Two novel inhibitory anti-human factor XI antibodies prevent cessation of blood flow in a murine venous thrombosis model

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Summary
Coagulation factor XI (FXI) is a promising target for anticoagulation, because of its major role in thrombosis and relatively minor role in haemostasis. This implies that inhibition of FXI can prevent thrombosis without causing bleeding. It was our aim to investigate the antithrombotic properties of two novel inhibitory anti-human FXI antibodies (aFXI-175 and aFXI-203). The in vitro properties of both antibodies were analysed using standard clotting assays and calibrated automated thrombography. For the in vivo model we used FXI knockout mice, in which FXI plasma levels were restored with purified human FXI. Thrombosis was induced by applying ferric chloride to the vena cava inferior, after which time to occlusion was analysed. A tail bleeding assay was used to investigate the safety of both antibodies. Using calibrated automated thrombography, both antibodies inhibited thrombin generation initiated via the intrinsic pathway. In contrast, upon tissue factor (TF)-initiated thrombin generation, aFXI-203 did not inhibit thrombin generation, while aFXI-175 inhibited thrombin generation only at low concentrations of TF. In the murine thrombosis model, the vena cava inferior remained patent for 25 minutes (min) in mice treated with aFXI-175 and for 12.5 min in aFXI-203 treated animals, which was significantly longer than in placebo-treated animals (5 min, p<0.05). Neither antibody caused severe blood loss in a tail bleeding assay. In conclusion, the two inhibitory antibodies against FXI prevented cessation of blood flow in a murine thrombosis model without inducing a bleeding tendency.

Keywords
Coagulation, factor XI, thrombosis, murine models

Introduction
Currently available anticoagulant medication, such as vitamin K antagonists (VKA), low-molecular-weight heparin (LMWH) and small molecule factor (F) Xa or thrombin inhibitors all target the extrinsic or common pathways of coagulation (1, 2). These drugs are all associated with an increased risk for severe bleeding even in spite of careful dose monitoring (3). Several lines of evidence suggest that inhibition of the intrinsic pathway, and in particular FXI, might be an attractive target of anticoagulation to overcome these difficulties (4, 5). Arguments in favour of this strategy are the lower incidence of ischaemic stroke and deep-vein thrombosis (DVT) in FXI-deficient patients when compared to the normal population (6, 7). Furthermore, increased plasma FXI levels have been associated with DVT, ischaemic stroke and myocardial infarction, indicating that FXI contributes to pathological thrombus formation (8-10). In addition, FXI-deficient mice are protected against several forms of artificially induced arterial and venous thrombosis (11-14). On the other hand FXI deficiency in humans is associated with a mild bleeding tendency and in some individuals even may be unnoticed (15). One may therefore postulate that inhibition of FXI may be an effective therapeutic approach for anticoagulation without a risk for severe bleeding.

FXI plays a minor role during normal haemostasis; normal clot formation occurs via the tissue factor (TF)-FVIIa complex, which can activate FX and FIX leading to prothrombin activation and ultimately producing a fibrin clot at the site of injury (1). FXI can be activated by thrombin creating a positive feedback loop which forms additional thrombin and thereby stabilises the clot via the activation of thrombin-activatable fibrinolysis inhibitor (TAFI), which protects the clot against fibrinolysis (16, 17). Furthermore, FXI can also be activated by FXII during the intrinsic or contact pathway of coagulation (5, 18).

The most common indication for anticoagulation is atrial fibrillation (AF), since VKA and FXa or thrombin inhibitors can significantly reduce the incidence of ischaemic stroke in these patients (2, 19). A second major indication for anticoagulation is the treatment and prevention of DVT and pulmonary embolism (PE) (20, 21). The pathophysiology of these two entities, however, is significantly different: AF and subsequent stroke arises in the arterial
vasculature and is primarily the consequence of atherosclerotic plaque rupture, whereas thrombus formation in the venous vasculature (DVT and PE) is caused by alterations in blood flow, composition of the blood and damage to the endothelial alignment of the vessel wall. Furthermore, risk factors for arterial thrombosis, like smoking, hypercholesterolaemia and hypertension do not apply to venous thrombosis, indicating that the underlying mechanism is different. Most of the previously mentioned murine models investigated the influence of FXI inhibition on ferric chloride (FeCl₃) induced carotid arterial thrombosis (22), while in this study we focused on the venous vasculature and blood flow, which is substantially different from the arterial vasculature. Here, we evaluated the potential of two novel inhibitory anti-human FXI antibodies (αFXI-175 and αFXI-203) in vitro and in vivo.

