Factor XI–Dependent Reciprocal Thrombin Generation Consolidates Blood Coagulation when Tissue Factor Is Not Available

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Objective—Feedback activation of factor XI by thrombin is a likely alternative for tissue factor-dependent propagation of thrombus formation. However, the hypothesis that thrombin can initiate and propagate its formation in a factor XI-dependent and platelet-dependent manner has not been tested in a plasma milieu.

Methods and Results—We investigated thrombin generation in recalcified platelet-rich plasma activated with varying amounts of thrombin or factor VIIa. Thrombin initiates and propagates dose-dependently thrombin generation only when platelets and plasma factor XI are present. Incubation of thrombin-activated platelets with a tissue factor neutralizing antibody had no effect on thrombin formation, indicating that platelet-associated tissue factor, if present at all, is not involved. In the absence of factor VIII, thrombin could not initiate its own formation, whereas factor VIIa-induced thrombin generation was reduced. Collagen strongly stimulated both thrombin-initiated and factor VIIa-initiated thrombin generation.

Conclusions—These findings support the notion that platelet-localized feedback activation of factor XI by thrombin plays an important role in maintaining normal hemostasis as well as in sustaining thrombus formation when the TF pathway is inhibited by tissue factor pathway inhibitor. (Arterioscler Thromb Vasc Biol. 2004;24:1138-1142.)

Key Words: factor XI • thrombin • clot formation • activated platelets • factor VIIa • collagen

It is generally appreciated that thrombin-mediated feedback activation of factor XI contributes to the consolidation phase of clot formation.1–3 In this process, thrombin that is initially generated via the tissue factor (TF) pathway, activates factor XI on the surface of (thrombin-activated) activated platelets.4 The accelerating role of activated platelets in thrombin-catalyzed factor XI activation is thought to result from the colocalization of thrombin and factor XI on the platelet membrane glycoprotein (GP) Ibα within the GP Ib-IX-V complex.5,6 Thrombin-activated factor XI, therefore, could be critical to the propagation of thrombin generation once the TF pathway is inhibited by TF pathway inhibitor (TFPI).

Hypothetically, thrombin-catalyzed factor XI activation could play a major role in the development of growing thrombi. Besides circulating cell-bound TF, the continuous supply of factor XI by blood flow to a thrombin-containing thrombus surface might result in a factor Xla-driven thrombin production that in turn will accelerate the rate of thrombus growth.7 An additional argument for an important contribution of a factor XI-dependent thrombus growth is found in the observation that factor XI activation seems to be confined to the surface of an activated platelet.8 The latter optimizes the thrombin generating potency because of maintaining high local concentrations of reactants and the inability to be inactivated by plasma protease inhibitors.

The present work was undertaken to explore the role of thrombin in initiating its own production by feedback activation of factor XI under physiological conditions as in platelet-rich plasma (PRP) and to know how effective collagen and factor VIIa are in promoting thrombin generation under these conditions. In an attempt to answer these questions, we used a continuous thrombin generation assay9,10 in which thrombin generation is not driven by factor Xla and TF.

Methods

Preparation of PRP and Washed Platelet Suspensions

Blood (9 volumes) from healthy donors was collected on 0.13 mol/L trisodium citrate (1 volume). PRP was obtained by centrifugation of the blood at 260g for 15 minutes at 20°C. The platelets were counted (Beckman Coulter Counter) and adjusted to 9×10⁸ platelets/mL using autologous platelet-poor plasma (PPP) that was prepared from citrated blood by centrifuging twice at 2900g for 10 minutes at 20°C. Suspension of washed platelets (6.6×10⁸ platelets/mL) were prepared as previously described.11
Preparation of Plasma Factor XI–Deficient and Plasma Factor VIII–Deficient PRP
Factor XI-deficient PPP (60 µL) and factor VIII-deficient PPP (60 µL) were mixed with a suspension of washed platelets (10 µL; 6.6×10^4 platelets/µL) that were isolated from normal PRP. Severe factor XI-deficient patient PPP (homozygote for type II mutation with factor XI level of <1%) was a kind gift from Dr Uri Seligsohn,¹ and congenital factor VIII-deficient plasma (factor VIII level of <1%) was purchased from George King Bio-Medical Inc.

