Factor XI as target for antithrombotic therapy
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Coagulation factor XI affects atherosclerosis in Apoe-deficient mice

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Preliminary report
Abstract

**Background:** Coagulation factors may alter atherosclerosis and atherosclerotic plaque progression. It is known that thrombin inhibitors reduce atherosclerosis in an anti-inflammatory manner. Thrombin is a key player during coagulation and a major activator of coagulation factor XI. Therefore, we hypothesized that factor XI inhibition could prevent atherosclerotic plaque progression, since factor XI has pro-inflammatory properties.

**Methods and Results:** Apolipoprotein E (Apoe)-deficient mice were fed a high-cholesterol diet for 12 weeks, during which they were treated either with dabigatran etexilate (a thrombin inhibitor), factor XI antisense oligonucleotides or nonsense oligonucleotides (control). As anticipated, atherosclerotic plaque formation was reduced in mice treated with dabigatran or factor XI antisense oligonucleotides as shown by histological analysis. Flow cytometry revealed a significant decrease in inflammatory monocytes in mice treated with dabigatran, while this was unaffected by treatment with factor XI antisense oligonucleotides. Furthermore, macrophage infiltration into atherosclerotic plaques was significantly reduced in dabigatran-treated mice when compared to placebo or factor XI antisense oligonucleotides-treated animals. Dabigatran or factor XI antisense oligonucleotide treatment prevented both arterial and venous thrombosis in atherosclerotic mice.

**Conclusion:** We here identify factor XI as determinant of atherosclerotic plaque progression. Furthermore, thrombin inhibition by dabigatran etexilate or factor XI reduction by factor XI antisense oligonucleotides prevented artificially induced arterial and venous thrombosis in atherosclerotic mice. These studies suggest that anticoagulants might have a beneficial effect on the development and progression of cardiovascular disorders like myocardial infarction and stroke.
Introduction

Atherosclerosis is a chronic inflammatory disorder, which can be complicated by atherosclerotic plaque rupture and subsequent thrombus formation (1,2). These atherothrombotic events, like myocardial infarction or stroke, are still the primary cause of death in the Western world (3). Cornerstone of treatment of these disorders are lipid-lowering and anti-hypertensive drugs, and platelet inhibitors (4). However, it is questionable whether these interventions influence plaque vulnerability and plaque rupture (5). To date, there are no therapeutic agents which stabilize the atherosclerotic plaque and thereby preventing atherothrombotic disease. It is hypothesized that inhibition of the coagulation system influences this process (6). Substantial evidence suggests that repeated subclinical plaque ruptures and associated microthrombosis contribute to plaque progression and finally to massive plaque rupture (7-9). This process can be impaired via inhibition of the coagulation system in particular via inhibition of thrombin. Several animal studies demonstrated that direct thrombin inhibitors like dabigatran attenuate atherosclerotic plaque progression by reducing the level of inflammation (10-13). These data indicate that thrombin is involved in the atherosclerotic process probably by influencing inflammatory pathways and leukocyte infiltration. Thrombin is a key player during coagulation and is responsible for activation and regulation of several coagulation proteins including coagulation factor XI (14,15). Therefore, by inhibition of thrombin, an important activator of factor XI is also inhibited and we hypothesized that this factor XI inhibition contributes to impaired atherosclerosis. Factor XI is a coagulation factor which plays a minor role during normal hemostasis, but a major role during thrombosis (16). For instance, patients with a deficiency of factor XI have a reduced incidence of ischemic stroke, which might indicate that inhibition of factor XI has a protective effect on this disease (17). Furthermore, it is suggested that factor XI inhibition attenuates inflammation (18) and leukocyte function (19), important factors in the development of atherosclerosis. Previously, we have shown that lowering the level of factor XI can reduce the atherothrombotic burden after acutely ruptured atherosclerotic plaques in mice (20). The aim of this study was to investigate whether factor XI reduction also influences chronic atherosclerosis. Furthermore, we studied the effect of factor XI reduction on acute arterial and venous thrombosis in mice with atherosclerosis.
Methods

Materials
Factor XI antisense and nonsense oligonucleotides were provided by ISIS Pharmaceuticals (Carlsbad, CA, United States). Dabigatran etexilate-supplemented high-fat and placebo high-fat chow diets (20 kcal% protein; 35 kcal% carbohydrate; 45 kcal% fat) were prepared at the Department of CardioMetabolic Disease Research, Boehringer Ingelheim Pharma GmbH (Biberach an der Riss, Germany). White blood cell count and platelet count were measured on a Coulter analyzer with reagents and protocols from the manufacturer (Beckman Coulter Inc., Brea, CA, United States).

