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van Montfoort, M.L.

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Chapter 5

Factor XI regulates pathological thrombus formation on acutely ruptured atherosclerotic plaques

M.L. van Montfoort
M.J. Kuijpers
V.L. Knaup
S. Bhanot
B.P. Monia
J.J. Roelofs
J.W. Heemskerk
J.C.M. Meijers

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Abstract

Objective: Coagulation factor XI is proposed as therapeutic target for anticoagulation. However, it is still unclear whether the antithrombotic properties of factor XI inhibitors influence atherosclerotic disease and atherothrombosis. Our aim is to investigate whether factor XI antisense oligonucleotides could prevent thrombus formation on acutely ruptured atherosclerotic plaques.

Approach and Results: Atherosclerotic plaques in the carotid arteries of Apoe⁻/⁻ mice were acutely ruptured using ultrasound. The subsequent thrombus formation was visualized and quantified by intravital microscopy and immunohistochemistry. Mice were pre-treated with either factor XI antisense or nonsense oligonucleotides (50 mg/kg) to lower factor XI plasma levels. A tail bleeding assay was used to determine safety. On plaque rupture, initial platelet adhesion and platelet plug formation was not impaired in animals treated with factor XI antisense oligonucleotides. However, the ensuing thrombus formation and fibrin deposition was significantly lower after 5 to 10 minutes (P<0.05) in factor XI antisense oligonucleotide treated animals without inducing a bleeding tendency. Furthermore, thrombi from antisense treated animals were less stable than thrombi from placebo treated animals. Moreover, macrophage infiltration and collagen deposition was lower in the carotid arteries of factor XI antisense treated animals. No neutrophils were present.

Conclusions: Factor XI antisense oligonucleotides safely prevent thrombus formation on acutely ruptured atherosclerotic plaques in mice. Furthermore, perturbed carotid arteries from factor XI antisense treated animals show a less severe inflammatory response.
Introduction

Atherosclerosis is the underlying cause of approximately 50% of all deaths in developed countries(1). An important reason for this chronic inflammatory disorder to become life-threatening is thrombus formation on ruptured atherosclerotic plaques, resulting in myocardial infarction, peripheral artery disease and stroke. Primary treatment of these disorders is with antiplatelet (e.g. aspirin, clopidogrel) and anticoagulant drugs (e.g. warfarin, heparins, factor Xa and thrombin inhibitors)(2). These drugs are undeniably good in terms of effectiveness, but have serious side effects of which bleeding is the most pronounced(3). To overcome these bleeding problems, coagulation factor XI has been proposed as alternative target for anticoagulation(4-7), because factor XI is associated with thrombosis while it has a relatively minor role during normal hemostasis. This means that normal clot formation is still preserved in the absence of factor XI, while thrombosis is prevented. For example, inhibition of factor XI protects rodents and primates from experimentally induced thrombosis in both arteries and veins(8-12). Another argument for factor XI as anti-thrombotic target is the epidemiological evidence that factor XI deficient patients have a lower incidence of deep vein thrombosis and ischemic stroke(13;14).

So far, little is known about the role of factor XI inhibition in atherosclerosis and subsequent atherothrombosis. This is a very important extension, because atherosclerotic plaques contain many components and cells with a thrombogenic potential beyond the coagulation cascade, such as oxidized low-density lipoproteins, lysophosphatidic acid and macrophages(15;16). In addition, tissue factor (TF) and collagen are important determinants of thrombus formation on ruptured plaques(15). The question is whether factor XI inhibition is capable to overcome this pro-thrombotic environment. Since coagulation is not dependent on factor XI when high concentrations of TF are present(17;18), we questioned whether factor XI inhibition was still effective to prevent atherothrombosis.

Furthermore, atherothrombotic disorders like myocardial infarction and stroke are diseases of the elderly and occur in patients with pronounced atherosclerotic vessels. In contrast, many animal experiments have been performed in young (4-8 weeks) mice with healthy non-atherosclerotic vessels. In this study, we investigated the antithrombotic potential of factor XI antisense oligonucleotides in a model of plaque rupture which is sensitive to both anti-platelet and antithrombotic therapy(15), in older (18-20 weeks) mice with pronounced atherosclerotic vessels.
Materials and Methods

