Thrombin-mediated Activation of Factor XI Results in a Thrombin-activatable Fibrinolysis Inhibitor-dependent Inhibition of Fibrinolysis

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Abstract

Recently, it has been shown that Factor XI can be activated by thrombin, and that Factor Xla significantly contributes to the generation of thrombin via the intrinsic pathway after the clot has been formed. This additional thrombin, generated inside the clot, was found to protect the clot from fibrinolysis. A plausible mechanism for this inhibitory effect of thrombin involves TAFI (thrombin-activatable fibrinolysis inhibitor, procarboxypeptidase B) which, upon activation, may inhibit fibrinolysis by removing carboxy-terminal lysines from fibrin.

We studied the role of Factor XI and TAFI in fibrinolysis using a clot lysis assay. The lysis time was decreased two-fold when TAFI was absent, when TAFI activation was inhibited by anti–TAFI antibodies, or when activated TAFI was inhibited by the competitive inhibitor (2-guanidinoethylmercapto)succinic acid. Inhibition of either TAFI activation or Factor Xla exhibited equivalent profibrinolytic effects. In the absence of TAFI, no additional effect of anti–Factor XI was observed on the rate of clot lysis.

We conclude that the mechanism of Factor XI–dependent inhibition of fibrinolysis is through the generation of thrombin via the intrinsic pathway, and is dependent upon TAFI. This pathway may play a role in determining the fate of in vivo formed clots. (J. Clin. Invest. 1997. 99:2323–2327.) Key words: coagulation • fibrinolysis • Factor XI • thrombin • carboxypeptidase

Introduction

The activation of Factor XI by thrombin was first demonstrated in the presence of the nonphysiological glycosaminoglycan dextran sulfate (1, 2). Later it was shown that thrombin-mediated Factor XI activation could also take place in the absence of an activating surface, although only trace amounts of Factor Xla were formed (3). Activation of Factor XI by thrombin is potentially important because these traces of Factor Xla contribute to the formation of large amounts of activated Factor X by the dramatic amplification in the coagulation cascade (3).

The activation of Factor XI by thrombin was also demonstrated in a plasma environment. In a tissue factor–induced clotting system using sulfatides as a surface, Factor XI was found to induce activation of Factor IX independent of Factor XII (4). More interestingly, Factor XI was also shown to be activated by thrombin in the absence of a surface (5). In a tissue factor–induced clotting system, Factor XI increased the rate of fibrin formation, confirming the contribution of Factor Xla to the coagulation system (5). Furthermore, the thrombin-mediated activation of Factor XI inhibited tissue type plasminogen activator (t-PA)–induced clot lysis (5). Trace amounts of activated Factor XI, representing 0.01% activation, were sufficient to inhibit fibrinolysis maximally. Exhaustive activation of prothrombin takes place predominantly after the formation of the fibrin clot, however, only in the presence of Factor XI (5). Therefore, protection of the clot from fibrinolytic attack was dependent on the thrombin that was generated in a Factor XI–dependent manner via the intrinsic pathway. The inhibitory effect of Factor XI on fibrinolysis provides an explanation for why patients with a Factor XI deficiency bleed from tissues with high local fibrinolytic activity (6).

The mechanism behind the inhibitory effect of thrombin is unclear. A potential candidate is TAFI (thrombin-activatable fibrinolysis inhibitor) (7). TAFI was isolated in the search for a mechanism behind the profibrinolytic effect of activated protein C (7–11). This protein C–dependent profibrinolytic effect had been attributed to downregulation of thrombin activation (12); TAFI, therefore, provides a connection between thrombin generation and inhibition of fibrinolysis. Amino-terminal sequence analysis showed that TAFI is similar to plasma procarboxypeptidase B (7). Plasma procarboxypeptidase B was isolated in 1991 as a plasminogen-binding protein (13), and was found to be very similar to the tissue form isolated some 30 yr earlier (14). In addition, a carboxypeptidase was purified from serum, and was called carboxypeptidase U because it was unstable (15). Carboxypeptidase U was later shown to be identical to plasma carboxypeptidase B (16). The mechanism by which activated TAFI inhibits fibrinolysis is still unclear. It is hypothesized that activated TAFI removes carboxy-terminal lysines from fibrin that play a key role in plasminogen binding of Factor XIa.

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Abbreviations used in this paper: TAFI, thrombin-activatable fibrinolysis inhibitor; t-PA, tissue type plasminogen activator.

