Feedback Activation of Factor XI by Thrombin in Plasma Results in Additional Formation of Thrombin That Protects Fibrin Clots From Fibrinolysis

By Peter A. Kr. von dem Borne, Joost C.M. Meijers, and Bonno N. Bourna

Recently, an alternative pathway for factor XI activation has been described in which factor XI is activated by thrombin. Patients with a factor XI deficiency bleed mostly from tissues with high local fibrinolytic activity. Therefore, the role of thrombin-mediated factor XI activation in both fibrin formation and fibrinolysis was studied in a plasma system. Clotting was induced by the addition of tissue factor or thrombin to recalcified plasma in the presence or absence of tissue-type plasminogen activator, after which clot formation and lysis were measured using turbidimetry. Thrombin-mediated activation of factor XI was found to take place in plasma under physiologic conditions in the absence of a dextran sulfate-like cofactor. At high tissue factor concentrations, no effect of factor XI was seen on the rate of fibrin formation. Decreasing amounts of tissue factor resulted in a gradually increasing contribution of factor XI to the rate of fibrin formation. In addition, thrombin-mediated factor XI activation resulted in an inhibition of tissue-type plasminogen activator-induced lysis of the clot. This inhibition occurred even at tissue factor concentrations at which no effect of factor XI was observed on fibrin formation. Trace amounts of activated factor XI (1.25 pmol/L, representing 0.01% activation) were capable of completely inhibiting fibrinolysis in our system. The inhibitory effect was found to be mediated by thrombin that is additionally generated in a factor XI-dependent manner via the intrinsic pathway and is capable of protecting the clot against lysis. We also observed that formation of additional thrombin continued after the clot had been formed. We conclude that thrombin-mediated factor XI activation can take place in plasma. The presence of factor XI during coagulation results in the formation of additional thrombin within the clot capable of protecting this clot from fibrinolytic attack. The large amounts of thrombin that are formed by the intrinsic pathway via factor XI may play an important role in the procoagulant and thrombogenic state of clots and may therefore have important clinical and therapeutic implications.

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MATERIALS AND METHODS

Materials. Fresh frozen plasma and citrated whole blood were obtained from the local blood bank. Bovine serum albumin (BSA; fraction V) was purchased from Sigma (St Louis, MO). Enzymoost F1+2 kits were obtained from Behring (Marburg, Germany). A monoclonal antibody (MoAb) capable of blocking activated factor XII (OT2) was a kind gift of Dr Erik Hack (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands). An MoAb against factor VII (2G3-2c) has been described. A MoAb against factor IX (2G4) and factor X (10F1) and rabbit polyclonal antibodies against factor II were prepared according to standard procedures. The rabbit polyclonal antiserum was immunopurified on a Sepharose column to which purified prothrombin was immobilized. Other chemicals obtained were the best grade available.

Proteins. Factor XI was purified from fresh frozen plasma using murine anti-factor XI MoAb (XI-5) immobilized on CNBr-activated Sepharose, as described earlier. Factor IX and X were isolated from fresh frozen plasma. All preparations appeared homogeneous using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
PACE). The specific coagulant activities for factor IX, X and XI were 210, 90, and 235 U/ml, respectively. Factor XIa was prepared from factor XI using β-factor XIIa, as previously described. Factor IX was activated using factor XIa,11 after which factor XIa was removed from the factor IXa preparation using Q-Sepharose chromatography. Factor X was activated with Russell’s Viper Venom.12 Recombinant human tissue factor (Innovin) was obtained from Baxter (Unterschleißheim, Germany). t-PA was purchased from Chromogenix (Möln达尔, Sweden). Purified human thrombin was a kind gift from Dr Walter Kissi (University of New Mexico, Albuquerque, NM). Recombinant hirudin (12,000 U/ml) was a generous gift from R. Wallis (Ciba Geigy, Horsham, UK).