Materials
Factor XI antisense and nonsense oligonucleotides were provided by ISIS Pharmaceuticals (Carlsbad, CA, United States)(1,2). Antisense therapy is based on base-pair hybridization through which antisense oligonucleotides selectively bind to their complementary mRNA target. This binding typically results in the selective and catalytic degradation of the targeted mRNA and leads to a corresponding reduction in target protein levels(3). Factor XI antisense oligonucleotides have been proposed as preventive strategy for thrombosis. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Leiden, the Netherlands). Human fibrinogen labeled with alexa fluor 546 was from Invitrogen (Leiden, the Netherlands). Prothrombin and factor XI levels were measured on an automated coagulation analyzer (Behring Coagulation System, BCS) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany). D-dimer levels were determined using a commercially available ELISA (Enzygnost, Siemens Healthcare Diagnostics, Marburg, Germany).

Animal experiments
Four-week-old male Apoe−/− mice on C57Bl/6 background were obtained from Charles River (Maastricht, the Netherlands). Plaque rupture and measurement of acute thrombus formation was performed as described earlier(4). In short, Apoe−/− mice were fed a Western-type diet with 0.25% cholesterol (Arie Blok, Woerden, the Netherlands) for 18-20 weeks. Then, mice received an intraperitoneal injection (50 mg/kg) of either factor XI antisense or nonsense oligonucleotides every 3-4 days for a total of 10 days (3 injections). The surgical procedure was performed 3 days after the final injection and proceeded as follows. After the mice were anesthetized (subcutaneous injection of ketamin and xylazin (0.1 mg/g and 0.02 mg/g body weight, respectively)), the carotids were dissected free from surrounding tissue and the animal was injected intravenously with CFSE-labeled platelets obtained from a donor mouse with the same genetic background(5). A subset of mice was also injected with fluorescently labelled human fibrinogen. Using intravital fluorescence microscopy, a plaque was selected and rupture was induced by ultrasound application using a VibraCell VCX130 processor (Sonics, Newtown, CT, United States). Thrombus formation was recorded as soon as possible by capturing fluorescent images for at least 10 minutes. Images were analyzed by selecting a region of interest (ROI) representing the site of thrombus formation, total pixel intensity was calculated and corrected for background. To quantify thrombus size after specific time intervals, fluorescent images were processed using ImageJ software. Within the image of a carotid artery, two similar ROIs were defined, one representing the background and
one representing the thrombus. Subsequently, a threshold level was set by eliminating all pixels with intensity lower than 99.0% of the pixels of the background ROI. Intensities of all pixels in the thrombus ROI were then integrated.

**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 (citrate-rinsed) 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.

**Tail vein bleeding assay**
A mouse tail bleeding assay was used as described(6). Bleeding was assessed by determining the time until bleeding stopped with a maximum recording time of 30 minutes.

**Histology and Immunohistochemistry**
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). Arteries were removed 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).

Carotid arteries were histomorphologically visualized using hematoxylin (Merck, KGaA, Darmstadt, Germany) and eosin (Sigma Aldrich, St. Louis, MO, USA) stains according to standard protocols. Tissue specimens were stained with antibodies against fibrinogen (rabbit anti mouse fibrinogen IgG, Gentaur, Kampenhout, Belgium), neutrophils (monoclonal antibody Ly-6G, clone 1A8, BD Biosciences Pharmingen, San Jose, CA, USA) and macrophages (rat anti mouse Mac-3, clone M3/M4, BD Biosciences). Sirius red staining (Polysciences Inc, Warrington, PA, USA) determined the relative collagen content in the atherosclerotic lesions. Both bright-field as polarized light was used to visualize collagen content.

Morphometric analysis using ImageJ analysis software was performed on the various 5 µm sections of the carotid arteries at the site of the thrombus. Additional information regarding staining is available in the supplement.

**Statistical analysis**
Statistical comparisons were made using non-parametric Mann-Whitney U tests. P values less than 0.05 were considered significant. Data were analyzed using SPSS software.
package for Windows, Version 19.0. Graphics were constructed using GraphPad Prism, Version 5 for Windows (GraphPad Software).

