platelet-rich plasma (PRP) supplemented with corn trypsin inhibitor (CTI) (to inhibit factor XIIa) was studied in well plate assays with a fluorogenic thrombin substrate or in flow assays by fibrin visualization. *Results:* Pretreatment of convulxin (CVX)-stimulated, fibrinogen-adherent, gel-filtered platelets with anti-TF, anti-FVII/VIIa, or 1 nM PPACK [inhibitor of FVIIa, factor XIa and factor (F)IIa] delayed fibrin deposition on platelets perfused with PFP/CTI at 62.5 s^{-1} . Anti-TF or anti-FVII/VIIa also attenuated thrombin generation in plate assays using recalcified PRP/CTI treated with CVX. Anti-TF or anti-FVII/VIIa (but not inhibited factor IXa) delayed the burst in thrombin production by gel-filtered platelets suspended in prothrombin and CVX by 14 min and 40 min, respectively. Anti-FVII/VIIa completely eliminated thrombin generation on fibrinogen-adherent, gel-filtered platelets pretreated with $10 \mu\text{M}$ PPACK and $10 \mu\text{M}$ EGR-CK [inhibitor of factor (F)Xa], rinsed, and then supplemented with CVX, prothrombin, and FX. Addition of anionic phospholipid to PFP/CTI or to a mixture of prothrombin, FX, and recVIIa was not sufficient to generate detectable tenase activity. Lastly, isolated, unactivated neutrophils suspended in FX, FII and recVIIa supported a very low level of thrombin generation sensitive to antagonism of P-selectin, CD18, and TF.

Conclusions: Activated platelets supported tenase and prothrombinase activity by elevating the function or level of FVIIa and exposing active FVIIa or FVIIa-cofactor(s), distinct from anionic lipid, that may be, in part, TF.

Keywords: convulxin, fibrin, GPVI, thrombin.

Introduction

Activated platelets provide anionic phospholipid binding sites for coagulation proteins and can release factor (F)V/Va for coagulation amplification. However, it is not fully clear how platelets, upon activation, are capable of initiating a coagulation response on their own without wall-derived tissue factor (TF). In the current view of coagulation [1,2], a small amount of thrombin, initially produced through TF/factor (F)VIIa activation of factor (F)IX and factor (F)X, is needed to activate the platelet and the coagulation factors FV, factor (F)XI, and factor (F)VIII. This is followed by FIXa/FVIIIa-mediated production of FXa, which then forms the prothrombinase complex on the activated platelet with FVa. Recent studies indicate that TF, stored in the α granules of resting platelets, may become exposed on the platelet membrane following activation by collagen [3,4]. Zillman *et al.* [5] reported the possibility that platelets contain TF (potentially transferred from plasma or leukocytes) that can be activated by interaction with leukocytes. Interestingly, FXa activity on the surface of activated platelets and platelet-derived microparticles has been found in assays that lack factor FX/Xa from external sources [6]. The relationships between 'blood-borne' TF [7], platelets, and neutrophils with respect to the initiation of coagulation remain a subject of investigation.

Collagen-adherent platelets are clearly more procoagulant than fibrinogen-adherent platelets. Heemskerk *et al.* [8] showed that while fibrinogen-adherent platelets extensively spread without detectable Ca^{2+} or secretion signals, collagen-adherent platelets demonstrate a sustained increase in cytosolic Ca^{2+} accompanied by bleb formation, phosphatidylserine (PS) exposure, and procoagulant activity. Higher thrombin

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generation on collagen-adherent platelets than on fibrinogen-adherent ones has been associated with the presence of platelet-bound FVa [9]. GPVI, which is associated with the Fc receptor γ -chain (FcR γ) [10], is the likely mediator of the signaling response to collagen [11,12]. Adhesion and activation of platelets through GPVI has been found to be a trigger for inducing PS exposure on platelets [13]. Convulxin (CVX), a C-type lectin isolated from *Crotalus durissus terrificus* venom, is a potent GPVI activator [10,14]. The stimulation of platelets with CVX is accompanied by an increased procoagulant activity, as demonstrated in our previous studies by increased thrombin generation under static conditions as well as increased fibrin accumulation on platelets under flow conditions [15]. Through experiments conducted with neutrophil cathepsin G-stimulated platelets, we have shown that cathepsin G can promote fibrin deposition under flow by elevating the activation state of fibrinogen-adherent platelets. This enhancement of coagulation by neutrophil cathepsin G was found to occur through tissue factor-independent pathways. Individual fibrinogen-adherent neutrophils can also support marked fibrin deposition on their own when factor (F)XIIa (contact pathway) is present [16].

The objective of this study was to examine if platelets, following activation of GPVI, could support tenase and prothrombinase activity without any exogenously added TF. We used gel-filtered platelets, platelet-rich plasma (PRP), plasma, and purified clotting factors to study procoagulant function of CVX-activated platelets. The effects of TF or FVIIa antagonism on thrombin generation were quantified using well-plate assays and flow assays aimed at understanding the function of platelet activation in the initiation of thrombosis under conditions of stasis or venous flow conditions.

Methods

Murine antihuman FVII/VIIa mAB IgG (American Diagnostica, Stamford, CT, USA), corn trypsin inhibitor (CTI) and FIXa-EGR (active site inhibited human FIXa; Hematologic Technologies Inc., Essex Junction, VT, USA), sheep antihuman TF pAB IgG (TF pAB), human FX, human FVIIa, fibrinogen and human prothrombin (Enzyme Research Laboratories, South Bend, IN, USA), anti-CD18 mAB IB4 (Ancell, Bayport, MN, USA), sheep antihuman P-selectin pAB IgG (R&D Systems, Minneapolis, MN, USA), human serum albumin (Golden West Biologicals, Temecula, CA, USA), bovine brain L- α -phosphatidyl-L-serine, and bovine brain L- α -phosphatidylcholine (Sigma, St Louis, MO, USA), and CVX (Centerchem, Norwalk, CT, USA) were stored following the manufacturers' recommendations. D-Phe-Pro-Arg-CMK, HCl (PPACK) and H-Glu-Gly-Arg-CMK (EGR-CK) were obtained from Calbiochem (San Diego, CA, USA). NovoSeven [recombinant FVIIa (recVIIa)] was from Novo Nordisk (Princeton, NJ, USA). The fluorogenic substrate for thrombin, boc-Val-Pro-Arg-methylcoumarin amide (boc-VPR-MCA), was obtained from Peninsula Laboratories (San Carlos, CA, USA).