Animals
Four-week-old female Apoe-/- mice (n=27 per treatment group) on C57Bl/6 background were obtained from Charles River (Maastricht, the Netherlands). The study consisted of two interventions and a control: in the first intervention arm, mice received a high-fat diet supplemented with dabigatran etexilate (7.5 mg/gram chow) for 12 weeks. In a second intervention arm, mice were fed a high-fat diet for 12 weeks and received intraperitoneal (i.p.) administration of 50 mg/kg factor XI antisense oligonucleotides (FXI ASO) every 3-4 days for a total of 12 weeks. Control animals were also fed a high-fat diet and received injection of 50 mg/kg nonsense oligonucleotides (placebo) every 3-4 days for 12 weeks. Diets and water were provided ad libitum throughout the experiment and all animal experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, the Netherlands). At the end of the study period, the mice were killed by cervical dislocation.

\( \text{FeCl}_3 \)-induced carotid artery thrombosis
The antithrombotic properties of FXI ASO and dabigatran were studied using a well-established ferric chloride induced carotid artery thrombosis model. In short, mice anesthetized with 2.5% inhalant isoflurane and a mixture of ketamin/xylazin (2:1), received an incision directly over the right common carotid artery, after which the carotid artery was exposed by blunt dissection. A filter paper soaked in a 10% \( \text{FeCl}_3 \) solution was placed on the artery for 3 minutes after which the paper was removed and arterial flow was measured for 30 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.
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Inferior vena cava stenosis model
Mice were anesthetized with 2-5% inhalant isoflurane mixed with oxygen during the entire procedure. Then, a median laparotomy was performed, the intestines were exteriorized and sterile saline was applied to prevent drying. The inferior vena cava was dissected free from all surrounding tissue and ligation by a 7.0 polypropylene suture, just below the renal veins, was performed over a 30-gauge needle after which the needle was removed. Intestines were placed back in the abdominal cavity and peritoneum and skin were closed by 6.0 silk. Mice were euthanized after 48 hours and the inferior vena cava was taken out for analysis.

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 5 times to ensure proper anticoagulation and then centrifuged for 15 minutes at 600g at room temperature. Plasma was stored at -80°C until assayed.

Flow cytometry
For FACS analysis, whole blood was combined with a red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to allow isolation of white blood cells. After centrifugation, the resulting cell suspensions were incubated with a mixture of specific antibodies to CD11b, Ly6G, MHCII, CD11c, Gr1 and CD45 for 30 minutes at 4°C. Data were acquired with a FACS Canto II (BD Biosciences) and leukocyte subsets were gated using FlowJo software (Treestar Inc.). Neutrophils were identified as CD45+, Ly6G, Gr1; dendritic cells as CD45+, MHCII; resident monocytes as CD45+, Gr1, CD11b and inflammatory monocytes as CD45+, Gr1, CD11c.

Serum cholesterol measurement
Total cholesterol levels in serum were analysed using a total cholesterol assay (Roche diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Immunohistochemical analysis of atherosclerosis
Mice were perfused with 25 ml of 0.9% NaCl (2 ml/min) followed by 15 ml of 4% paraformaldehyde in phosphate-buffered saline (0.5 ml/min). The heart and aortic arch were removed and cleaned of fat and fixed for 48-72 hours in 4% paraformaldehyde, embedded in paraffin (Leica EG1160, Wetzlar, Germany) and cut into 5 µm sections using a microtome (Leica E2235, Wetzlar, Germany).
Plaque formation in aortic root sections were histomorphologically visualized using hematoxylin (Merck, KGaA, Darmstadt, Germany) and eosin (Sigma Aldrich, St. Louis, MO, USA) stains according to standard protocols. Adjacent sections were immunofluorescently stained with antibodies against fibrinogen (rabbit anti mouse fibrinogen IgG, Gentaur, Kampenhout, Belgium), macrophages (rat anti mouse Mac-3, clone M3/M4, BD Biosciences) and smooth muscle cells (mouse anti human smooth muscle actin, clone 1A4, DAKO). All images were recorded with a Leica microscope and camera. Morphometric analysis using Adobe Photoshop analysis software was performed on the various aortic root sections.

Statistical analysis
Statistical comparisons were made using non-parametric 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. Graphics were constructed using GraphPad Prism, Version 5 for Windows (GraphPad Software).