Deficient plasma. Factor XII-deficient plasma was obtained from a congenitally deficient patient. Plasma deficient in both factor XII and factor XI was prepared by passing this factor XII-deficient plasma over a Sepharose column to which a murine anti-factor XI antibody (XI-5) was immobilized. Less than 0.01% of factor XI was present in the factor XI and XII double-deficient plasma as determined using enzyme-linked immunosorbent assay (ELISA). Plasmas deficient in either factor VIII, IX, or X were obtained by passing plasma from a healthy donor over Sepharose columns to which murine MoAbs against these factors were coupled. Plasma deficient in factor II was obtained by passing plasma from a healthy donor over a Sepharose column to which immunopurified rabbit polyclonal antibodies against factor II were coupled. The undiluted flow-through fractions of the columns were pooled and used as deficient plasma. The deficient plasmas were tested with clotting assays and found to contain less than 1% of the deficient factor, whereas the other coagulation factors remained normal.

Turbidimetry. Thrombin-mediated factor XI activation in plasma was studied by monitoring the change in turbidity during fibrin formation and lysis at 405 nm in a microplate reader.13 An increase in turbidity indicated fibrin gel assembly, whereas a decrease in turbidity indicated clot lysis.14 All experiments were performed in citrated plasma recalcified with CaCl₂ (final concentration, 17 mM) resulting in a free Ca²⁺ concentration of 2.3 mM/L. A mixture of tissue factor (different dilutions of Innovin) or thrombin (20 mM/L calcium necessary for recalcification) was added to 75 μL of plasma to initiate clotting. The volume was adjusted to 150 μL with HEPES buffer (25 mM/L HEPES, 137 mM/L NaCl, 3.5 mM/L KCl, 3 mM/L CaCl₂, and 0.1% BSA), resulting in a final plasma concentration of 50%. After mixing, 100 μL of the reaction mixture was spotted into a microplate and then incubated at 37°C, during which turbidity was measured at different time points. To study the effect of factor XI on fibrin formation, plasma deficient in both factor XI and XII was reconstituted with factor XI and preincubated for 30 minutes at room temperature before use. The final concentration of factor XI was 12.5 nmol/L (approximately 0.5 U/mL). In some experiments, a murine MoAb (XI-1) that blocks the activation of factor IX by factor Xa15 was preincubated with the plasma. In addition, experiments were performed in minimally diluted plasma with a final plasma concentration of 94%.

Fibrinolysis was studied using the same assay as described above except that 30 U/mL of t-PA was added. To study the effect of factor XI we used plasma deficient in both factor XI and XII that was reconstituted with factor XI and normal plasma that was preincubated for 30 minutes at room temperature with the murine MoAb (XI-1) that blocks factor Xa activity. In several experiments, factor IXa, Xa, or Xa or additional thrombin was also included, as described in the legends to the figures. The concentrations of the components are indicated as final concentrations.

F1+2 assay. Fibrin formation was induced in plasma by thrombin in the presence of t-PA, as described above. The reaction mixture was incubated at 37°C in polypropylene tubes instead of microtiter plates. At different time intervals, 100 μL of HEPES buffer containing 10 mM/L of EDTA and 50 U/mL of hirudin was added to the fibrin clot. After thorough mixing for 1 minute and centrifugation (2 minutes at 15,000g), the amount of prothrombin activation was determined in the supernatant by measuring the concentration of fragment F1+2 with a commercial ELISA.