Cell isolation

Human blood was collected from healthy adult donors by venepuncture and anticoagulated with Na-citrate (nine parts blood to one part Na-citrate) or Na-citrate supplemented with CTI (50 $\mu\text{g mL}^{-1}$), PPACK (10 μM) and/or EGR-CK (10 μM). PRP was obtained by centrifugation of anticoagulated whole blood at $130 \times g$ for 15 min. Platelet singlets were prepared by gel filtration [16,17]. Neutrophils were isolated by centrifugation over neutrophil isolation medium (Robbins Scientific, Sunnyvale, CA, USA) as previously described [16,18]. Following isolation, neutrophils or platelets were diluted to final concentrations of 10^6 or 10^8 cells mL^{-1} , respectively.

Microcapillary flow chambers

Rectangular glass capillaries (Vitrocom, Mountain Lakes, NJ, USA) with a cross section of 0.2×2.0 mm, a length of 7 cm and a wall thickness of 0.15 mm were used as flow chambers as previously described [15–17]. To enable adhesion of platelets, microcapillary flow chambers were incubated with human fibrinogen solution (100 $\mu\text{g mL}^{-1}$) for 120 min at room temperature. The chambers were rinsed and platelets were allowed to adhere under no-flow conditions for 30 min. To activate fibrinogen-adherent platelets in selected experiments, CVX (10 nM) was perfused over the platelets for 5 min. In some experiments, a mixture of PPACK (1 nM) and CVX was perfused over fibrinogen-adherent platelets for 5 min to inhibit any FVIIa, FXIa, or factor (F)IIa on the activated platelet surface that may possibly be present following CVX-mediated stimulation of platelet GPVI. In selected experiments, platelets were pretreated with TF pAB (50 $\mu\text{g mL}^{-1}$) or FVII/VIIa mAB (25 $\mu\text{g mL}^{-1}$) for 10 min. Stimulation with CVX and treatment with PPACK, TF pAB, or FVII/VIIa mAB were concurrent. Platelet-free plasma (PFP) treated with CTI (50 $\mu\text{g mL}^{-1}$) was perfused into the flow chambers containing defined surface compositions at a controlled flow rate using a syringe pump (Harvard Apparatus, Holliston, MA, USA). The wall shear stress (τ_w) was calculated by $\tau_w = (6Q\mu)/(B^2W)$, where Q represents the flow rate ($\text{cm}^3 \text{s}^{-1}$), μ represents the viscosity (0.01 Poise at room temperature), B represents the total plate separation (0.02 cm), and W represents the width (0.2 cm). The wall shear rate, γ_w (s^{-1}) was calculated as $\gamma_w = 6Q/B^2W$. During flow experiments, the flow chambers were mounted on a Zeiss Axiovert 135 microscope and a 63X (NA 1.40) oil immersion objective lens (Plan Apochromat) was used to conduct differential interference contrast (DIC) microscopy to visualize fibrin fibers.

Fluorogenic measurement of thrombin generation

TF pAB and FVII/VIIa mAB were mixed with either PRP or recalcified gel-filtered platelets (10^8 cells mL^{-1}) that had been supplemented with prothrombin (1.5 μM) in a 96-well plate. FX (2 nM) was also added to gel-filtered platelets in some experiments. The FX concentration was selected to be much lower than the plasma concentration to slow down the

amplification routes of coagulation. Additionally, there were no inhibitors to antagonize FXa or TF-FVIIa in the purified systems used. Thus a lower level of FX was used compared with levels in blood. Fluorogenic substrate for thrombin (tbo-Val-Pro-Arg-MCA; 20 μM final concentration) was added to the reaction mixture (100 μL per well) for kinetic measurements of thrombin generation. Because picomolar levels of thrombin were present in prothrombin as an impurity (detected by the thrombin substrate), prothrombin was titrated with fresh PPACK to find the ideal PPACK concentration for complete thrombin inhibition. Prothrombin (II) was then pretreated with excess PPACK (150 μM or 400 μM) and incubated for at least 1 h to eliminate FIIa contamination of FII. In selected experiments, CVX (10 nM) was added to the reaction mixture to activate platelets. To enable cell adhesion, wells were incubated with human fibrinogen solution (100 $\mu\text{g mL}^{-1}$) for 120 min at room temperature. Gel filtered platelets were allowed to settle and adhere to the fibrinogen-coated surface for 30 min. Following adhesion, in selected experiments the cells were washed with PPACK (10 μM ; inhibitor of FVIIa, FIIa and FXIa) and EGR-CK (10 μM ; inhibitor of FXa) to inhibit any FVIIa, FIIa, FXIa, or FXa that may be present on the surface of gel-filtered platelets. Finally, platelets were washed three times with HBSS. Fluorescence in the 96-well plate was measured using a Fluoroskan Ascent fluorometer (Ex 390 nm; Em 460 nm). All well-plate experiments were performed at 37 $^{\circ}\text{C}$.