Methods

Materials

Enoxaparin (Clexane) was from Sanofi-Aventis (Paris, France). Human coagulation FXI (Hemoleven) was purchased from LFB Biomedicaments (Les Ulis, France). FXI- and FXII-deficient plasmas were obtained from Siemens Healthcare Diagnostics (Marburg, Germany). Purified FXII and high-molecular-weight kininogen (HK) were from Haematologic Technologies Inc. (Essex Junction, VT, USA). Chromogenic substrate S-2366 was from Chromogenix (Milano, Italy). Thrombin was provided by Dr. W. Kisiel (University of Albuquerque, NM, USA). FXI was purified from human plasma as described before (17). Corn trypsin inhibitor (CTI) was from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies

Monoclonal antibodies were generated by conventional methods after injection of Balb/c mice with a mixture of purified human FXI and FXIa. Antibody-producing clones were tested for coagulation inhibitory properties in an aPTT coagulation test. Two antibodies were identified, designated αFXI-175 and αFXI-203.

Coagulation assays

Normal pooled plasma, derived from more than 200 healthy volunteers, was supplemented with antibodies and incubated for 30 minutes (min) at room temperature before analysis. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and FXI levels were measured on an automated coagulation analyser (Behring Coagulation System, BCS) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics).

The Calibrated Automated Thrombogram assays the generation of thrombin in clotting plasma. The assay was carried out as described (23) and the Thrombinoscope manual.

FXI activation by thrombin or FXIIa

A total of 50 nM FXI was incubated with either αFXI-175 or αFXI-203 (500 nM) in Tris-buffered saline (TBS; pH 7.4) and incubated for 30 min at room temperature. Then, 10 nM of thrombin or 5 nM of FXIIa was added, thrombin was incubated overnight at room temperature and FXIIa was incubated for 2 hours (h) at 37°C. Subsequently, 1 unit of hirudin was added to inhibit thrombin or 0.2 units of CTI to inhibit FXIIa and incubated for 20 min at room temperature. Chromogenic substrate S-2366 (f.c. 0.5 mM) was added and changes in OD 405 nm were monitored for 5 min. Activation of FXI by FXIIa was performed in absence or presence of 50 nM HK.
Solid-phase binding assay

The interaction of the monoclonal antibodies with FXI was determined with a solid-phase binding assay. The individual apple domains of FXI as fusion protein with tPA (24) were coated on microtitre wells (100 μl, 5 μg/ml in coating buffer; 50 mM Na₂CO₃, pH 9.6) and incubated overnight at 4°C. After washing the plate three times with TBS, the plate was blocked with 200 μl blocking buffer (TBS/1.5% BSA) for 30 min at room temperature, followed by incubation with the different antibodies (αFXI-175 and 203, 2 μg/ml in TBS/1.5% BSA) for 1 h at room temperature. Antibodies were diluted to 2 μg/ml in blocking buffer. Then, the plate was washed three times with TBS/Tween-20 (0.1%), followed by detection with peroxidase-labelled goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark). O-phenylenediamine dihydrochloride (OPD) tablets (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-citrate buffer (50 mM citric acid, 114 mM NaH₂PO₄, pH 4.5) was used as substrate after which the reaction was stopped with 50 μl 1.0 M H₂SO₄. Absorption was read at 490 nm.