Results

Factor XI and thrombin demonstrate different effects on inflammation and lipid metabolism. Apoe<sup>-/-</sup> mice were fed a high fat diet for 12 weeks during which they were either treated with dabigatran, factor XI antisense oligonucleotides or nonsense oligonucleotides (placebo). After this treatment period, mice were initially characterized on white blood cell count and cholesterol (figure 1 and 2), since it is suggested that thrombin and factor XI influence inflammation and leukocyte function. There was a significant reduction in white blood cells in the dabigatran treated animals, while there was no difference between the placebo group and the factor XI antisense oligonucleotides group (figure 1a). We performed flow cytometric analyses of whole blood to further evaluate this observation. There was no difference between the relative number of neutrophils, dendritic cells or resident monocytes between the three treatment groups (figures 1b-d). However, there was a significant reduction in inflammatory monocytes as percentage of non-neutrophils in peripheral blood from animals treated with dabigatran when compared to animal treated with placebo or factor XI antisense oligonucleotides (figure 1e). Since cholesterol is an important determinant of atherosclerosis and atherosclerotic plaque progression, we analysed total cholesterol levels in the three treatment groups (figure 2). The dabigatran-treated mice showed a slight but significant decrease in plasma cholesterol. Further analysis using HPLC revealed that most of the cholesterol was present in very low-density and low-density lipoproteins (data not shown).
Figure 1. Factor XI has no influence on leukocyte number and functioning in hypercholesterolemic mice. Apoe<sup>−/−</sup> mice were fed a high fat diet for 12 weeks and were treated with either dabigatran, factor XI antisense oligonucleotides or nonsense oligonucleotides (placebo). White blood cell count was significantly reduced in mice treated with dabigatran, while treatment with factor XI antisense oligonucleotides (FXI ASO) had no effect on white blood cell levels (a). Flow cytometric analysis of peripheral blood revealed no difference in relative numbers of neutrophils (b) as percentage of Cd45+ leukocytes, dendritic cells (DC) (c) or resident monocytes (d) as percentage of non-neutrophils. We did observe a lower relative number of inflammatory monocytes as percentage of non-neutrophils in dabigatran treated mice (e). All graphs represent the median with interquartile ranges (n=10-15); *P<0.05, **P<0.01.
**Figure 2.** Factor XI did not affect plasma cholesterol levels in hypercholesterolemic mice. ApoE<sup>-/-</sup> mice were fed a high-cholesterol diet for 12 weeks before analysis. Serum cholesterol levels were significantly reduced in mice treated with dabigatran for 12 weeks, while treatment with factor XI antisense oligonucleotides (FXI ASO) had no effect on serum cholesterol levels. Graph represents the median (n=9-10); ns=non-significant, *P<0.05.

Effect of factor XI antisense oligonucleotides on atherosclerotic plaque burden

Atherosclerotic plaque formation in the aortic root of mice treated with either dabigatran or factor XI antisense oligonucleotides was significantly reduced when compared to mice treated with placebo (figure 3a and b). The effect appeared to be more pronounced in the dabigatran treated animals than in animals treated with factor XI antisense oligonucleotides (figure 3b). Since dabigatran lowered the relative number of circulating inflammatory monocytes in blood, we investigated whether this was also reflected in the atherosclerotic plaques. Indeed, macrophage infiltration (MAC-3<sup>+</sup> cells) was significantly reduced in atherosclerotic plaques from mice treated with dabigatran (figure 3c and d). Interestingly, macrophage infiltration in atherosclerotic plaques from mice treated with factor XI antisense oligonucleotides was unaffected. Furthermore, the dabigatran-treated mice revealed more fibrotic lesions, since these small lesions had relatively more smooth muscle cell accumulation (Sma<sup>+</sup> cells), while smooth muscle cell accumulation was unaffected by treatment with factor XI antisense oligonucleotides (figure 3e and f). Thus, while only dabigatran decreased macrophage influx into the atherosclerotic lesion, both dabigatran- and factor XI antisense oligonucleotides-treated mice revealed decreased atherosclerosis.
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**a.**
Placebo  Factor XI antisense  Dabigatran

**b.**

![Plaque area (mm²)](image)

**c.**
Placebo  Factor XI antisense  Dabigatran

**d.**

![MAC-3 positive cells (% of plaque area)](image)
Chapter 6

Figure 3. Factor XI influences atherosclerosis in Apoe^-mice. Atherosclerotic lesions in the aortic root of Apoe^- mice were significantly reduced in animals treated with either factor XI antisense oligonucleotides (FXI ASO) (n=11) or dabigatran (n=9) when compared to placebo (n=10) treated animals (a,b). Shown are representative images of hematoxylin and eosin (H&E) stained sections, scale bar=500 µm. Quantification of plaque content relative to total plaque area as evidenced by MAC-3+ macrophage infiltration (c,d) (n=9-11) and α-smooth muscle actin+ smooth muscle cells (e,f) (n=9-11). Representative images are shown, scale bar=500 µm. Graphs represent mean ± SD; *P<0.05, ***P<0.001.