RESULTS

The role of factor XI in tissue factor-induced fibrin formation in plasma. The role of factor XI in tissue factor-induced coagulation was studied using a fibrin formation assay. In this assay, tissue factor was added to recalcified plasma and the rate of fibrin formation was followed by measuring the increase in turbidity. Tissue factor-induced fibrin formation was measured in plasma deficient in both factor XI and factor XII to avoid any interference of contact activation. To study the influence of factor XI on the rate of fibrin formation, this plasma was reconstituted with factor XI. At high tissue factor concentrations, no difference in fibrin formation was observed in the presence or absence of factor XI (Fig 1A). With decreasing amounts of tissue factor, a gradual increase in the rate of fibrin formation was observed in the presence of factor XI compared with the rate of fibrin formation in the absence of factor XI. Because the recombinant tissue factor preparation contains phosphatidylserine/phosphatidylcholine phospholipids that might contribute to the rate of fibrin formation in this assay, control experiments were performed in which extra phospholipids were added, showing identical results as those depicted in Fig 1A (data not shown). Preincubation of factor XI with the antibody that blocks factor Xa activity (XI-1) resulted in a complete abolition of the effect of factor XI on fibrin formation (Table 1), indicating that this effect is caused by factor XI and not by a contaminant in the factor XI preparation. Control experiments were performed with minimally diluted plasma (94% plasma concentration) to investigate the effect of varying concentrations of HK, fibrinogen,3 or other inhibitory factors in plasma. An identical effect of factor XI on fibrin formation was observed, fibrin formation rates in the presence or absence of factor XI were 56.2 ± 3.7 and 41.2 ± 2.4 mOD/min, respectively. From these experiments it was concluded that factor XI contributes to the formation of thrombin at low tissue factor concentrations.

The effect of factor XI on the lysis of a tissue factor-induced clot. To study the effect of factor XI on clot lysis, we included t-PA in the tissue factor-induced fibrin formation assay. Formation and subsequent lysis of the fibrin clot was determined by following the increase and decrease in turbidity. In separate experiments, we studied the effect of the t-PA concentration on the clot lysis time. A t-PA concentration of 30 U/mL was chosen because it induced complete lysis of the fibrin clot in a reasonable amount of time (60 to 90 minutes). At the highest tissue factor concentration tested, almost no clot lysis was observed in 80 minutes, either in the presence or absence of factor XI (Fig 1B). Decreasing concentrations of tissue factor resulted in an increase of the rate of clot lysis in the absence of factor XI, whereas in the presence of factor XI only a minor effect on clot lysis was observed. This finding indicates that clot lysis is inhibited in the presence of factor XI. Effects of factor XI on clot
FACTOR XI ACTIVATION BY THROMBIN IN PLASMA

Fig 1. The role of factor XI in tissue factor-induced fibrin formation and lysis in plasma. Plasma deficient in both factor XI and XII was preincubated in the presence (solid symbols) or absence (open symbols) of factor XI (12.5 nmol/L). (A) Coagulation was initiated by adding tissue factor and calcium necessary for recalcification. Fibrin formation was measured in time as the change in turbidity at 405 nm. For clarification, the areas between the curves representing identical tissue factor dilutions were shaded. (B) To study the influence of fibrinolysis, t-PA (30 U/mL) was included in the incubation mixture used in (A). Fibrin formation and subsequent lysis were measured in time as the change in turbidity at 405 nm. Tissue factor (dilutions of Innovin): \(10^{-3}\) (○), \(10^{-3}\) (△), \(3 \times 10^{-4}\) (●), \(10^{-2}\) (●), \(3 \times 10^{-4}\) (●), buffer (M). Each point represents the mean ± SEM of three independent measurements.

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lysis could be observed at tissue factor concentrations at which factor XI had no effect on fibrin formation.

The effect of factor XI on the lysis of a thrombin-induced clot. The use of varying tissue factor concentrations results in formation of different amounts of thrombin. Thrombin can activate not only factor XI but also factor V and VIII, resulting in amplification of the intrinsic pathway. Therefore, we repeated the experiments using a fixed concentration of thrombin (20 nmol/L) to induce clotting. Clot formation was observed within a few minutes and lysis of the clot was studied in the presence and absence of factor XI. Again, an antifibrinolytic effect of the plasma concentration of factor XI was observed (Fig 2A). Essentially the same results were obtained with minimally diluted plasma (94% plasma concentration) instead of the normally used 50% plasma concentration (data not shown).

The antifibrinolytic effect of factor XI results from factor Xla generated by thrombin-mediated activation. Traces of factor Xla in our factor XI preparation instead of factor Xla generated by thrombin-mediated factor XI activation might be responsible for the observed antifibrinolytic effect of factor XI. Therefore, we repeated the experiments with different
factor by adding different concentrations of factor XIa or 12.5 fmol/L of factor XIa, indicating that the factor XIa concentration is similar to the clotting time induced by 1.25 to 12.5 factor XIa needed to induce a direct antifibrinolytic effect,

indicating that the observed antifibrinolytic effect is the result of newly formed factor XIa generated by thrombin-mediated activation.

