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Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor.

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Research Article

Recent in vitro studies have shown that fibrinolytic activity may be attenuated by a thrombin-activatable fibrinolysis inhibitor (TAFI), which is activated by thrombin, generated via the intrinsic pathway of coagulation in a factor XI-dependent way. Thus factor XI may play a role in the regulation of endogenous fibrinolysis. The aim of this study was to investigate the effect of in vivo inhibition of factor XI and TAFI in an experimental thrombosis model in rabbits. Incorporation of anti-factor XI antibodies in jugular vein thrombi resulted in an almost twofold increase in endogenous thrombolysis compared with a control antibody. A similar effect was observed when the anti-factor XI antibody was administered systemically. Inhibition of TAFI activity also resulted in a twofold increase in clot lysis whereas inhibition of both factor XI and TAFI activity had no additional effect. Thus, we provide the first in vivo evidence for enhanced thrombolysis through inhibition of clotting factor XI, demonstrating a novel role for the intrinsic pathway of coagulation. Furthermore we demonstrate that inhibition of TAFI had a similar effect on thrombolysis. We postulate that inhibition of factor XI activity enhances thrombolysis because of diminished indirect [...]

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Enhancement of Rabbit Jugular Vein Thrombolysis by Neutralization of Factor XI In Vivo Evidence for a Role of Factor XI as an Anti-fibrinolytic Factor

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Abstract

Recent *in vitro* studies have shown that fibrinolytic activity may be attenuated by a thrombin-activatable fibrinolysis inhibitor (TAFI), which is activated by thrombin, generated via the intrinsic pathway of coagulation in a factor XI-dependent way. Thus factor XI may play a role in the regulation of endogenous fibrinolysis. The aim of this study was to investigate the effect of *in vivo* inhibition of factor XI and TAFI in an experimental thrombosis model in rabbits.

Incorporation of anti-factor XI antibodies in jugular vein thrombi resulted in an almost twofold increase in endogenous thrombolysis compared with a control antibody. A similar effect was observed when the anti-factor XI antibody was administered systemically. Inhibition of TAFI activity also resulted in a twofold increase in clot lysis whereas inhibition of both factor XI and TAFI activity had no additional effect.

Thus, we provide the first *in vivo* evidence for enhanced thrombolysis through inhibition of clotting factor XI, demonstrating a novel role for the intrinsic pathway of coagulation. Furthermore we demonstrate that inhibition of TAFI had a similar effect on thrombolysis. We postulate that inhibition of factor XI activity enhances thrombolysis because of diminished indirect activation of TAFI. (*J. Clin. Invest.* 1998. 101:10–14.) Key words: factor XI • TAFI • fibrinolysis • factor XI deficiency • rabbits

Introduction

Activation of coagulation *in vivo* predominantly proceeds by tissue factor/factor VIIa-mediated activation of clotting fac-

tors X and IX (1, 2). Consequently, the role of the contact activation system in the initiation of coagulation (intrinsic pathway) for normal hemostasis is considered to be less important, although a role of this system in the activation of fibrinolysis and in other regulatory processes has been postulated (3, 4). In this regard, the role of factor XI, which is supposed to be the intermediate between the contact system and factor IX, remains obscure. Patients with an inherited or acquired deficiency of factor XI do show a bleeding tendency, which is variable in its expression and often mild regarding its natural course (5–7). Recent studies have shown that factor XI is activated during blood coagulation and that even small amounts of activated factor XI may induce the formation of thrombin (8, 9). This additional thrombin can protect a fibrin clot from fibrinolysis by activating a recently identified protein, the thrombin-activatable fibrinolysis inhibitor (TAFI)¹ (10, 11). Thereby, factor XI may be important for clot stability since *in vitro* experiments demonstrated that inhibition of factor XI activity resulted in enhanced fibrinolysis of fibrin clots (11), indicating that factor XI may play a role as regulator of fibrinolysis during activation of blood coagulation. In order to establish the significance of these observations for fibrinolysis *in vivo*, we have studied the effect of the neutralization of factor XI activity as compared with inhibition of TAFI activity on endogenous thrombolysis in an experimental thrombosis model in rabbits.