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and activation (7, 13, 17–19). Many interactions in the fibrinolytic system, however, are promoted by the presence of lysine binding sites which could be inhibited by the action of carboxypeptidases. We investigated the inhibition of fibrinolysis by thrombin (reciprocally generated via the intrinsic pathway initiated by thrombin-mediated Factor XI activation) in clots formed from plasma. Our results indicate that Factor XI–dependent inhibition of fibrinolysis is TAFI dependent.

**Methods**

**Materials.** Bovine serum albumin (fraction V) was purchased from Sigma (St. Louis, MO). GEMSA (2-guanidinoethylmercaptosuccinic acid) was obtained from Fluka AG (Buchs, Switzerland). Other chemicals obtained were the best grade available. Factor XI was purified from fresh frozen plasma using a murine anti–Factor XI monoclonal antibody (XI-5) immobilized on CNBr-activated Sepharose, as described earlier (3). The specific coagulant activity of Factor XI was 235 U/mg. Factor XIa was purified on CNBr-activated Sepharose, as described earlier (3). The specific clotting activity of Factor XI was 235 U/mg. Factor XIa was prepared from Factor XI using β-Factor XIIa as described previously (20). A monoclonal antibody against Factor XI capable of blocking Factor IX activation (XI-1) has been described (21). A monoclonal antibody against TAFI (mAbTAFI#16) was prepared as described (22). A polyclonal antibody against TAFI was prepared in rabbits. The antiserum was purified on protein G–Sepharose, and was able to completely inhibit TAFI in a clot lysis assay.

TAFI was purified from fresh frozen plasma as described (7). TAFI-deficient plasma was obtained by passing plasma from a healthy donor over a Sepharose column to which mAbTAFI#16 (2.0 mg/ml) was coupled. The undiluted flow-through fractions of the columns were pooled and used as deficient plasma. Less than 0.1% of TAFI was present in the deficient plasma as determined by ELISA. Factor XII–deficient plasma was obtained from a congenitally deficient patient. Plasma deficient in both Factor XII and Factor XI was prepared by passing the Factor XII–deficient plasma over a Sepharose column to which a murine anti–Factor XI antibody (XI-5) was coupled. The undiluted flow-through fractions of the column were pooled and used as deficient plasma. Less than 0.1% of Factor XI was present in the Factor XI and XII double-deficient plasma. Purified human thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, Albuquerque, NM) (23).

Clot lysis assay in clots formed from plasma. The inhibition of t-PA-induced clot lysis by thrombin-mediated Factor XI activation was studied in clots formed from human plasma by monitoring the change in turbidity during fibrin formation and lysis at 405 nm in a microplate reader as described earlier (5). All experiments were performed in citrated plasma recalcified with CaCl₂ (final concentration of 17 mM), resulting in a free calcium concentration of 2.3 mM. The plasmas contained sufficient endogenous phospholipids to sustain coagulation. Thrombin (20 nM), calcium necessary for recalcification, and t-PA (10–30 U/ml) were added to 75 μl of the reaction mixture. After 3 min, coagulation was initiated with Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl₂, 0.1% BSA, pH 7.4) resulting in a final plasma concentration of 50%. After mixing, 100 μl of the reaction mixture was pipetted in a microtiter plate, and was then incubated at 37°C during which turbidity was measured at different time points. Lysis time (defined as the time to achieve a 50% reduction of the maximum turbidity) was determined from the plot of A_{405} versus time of three independent experiments. A monoclonal anti-Factor XI antibody (XI-1) (21), capable of blocking the activation of Factor IX by Factor Xla, was preincubated for 30 min with the plasma to determine the effect of Factor XI. To assess the requirement for TAFI in the effect of Factor XI or Factor Xla on clot lysis in this system, mAbTAFI#16, or a rabbit antibody against TAFI (antibodies that both inhibit the activation of TAFI) was also preincubated for 30 min with the plasma. Alternatively, the effect of XI-1 on clot lysis was also studied with TAFI-deficient plasma in the presence and absence of a reconstituting amount of purified TAFI. All experiments (except those with the TAFI-deficient, or Factor XI and XII double-deficient plasma) were performed in pooled plasma from 40 healthy volunteers.