Lysis of a thrombin-induced clot: effect of an antibody blocking factor XIa activity. The experiments described above were performed with plasma from a single factor XII-deficient donor. To exclude donor-specific effects, experiments were performed with citrated plasma from a pool of 40 healthy volunteers that was preincubated with a murine anti-factor XI antibody (XI-1) that blocks activation of factor IX by factor XIa. In addition, an MoAb capable of blocking factor XIla activity (OT2) and a control antibody against protein C inhibitor (API-93) were also included. After preincubation with the antibodies, the plasma was recalcified, thrombin and t-PA were added, and turbidity was measured. An increased rate of clot lysis was observed in the presence of XI-1 (Fig 3), again confirming the antifibrinolytic effect of factor XI. The presence or absence of OT2 had no influence, suggesting that factor XII, which is known to play a role in fibrinolysis, apparently has no part in the effects we observe.

Inhibition of fibrinolysis by factor XIa is dependent on the intrinsic pathway of coagulation. The antifibrinolytic effect of factor XI can be caused either by a direct effect of activated factor XI or by an indirect effect caused by the generation of another activated clotting factor in the intrinsic pathway. Therefore, we studied the effect of addition of activated factor IX (50 pmol/L, representing 0.1% activation) to factor XI-deficient plasma on clot lysis. Factor IXa was also found to inhibit fibrinolysis (Fig 4A), indicating that the presence of factor XIa itself is not a prerequisite for this effect.

The lysis of a thrombin-induced clot was also studied in plasmas deficient in either factor II, VII, IX, or X in the presence or absence of XI-1 (Fig 4B). The antibody did not influence clot lysis in plasmas deficient in factor II, IX, or X. Only in factor VII-deficient plasma was an effect of the antibody found. It was concluded that an intact intrinsic pathway is necessary for the inhibition of fibrinolysis by factor XIa.

To determine which factor of the intrinsic pathway was responsible for inhibition of fibrinolysis, lysis of a thrombin-induced clot was studied in prothrombin-deficient plasma to which also factor IXa, Xa, or additional Ha was added. Concentrations of activated factor IX and X representing complete activation had no influence on fibrinolysis, whereas a concentration-dependent antifibrinolytic effect of factor Ha was observed (Fig 4C). We concluded that formation of additional thrombin by activation of the intrinsic pathway of coagulation is responsible for the inhibition of fibrinolysis.

F1 + 2 formation during coagulation and fibrinolysis. Because the inhibitory effect on fibrinolysis was found to be mediated by additionally generated thrombin, the activation of prothrombin during clotting and clot lysis was studied in our system by measuring the activation fragment 1 + 2. After preincubation with or without XI-1, normal plasma was recalcified and both thrombin and t-PA were added. After the addition of EDTA and hirudin, fibrin was removed by centrifugation and F1 + 2 was measured in the supernatant. Significant differences in prothrombin activation were observed in the presence or absence of the antibody, indicating an important contribution of the intrinsic pathway (Fig 5). Prothrombin activation was found to continue after clot formation, indicating ongoing thrombin generation within the clot. The presence of t-PA did not affect prothrombin activation, showing that this activation is not influenced by clot lysis.

DISCUSSION

In this report, we show that factor XI activation by thrombin can take place in plasma under physiologic conditions in the absence of a dextran sulfate-like cofactor. This activation was studied by monitoring tissue factor-induced fibrin formation in plasma using turbidimetry. At high tissue factor concentrations, no effect of the presence or absence of factor XI was seen on fibrin formation, probably because sufficient amounts of thrombin had already been formed via the extrinsic pathway to induce rapid fibrin formation. At low tissue factor concentrations, thrombin formation was probably limited and additional fibrin formation could now be provided by thrombin generated via the intrinsic pathway initiated by thrombin-mediated factor XI activation. Only differences in