Methods

In vitro inhibition of rabbit factor XI activity. Normal citrated rabbit plasma was diluted 1:3 with Imidazolcitrate buffer (0.05 M Imidazol, 0.1 M NaCl, 0.06% citrate, pH 7.4) to the same level of clotting activity as human factor XI (12). Diluted rabbit plasma was incubated with increasing concentrations (range 0.005–0.5 mg/ml) of a polyclonal goat anti-rabbit factor XI antibody (IgG fraction, purified by affinity chromatography [DEAE Affigel Blue]), generously supplied by Dr. S.I. Rapaport (Department of Medicine, University of California at San Diego, La Jolla, CA) (13). Samples were tested in a one-stage clotting assay using human factor XI-deficient plasma (DiaMed AG, Cressier sur Morat, Switzerland) (14). The inhibition of factor XI activity at different concentrations of the antibody was expressed as

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1. Abbreviations used in this paper: PCI, potato carboxypeptidase inhibitor; TAFI, thrombin-activatable fibrinolysis inhibitor.

percentage of factor XI activity in human plasma, which was used as standard.

Clot lysis assay in clots formed from rabbit plasma. Fibrin clot lysis *in vitro* was studied by monitoring the decrease in turbidity at 405 nm in a microtiter plate reader at 37°C as described (9). Experiments were performed in citrated rabbit plasma recalcified with CaCl₂ (17 mmol/liter). A mixture of purified human thrombin (20 nmol/liter; kindly supplied by Dr. W. Kiesel, University of Mexico, Albuquerque, NM) and t-PA (20 U/ml; Chromogenix, Mölndal, Sweden) was added to plasma to initiate clotting and subsequent lysis. The anti-factor XI antibodies were preincubated for 30 min with the plasma at increasing concentrations (range 0.1–0.5 mg/ml). Inhibition of TAFI activity in the clot lysis assay was studied with a potato carboxypeptidase inhibitor (PCI; Calbiochem Corp., La Jolla, CA) in increasing concentrations (range 1–20 µg/ml).

Measurement of plasma factor XI activity. Blood samples were collected in vacutainer tubes containing sodium citrate (concentration 0.105 mol/liter) via the carotid cannula at the start of the experiment (t = 0) and at 30, 60, and 120 min after the start of the infusion of the study medication. Platelet poor plasma was obtained by centrifugation of the blood samples at 1,600 g for 20 min at room temperature. Plasma samples were stored at –70°C until assayed. Factor XI activity was measured in a one stage clotting assay using human factor XI deficient plasma and expressed as percentage of factor XI activity in human plasma, which was used as standard (14).

Rabbit jugular vein endogenous thrombolysis model. New Zealand white rabbits (~2.5 kg) were anesthetized with 9 mg ketamine (Aescocet, Boxtel, The Netherlands) and 0.5 ml Rompun 2% (Bayer AG, Leverkusen, Germany) intramuscularly. Anesthesia was maintained by repeated administration of ketamine. The carotid artery and jugular veins were exposed by a median incision in the neck. In the carotid artery a cannula (Baby Feeding Tube, 1.6 mm Ø) was introduced for the administration of the study agents and collection of blood samples. The jugular veins were exposed on both sides for a distance of 2 cm and all side branches were ligated. The venous segments were isolated by application of vessel clamps proximally and distally.

To assess the extent of thrombolysis, ¹²⁵I-radiolabeled thrombi were injected into the isolated venous segments. The labeled thrombi were prepared by incubating homologous citrated rabbit blood with ¹²⁵I-radiolabeled human fibrinogen (Amersham, Den Bosch, The Netherlands; final radioactivity 2.5 µCi/ml). An aliquot of 150 µl of this mixture was aspirated in a syringe containing 25 µl thromboplastin (Thromboplastin IS; Baxter, Maarsse, The Netherlands), 45 µl CaCl₂ (0.25 mmol/liter) and either polyclonal goat anti-rabbit factor XI antibodies (0.1 mg/ml), PCI (20 µg/ml), or polyclonal goat anticuloplasmin (0.1 mg/ml, control; CLB, Amsterdam, The Netherlands). Hereafter, the mixture was quickly injected in the isolated venous segment. The procedure was repeated for the contralateral site. After 30 min of aging, the vessel clamps were removed, blood flow was restored, and a systemic injection of the anti-factor XI antibodies (0.1 or 0.01 mg/ml), control antibodies, or no systemic injection was given. 2 h later, both thrombi were removed and washed. The extent of thrombolysis was assessed by comparing the remaining radioactivity of the thrombi at the end of the experiment with the initial radioactivity of the preformed clots. Thrombolysis was expressed as a percentage of the initial thrombus radioactivity.