Continuous Thrombin Generation Assay
Wells of Immulon 2 HB, round-bottom 96-well plates (Thermo Labsystems), were supplied with 20 µL 2.4 mmol/L fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl (Bachem) in HEPES buffer (pH 7.45; 10 mmol/L HEPES, 136 mmol/L NaCl, 2 mmol/L MgCl₂, 2.7 mmol/L KCl, 1 mg/mL bovine serum albumin), 70 µL PRP (9×10^4 platelets/µL), and 10 µL HEPES buffer containing 0.6 mg/mL corn trypsin inhibitor (CTI; Hematologic Technologies), and 20 µL fluorogenic substrate (2.4 mmol/L). Reaction was started by the addition of thrombin or recombinant factor VIIa (Novo Nordisk) with 20 µL HEPES buffer containing CaCl₂ (0.1 mol/L). Fluorescence tracings were recorded in a 96-well plate spectrofluorometer (Molecular Devices, Spectra Max Gemini XS) with 100 µL of the incubation mixture adding thrombin or recombinant factor VIIa (Novo Nordisk Pharma) in 20 µL HEPES buffer containing CaCl₂ (0.1 mol/L). Fluorescence tracings were recorded in a 96-well plate spectrofluorometer (Molecular Devices, Spectra Max Gemini XS) with absorbance at 368 nm and emission at 460 nm. A cutoff filter of 455 nm was used. The fluorescence intensity after correction for the inner filter effects and substrate depletion were converted into molar concentrations thrombin as described.¹⁰ Thrombin generation curves are the mean of three independent experiments. All procedures were performed at 37°C.

Continuous Thrombin Generation Assay in Plasma Factor VIII–Deficient PRP
A suspension of washed platelets (10 µL, 6×10^4/µL) was added to a mixture containing 60 µL factor VIII-deficient PPP, 6 µL CTI (1 mg/mL), 4 µL collagen (0.15 mg/mL; Horm, Nycomed Pharma), and 20 µL fluorogenic substrate (2.4 mmol/L). Reaction was started by adding 20 µL CaCl₂ (0.1 mol/L) containing 120 mmol/L factor VIIa or 30 nmol/L thrombin.

Detection of Platelet-Associated TF
A suspension of washed platelets (100 µL; 6×10^4/µL) was incubated for 10 minutes at 37°C with thrombin (5 nmol/L) and CaCl₂ (3 mmol/L) in the presence of 20 µg/mL of a neutralizing polyclonal rabbit antibody against human TF (American Diagnostics) or 24 pmol/L TF (Innovin, Dade Behring) either in the presence or in the absence of anti-TF (20 µg/mL). A similar incubation was performed with 20 µg/mL rabbit immunoglobulin (Dako) as a negative control for rabbit antibodies. An aliquot (10 µL) of the incubation mixture was transferred to a solution containing PPP (60 µL), CTI (10 µL), 0.6 mg/mL, and 20 µL fluorogenic substrate (2.4 mmol/L). The reaction was started by the addition of 20 µL CaCl₂ (0.1 mol/L). Thrombin generation was monitored as described.

Results
Thrombin Initiates Thrombin Generation in PRP
We hypothesized that thrombin-catalyzed factor X activation at the platelet surface is the sole contributor to thrombin generation in PRP when TF is not available. To test this hypothesis, we added increasing amounts of thrombin to PRP and continuously monitored thrombin generation using a fluorogenic thrombin substrate.¹⁰ Initial experiments showed that recalcification of PRP resulted after a lag phase of ~20 minutes in a sudden onset of thrombin generation, which could be fully inhibited by CTI (50 µg/mL), a selective factor Xa inhibitor.¹⁰ Therefore, all next experiments were performed in the presence of CTI to avoid contact activation-driven thrombin generation.

The addition of increasing amounts of thrombin (0 to 5 nmol/L) to PRP yielded increasing amounts of in situ-formed thrombin (Figure 1A). That is, thrombin dose-dependently increased the time to thrombin peak and increased the peak thrombin values. Platelets appeared to be essential because in PPP, thrombin was not formed. We note that the thrombin that was added to initiate thrombin generation in PPP was also not detectable. It is assumed that this exogenous thrombin is neutralized by its plasma inhibitors within the mixing time and first read out by the fluorometer (typically <30 s). These findings thus indicate that thrombin initiates and dose-dependently propagates thrombin generation only in PRP. Indeed, when plasma is activated with thrombin (5 nmol/L), thrombin peak values increased with the platelet concentration and reached a maximum with 3×10⁶ platelets/mL (data not shown).

To investigate the role of factor XI, thrombin generation was monitored in PRP that was reconstituted from factor XI-deficient plasma and washed normal platelets and compared with thrombin generation in PRP reconstituted from normal plasma and washed normal platelets. In reconstituted normal PRP, thrombin generation showed a short lag phase of ~5 minutes and reached after 40 to 50 minutes incubation with thrombin (1 nmol/L) an apparent peak value of 20 nmol/L (Figure 1B). In PRP, lacking plasma factor XI, the
time of onset of thrombin appearance was ≈30 minutes and thus greatly prolonged. At the end of the experiment, a thrombin level ≈2 nmol/L was measured. Thrombin generation could be restored by mixing factor XI-deficient patient plasma with 10% normal PPP (data not shown).