In some experiments, anionic phospholipid vesicles (50 μM) were mixed with PFP or with FX (2 nM), prothrombin (700 nM), and recFVIIa (0.2 nM) prior to measurement of thrombin production. To prepare vesicles, L- α -phosphatidyl-L-serine (PS) and L- α -phosphatidylcholine (PC) were mixed (20 : 80 wt percentage) and dried under nitrogen. As previously described [15], the dry film was hydrated with buffer (20 mM HEPES, 150 mM NaCl) and sonicated for 15 min. To measure thrombin generation on neutrophils, individual neutrophils, isolated from whole blood drawn in a mixture of Na-citrate, CTI, PPACK and EGR-CK, were mixed in prothrombin, FX and/or recFVIIa. In selected experiments, neutrophils were pretreated (10 min) with P-sel pAB (25 $\mu\text{g mL}^{-1}$), TF pAB (1 : 20 dilution), or CD18 mAB (25 $\mu\text{g mL}^{-1}$).

Results

Fibrin formation on GPVI-activated fibrinogen-adherent platelets

To investigate if platelet-associated TF or FVIIa can facilitate fibrin deposition on CVX-activated platelets under flow, fibrinogen-adherent platelets were pretreated for 10 min with CVX (Fig. 1A), CVX and FVII/VIIa mAB (Fig. 1B), or CVX and TF pAB (Fig. 1c) and then were thoroughly rinsed with HBSS under flow (5 min at $\gamma_w = 200 \text{ s}^{-1}$). Finally, recalcified citrated PFP/CTI was perfused ($\gamma_w = 62.5 \text{ s}^{-1}$) over all three

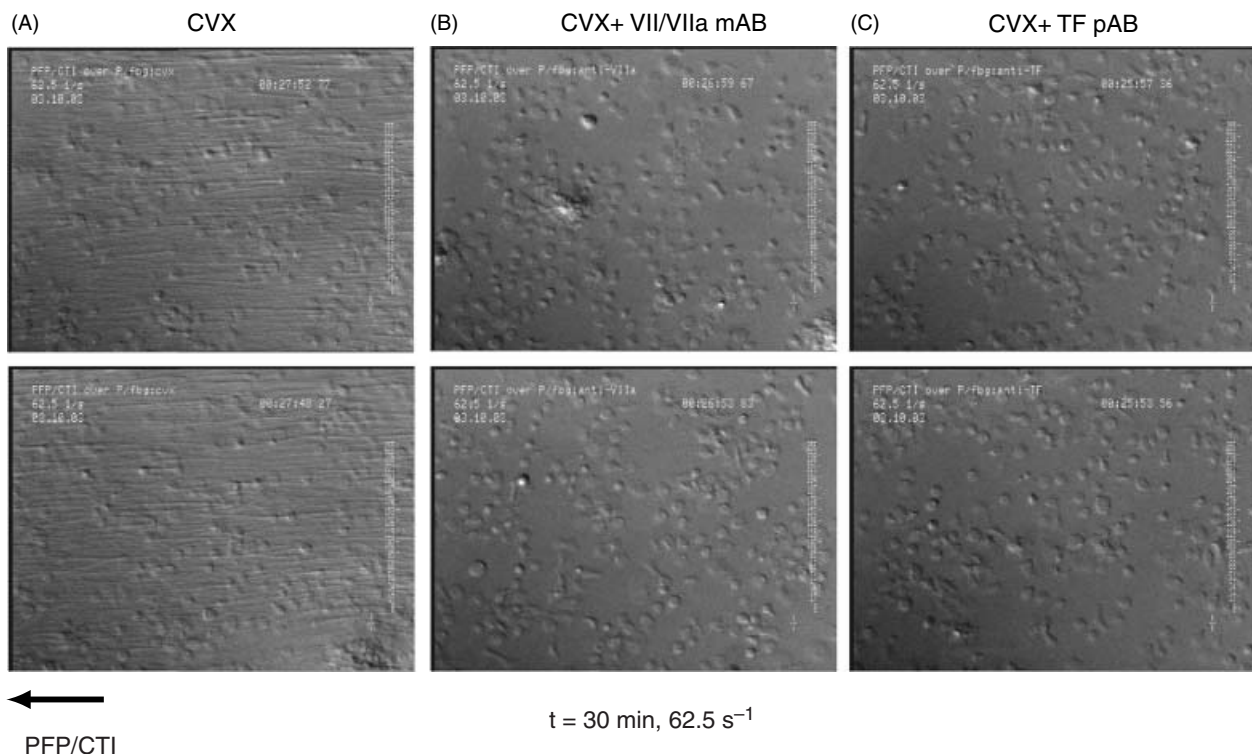


Fig. 1. Antibodies against tissue factor (TF) or factor (F)VII/VIIa delay fibrin formation on convulxin (CVX)-stimulated fibrinogen-adherent platelets under flow. When recalcified citrated platelet-free plasma containing corn trypsin inhibitor (CTI) (50 $\mu\text{g mL}^{-1}$) was perfused ($\gamma_w = 62.5 \text{ s}^{-1}$) over fibrinogen-adherent platelets activated with CVX, fibrin formation could be observed at 30 min. In contrast, when platelets were pretreated with anti-FVII/VIIa (25 $\mu\text{g mL}^{-1}$) or TF pAB (50 $\mu\text{g mL}^{-1}$), no fibrin was observed by 30 min. Flow is from right to left.

surfaces. Fibrin accumulated by 30 min on platelets stimulated with CVX alone (Fig. 1A), while negligible fibrin appeared on platelets pretreated with both CVX and blocking antibodies (Fig. 1B,C). Because all the platelet surfaces had been thoroughly rinsed with buffer prior to the perfusion of PFP and no antibodies were added to the PFP, these results indicate that some amount of functional FVIIa and/or TF was initially displayed on CVX-activated platelets before perfusion and was responsible for the massive fibrin deposition seen on the surface in Fig. 1A. Fibrin fibers did not begin to appear on surfaces in Fig. 1B,C until 38 min. No fibrin was formed on fibrinogen-adherent platelets without CVX treatment by 40 min (not shown) when the experiment was terminated.