**Results**

**Effect of TAFI and Factor XI on the lysis of a thrombin-induced clot.** To study the effect of both Factor XI and TAFI on clot lysis, thrombin (20 nM) and t-PA (30 U/ml) were added to recalcified plasma preincubated with antibodies that inhibit Factor XIa (XI-1), or antibodies that inhibit activation of TAFI (mAbTAFI#16). The presence of 20 nM of thrombin produced a clot within 10 min (5). Subsequent t-PA–induced clot lysis was followed in time by measuring turbidity of the formed clot. In the presence of the Factor XIa blocking antibody XI-1, an enhancement of clot lysis was observed that was caused by inhibition of thrombin generation by the intrinsic pathway (Fig. 1). The antibody XI-1 was found to have a maximal effect on clot lysis at a concentration of 25 μg/ml (data not shown). mAbTAFI#16 also enhanced lysis of the clot, an effect that was also maximal at a concentration of 25 μg/ml (data not shown). Therefore, the presence of mAbTAFI#16 and XI-1 resulted in a rapid lysis of the clot (Fig. 1). The lysis that was observed with XI-1 did not differ from the lysis observed with TAFI-16 (lysis times 42±4 and 41±3 min, respectively; lysis time in the absence of antibodies: 92±5 min). Lysis could not be enhanced further by the addition of both antibodies together (lysis time: 42±4 min).

The effect of both Factor XI and TAFI on the lysis of a thrombin-induced clot was also studied in TAFI-deficient plasma to which XI-1 and a reconstituting amount of purified TAFI (60 nM) were added. Essentially the same effects of Factor XI and TAFI were observed (Fig. 2). Again, in the absence of TAFI, no effect of XI-1 on clot lysis was observed.

**The effect of Factor XIa and TAFI on clot lysis.** The effect of Factor XIa and TAFI on the lysis of a thrombin-induced clot was assessed by monitoring the change in turbidity during fibrin formation and lysis at 405 nm in a microplate reader as described earlier (5). All experiments were performed in citrated plasma recalcified with CaCl₂ (final concentration of 17 mM), resulting in a free calcium concentration of 2.3 mM. The plasmas contained sufficient endogenous phospholipids to sustain coagulation. Thrombin (20 nM), calcium necessary for recalcification, and t-PA (10–30 U/ml) were added to 75 μl of the reaction mixture. After 3 min, coagulation was initiated with Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl₂, 0.1% BSA, pH 7.4) resulting in a final plasma concentration of 50%. After mixing, 100 μl of the reaction mixture was pipetted in a microtiter plate, and was then incubated at 37°C during which turbidity was measured at different time points. Lysis time (defined as the time to achieve a 50% reduction of the maximum turbidity) was determined from the plot of A_{405} versus time of three independent experiments. A monoclonal anti-Factor XI antibody (XI-1) (21), capable of blocking the activation of Factor IX by Factor Xla, was preincubated for 30 min with the plasma to determine the effect of Factor XI. To assess the requirement for TAFI in the effect of Factor XI or Xla on clot lysis in this system, mAbTAFI#16, or a rabbit antibody against TAFI (antibodies that both inhibit the activation of TAFI) was also preincubated for 30 min with the plasma. Alternatively, the effect of XI-1 on clot lysis was also studied with TAFI-deficient plasma in the presence and absence of a reconstituting amount of purified TAFI. All experiments (except those with the TAFI-deficient, or Factor XI and XII double-deficient plasma) were performed in pooled plasma from 40 healthy volunteers.

**Figure 1.** Lysis of a thrombin-induced clot in the presence or absence of antibodies against Factor XI or TAFI. Normal plasma was preincubated with XI-1 (△, 160 nM), mAbTAFI#16 (▼, 160 nM), XI-1 and mAbTAFI#16 (▲, 160 nM), or buffer (▼). Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM), and t-PA (30 U/ml). Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm. The means±SD of three independent experiments are given.
Inhibition of Fibrinolysis by a Factor XI- and TAFI-dependent Mechanism

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Addition of trace amounts of Factor XIa (12.5 fM) already resulted in an increase in the lysis time (from 47 to 66 min). The lysis time was further increased (to 110 min) by the addition of higher concentrations of Factor XIa. Preincubation of the plasma with an anti-TAFI antibody completely abolished the effect of Factor XIa on the rate of clot lysis. This indicates that the effect of TAFI on clot lysis is dependent on the amount of Factor XIa generated in a normally functioning coagulation cascade.

**Figure 2.** Effect of TAFI on the lysis of a thrombin-induced clot in the presence or absence of a Factor XIa–inhibiting antibody. Plasma deficient in TAFI was preincubated with TAFI (∇, 60 nM), XI-1 (▲, 160 nM), TAFI and XI-1 (Δ, 60 and 160 nM, respectively), or buffer (●). Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM), and t-PA (30 U/ml). Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm. Each point represents the mean ± SD of three independent experiments.