Study design. All animal studies were approved by the Institutional Review Board for Animal Experiments and were performed according to the guidelines of the American Physiological Society and Dutch Law for Animal Experiments.

In the first series of experiments, the rabbits were randomly assigned to one of six groups, in each group six thrombi were studied. In the first three groups, the anti-factor XI antibody (0.1 mg/ml) was incorporated in the preformed clot. In group 1, no systemic anti-factor XI was given, whereas rabbits in groups 2 and 3 were administered anti-factor XI antibodies systemically, at a dose of 0.01 mg/ml and 0.1 mg/ml, respectively. Rabbits in group 4 and 5 only received the anti-

factor XI antibody systemically at a dose of 0.01 mg/ml and 0.1 mg/ml, respectively. In group 6, rabbits did not receive anti-factor XI antibody at all (control group).

In the second series of experiments, the rabbits were randomly assigned to one of four groups, in each group six thrombi were studied. In group 7, again the anti-factor XI antibodies (0.1 mg/ml) were incorporated in the preformed clots and in group 8 the PCI (20 µg/ml) was incorporated. In group 9 both the anti-factor XI antibodies as well as PCI were incorporated and group 10 served as a control group, i.e., incorporation of control antibodies. No systemic injections were given.

Statistical analysis. Counting of the thrombi and calculation of the degree of clot lysis was performed by an independent investigator, blinded for the group assignment. Statistical analysis was performed by ANOVA and Newman-Keuls test. A *P* value < 0.05 was considered statistically significant. All values are presented as mean ± SD.

Results

***In vitro* inhibition of rabbit factor XI coagulant activity.** Increasing concentrations of the polyclonal anti-factor XI rabbit antibodies resulted in a dose-dependent inhibition of rabbit plasma factor XI (Fig. 1). At concentrations of 0.25 mg/ml or higher, rabbit plasma factor XI activity was virtually completely blocked.

Clot lysis assay in clots formed from rabbit plasma. The clot lysis of rabbit plasma *in vitro* could be enhanced by incorporation of anti-factor XI antibodies in the clot (Fig. 2). To demonstrate that TAFI was involved, clot lysis was studied in the presence of PCI. Initial experiments (not shown) had demonstrated that incorporation of more than 10 µg/ml of PCI in the clot resulted in maximally enhanced lysis. The lysis in the

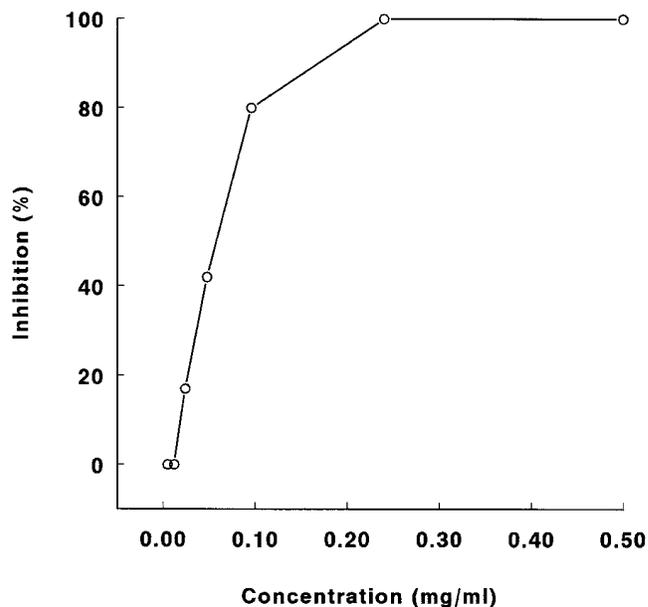


Figure 1. Inhibition of factor XI activity after *in vitro* incubation with anti-factor XI antibody. The polyclonal anti-rabbit factor XI antibody was incubated with an equal volume of normal citrated rabbit plasma (1:3 diluted) for 2 h at 37°C. Samples were tested in a one stage clotting assay to establish the inhibition of factor XI activity at different concentrations of the antibody. 501% inhibition was demonstrated at a concentration of 0.05 mg/ml.