In a related experiment, recalcified citrated PFP containing CTI was perfused at a wall shear rate of 62.5 s^{-1} over two different surfaces: fibrinogen-adherent CVX-stimulated plate-

lets and fibrinogen-adherent CVX-stimulated platelets also pretreated with low-level PPACK (1 nM). Both surfaces were rinsed before PFP/CTI perfusion. By $t = 40 \text{ min}$, fibrin formation was detected (as expected) on the first surface; however, no fibrin deposition could be observed on the PPACK-treated surface (Fig. 2). This initial treatment of GPVI-activated platelets with PPACK, an inhibitor of FVIIa, FXIa and FIIa, abolished fibrin deposition under flow on these platelets at $t = 40 \text{ min}$. It remains unclear whether this fibrin reduction is due to FVIIa inhibition or due to thrombin inhibition by residual PPACK because a low level (1 nM) of PPACK is not likely to inhibit FVIIa fully in 5 min. However, fibrin reduction under similar conditions by an antibody against FVII/VIIIa (Fig. 1B), and as seen in the next section, demonstrated that such fibrin reduction on platelets can result from an initial inhibition of FVIIa. Results in Fig. 4 will show

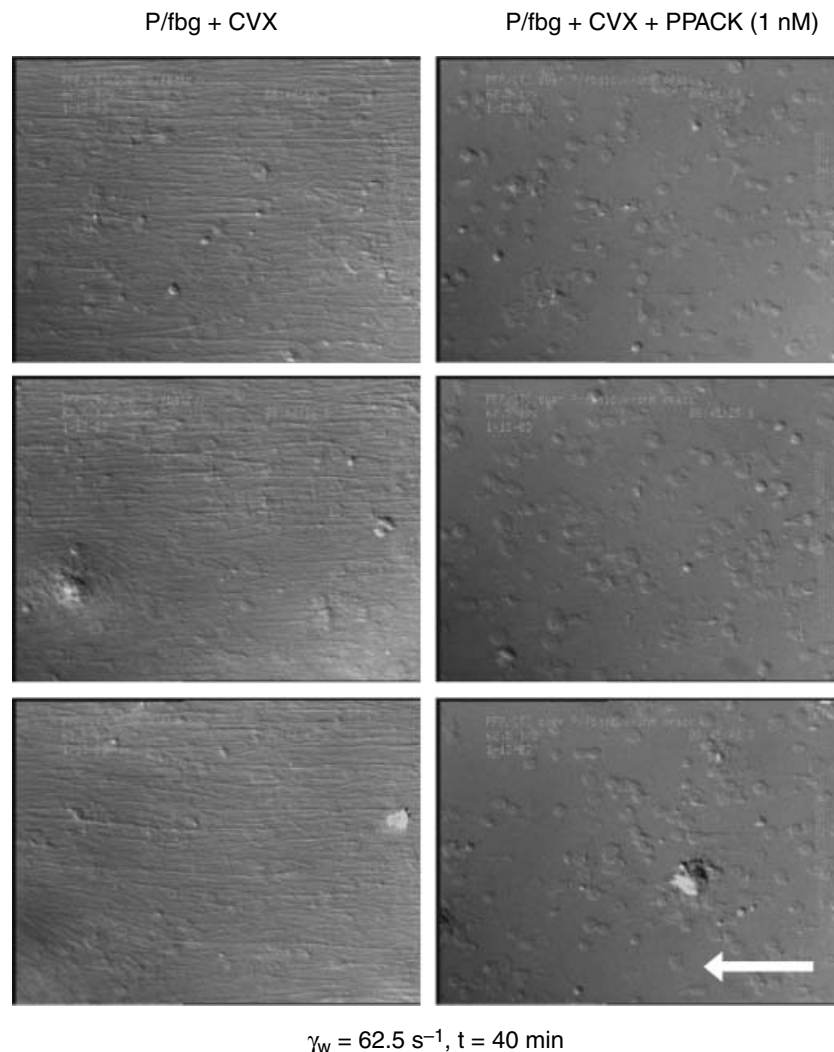


Fig. 2. Fibrin deposition on adherent GPVI-activated platelets was abolished by PPACK pretreatment. Recalcified citrated platelet-free plasma (PFP)/corn trypsin inhibitor (CTI) was perfused at a shear rate of 62.5 s^{-1} for 40 min over convulxin (CVX)-treated (10 nM) fibrinogen-adherent platelets, and fibrinogen-adherent platelets treated with both CVX and PPACK (1 nM). Fibrin accumulation was observed only on the former surface, demonstrating that initial pretreatment of activated platelets by PPACK (inhibitor of factors VIIa, XIa and IIa) prevented fibrin formation on these cells. Flow is from right to left.

that FXIa was least likely to have a role in this procoagulant activity over CVX-stimulated, gel-filtered platelets.

Procoagulant activity in CVX-activated PRP

When recalcified citrated PRP (1 : 2 dilution; obtained from whole blood drawn into citrate, CTI and 100 μM PPACK) was treated with CVX, peak thrombin generation (t_{50} , the time to reach 50% thrombin substrate conversion) was observed at 69 ± 6 min (Fig. 3). When PRP, pretreated with TF pAB (1 : 20 dilution) or FVII/VIIa mAB (50 $\mu\text{g mL}^{-1}$), was stimulated with CVX (total mixture volume per well = 150 μL), thrombin generation was greatly reduced at even 90 min. This observation supports the role of TF and FVIIa in the initiation and/or enhancement of coagulation in diluted PRP/CTI under static conditions, but does not specify if TF and FVIIa originated with the platelet or were present in plasma. As a control, progress curves for thrombin generation in diluted PRP/CTI without CVX treatment showed minimal thrombin production even after 90 min.