Animal experiments

Eight-week-old male and female FXI knockout mice on C57BL/6 background (11) were given plasma-derived human FXI concentrate (Hemoleven) prior to the surgical procedure, which completely restores FXI levels (14). Dosage of Hemoleven was based on our previous experiments with this compound (14). Thereafter, the mice were intravenously injected with either αFXI-175, αFXI-203 or saline. Enoxaparin (LMWH) was injected subcutaneously 6 h before the procedure. Dosages for the antibodies were based on the in vitro experiments presented here. Enoxaparin concentration was based on the dosage used in humans treated for DVT or PE (1 mg/kg).

Mice were housed in micro-isolator cages on a constant light-dark cycle and were given access to food and water ad libitum. All animal procedures were performed at the Academic Medical Center (Amsterdam, The Netherlands) and approved by the Animal Care and Use Committee of the institute. At the end of the study period the mice were killed by cervical dislocation.

Collection of mouse plasma samples

Blood samples were collected by cardiac puncture under anaesthesia. Blood was quickly withdrawn from the heart using a 1-ml plastic syringe with a 27-G needle and collected into a final ratio of 9 parts of whole blood to one part of 3.2% sodium citrate. Blood samples were immediately mixed by tapping and inverting the tube five times to ensure proper anticoagulation and then centrifuged for 15 min at 600 g at room temperature. Plasma was stored at -80°C until assayed.

FeCl₃-induced IVC thrombosis

The antithrombotic properties of αFXI-175 and αFXI-203 were studied using a well established ferric chloride induced inferior vena cava (IVC) thrombosis model (13). In short, mice anesthetised with 2.5% inhalant isoflurane and a mixture of ketamin/xylazine (2: 1), received a midline incision after which the IVC was exposed by blunt dissection. A filter paper soaked in a 10% FeCl₃ solution was placed below the renal veins on the IVC for 3 min after which the paper was removed and venous flow was measured for 45 minutes using a tissue perfusion monitor (type BLF22; Transonic Systems Inc. Ithaca, NY, USA). The flow before administration was set at 100% after which the decline in flow was calculated accordingly.

Tail vein bleeding assay

A mouse tail bleeding assay was used as described (13). Bleeding was assessed by determining the time until bleeding stopped with

![Figure 2: Binding properties of αFXI-175 and αFXI-203 to FXI. The binding site of both antibodies was assessed with solid-phase binding assays using individual human FXI apple domains linked to tPA. Individual apple domains were coated on microtitre wells and incubated with either αFXI-175 (2 μg/ml; a) or αFXI-203 (2 μg/ml; b) after which binding to the apple domains was determined. The experiment was performed three times, and a representative experiment is depicted.](image-url)
a maximum recording time of 30 min. Furthermore, the total amount of blood loss was analysed as described (25).

Statistical analysis
Statistical comparisons were made using Student t-tests or Mann-Whitney U tests. P-values less than 0.05 were considered significant. Data were analysed using SPSS software package for Windows, Version 19.0 (SPSS, Chicago, IL, USA). Graphics were constructed using GraphPad Prism, Version 5 for Windows (GraphPad Software, San Diego, CA, USA).

Figure 3: Effect of aFXI-175 and aFXI-203 on thrombin generation. The antibodies were added to normal human plasma. Thrombin generation was assessed in recalcified plasma in the presence of antibodies (a, c, e and f; aFXI-175 and b, d, e and f; aFXI-203). Coagulation was initiated by aPTT reagent (a and b) or different concentrations of TF (1 pM in panels c and d; 5 pM in panel e and 20 pM in panel f). Concentrations of the antibodies in plasma are indicated, except for e and f, where 100 μg/ml antibody was used. The experiment was performed twice, and a representative experiment is depicted.
Results

Both antibodies were able to (dose-dependently) prolong coagulation in normal plasma with a maximum inhibition of ~85% (▶Figure 1). Complete inhibition of FXI (<1% FXI activity) was achieved by adding the combination of αFXI-175 and αFXI-203 to normal human plasma (data not shown). The antibodies reacted with both FXI and FXIa with dissociation constants of 3-5 nM (data not shown). FXI is a homodimer with a molecular size of 160-kDa, consisting of 4 tandem repeats called apple domains (apple1-4) and a catalytic domain. We tested the binding of the antibodies to the individual apple domains (▶Figure 2) using solid-phase binding assays. αFXI-175 predominantly binds to apple domain 4 and αFXI-203 interacts with apple domain 2.