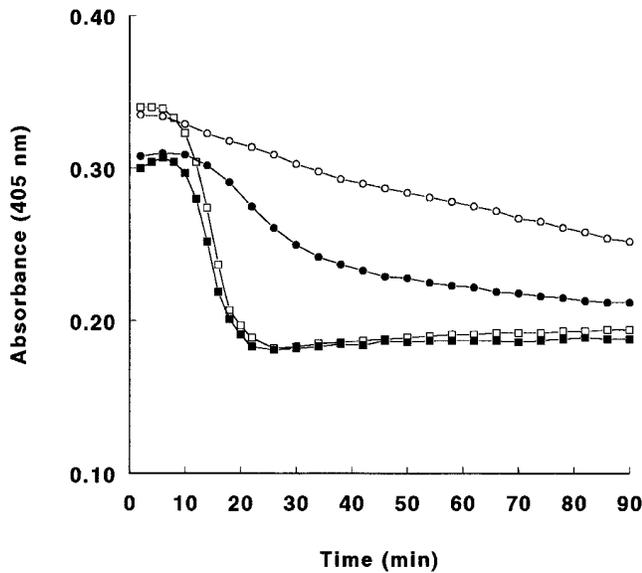


Figure 2. Lysis of a thrombin induced clot in the presence or absence of anti-factor XI antibodies or PCI. Rabbit plasma was incubated with buffer (white circles), anti-factor XI antibodies, 0.5 mg/ml, (black circles), PCI, 20 μ g/ml, (white squares), or both (black squares). Coagulation and subsequent fibrinolysis was initiated by adding calcium, thrombin (20 nmol/liter), and t-PA (20 U/ml). Fibrinolysis was measured in time as the change in turbidity at 405 nm.

presence of 20 μ g/ml of PCI could not be further enhanced by the addition of anti-factor XI antibodies (Fig. 2) indicating that also in rabbit plasma, TAFI needs to be activated by thrombin in a factor XI-dependent manner via the intrinsic pathway (11).

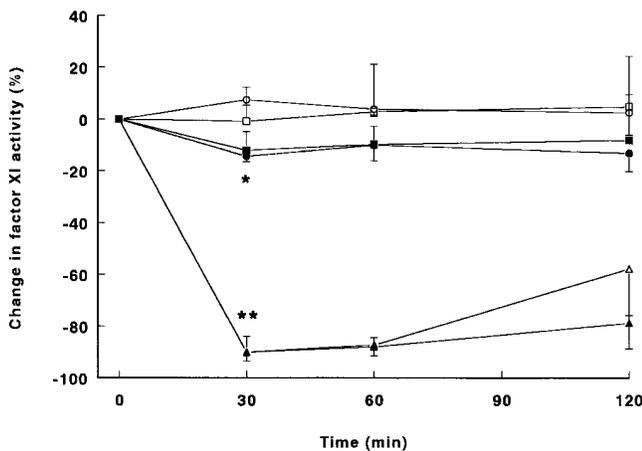


Figure 3. Effect of a bolus injection of anti-factor XI antibody on plasma factor XI activity in rabbits. Anti-factor XI antibodies or control antibodies were injected through a cannula in the carotid artery. Blood samples were drawn before ($t = 0$) and after 30, 60, and 120 min in citrated vacutainer tubes and factor XI activity was measured as described. The mean and SD are plotted as percentile change of baseline levels of three rabbits in each group. Control antibody in clot and systemic (white circles); anti-FXI antibody in clot (0.1 mg/ml), control antibody systemic (white squares); control antibody in clot, and anti-factor XI antibody systemic 0.01 mg/ml (black circles), or 0.1 mg/ml (black triangles). Anti-factor XI antibody in clot (0.1 mg/ml) and anti-factor XI antibody systemic 0.01 mg/ml (black squares) or 0.1 mg/ml (white triangles). Statistical significant difference as compared with control antibody (* $P < 0.05$, ** $P < 0.001$) is indicated.