**Figure 3.** Effect of Factor XIa on the lysis of a thrombin-induced clot. Factor XI and XII double-deficient plasma was preincubated with buffer (●), Factor XIa: 12.5 fM (▲), 125 fM (■), 12.5 pM (●), and Factor XIa: 12.5 pM in the presence of a polyclonal anti-TAFI antibody (○). Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM), and t-PA (30 U/ml). Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm.

**Figure 4.** Lysis of a thrombin-induced clot in the presence or absence of antibodies against Factor XI or TAFI: effect of various t-PA concentrations. Normal plasma was preincubated with 160 nM mAbTAFI#16 in the presence (closed symbols) or absence (open symbols) of 160 nM XI-1. Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM), and t-PA (10–30 U/ml). Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm. t-PA: 30 U/ml (△), 25 U/ml (○), 20 U/ml (●), 10 U/ml (∇). The mean ± SD of three independent experiments are given.

The effect of XI-1 and mAbTAFI#16 on clot lysis using various concentrations of t-PA. To exclude the possibility that, in the absence of TAFI, lysis may take place so fast that no effect of Factor XI can be observed, we studied the effect of XI-1 and mAbTAFI#16 on the lysis of a thrombin-induced clot using various concentrations of t-PA (10–30 U/ml). At all t-PA concentrations used, no additional Factor XI effect was discernible in the presence of the antibody that inhibits activation of TAFI (Fig. 4).

**Figure 4.** Lysis of a thrombin-induced clot in the presence or absence of antibodies against Factor XI or TAFI: effect of various t-PA concentrations. Normal plasma was preincubated with 160 nM mAbTAFI#16 in the presence (closed symbols) or absence (open symbols) of 160 nM XI-1. Coagulation was initiated by adding calcium necessary for recalcification, thrombin (20 nM), and t-PA (10–30 U/ml). Fibrin formation and lysis were measured in time as the change in turbidity at 405 nm. t-PA: 30 U/ml (△), 25 U/ml (○), 20 U/ml (●), 10 U/ml (∇). The mean ± SD of three independent experiments are given.

Effect of GEMSA on the clot lysis assay. GEMSA, a competitive inhibitor of carboxypeptidase B (7, 13), was also used to study the contribution of TAFI on the effect of Factor XI on clot lysis. By using different concentrations of GEMSA, it was determined that a maximum effect on clot lysis was reached at 0.5–1 mM (data not shown). Therefore, the effect of the Factor XIa–inhibiting antibody XI-1 on the lysis of a thrombin-induced clot was studied in the presence and absence of 1 mM GEMSA. In the presence of XI-1, GEMSA could not enhance the rate of clot lysis (Fig. 5), suggesting that to inhibit fibrinolysis, TAFI needs thrombin that is generated in a Factor XI–dependent manner via the intrinsic pathway. In the presence of GEMSA however, the addition of XI-1 resulted in a faster lysis of the clot. This result can be explained by the fact that GEMSA is a competitive inhibitor incapable of completely inhibiting activated TAFI.

**Discussion**

Patients with a deficiency of Factor XI are prone to bleed from tissues with a high local fibrinolytic activity (6). A possible explanation for this clinical observation was recently provided when it was demonstrated that the presence of Factor XI during coagulation has an inhibitory effect on clot lysis (5). An intact intrinsic pathway was found to be necessary for the inhibi-
tion of fibrinolysis by Factor XI because an increased rate of clot lysis was observed in Factor XI–deficient plasma as well as in Factor II–, IX–, or X–deficient plasma, whereas in Factor VII–deficient plasma, a normal lysis rate was observed (5, 24).

To determine which Factor of the intrinsic pathway was responsible for inhibition of fibrinolysis, clot lysis was studied in prothrombin-deficient plasma to which Factor IXa, Xa, or thrombin was added. Factor IXa and Xa had no effect on fibrinolysis, whereas a concentration-dependent antifibrinolytic effect of thrombin was observed. We concluded that the antifibrinolytic effect of Factor XI was mediated by the formation of additional thrombin in a reciprocal activation process involving the activation of Factor XI by thrombin in the intrinsic pathway (5). These effects were observed in the absence of an activating surface (e.g., dextran sulfate). Previous studies had shown that activation of Factor XI by thrombin takes place in the absence of such a surface (2, 3, 5). Small amounts of activated Factor XI are generated, but even very small amounts of activated Factor XI, representing 0.01% activation, are capable of inhibiting clot lysis (5), showing not only the amplification power of the coagulation cascade, but also the potential importance of traces of Factor XIa. An activating surface on which activation of Factor XI takes place, however, may further potentiate this process.