Effect of αFXI-175 and αFXI-203 on thrombin generation in human plasma

Both antibodies inhibited thrombin generation initiated via the intrinsic pathway in normal plasma (▶Figure 3a, b), indicating that the antibodies inhibited FXIIa-dependent activation of FXI, FXIa-mediated activation of FIX or both. Upon TF-initiated thrombin generation, αFXI-203 did not inhibit thrombin generation (▶Figure 3d-f), while αFXI-175 was able to inhibit thrombin generation at TF concentrations up to 1 pM (▶Figure 3c), but not at higher concentrations of TF (▶Figure 3e, f). This suggests that αFXI-203 inhibits FXIIa-mediated activation of FXI, and that αFXI-175 inhibits FXIa-mediated activation of FIX or activation of FXI by either FXIIa or thrombin. To further elucidate the working mechanism, we tested the effects of the antibodies in a FXIa-initiated thrombin generation assay in the absence or presence of endogenous FXI. As expected, no effect of αFXI-203 could be observed on thrombin generation, whereas αFXI-175 inhibited FXIa-initiated thrombin generation either in normal plasma (a) or in FXI-deficient plasma (b) was initiated by addition of FXIa (50 ng/ml) in presence or absence of anti-FXI antibody (60 μg/ml). The experiment was performed three times, and a representative experiment is depicted.

Figure 4: Effect of αFXI-175 and αFXI-203 on thrombin generation in plasma initiated by FXIa. Thrombin generation either in normal plasma (a) or in FXI-deficient plasma (b) was initiated by addition of FXIa (50 ng/ml) in presence or absence of anti-FXI antibody (60 μg/ml). The experiment was performed three times, and a representative experiment is depicted.

Figure 5: Effect of αFXI-175 and αFXI-203 on FXI activation by thrombin and FXIIa in a purified system. FXI (50 nM) was incubated with αFXI-175 or αFXI-203 and 10 nM thrombin, and subsequently FXIa activity was measured using a chromogenic substrate S-2366 (a). A similar experiment was performed using FXIIa (5 nM) as activator of FXI in the absence or presence of HK (50 nM) (b). The experiment was performed twice, and a representative experiment is depicted.
thrombin generation, both in normal plasma (with an intact thrombin-mediated FXI feedback loop) and in FXI-deficient plasma (▶Figure 4). αFXI-175 inhibits thrombin generation in both systems, most likely indicating that this antibody interferes with factor IX activation by FXIa.

FXI activation studies were performed in a purified system. αFXI-175, but not αFXI-203, inhibited FXI activation by thrombin (▶Figure 5a). On the other hand, both antibodies were able to inhibit FXI-mediated activation of FXI (▶Figure 5b, closed bars) in the presence of HK. Strikingly, in the absence of HK, αFXI-203 was not able to inhibit FXI activation by FXIIa (▶Figure 5b), indicating that this antibody prevents the binding of FXI (a) to HK.

This could be confirmed in direct binding assays with HK (data not shown).

FXI suppression by inhibiting antibodies in mice

The antibodies were raised in mice against human FXI (a), and in order to perform animal experiments in mice, it was necessary to

Figure 6: Reconstitution of FXI in FXI−/− mice. FXI−/− mice were injected with human-derived FXI concentrate, which restored aPTT (a) and FXI levels (b). Subsequent administration of the inhibiting FXI antibodies or enoxaparin increased aPTT and decreased FXI activity. Each symbol represents one animal, and the horizontal line indicates median.