Prothrombinase activity on CVX-activated, gel-filtered platelets in suspension

Further studies were conducted using gel-filtered platelets in suspension in place of PRP. To verify that stimulation by CVX turns platelets procoagulant, thrombin generation on recalcified gel-filtered platelets supplemented with prothrombin (pretreated with 150 μM PPACK) was compared in the presence or absence of CVX (10 nM). Again, thrombin generation was measured by conversion of a fluorogenic

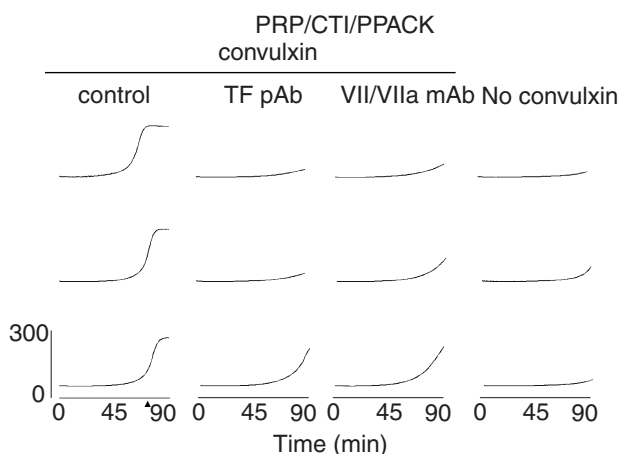


Fig. 3. Antibodies against tissue factor (TF) or factor (F)VII/VIIa delay thrombin generation in platelet-rich plasma (PRP). Measurements of thrombin generation in convulxin (CVX)-stimulated recalcified citrated PRP [containing corn trypsin inhibitor (CTI)] were made following pretreatment with TF pAB (1 : 20 dilution), pretreatment with anti-FVII/VIIa (50 $\mu\text{g mL}^{-1}$), or in the absence of antibodies. When PRP, mixed with TF pAB or FVII/VIIa mAB, was stimulated with CVX, thrombin generation was greatly reduced at 69 ± 6 min. Minimal thrombin production was observed in PRP not stimulated with CVX. Experiments were conducted in triplicate.

thrombin substrate in a 96-well plate assay (total mixture volume per well = 100 μL). Whereas negligible thrombin production was observed in gel-filtered platelets without CVX, dramatic thrombin was produced on CVX-stimulated platelets (Fig. 4A). Peak thrombin generation on CVX-activated platelets (t_{50}) was observed at 16 min, considerably faster than seen in Fig. 3 with diluted PRP probably due to the lack of plasma inhibitors (TFPI and AT) in Fig. 4A. The addition of antibodies against tissue factor (TF pAB; 25 $\mu\text{g mL}^{-1}$) or FVIIa (FVII/VIIa mAB; 25 $\mu\text{g mL}^{-1}$) significantly delayed the t_{50} by over 14 min and 40 min, respectively, and decreased the maximum rate of thrombin generation by 1.6 times and 2.3 times, respectively. The presence of FIXa-EGR (active site inhibited FIXa; 20 nM) did not delay thrombin generation in the platelet/prothrombin mixture (Fig. 4B), indicating that the intrinsic pathway of coagulation was not participating in prothrombinase formation on CVX-stimulated, gel-filtered platelets maintained in buffer essentially free of plasma. Thrombin activity was not detected in PPACK-treated prothrombin (Fig. 4B). This experiment demonstrated the roles of FVIIa and TF in GPVI-signaled tenase and prothrombinase activity on activated platelets in a minimal (but non-zero) plasma background. While no FX was added in this assay, thrombin generation on activated platelets in prothrombin alone indicated that FX must have been present with gel-filtered platelet and/or the added prothrombin. Results from the next experiment will clarify that this FX was present with gel-filtered platelets, not delivered with the added prothrombin.

To ensure that no activated factors (FVIIa, FXa, FIIa, or FXIa) were carried into the system during platelet isolation by gel filtration, platelets (isolated from whole blood drawn in Na-citrate, CTI, 10 μM PPACK and 10 μM EGR-CK) were adhered to fibrinogen-coated wells in a 96-well plate, incubated for 10 min with PPACK (10 μM ; inhibitor of FVIIa, FIIa and FXIa) and EGR-CK (10 μM ; inhibitor of FXa), and rinsed three times with HBSS. Finally, prothrombin (pretreated with 150 μM PPACK), CaCl_2 and the thrombin substrate were added in the wells over the adherent platelets (total volume of mixture = 100 μL). In the absence of FX supplementation and with high-dose PPACK/EGR-CK pretreatment, no thrombin generation was observed, demonstrating that the rinse steps had left platelets free of FXa (and FIIa) and no significant FX impurity was present in prothrombin (Fig. 5). However, when 2 nM FX was added, peak thrombin generation at 62 ± 8 min demonstrated substantial tenase and prothrombinase activity in a pure system consisting only of CVX-activated gel-filtered platelets (fibrinogen-adherent and PPACK/EGR-CK pretreated), calcium, prothrombin (II), FX, and the fluorogenic thrombin substrate. Thrombin generation in this platelet/FII/FX was completely eliminated by a monoclonal antibody against FVIIa. This observation not only confirmed the role of FVIIa in the observed tenase/prothrombinase activity on GPVI-activated platelets, but also raised a question regarding the source of this tenase activity in the absence of activated coagulation factors and TF from plasma. It is reasonable to conclude that FVIIa and/or TF-FVIIa, prestored inside