Table 1. Tissue Factor-Induced Fibrin Formation in the Presence and Absence of Factor XI: Effect of an Antibody That Blocks Factor XIa Activity (XI-1)

<table>
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<tr>
<th>Factor XI</th>
<th>XI-1 Antibody</th>
<th>Fibrin Formation Rate [mOD/min]</th>
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<tr>
<td>-</td>
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<td>39.0 ± 3.0</td>
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<td>38.1 ± 1.5</td>
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<td>+</td>
<td>+</td>
<td>58.4 ± 0.7</td>
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Plasma deficient in both factor XI and XII was preincubated in the presence (+) or absence (-) of factor XI or MoAb XI-1. Coagulation was initiated by adding tissue factor (dilution of Innovin, 100 ng/ml) and calcium necessary for recalcification. Fibrin formation was measured in time as the change in turbidity at 405 nm. Results are depicted as fibrin formation rate, which is defined as the slope of the linear part of the fibrin formation curve. The means ± SD of three independent experiments are shown.
Fig 2. The antifibrinolytic effect of factor XI results from factor Xla generated by thrombin-mediated activation. (A) To determine the concentration of factor Xla necessary for an antifibrinolytic effect, plasma deficient in both factor XI and XII was preincubated with factor Xla (12.5 nmol/L) or buffer. Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nmol/L), t-PA (30 U/mL), and factor Xla to the plasma. Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm. Factor XI (■); buffer (○); and factor Xla at 1.25 pmol/L (△), 12.5 fmol/L (□), 125 fmol/L (◇), or 1.25 fmol/L (▲). (B) To determine the amount of factor Xla in our factor XI preparation, plasma deficient in both factor XI and XII was preincubated with factor Xla (12.5 nmol/L). Calcium necessary for recalcification and factor Xla were added to the plasma. Fibrin formation was measured in time as the change in turbidity at 405 nm. The same symbols as depicted in (A) were used in (B). The means ± SD of three independent experiments are shown.

In contrast to our results, no significant activation of factor XI by thrombin in plasma could be measured in an earlier study. However, in this study, autoradiography was used to detect factor XI activation. This technique is not sensitive enough to detect the small amounts of activation that were observed in this study.

Coagulation is thought to be initiated by exposure of blood to tissue factor. While thrombin and fibrin are being formed via the extrinsic pathway, tissue factor pathway inhibitor quickly turns off the activation by inhibiting the factor VIIa-tissue factor complex. Continuation of coagulation is now dependent on the formation of additional thrombin via the intrinsic pathway. According to our findings, maintenance of thrombin formation can take place in plasma as a result of thrombin-mediated factor XI activation leading to additional fibrin formation.

The effect of factor XI on clot lysis was studied by adding a fibrinolytic component (t-PA) to our plasma system. Clot
Fig 3. Lysis of a thrombin induced clot: effect of an antibody blocking factor XIa activity. Normal plasma was preincubated with XI-a (250 nM/L; A, Δ), API-93 (250 nM/L; Y, ▼), or buffer (E, O) in the presence (solid symbols) or absence (open symbols) of DT (675 nM/L). Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM/L), and t-PA (30 U/mL). Fibrin formation and lysis were measured in time as the change in absorbance at 405 nm. The means ± SD of three independent experiments are given.

Fig 4. Inhibition of fibrinolysis by factor XIa is dependent on the intrinsic pathway of coagulation. Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM/L), and t-PA (30 U/mL). Fibrin formation and lysis were measured in time as the change in absorbance at 405 nm. In this figure the amount of fibrinolysis is shown and is defined as the change in absorbance between 10 and 60 minutes. Each bar represents the mean ± SD of three independent experiments. (A) Factor XI-deficient plasma with or without factor X (50 nM/L). (B) Factor II-, VII-, IX-, or X-deficient plasma preincubated with XI-a (250 nM/L; solid bars) or buffer (open bars). (C) Factor II-deficient plasma with factor X (45 nM/L), Xa (75 nM/L), or IIa (20 to 160 nM/L).