Figure 7: Mouse inferior vena cava thrombosis model. FXI−/− mice were given human derived FXI concentrate followed by a single dose of either saline (control), enoxaparin (1 mg/kg), αFXI-175 (8 mg/kg) or αFXI-203 (8 mg/kg). Thrombosis was induced by applying a filter paper soaked in 10% ferric chloride for 3 min to the inferior vena cava, and venous blood flow was measured for 45 min. Each symbol represent the mean value of six animals, bars indicate SEM, *p<0.05, ns=non-significant.
check cross-reactivity of the antibodies for mouse FXI. In functional assays, it was established that the antibodies did not inhibit mouse FXI (data not shown). To allow testing of αFXI-175 and αFXI-203 in a mouse thrombosis model, we reconstituted FXI−/− mice with human FXI. Thus, all the circulating FXI in these mice is human derived and receptive for both antibodies. This reversal of FXI plasma levels in FXI−/− mice with human-derived FXI concentrate was investigated prior to the thrombosis model. Intravenous administration of 5 U FXI immediately restored FXI plasma levels to more than 100% (▶Figure 6b). The subsequent administration of inhibiting FXI antibodies and enoxaparin decreased FXI activity levels in mice, with a concomitant increase in aPTT (▶Figure 6a, b).

**Ferric chloride induced IVC thrombosis**

Enoxaparin treatment of mice completely prevented FeCl₃-induced IVC thrombosis, while animals treated with saline revealed a rapid decline in flow (▶Figure 7a). ▶Figure 7b and c show the influence of αFXI-175 and αFXI-203 on venous blood flow after applying FeCl₃. Both inhibitory antibodies significantly prolonged the time to obstruction, 12.5 min for αFXI-203 and 25 min for αFXI-175 (p<0.05).

**Tail bleeding assay**

Infusion of the antibodies did not result in prolonged bleeding times compared to the saline control (▶Figure 8a). This is in contrast to the markedly prolonged bleeding times in the enoxaparin-treated animals. Also, the total amount of blood loss (▶Figure 8b) was increased with enoxaparin and if anything, decreased in the animals treated with the FXI antibodies.

**Discussion**

The last two decades the medical need for effective antithrombotic drugs with a greater safety profile than VKA and LMWH led to the development of several small molecule FXa and thrombin inhibitors (2). However, these drugs have some major drawbacks, paving the way for new innovations (3). Coagulation FXI has been a target of interest for many years and there was steady increase in publications dealing with this subject (4, 5, 18), most of which used mice as experimental animals to obtain preclinical data. Because mice do not develop spontaneous thrombosis, an artificial trigger is warranted (26). The most commonly used trigger in drug development studies is ferric chloride, because it works immediately while leaving the vasculature and circulation intact (22).

In this study we focused on the venous vasculature and the influence of FXI inhibition on thrombus formation. We investigated two novel antibodies, one of which inhibited the FXIIa-mediated FXI activation (αFXI-203) and the other interfered with the activation of factor IX by FXIa and thrombin-mediated FXI activation (αFXI-175). In a mouse model for venous thrombosis, we have shown a rapid decline in venous blood flow after inducing thrombosis with a 10% FeCl₃ solution placed for 3 min on the IVC, which was prevented completely by LMWH and prevented partially with the two inhibiting FXI antibodies. Previous experiments with FXI−/− mice and FeCl₃ applied to the carotid arteries revealed 30 min as mean time to occlusion (27). Our αFXI-175 data are consistent with these observations; αFXI-175 prolonged the time to 50% of flow significantly for 25 min. At 30 min, venous blood flow was still higher in the αFXI-175 group than in the saline group, but this difference was not significant (p=0.25). Also, αFXI-203 significantly delayed flow reduction, but the delay induced by the antibody was considerably shorter than that by

![Figure 8: Effect of inhibiting FXI antibodies and enoxaparin on bleeding in mice. FXI−/− mice were given human-derived FXI concentrate followed by a single dose of either saline (control), enoxaparin (1 mg/kg), αFXI-175 (8 mg/kg) or αFXI-203 (8 mg/kg). Subsequently, tail bleeding (a) and amount of blood loss (b), depicted as haemoglobin (Hb) content, were evaluated as described in Methods. Each symbol represents one animal, and the horizontal line indicates median. ***p<0.0001, ns=non-significant.