Factor XI antisense oligonucleotides prevent arterial thrombosis in atherosclerotic mice

The effects of dabigatran and factor XI antisense oligonucleotides on arterial thrombosis are shown in figure 4. Both thrombin inhibition and factor XI inhibition prevented vessel occlusion in the ferric chloride induced thrombosis model in mice with atherosclerosis (figure 4a-c). The areas under the curve of the blood flow studies over 30 minutes (reflecting continuous monitoring from each animal) for factor XI antisense oligonucleotides and dabigatran treated animals were not significantly different (figure 4b). In the animals treated with nonsense oligonucleotides (placebo group), all carotid arteries were occluded after 30 minutes, while only 33% of the carotids of the factor XI antisense treated animals and 17% of the carotids of the dabigatran group were occluded after 30 minutes (figure 4c).
Factor XI antisense oligonucleotides prevent venous thrombosis in atherosclerotic mice

The inhibitory effects of dabigatran and factor XI antisense oligonucleotides have been studied extensively in various venous thrombosis models. However, little is known about the antithrombotic effects of these therapeutics on venous thrombosis in mice with atherosclerosis. As shown in figure 5 both factor XI antisense oligonucleotides and dabigatran prevent venous thrombosis in the inferior vena cava stenosis model. Thrombi had a lower weight (figure 5a) and were smaller (figure 5b) in the intervention groups when compared to the placebo treated animals. In total, 87% of the animals in the placebo group developed a thrombus 48 hours after ligation of the inferior vena cava, versus 42% in the factor XI antisense oligonucleotides treated animals and 28% of the dabigatran treated animals; a difference that was statistically significant (figure 5c).

Figure 4. Ferric chloride induced carotid artery thrombosis was reduced in mice treated with either dabigatran or factor XI antisense oligonucleotides. Treatment with dabigatran or factor XI antisense oligonucleotides (FXI ASO) prevented ferric chloride induced carotid artery occlusion in mice with atherosclerosis. (a) Time courses of relative blood flow. (b) Mean areas under the curve (AUC) ± standard errors for relative flow following ferric chloride application. (c) After 45 minutes, 100% of the carotid arteries of placebo-treated animals were occluded versus 33% in the factor XI antisense oligonucleotides group and 17% of the dabigatran-treated animals. N=6, ***P<0.001.
Administration of factor XI antisense oligonucleotides profoundly diminished atherosclerotic plaque formation in Apoe<sup>−/−</sup> mice. In animals treated with the thrombin inhibitor dabigatran, this was accompanied by a reduced number of circulating inflammatory monocytes in blood and less macrophage infiltration in the plaque area, which suggests that dabigatran induces an anti-inflammatory phenotype as has been
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previously described in literature(11). Furthermore, plaques from animals treated with dabigatran were more fibrotic, due to the relatively enhanced smooth muscle cell content. Smooth muscle cells are one of the most prevailing cell types in atherosclerotic plaques and are considered important for the tissue integrity and stability of the plaque(21;22). The observation that plaques from dabigatran treated animals contained relatively more smooth muscle cells might indicate that these plaques are more stable and less prone to rupture than plaques from placebo treated animals. Interestingly, none of these features were found in the blood and plaques from animals treated with factor XI antisense oligonucleotides despite the reduced total atherosclerotic plaque content in these animals. This suggests that thrombin influences atherosclerosis presumably by more than one mechanism. However, the undefined mechanism by which factor XI inhibition decreases atherosclerosis does not include lowering of cholesterol or decreased inflammatory monocytes and thus a reduction in monocyte influx into the plaque. Factor XI activity has been associated with leukocyte function and trafficking, as well as a reduction in inflammatory plasma markers like TNF-α and IL-6(18). Therefore, we hypothesized that inhibition of factor XI could have a beneficial effect in an inflammatory disorder like atherosclerosis. However, in this study we found no evidence for factor XI as determinant of inflammation. The underlying mechanism behind reduced atherosclerosis and factor XI inhibition is the objective of future research and might be influenced by the following pathways. Coagulation proteases and their receptors (protease-activated receptors, PARs) have the capacity to control inflammatory and reparative processes(6). However, the effect of factor XI on for instance PAR-1 signalling is largely unknown and this might be the onset for new studies. Another important determinant of atherosclerosis is the blood platelet. We have previously shown that reduced factor XI plasma levels have no effect on platelet adhesion and aggregation in the acute phase of atherosclerotic plaque rupture(20), however the long term effects of factor XI inhibition on platelet activation and subsequent atherosclerosis progression are not known. Finally, factor XI may also be related to atherogenesis via the contact system. The contact system consists of factor XI, factor XII, prekallikrein and high-molecular-weight kininogen. Activation of this system via negatively charged surfaces like misfolded proteins or polyphosphates induces activation of the complement and fibrinolytic systems, for instance activation of complement C3 and C5(6;23). Furthermore, contact activation results into kallikrein formation which is associated with the severity of cardiovascular disease(24). Together, these pro-inflammatory processes might influence the development and course of atherosclerosis.