Inhibition of plasma factor XI activity after administration of the anti-factor XI antibody. As shown in Fig. 3, the systemic administration of the anti-factor XI polyclonal antibodies to rabbits resulted in a significant decline in the plasma level of factor XI activity. At 30 min after the bolus administration of the antibodies at a concentration of 0.01 mg/ml, plasma factor XI activity levels were decreased with $13.2\% \pm 4.9$ ($P < 0.05$ as compared with the control group). Levels remained decreased up to 2 h after the administration, with $9.8\% \pm 5.9$ and $10.5\% \pm 12.4$ at 60 and 120 min, respectively. The intravenous bolus injection of the anti-factor XI antibodies at a concentration of 0.1 mg/ml resulted in a further reduction of plasma factor XI activity. At 30 min after the injection, factor XI activity was reduced by $90.0\% \pm 4.8$ ($P < 0.001$ as compared with the control group), and remained depressed up

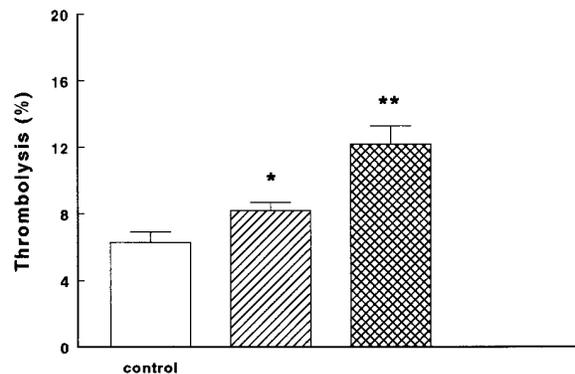
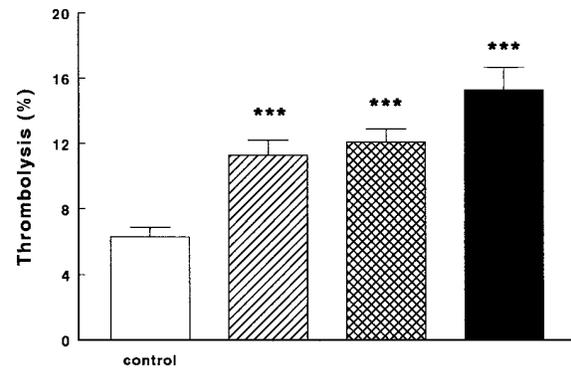


Figure 4. Effect of anti-factor XI antibody on endogenous thrombolysis. Top, endogenous thrombolysis after incubation of the thrombi with the anti-factor XI antibody with or without systemic injection of anti-factor XI antibody. The white bar represents control antibody (in thrombi and systemic), the shaded bar represents incubation of 0.1 mg/ml anti-factor XI antibody in thrombi and control antibody systemic. The hatched bar represents 0.1 mg/ml anti-factor XI in thrombi and 0.01 mg/ml systemic; the black bar represents 0.1 mg/ml in thrombi and 0.1 mg/ml systemic. Bottom: endogenous thrombolysis after systemic injection of the anti-factor XI antibody or a control antibody (white bar). The shaded bar represents systemic injection of 0.01 mg/ml anti-factor XI antibodies; the hatched bar represents systemic injection of 0.1 mg/ml. Thrombolysis is expressed as a percentage of the initial thrombus radioactivity. The mean and SD are given of six thrombi in each group. Statistically significant difference, as compared with control antibody (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), is indicated.