Lysis was found to be inhibited by the presence of factor XI, even at low factor concentrations at which factor XI had no effect on fibrin formation. We also observed that the inhibitory effect of factor XI on fibrinolysis was caused by the generation of additional thrombin. Additional thrombin formation continued after clot formation had taken place. In the presence of factor XI, the intrinsic loop of coagulation could generate large amounts of thrombin within the clot that were capable of inhibiting fibrinolysis. So, on the one hand only small amounts of thrombin provided by the extrinsic pathway were sufficient for clot formation, whereas on the other hand large amounts of thrombin formed by the intrinsic pathway were necessary for protecting the clot against lysis.

By directly adding activated factor XI to the plasma, we could show that even a very small amount of factor XIa (1.25 pM/L, representing 0.01% activation) was capable of completely inhibiting fibrinolysis. This finding corresponds very well with our earlier findings that trace amounts of factor XIa can play a role in blood coagulation because of the amplification potential of the coagulation cascade. This amplification could also easily overcome any inhibitory effect of high molecular weight kininogen and fibrinogen on the thrombin-mediated factor XIa activation.

At this moment it is not clear how thrombin exerts its inhibitory effect on fibrinolysis. Several possible explanations exist, such as a direct effect of thrombin on the quantity or structure of the formed fibrin or enhanced cross-linking of the fibrin clot by activation of factor XIII. Activated protein C is a known accelerator of clot lysis in vitro. It was recently proposed that enhancement of clot lysis by activated protein C is mediated by downregulation of the generation of thrombin and factor XIIIa, which is in agreement with our findings and suggests an important role for factor XIII. Further investigations are needed to elucidate the mechanism of inhibition.
The findings in this report may explain the clinical manifestations of factor XI deficiency. Bleeding is mostly seen after surgery or trauma, often with a delay. The formation of the hemostatic plug is probably not inhibited, but the fibrin that replaces the platelet plug after fibrinous transformation may be more susceptible to fibrinolysis. This susceptibility is most important in places with high local fibrinolytic activity, i.e., at those sites where bleeding in patients with a factor XI deficiency is seen. As might be expected, fibrinolytic inhibitors such as tranexamic acid and e-aminocaproic acid are successfully being used for the treatment of bleeding in patients with a factor XI deficiency.

Our findings may have some important implications. Thrombin is believed to play a very important role in both venous and arterial thrombosis. Thrombin bound to a clot retains coagulant activity, although it is relatively inaccessible to inhibition by heparin-antithrombin III, turning the clot into a powerful thrombogenic surface. For this reason, inhibitors of thrombin (heparin or hirudin) are used alone as antithrombotics or as adjuvants in fibrinolytic treatment of these thrombotic states. A major disadvantage of these agents is that their local antithrombotic effect parallels a systemic anticoagulant effect. Therefore, treatment is presently hampered by bleeding complications, although failure to achieve reperfusion and occurrence of subsequent reocclusion demand higher doses of heparin or hirudin.

Activation of the intrinsic pathway by thrombin-mediated factor XI activation may play an important role in venous and arterial thrombosis. The factor XI-dependent thrombin generation that was observed to continue within the clot is especially of interest, because it may contribute to the thrombogenic state of the clot. Inhibition of thrombin-mediated factor XI activation, e.g., with antibodies or peptides, may result in clots that are less thrombogenic and more susceptible to fibrinolysis. Furthermore, the risk of bleeding complications may be low, because this therapy would not be accompanied by strong anticoagulant effects. Thrombin-dependent coagulation at the site of the clot would be inhibited, whereas tissue factor-dependent hemostasis elsewhere would be less affected. Inhibition of coagulation may turn out to be more effective at the start of the coagulation cascade by interfering with thrombin-mediated factor XI activation than at the end by using heparin or hirudin.

NOTE ADDED IN PROOF

Recently, Bajzár et al published an article describing the existence of a thrombin-activatable fibrinolysis inhibitor (TAFI), which may be a plausible explanation for the thrombin-dependent inhibition of fibrinolysis in our system.

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