Recently, a thrombin-activatable fibrinolysis inhibitor (TAFI) has been described (7) that may provide an explanation for the observed inhibitory effect of Factor XI on clot lysis (5). Therefore, in this report we investigated the Factor XI–dependent inhibition of fibrinolysis in the presence and absence of TAFI activation.

Experiments were performed with the same clot lysis assay that was used to show the inhibitory effect of Factor XI on clot lysis (5). In this assay, fibrin formation is induced by the addition of thrombin to recalcified plasma, after which t-PA–mediated lysis of the clot is monitored by change in turbidity. In this way, interference of the extrinsic pathway is excluded. Furthermore, we have shown previously that the contact activation does not play a role in this system, since Factor XIIa–blocking antibodies did not affect lysis time (5). The effect of Factor XI on lysis was determined by the addition of a Factor XIa–inhibiting antibody (XI-1). The presence or absence of Factor XI had no effect on clotting time in our system, nor had it an effect on the structure of the clot (data not shown). The plasmas contained sufficient endogenous phospholipids to sustain coagulation. Addition of exogenous phospholipids gave identical results.

First, the effect of TAFI in the lysis assay was studied with a monoclonal antibody that inhibits activation of TAFI. Lysis in the presence of this antibody took place at the same rate as in the presence of Factor XIa–inhibiting antibody. An additive or synergistic effect was not observed in the presence of both antibodies. We conclude that the Factor XI–dependent inhibition of clot lysis is mediated through TAFI. Second, it was shown that addition of increasing amounts of Factor XIa resulted in an increase in the lysis time of a thrombin-induced fibrin clot in plasma deficient in both Factor XI and XII. The effect of Factor XIa was abolished by preincubation with anti–TAFI antibodies, indicating that the effect of TAFI on clot lysis is dependent on the amount of Factor XIa generated. Then, lysis experiments were performed with TAFI-deficient plasma in the presence and absence of a reconstituting amount of TAFI. The effects of XI-1 on lysis were comparable to those observed with the antibody that inhibits activation of TAFI. In the absence of TAFI, XI-1 had no effect on clot lysis. Finally, lysis experiments were performed in clots formed from plasma in the presence and absence of GEMSA, a competitive inhibitor of the carboxypeptidase B family of enzymes, which includes activated TAFI. In the presence of GEMSA, lysis times were reduced (relative to the lysis time in the absence of GEMSA), but XI-1 did not reduce lysis time in the presence of GEMSA. These results further indicate that TAFI plays a central role in the Factor XI–dependent inhibition of clot lysis.

In contrast to the small amounts of thrombin that are needed for clot formation, high concentrations of thrombin are needed for both the inhibition of clot lysis (5) and the activation of TAFI (7). Earlier studies in which thrombin activity was measured during coagulation showed that relatively small amounts of thrombin (10–20 nM) are present at the moment of clotting, whereas high concentrations of thrombin (100–200 nM) are generated within the clot (25). We also found that thrombin activation continued inside the fibrin clot (5) by measuring generation of F1+2. Exhaustive thrombin activation was observed in a Factor XI–dependent way in parallel to the antifibrinolytic effect (5). These data indicated that the concentrations of thrombin that are generated inside the clot in a Factor XI–dependent way are sufficient for the activation of TAFI. Activated TAFI is then able to protect the clot from fibrinolytic attack.

In the presence of endothelial cells, the thrombin that is generated by the intrinsic pathway will also activate protein C on thrombomodulin. Activated protein C can reduce thrombin generation by inactivating Factor Va and VIIIa, resulting in a reduced activation of TAFI and increased clot lysis. This mechanism may explain the reported profibrinolytic effects of activated protein C (8–11). Thrombomodulin, however, is also capable of enhancing the activation of TAFI by thrombin by three orders of magnitude (26). It still remains to be demon-
strated whether the protein C– or TAFI–stimulating effect of thrombomodulin dominates in vivo.

We conclude that TAFI plays a crucial role in the inhibition of fibrinolysis by thrombin generated via the intrinsic pathway through thrombin-mediated Factor XI activation. This pathway may thereby play an important role in determining the fate of clots that are formed in vivo. Inhibition of this pathway may result in clots that are more susceptible to fibrinolysis, and may therefore prove to be a valuable addition to fibrinolytic therapy. This therapy can be done directly by inhibition of TAFI with antibodies or peptides, or indirectly by inhibiting thrombin generation via the intrinsic pathway by interfering with thrombin-mediated Factor XI activation.

Future research will have to show which approach will render the best results with the fewest (bleeding) complications.

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