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αFXI-175. In contrast to αFXI-175, αFXI-203 did not inhibit TF driven thrombin generation in vitro (Figure 3). This suggests a relatively minor role for FXIIa-mediated FXI activation in FeCl₃-induced thrombosis models, which is in accordance with the publication by Cheng et al. (27). In this study, the antibody 14E11 (with a similar working mechanism as αFXI-203) was unable to protect mice from carotid artery occlusion induced by 10% FeCl₃.

Since FXI⁺/⁻ mice do not have a bleeding tendency (28), we did not expect an effect of our inhibiting FXI antibodies in a tail bleeding assay. Indeed, bleeding in mice was unaffected by administration of αFXI-175 or αFXI-203 as compared to control mice treated with saline. In contrast, animals who received enoxaparin revealed a significantly longer bleeding time (Figure 8a), indicating that FXI inhibition may have a better safety profile over LMWH drugs like enoxaparin. The tail bleeding assay consists of cutting the tip of the mouse tail, followed by measuring the time until bleeding stops. In our hands, making a big wound induces major blood loss with a relatively short bleeding time, while a small wound causes less blood loss with a longer bleeding time. To correct for this, we also quantified the blood loss by analysing the haemoglobin (Hb) content as described (25). A similar, albeit not identical, pattern as the bleeding time was observed when the Hb content was analysed (Figure 8b).

Inhibiting FXI antibody αFXI-203 prevented the activation of FXI by FXIIa in the presence of HK. Since FXII displays a negligible role during normal human haemostasis (29), it is difficult to directly translate this observation to the human situation; further research is needed to establish whether inhibition of FXIIa-mediated FXI activation is an effective strategy to treat thrombosis. Antibody αFXI-175 appears to be a more suitable candidate for anticoagulation, since this antibody inhibits FXI activation by FXIIa, which is linked to pathological thrombus formation. Furthermore, αFXI-175 inhibits FXI activation by thrombin, thereby blocking the feedback amplification loop in coagulation. Initiation of thrombin formation is still preserved, allowing the fibrin network to be formed. Interestingly, αFXI-175 showed a high affinity for the apple 4 domain of FXI. Apple 4 is not involved in the binding of either FIX or thrombin; therefore the exact working mechanism of αFXI-175 is not entirely clear. Puy et al. described an antibody (Ab 1A6) with a similar working mechanism as αFXI-175, but with a different binding site on FXI (apple 3) (30). Both antibodies block FIX activation by FXIIa, as well as FXI activation by FXIIa. However, αFXI-175 also inhibits thrombin-mediated FXI activation, suggesting that the apple 4 domain is involved in the activation of FXI by thrombin.

This is not the first study which proposes FXI as alternative target for anticoagulation. We previously reported the use of antisense FXI oligonucleotides (14), other research groups also used an antibody approach (27, 31-33). The advantage of antibodies is the fast mechanism of action, which is particularly convenient in the treatment of DVT and/or PE. A possible disadvantage is the immunological reaction towards the antibodies. The novel FXa and thrombin inhibitors are small molecule inhibitors; the major drawback of these types of drugs is the lack of an antidote. We did not perform reversal studies in this research, but antibodies could have similar problems concerning an antidote as the small molecule inhibitors although FXI concentrate is available as potential antidote. Furthermore, antibodies require parenteral administration which is less desirable in thrombosis patients who often need treatment for a long period of time. However, due to the long half-life of the antibodies, administration once weekly would suffice, which also ensures patient compliance.

In conclusion, we have generated two inhibitory antibodies against FXI with different specificities. Both antibodies prevent flow restriction in a mouse IVC thrombosis model.

Conflicts of interest
Prof. C. E. Hack is shareholder of Prothix BV. None of the other authors declares any conflicts of interest.

References

What is known about this topic?
• Patients with a severe factor XI (FXI) deficiency have a reduced incidence of deep-vein thrombosis and ischaemic stroke.
• FXI inhibition is an effective strategy for anticoagulation in rodents and primates.

What does this paper add?
• We present two novel inhibiting FXI antibodies, which have a distinct working mechanism and different specificities.
• Both antibodies prevent thrombosis in a murine thrombosis model.