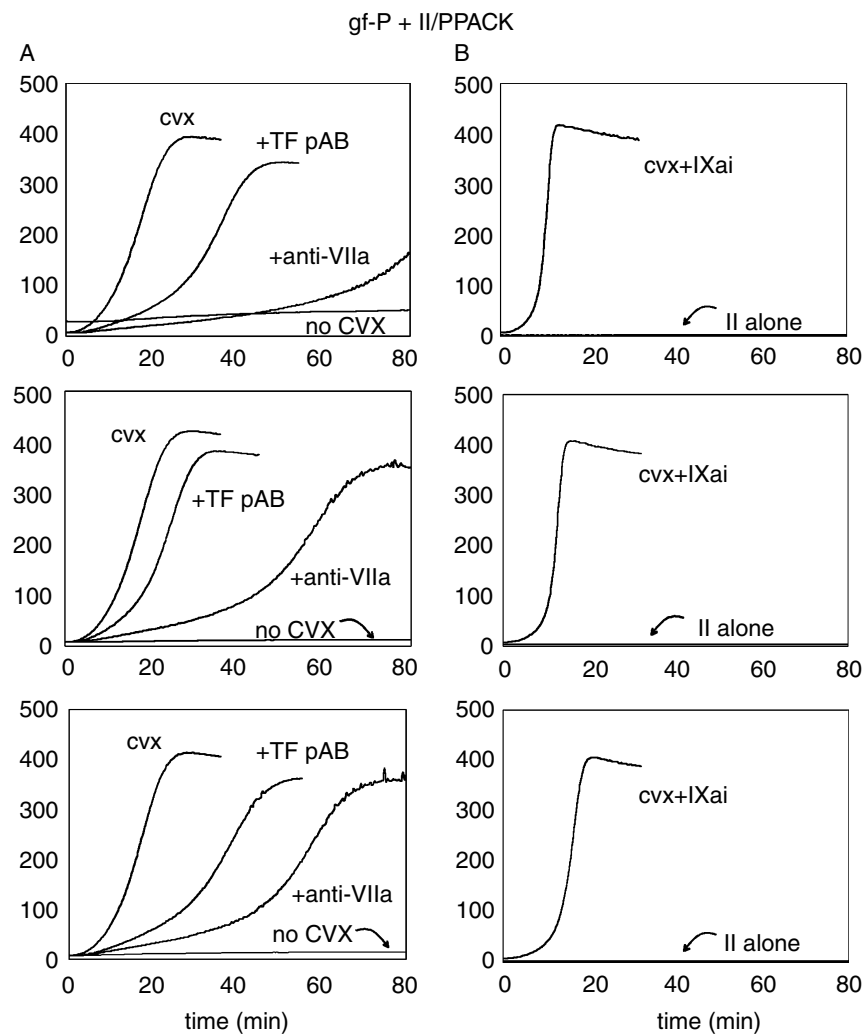


Fig. 4. Factor (F)VII/VIIa and tissue factor mediate prothrombinase activity on convulxin (CVX)-stimulated gel-filtered platelets. Progress curves of fluorescence intensity for fluorogenic thrombin substrate (boc-VPR-MCA) conversion in gel-filtered platelets supplemented with prothrombin ($1.5 \mu\text{M}$) were obtained in the absence of CVX, presence of CVX (10 nM), presence of CVX and polyclonal tissue factor antibody ($25 \mu\text{g mL}^{-1}$), and presence of CVX and FVIIa/VII antibody ($25 \mu\text{g mL}^{-1}$). The addition of either antibody reduced thrombin generation by CVX-stimulated platelets and minimal thrombin was produced in the absence of CVX stimulation (A). Active site inhibited factor IXa (IXa-EGR; 20 nM) did not affect thrombin generation (B). Experiments were conducted in triplicate.

platelets, were being presented following platelet activation by CVX. The block by an antibody against FVIIa also verified that no kinetically significant FXa or other activated coagulation factors (distal of FVIIa) were present on the platelet surface or carried with FII and FX as impurity at $t = 0 \text{ min}$. Also, when recFVIIa was added to the system, an enhanced thrombin generation was observed at $23 \pm 2 \text{ min}$. TF pAB produced highly variable effects (no effect, partial block, or strong block) in this experimental configuration, whereas the FVII/VIIa mAb always gave a strong block in this experiment.

Phospholipid exposure is not sufficient for initiation of coagulation

To test if anionic phospholipid exposure is sufficient for activation-enhanced coagulation, thrombin generation in

recalcified PFP/CTI mixed with PSPC vesicles was measured. Even though PFP already contains some FVIIa, no thrombin formation was observed in PFP or in PFP/PSPC after 2 h (not shown). Moreover, essentially no thrombin production was observed in a mixture of PSPC vesicles, FX, FII and recFVIIa. The maximum rate of thrombin generation on PSPC/FX/FII/recFVIIa was about 140 times or 675 times lower than that on gel-filtered platelets in the presence of FX alone or FX/FVIIa, respectively (seen in Fig. 5). These results confirm that mere anionic phospholipid exposure was not sufficient for initiation of coagulation.

Prothrombinase activity on isolated neutrophils

To investigate if neutrophils, like platelets, can support thrombin generation on their own, isolated neutrophils were