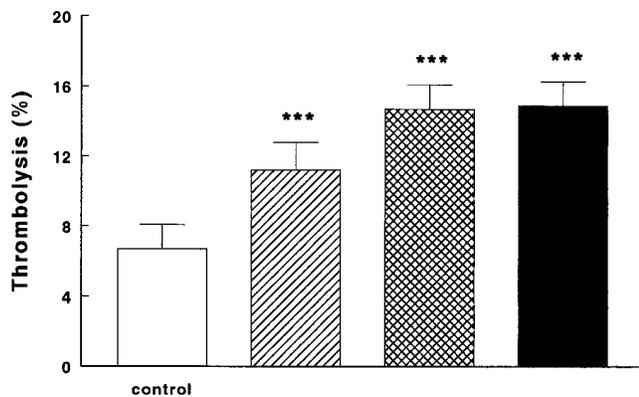


Figure 5. Effect of PCI on endogenous fibrinolysis. Endogenous fibrinolysis after incubation of the thrombi with the anti-factor XI antibodies and/or PCI. The white bar represents control antibody, the shaded bar represents incubation of 0.1 mg/ml anti-factor XI antibody, the hatched bar represents incubation of 20 µg/ml PCI, the black bar represents incubation of both the anti-factor XI antibodies and PCI. Thrombolysis is expressed as a percentage of the initial thrombus activity. The mean and SD of six thrombi are given in each group. Statistical difference as compared with control antibody (***) $P < 0.001$ is indicated.

to 2 h. At 120 min after the bolus administration, factor XI activity levels were still 70–80% lower than control values ($P < 0.005$). The plasma level of factor XI activity in the group receiving the control antibody remained stable during the experiment. The prior incorporation of anti-factor XI antibodies in the thrombi did not affect plasma factor XI activity levels in either experimental group.

Thrombolysis of clots with incorporated anti-factor XI antibodies. The incorporation of anti-factor XI antibodies in the thrombi resulted in an almost twofold increase in endogenous thrombolysis (Fig. 4, top). Clots that contained the anti-factor XI antibody showed $11.3\% \pm 0.9$ thrombolysis after 2 h as compared with $6.3\% \pm 0.6$ in control thrombi ($P < 0.001$). Systemic administration of anti-factor XI antibodies at a dose of 0.01 mg/ml or 0.1 mg/ml resulted in a small increase in thrombolysis of these clots to $12.1\% \pm 0.8$ and $15.3\% \pm 1.4$, respectively.

Thrombolytic effect of the systemic administration of anti-factor XI antibodies alone. The lysis of thrombi not preincubated with anti-factor XI antibodies, could be increased by the systemic administration of anti-factor XI antibodies only. As shown in Fig. 4, bottom, the systemic administration of 0.01 mg/ml anti-factor XI antibodies to the rabbits resulted in a 2-h thrombolysis of $8.2\% \pm 0.5$ (as compared with $6.3\% \pm 0.6$ thrombolysis of control thrombi, $P < 0.05$). Thrombolysis after administration of the higher dose of 0.1 mg/ml anti-factor XI antibodies was $12.2\% \pm 1.1$ ($P < 0.01$ as compared with the control thrombi and $P < 0.05$ as compared with the systemic dose of 0.01 mg/ml).

Thrombolysis of clots with PCI. In a second series of experiments the incorporation of anti-factor XI antibodies was repeated and compared with the effect of incorporation of PCI. As shown before, the incorporation of anti-factor XI antibodies resulted in enhanced lysis of clots compared with control thrombi ($11.2\% \pm 1.6$ and $6.7\% \pm 1.4$, respectively, $P < 0.001$, Fig. 5). Inhibition of TAFI activity by PCI, incorporated in the clots, also resulted in enhanced clot lysis ($14.7\% \pm 1.4$,

$P < 0.001$ as compared with control and anti-factor XI antibody thrombi). Incorporation of both the anti-factor XI antibodies and PCI had no additional effect ($14.9\% \pm 1.4$) as compared with incorporation of PCI alone.

Discussion

Recent studies have revealed a novel role of factor XI during activation of blood coagulation. These studies suggested that factor XI is an important mediator in the activation of TAFI, via its potency to generate additional thrombin, acting as a fibrinolytic inhibitor rather than as a coagulation factor (9, 11). TAFI is a plasma procarboxypeptidase that, upon activation by thrombin or the thrombin-thrombomodulin complex, attenuates fibrinolysis by removing the COOH-terminal lysine binding sites for plasminogen (15–18). In this study, we provide *in vivo* evidence for the anti-fibrinolytic effect of factor XI and the anti-fibrinolytic potency of TAFI in a thrombosis model in rabbits.