Results

Factor XI inhibition affects thrombus formation on ruptured plaques

The role of factor XI in mouse models has only been established in artificially damaged healthy vessels. Here we studied the role of factor XI in thrombus formation following acute rupture of an atherosclerotic plaque in diseased vessels. Factor XI inhibition was accomplished by injection of factor XI antisense oligonucleotides (50 mg/kg) in Apoe⁻/⁻ mice fed with a high fat diet to produce sufficient atherosclerosis. Three injections with factor XI antisense oligonucleotides significantly reduced plasma factor XI activity levels to 20% (Fig 1a). Because the factor XI levels in the control group showed some variation, we normalized these levels for prothrombin. As depicted in figure 1b, prothrombin levels were not significantly different between groups. Subsequently, we calculated the mean factor XI/prothrombin ratio for both controls (0.36) and factor XI antisense (0.13) treated animals. By dividing these ratios, we found that factor XI levels were 2.8 times lower in the plasma of factor XI antisense treated animals than in the control group, corresponding to a 64% reduction of factor XI plasma levels.

Figure 1. Effect of factor XI antisense on factor XI activity and prothrombin levels in mouse plasma. Male Apoe⁻/⁻ mice were fed on a high fat diet for 18-20 weeks, after which they were treated subcutaneously every 3-4 days with factor XI antisense oligonucleotides for 10 days (dose 50 mg/kg). Each symbol represents one animal and the horizontal line indicates median, ***P<0.0001, ns=non significant. N=9 for the control group and n=13 for the factor XI antisense treated group.
Initially, in vivo thrombus formation after plaque rupture occurred similarly in both control Apoe<sup>–/–</sup> mice and Apoe<sup>–/–</sup> mice pretreated with factor XI antisense oligonucleotides (Figs 2a and 2b). However, pretreatment with factor XI antisense oligonucleotides reduced thrombus propagation and fibrin deposition, an effect that became significant 5 minutes after plaque rupture and continued for 10 minutes (Figs 2a and 2b). Furthermore, the platelet aggregates appeared to be less stable than the thrombi formed in animals treated with nonsense oligonucleotides as depicted by the number of platelets shed from the initial thrombus (Fig 2c). This indicates that inhibition of factor XI results in less stable clots, which should be a local event as systemic D-dimer levels were not different between groups (data not shown). Treatment with factor XI antisense oligonucleotides is safe in mice as the bleeding time was unaffected by the oligonucleotides (Fig 2d).
a.

Control

Factor XI antisense
Factor XI regulates pathological thrombus formation on acutely ruptured atherosclerotic plaques. The carotid artery of Apoe\(^{-}\) mice fed on a Western-type diet for 18-20 weeks was dissected free from surrounding tissue. Visible plaques were ruptured using an ultrasound wave. Subsequent thrombus formation was registered using intravital microscopy for a maximum of 30 minutes. Mice were treated with either factor XI antisense (50 mg/kg; 3 injections) or nonsense oligonucleotides (control; 50 mg/kg; 3 injections). Depicted are representative fluorescent images after 30 minutes. Mice were treated with either factor XI antisense (50 mg/kg; 3 injections) or nonsense oligonucleotides (control). Tail bleeding was evaluated as described in the method section (d). Each bar graph represents the median value of 9 animals, error bars indicate interquartile ranges (IQR), *P<0.05. The number of fluorescent platelets shed were counted (independently by 2 investigators) for the first 3 minutes after plaque rupture and for 1 minute after t=5 and t=10 (c). Data are medians with IQR, *P<0.05, ns=non-significant. Atherosclerotic mice were given three doses of either factor XI antisense (50 mg/kg) or non-sense oligonucleotides (50 mg/kg). Tail bleeding was evaluated as described in the method section (d). Each symbol represents one animal (n=4 per group) and the horizontal line indicates median, ns=non-significant.
Reduced thrombus formation is partly due to a less severe inflammatory response

To study the thrombi and plaques after the rupturing procedure in more detail, we post-stained the carotid arteries and subjected these to immunohistological analysis. Thrombus phenotype characteristics were determined by staining the sections for fibrin, MAC-3 (macrophage infiltration), Ly-6G (neutrophil recruitment) and with Sirius red (collagen) and hematoxylin and eosin. In the arteries of control Apoe⁻/⁻ mice, thrombi contained many erythrocytes as a major component of a thrombus (Fig 3a). Factor XI antisense oligonucleotides significantly reduced thrombus size (14.1±4.0\times10^3 \, \mu m^2 \, total \, thrombus \, size) when compared to placebo treated animals (68.4±23.9\times10^3 \, \mu m^2) (n=4 per group, p<0.05) as shown in figure 3b. Furthermore, using an antibody against fibrinogen, we observed fibrin fibers in the same thrombus within the carotid artery (Fig 3c, in brown) (29.9±10.2\% \, of \, thrombus \, area, \, Fig. \, 3d). In contrast, hardly any thrombi were observed in the carotid arteries of animals treated with factor XI antisense oligonucleotides as shown in figures 3a and 3c (6.4±2.6\% \, of \, thrombus \, area, \, Fig 3d) (n=4 per group, p<0.05). Finally, to confirm that the thrombi are formed on a ruptured atherosclerotic plaque, we visualized the fibrous cap of the plaque using a Sirius red staining (Fig 3e). The fibrous cap with subsequent thrombus formation was only visible in control animals and not in the factor XI antisense treated animals.