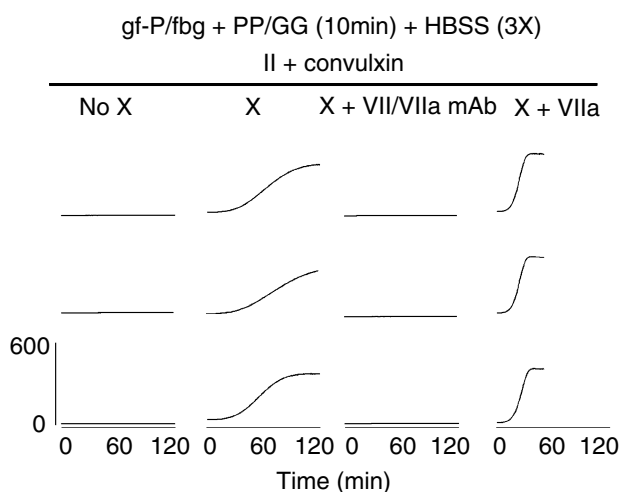


Fig. 5. Factor (F)VIIa-mediated prothrombinase activity on fibrinogen-adherent platelets. Thrombin generation was measured following addition of prothrombin ($1.5 \mu\text{M}$) to platelets that were adherent to fibrinogen-coated wells, washed with PPACK ($10 \mu\text{M}$ PP; inhibitor of FVIIa, factor IIa and factor XIa) and EGR-CK [$10 \mu\text{M}$ GG; inhibitor of factor (FX)a], and rinsed three times with HBSS (HBSS $3 \times$). While no thrombin generation was detected on convulxin (CVX)-stimulated adherent platelets (no FX), the addition of FX (2 nM) enhanced thrombin levels considerably. The presence of a monoclonal FVII/VIIa antibody ($25 \mu\text{g mL}^{-1}$) completely attenuated thrombin production by CVX-stimulated platelets in prothrombin and FX, whereas addition of FVIIa (0.2 nM) accelerated thrombin production. Experiments were conducted in triplicate.

mixed with FII, FX, and the thrombin substrate. A relatively low level of thrombin generation was detected (Fig. 6; note full scale = 100 fluorescence units). When recFVIIa was added to the neutrophil/FII/FX mixture, a detectable amount of thrombin was produced after a lag but without a non-linear, amplifying burst. This prothrombinase activity was considerably reduced by the pretreatment of isolated neutrophils with P-sel pAB, TF pAB, or CD18 mAB. Quantitatively, the

thrombin generated on neutrophils/FII/FX/FVIIa was sensitive to antagonism of P-selectin, TF, and CD18 and the total substrate conversion at 120 min was lower (by 2–5-fold) than that achieved on activated platelets in FII/FX (\pm FVIIa). Furthermore, the maximum rate of thrombin production on neutrophils at $0.5 \times 10^6 \text{ mL}^{-1}$ ($100 \mu\text{L}$ mixture per well) was 4.7 or 22.8 times lower than that of gel-filtered fibrinogen-adherent platelets (0.75×10^7 per well added for adhesion) in the presence of FII/FX or FII/FX/FVIIa, respectively. The inhibitory effect of P-sel pAB indicated a role for platelet membrane as a participant in neutrophil procoagulant function.

Discussion

Using suspension and adherent platelets (\pm flow), we used fluorogenic substrates and fibrin deposition to study mechanisms by which platelets make FXa and FIIa. We demonstrated that CVX activation of gel-filtered platelets turned them procoagulant through a FVIIa- and TF-dependent mechanism even in the absence of exogenously added TF. In an experiment where the possibility of FXa or FVIIa being initially present on the platelet surface before activation was eliminated by pretreatment with PPACK and EGR-CK, the existence of released active FVIIa and endogenous tenase activity on the activated platelet was evident (Fig. 5). Because no FVIIa from plasma was present in this experimental design, the most likely source of kinetically relevant FVIIa was the platelet itself. This conclusion was further supported by a delay in thrombin generation in PRP under static conditions and reduced fibrin deposition on adherent platelets under flow conditions by an antibody against FVII/VIIa. The conversion of FVII, which may be released from the platelet or may be present with the supplemented coagulation factors, to FVIIa on the platelet surface is a possibility, but this would again require FXa or

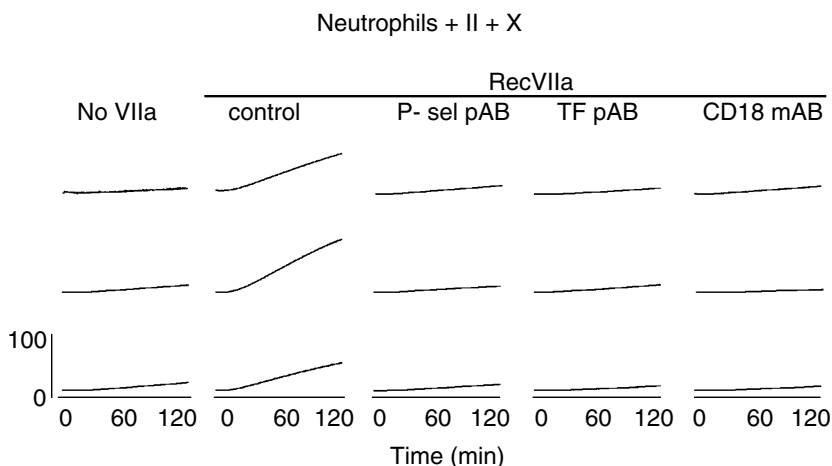


Fig. 6. Prothrombinase activity on isolated and unactivated neutrophils in suspension. Thrombin generation was measured following addition of prothrombin (II; 700 nM), factor X (2 nM), and recombinant factor (recF)VIIa (0.2 nM) to isolated neutrophils. While small amounts of thrombin were generated in the absence of FVIIa, considerable amounts were generated in the presence of recFVIIa. This thrombin generation was attenuated by pretreatment of neutrophils with P-selectin pAB ($25 \mu\text{g mL}^{-1}$), tissue factor (TF) pAB (1 : 20 dilution) or CD18 mAB ($25 \mu\text{g mL}^{-1}$). Experiments were conducted in triplicate.

Xase activity. Pretreatment of CVX-stimulated platelets with PPACK substantially delayed fibrin deposition on platelets under flow.

Initial treatment of CVX-stimulated platelets with an antibody against TF reduced fibrin accumulation on platelets under flow and addition of TF pAB to PRP attenuated thrombin generation in well-plate assays. The effect of TF pAB on thrombin generation on gel-filtered platelets prepared in the manner shown in Fig. 5 was variable, which is not inconsistent with a display of FVIIa in a prebound complex with TF [4] or other proteins. Our results do not support a role for production of FXa through the intrinsic pathway.