Clots formed in blood in which factor XI activity was neutralized, showed an enhanced lysis as compared with control clots. Also, clots formed in the presence of a potato carboxypeptidase inhibitor demonstrated increased lysis. PCI is an inhibitor of carboxypeptidases and TAFI is the only known carboxypeptidase capable of attenuating fibrinolysis (15). As shown in the clot lysis assay with rabbit plasma, PCI had a maximal effect at a concentration of 20 µg/ml, while factor XI activity was not completely inhibited using the anti-factor XI antibodies in a concentration of 0.1 mg/ml as shown *in vitro* (Fig. 1) and *in vivo* (Fig. 3). This probably explains the more pronounced lysis of the fibrin clots with the incorporation of PCI as compared with incorporation of the anti-factor XI antibodies.

Clots formed in blood with both PCI and the anti-factor XI antibodies showed no additional increment of fibrinolysis. This supports *in vitro* evidence that the anti-fibrinolytic mechanism of factor XI is primarily TAFI dependent and is not the result of an indirect effect of the anti-factor XI antibodies on fibrin formation during aging of the clots (11).

Incorporation of the anti-factor XI antibodies in the clot together with systemic neutralization of factor XI activity in the circulation, a situation best mimicking factor XI deficiency, showed an additional enhancement of thrombolysis. In these experiments, a 2.4-fold increase in thrombolysis in our model was observed. Systemic neutralization of factor XI activity alone resulted also in a similar increase in endogenous thrombolysis as compared with incorporation of the anti-factor XI antibodies in the fibrin clot. These results suggest that thrombin generation continues in a factor XI-dependent way after initial clot formation has taken place and that this thrombin is capable of activating TAFI. Clot-bound thrombin has been shown to be inaccessible to inhibition by the heparin-anti-thrombin complex and to retain its procoagulant activity on the surface of fibrin clots (19, 20). Furthermore, *in vitro* studies have shown that thrombin, bound to fibrin monomers, can still activate factor XI (21). Thus, in a factor XI-deficient plasma milieu, the diminished presence of clot-bound thrombin may have facilitated enhanced fibrinolysis, because of a lower degree of activation of TAFI. Indeed, therapeutic strategies that inhibited the activity of clot-bound thrombin enhanced endogenous thrombolysis in an experimental rabbit thrombosis model (22).

The importance of factor XI in thrombin-dependent inhibition of fibrinolysis can be explained by the amplification potential of the coagulation cascade. Upon formation of thrombin, factor XI is activated, inducing further conversion of intrinsic coagulation proteins. This process causes additional thrombin formation capable of inhibiting fibrinolysis through activation of TAFI.

These findings place the role of factor XI in hemostasis in a new perspective. The importance of factor XI for inhibition of fibrinolytic activity, and thereby clot stability, is illustrated by the clinical expression of factor XI deficiency. Patients with a congenital factor XI deficiency or acquired factor XI antibodies, show a variable bleeding tendency, predominantly localized at mucosal surfaces, in contrast to the clinical expression of deficiencies in other coagulation factors that play a role in thrombin generation and subsequent conversion of fibrinogen to fibrin (6, 23, 24). Interestingly, mucosal surfaces usually possess a high intrinsic fibrinolytic activity. It may be postulated that a deficiency of factor XI results in impaired fibrinolytic inhibition at these sites and in bleeding. In accordance with this hypothesis, bleeding in patients with factor XI deficiency can very often be managed with the administration of anti-fibrinolytic agents, such as tranexamic acid (25).

In conclusion, we have demonstrated *in vivo*, in an experimental thrombosis model, that inhibition of factor XI activity resulted in enhanced endogenous lysis of jugular vein clots in rabbits. This effect is probably due to diminished activation of TAFI upon inhibition of factor XI activity, given the comparable effects on clot lysis of inhibition of factor XI and TAFI. We propose that this effect of factor XI explains in part the bleeding tendency of patients with factor XI deficiency.

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