In view of a report demonstrating that platelet α-granules contain TF, the question had to be addressed whether a thrombin-induced release of functional TF could be (partly) responsible for thrombin generation in PRP. To explore a possible role of platelet-associated TF in initiating thrombin generation, a suspension of washed normal platelets was incubated with thrombin in the absence and presence of a TF activity neutralizing antibody. It is apparent that the anti-TF antibody shortened to some extent the lag phase (defined as the time to generate 2 nmol/L thrombin), and increased the thrombin and the thrombin peak value (Figure 2). However, incubation with a negative control for rabbit antibodies resulted in a similar stimulation of thrombin generation. We assume that the slightly stimulated thrombin generation is caused by an antibody-induced platelet activation reaction. To verify that the polyclonal anti-TF antibody does neutralize TF activity under the conditions of the experiment, thrombin-stimulated platelets were incubated with 24 pmol/L recombinant TF (Innovin, Dade Behring) in the presence and absence of anti-TF. Thrombin generation was then started by adding PPP. Figure 2 shows that TF greatly enhanced thrombin generation in PRP and that this TF-driven thrombin generation could be fully abolished by anti-TF. Altogether, these experiments strongly support the notion that PRP does not contain sufficient amounts of functional TF to contribute to thrombin generation in PRP.

Collagen Enhances Thrombin-Initiated and Factor VIIa–Initiated Thrombin Generation in PRP

We next investigated the ability of collagen to synergistically enforce the platelet-dependent function of thrombin in its own initiation and propagation. The time of onset of thrombin generation, initiated with 0.5 nmol/L thrombin, was dramatically shortened, and the thrombin peak value was greatly increased when PRP was preincubated with 5 μg/mL colla-
We next addressed the question whether platelet-dependent propagation of thrombin generation by factor VIIa, as reported by Monroe et al., is also amplified by feedback activation of factor XI. Figure 3B shows that factor VIIa dose-dependently initiates thrombin generation in PRP containing CTI. However, a supraphysiological amount of factor VIIa, namely twice the plasma factor VII level, was required to generate rather small amounts of thrombin (ie, \( \approx 5 \text{ nmol/L} \) thrombin after 60 minutes). Interestingly, collagen dramatically stimulated the factor VIIa-initiated thrombin generation. Under these conditions, a relatively low concentration of factor VIIa (2 nmol/L) rapidly initiates and sustains an extensive amount of thrombin, as indicated by a greatly reduced lag phase and increased maximal thrombin activity.

A previous experiment with a neutralizing TF antibody (see Figure 2) ruled out that platelet-associated TF is not involved in thrombin-initiated thrombin generation in PRP. Because factor VIIa-driven thrombin generation could be more sensitive to the presence of TF, the anti-TF antibody was used to investigate a contribution of TF in collagen-enhanced factor VIIa-driven thrombin generation. Figure 3C demonstrates that anti-TF did not inhibit factor VIIa-driven thrombin generation. As a matter of fact, both the anti-TF and a control antibody stimulated thrombin generation as previously observed in thrombin-initiated thrombin generation experiments (Figure 2). Evidently, TF, if present in PRP, does not contribute to factor VIIa-driven thrombin generation in collagen-stimulated PRP.

To dissect the contribution of the intrinsic (ie, thrombin-activated factor XI) and the extrinsic pathway (ie, factor VIIa activated factor X) in factor VIIa-initiated thrombin generation, thrombin generation was monitored in PRP reconstituted from normal platelets and congenital factor VIII-deficient plasma and in PRP reconstituted from factor XI-deficient plasma and normal platelets. Although thrombin generation was absent when plasma factor XI-deficient PRP was activated with thrombin (see Figure 1B), in the presence of 20 nmol/L factor VIIa and 5 \( \mu \text{g/mL} \) collagen a considerable amount of thrombin was formed (Figure 3C). In factor VIII-deficient PRP, thrombin did not initiate its own generation, whereas factor VIIa induced thrombin generation to approximately the same extent as observed in plasma factor XI-deficient PRP. Because factor VIII and factor XI deficiency resulted in similar but reduced thrombin generation, it is apparent that a large part of thrombin is directly generated via a factor VIIa-catalyzed activation of factor X, and an additional amount is supplied by the factor XI-dependent pathway.

Discussion

Extensive work from Walsh et al has revealed much of the molecular basis of thrombin-catalyzed XI activation at the surfaces of activated platelets. Yet the precise implications of this process for ex vivo and in vivo thrombin generation remain to be clarified. Our work demonstrates that low amounts of thrombin (< 1 nmol/L), despite thrombin’s short half life (<30 s), initiate and amplify thrombin generation in PRP. This reciprocal thrombin generation is solely mediated by factor XI and operates in the absence of platelet-derived TF. Yet platelets play a critical role, because initiation and propagation was not observed in PPP. In contrast, TF-independent factor VIIa-driven thrombin generation is only partly dependent on factor XI, suggesting that factor VIIa-catalyzed factor X activation initiates and contributes largely to thrombin generation in the propagation phase.