In this study, we only analysed the extent of atherosclerosis in the aortic root, because we used a relatively mild phenotype (Apoe⁻/⁻ mice on a 12-week high-fat diet) and atherosclerotic plaque formation did not extend into the aorta yet. We were primarily
interested whether factor XI had any influence on atherogenesis and therefore we wanted to identify early changes in size, phenotype and composition of atherosclerotic lesions. The atherosclerotic plaques we observed were relatively small and in an early stage, making it impossible to draw any conclusions on end-stage atherosclerosis. Prolonged treatment with high-fat diet (>12 weeks) would provide more information on the role of factor XI and thrombin during atherosclerosis progression and could reveal if treatment with dabigatran or factor XI antisense oligonucleotides would also be feasible in more advanced atherosclerosis. Such a model would generate more insights on features of plaque vulnerability at later stages of atherosclerosis development like necrotic cores, thin fibrous caps and plaque haemorrhages. Furthermore, a different model (e.g. Ldlr−/− mice) could be used to confirm these results and to investigate whether the LDL receptor is mechanistically involved. Nevertheless, our study indicates that both thrombin and factor XI are important determinants of (early) atherogenesis.

The effect of novel anticoagulant drugs is initially studied in animal thrombosis models. Researchers generally use young healthy mice in which thrombosis is induced with an artificial trigger like ferric chloride or laser(25). Subsequently, the time to vessel occlusion is used as primary outcome. However, cardiovascular disorders like stroke and myocardial infarction occur in patients with diseased atherosclerotic vessels. Thrombus formation on a ruptured atherosclerotic plaque is substantially different from venous thrombosis and therefore it is important to study the effects of anticoagulants both in arterial and venous thrombosis models(20). Furthermore, patients with atherosclerosis often suffer from associated disorders like dyslipidaemia, hypertension and/or diabetes mellitus. Whether these disorders are also a risk factor for venous thrombosis is currently under debate(26-28). In this study, we investigated the anti-thrombotic potential of dabigatran and factor XI antisense oligonucleotides in mice with an atherosclerotic phenotype. Arterial thrombosis was studied using the well-established ferric chloride induced thrombosis model(29). To study venous thrombosis, we used a novel ligation method(30). The vena cava inferior ligation model is a relatively new method to study venous thrombosis. By inducing blood stasis, this model might more closely resemble the pathophysiology of (human) venous thrombosis(30). Both dabigatran and factor XI antisense oligonucleotides prevented thrombosis in these animal models, which suggests that inhibition of thrombin and factor XI are appropriate interventions for both arterial and venous thrombosis in patients with atherosclerosis. Nevertheless, the anti-thrombotic effect of factor XI antisense oligonucleotides or other factor XI targeting therapy in humans remains to be established in clinical trials. Ferric chloride is a well-established substance to induce thrombosis and to study anticoagulant drugs in rodents. However it is an artificial trigger, which is not comparable to human atherothrombotic disease(25;29).
In conclusion, we provide evidence that pharmacological reduction of factor XI plasma levels can potentially treat atherosclerosis and associated disorders like stroke and myocardial infarction. Considering the promising safety profile of factor XI inhibitors(31), our findings have various clinical implications. Not only can these drugs potentially be used in the acute phase of atherothrombosis, for instance during stroke or acute coronary syndrome, but if indeed anticoagulants modify atherosclerosis phenotype, these drugs are also indicated for prevention and extended treatment of atherosclerosis. We now studied atherosclerosis in a standardized animal model, while atherosclerosis in humans is a multifactorial disorder with many unresolved issues. It is essential to investigate the efficacy and safety of anticoagulant drugs in patients with atherosclerosis in clinical trials.
References