We observed peak thrombin generation in CVX-treated PRP (1 : 2 dilution) at 69 ± 6 min. In contrast, Hemker *et al.* [19] observed peak thrombin generation at 20 min in 2 : 3 dilute PRP supplemented with 1 μM recombinant tissue factor (but no CTI), which corresponds to three TF molecules per platelet (assuming 2×10^8 platelets mL^{-1}). A significant delay in thrombin generation may also result from contact inhibition by CTIR which was absent in the Hemker study. While it may not be possible to specify how many TF molecules per platelet are present in our system from this comparison, one may conclude that the FVIIa-activating activity corresponds to TF levels that are low and in the sub μM range.

We also demonstrated that individual unactivated neutrophils (isolated from blood drawn into CTI) suspended in prothrombin, FX and FVIIa support a relatively low level of thrombin generation under static conditions through a mechanism that depends on CD18, P-selectin and TF. Reduction in thrombin production by anti-CD18 may be attributed to reduction in FX binding to Mac-1 [20]. The P-selectin function in neutrophil-mediated prothrombinase activity points towards the role of platelet membrane being present along with neutrophils. P-selectin blockage could have reduced the binding of microparticles to neutrophils, reducing transfer of TF-laden microparticles to the neutrophil surface. Reduction of thrombin generation in the presence of an antibody against TF also supports this concept. Our earlier studies [16] have shown that individual fibrinogen-adherent neutrophils in PFP/CTI (with TFPI) can support thrombin generation and subsequent fibrin deposition under flow through a mechanism that was dependent on FXIIa (contact pathway), Mac-1, cathepsin G and elastase, but not sensitive to TF pAB. Further studies would be required to study the neutrophil-platelet interaction in plasma under well-defined flow conditions to determine if and when TF-dependent initiation of coagulation on neutrophils is more or less significant than neutrophil cathepsin G-mediated pathways [16,20].

A significant question that remains unresolved is the original source of platelet-associated TF. Studies by Giesen *et al.* [7] demonstrated that an inhibitor of TF can greatly reduce thrombi formation on purified collagen surfaces devoid of TF. Neutrophils may transfer TF to platelets through microparticles [21]. Activated platelets may upregulate the expression of TF on monocytes via P-selectin [22]. Monocyte TF expression may be further enhanced by monocyte differentiation into

macrophages [23]. TF mRNA has also been detected by PCR in platelets. Additionally, it is unclear whether this TF resides on the platelet surface in an encrypted state or is located inside the α granules of resting platelets.

In the study of FVIIa functionality on platelets, the following qualifications are relevant: phlebotomy may introduce wall-derived TF into the drawn blood. It is not possible to determine if microparticles from platelets or neutrophils exist *in vivo* or are created during blood manipulation *ex vivo*. Monocyte expression of TF may occur within approximately 15–30 min of phlebotomy. The priority of platelet TF in various *in vivo* clotting scenarios, physiological or pathological, remains to be determined.

To conclude, Fig. 7 summarizes the 'layers' through which coagulation may be initiated *in vitro* in the absence of added TF. FXa may be formed through the contact pathway, but this pathway can be essentially eliminated by the addition of CTI. Adherent neutrophils can activate platelets via cathepsin G-mediated signaling [15] in platelets in the presence of plasma. Unactivated neutrophils alone in the absence of plasma (i.e. TFPI) demonstrated a low level of thrombin generation through a CD18, P-selectin and TF-dependent mechanism that may involve platelet microparticle interaction. We have shown that platelets, following activation via GPVI, can initiate coagulation on their own by exposing TF and kinetically significant FVIIa. The combination of anionic phospholipid exposure and FVIIa alone is not sufficient to initiate significant coagulation.

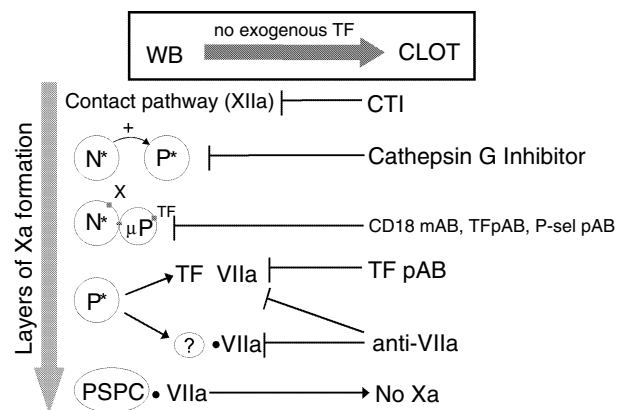


Fig. 7. Schematic of various pathways through which coagulation may be initiated in the absence of wall-derived tissue factor (TF), including: contact pathway (inhibitable by corn trypsin inhibitor), neutrophil cathepsin G-mediated activation of platelets (inhibitable by cathepsin G inhibitor), interaction between individual neutrophils and platelet microparticles (dependent on CD18, TF and P-selectin), display of TF and factor (F)VIIa by individual activated platelet (inhibitable by antibodies against TF and FVIIa), and TF-independent FVIIa activity (VIIa) by individual activated platelet (inhibitable by anti-FVIIa). PSPC alone with FVIIa is incapable of initiating coagulation. WB, Whole blood; CTI, corn trypsin inhibitor; N*, activated neutrophil; P*, activated platelet; μP , platelet microparticle; PSPC, phosphatidylserine/phosphatidylcholine; P-sel, P-selectin; pTF, platelet-associated tissue factor; IIa, thrombin; Xa, factor Xa; mAB, monoclonal antibody; pAB, polyclonal antibody.

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