That (platelet-derived) TF is not involved in platelet-enhanced thrombin-initiated thrombin generation is supported by the finding that when contact activation is inhibited no thrombin is formed. This notion was further corroborated by experiments in which platelets were incubated with thrombin in the presence of a polyclonal antibody against TF. Although this antibody inhibits TF-driven thrombin generation in PRP, it did not inhibit reciprocal thrombin generation. Furthermore, experiments with PRP that was reconstituted from factor XI-deficient plasma and platelets isolated from normal PRP clearly indicated that plasma factor XI is a prerequisite for a TF-independent thrombin generation. A factor XI-dependent pathway in thrombin generation was finally confirmed in similar experiments; however, in factor VIII-deficient plasma, thrombin-initiated thrombin formation could not be detected.

Intriguingly, in the presence of platelets, a factor XI plasma level of \( \approx 10\% \) is already sufficient to normalize factor XI-dependent reciprocal thrombin generation. Yet no significant thrombin generation was measured in PRP that was reconstituted from normal platelets and congenital factor XI-deficient plasma, indicating that platelet factor XI, under the conditions of the experiment, does not compensate for the lack in plasma factor XI. This finding is in good agreement with earlier observations from our laboratory but apparently contradicts the notion that platelet-derived factor XI may be important in maintaining normal hemostasis in patients with a severe plasma factor XI deficiency.

Pretreatment of resting platelets in PRP with collagen shortened the lag phase of thrombin generation as well as the thrombin peak value that was induced by thrombin or factor VIIa. The lag phase reflects a threshold mechanism of thrombin-dependent feedback reactions, eg, platelet activation and the activation of the essential cofactors factor V and VIII. The thrombin peak value is determined by the rate of thrombin production, which in turn reflects the concentration of the prothrombinase complex at the surface of activated platelets and the rate of thrombin inactivation. The interaction of collagen with its platelet receptor, GPVI, induces a signal pathway leading to exposure of anionic phospholipids that support blood coagulation. In addition, (in situ-formed) thrombin synergistically enhances the generation of platelet procoagulant phospholipid. Thus, a picture emerges in which the surface of collagen-treated platelets exert a dual role: (1) thrombin-activated platelets offer binding sites (GP Ib\( _\alpha \) for both factor XI and thrombin; and (2) collagen/thrombin-activated platelets provide procoagulant anionic phospholipids that support the assembly of prothrombin and factor X converting enzyme complexes on platelet surfaces. That these phospholipids alone or in combination with protein receptors on the platelet surface mediate thrombin generation is consistent with the fact that a protein with high...
affinity for anionic phospholipids, ie, annexin A5, prohibited in full factor XI-dependent reciprocal thrombin generation.

There is much debate about the role and function of platelets in factor VIIa-mediated clot formation.23,24 The question has been addressed to what extent high-dose factor VIIa supports thrombin generation in a TF-independent manner. Our results confirm that supraphysiologically factor VIIa concentrations increase dose-dependently thrombin generation only in the presence of platelets. Collagen dramatically accelerated the initiation and propagation of factor VIIa-driven thrombin generation. Using plasma factor XI-deficient or plasma factor VIII-deficient reconstituted PRP, we could demonstrate that factor VIIa-initiated thrombin generation was partly driven by the factor Xla-activated intrinsic pathway. The factor XI-dependent consolidation phase of the blood coagulation process is likely triggered by thrombin that is initially generated by factor VIIa-activated factor X. Furthermore, in these in vitro experiments, no evidence was found that TF is required in factor VIIa-initiated and factor XI-dependent propagation of thrombin formation at the surface of activated platelets. It is evident that care should be exercised in predicting the in vivo implications of these findings. The primary initiator of coagulation in vivo is TF, either located in the vessel wall and/or present in circulating blood.25–27 However, the question to what extent functional TF, if incorporated into a thrombus, determines thrombus growth has yet to be answered.28,29 Alternatively, FXI-dependent thrombus propagation could, after the initiation phase, limit the availability of TF at the blood/thrombus interface.7 It is an interesting thought that the continuous supply of factor XI to a thrombus that contains fibrin-bound thrombin is a potential alternative pathway to mediate thrombus growth once TF is no longer available because of its rapid neutralization by TFPI or the increasing distance between blood–thrombus interface and vessel wall-located TF.

Collectively, our investigations suggest an important role of thrombin-activated factor XI in maintaining normal hemostasis and driving thrombus growth. Our results may also explain how pharmaceutical doses of factor VIIa function in maintaining or restoring normal hemostasis.

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References

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