The (innate) immune system contributes to the initiation and amplification of venous thrombosis in mice(19). Especially monocytes and neutrophils appear to be responsible for thrombus formation. Therefore, we stained the carotid arteries for the presence of macrophages and neutrophils to investigate whether these cells are also involved during thrombus formation on ruptured atherosclerotic plaques. As shown in figures 3f and g, there was a higher degree of macrophage infiltration in thrombi from placebo treated animals than in factor XI antisense treated animals (18.5±1.1\% \, vs. \, 11.4±2.6 \, of \, thrombus \, area; \, n=4 \, per \, group, \, p=0.05). However, we did not observe any neutrophil infiltration in the thrombi from both control animals and factor XI antisense treated animals (Fig 3h).
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a. Control                                    Factor XI antisense

H&E

H&E

b. H&E

Thrombus size (x10^3 µm^2)

- Control
- FXI ASO

*
c. Control                                      Factor XI antisense

Fibrin

\[ \begin{align*}
\text{Fibrin area} & \quad \text{(% of thrombus area)} \\
\hline
\text{Control} & \quad 200 \, \mu m \\
\text{FXI ASO} & \quad 200 \, \mu m \\
\end{align*} \]

Fibrin

\[ \begin{align*}
\text{Fibrin area} & \quad \text{(% of thrombus area)} \\
\hline
\text{Control} & \quad 50 \, \mu m \\
\text{FXI ASO} & \quad 50 \, \mu m \\
\end{align*} \]

\[ \begin{align*}
\textbf{d. Fibrin} & \\
\text{Fibrin area (\% of thrombus area)} & \quad 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \\
\hline
\text{Control} & \quad \text{FXI ASO} \\
\end{align*} \]

- Control
- FXI ASO
Factor XI regulates pathological thrombus formation on acutely ruptured atherosclerotic plaques.

e. Control          Factor XI antisense

Sirius Red

f. Control          Factor XI antisense

MAC-3

MAC-3
Figure 3. The role of factor XI in coagulation and inflammation. (a) Images of the carotid artery stained with hematoxylin and eosin (H&E), used to visualize the extent of atherosclerotic plaque burden and erythrocyte infiltration. The arrow indicates a ruptured plaque. (b) There was a significant difference in thrombus size between factor XI antisense and placebo treated animals. (c) Sections were stained with an anti-fibrin(ogen) antibody to study thrombus formation. The arrow indicates the formed thrombus, with the fibrin threads in brown. (d) Fibrin deposition was significantly lower in thrombi from factor XI antisense treated animals when compared to placebo. (e) The fibrous cap was visualized with a collagen staining (Sirius red) both in bright-field (upper panels) and under polarized light (lower panels), the arrow indicates positive staining. (f) Macrophage infiltration at the ruptured site was analyzed using MAC-3 staining. The arrow indicates positive staining, macrophages in brown. (g) Macrophage infiltration was not significantly different between the two treatment groups. (h) Finally, the sections were stained for neutrophils using a Ly-6G antibody, however no positive staining in the thrombus was observed. Error bars represent mean±SEM; *p<0.05. Representative lower (bar 200 μm) and higher (bar 50 μm) magnification images are given.
Discussion

The results of our study demonstrate that initial clot formation on atherosclerotic plaques is not impaired in mice treated with factor XI antisense drugs. Platelet adhesion and aggregation was not significantly different between treatment groups, which imply that primary hemostasis is not influenced by factor XI inhibition. However, since clots were significantly smaller after 5 to 10 minutes in the factor XI antisense group when compared to controls, this shows that secondary hemostasis is impaired by factor XI inhibition.

This is the first study which focused on factor XI inhibition in diseased atherosclerotic vessels of older animals. Because atherosclerotic plaques contain a variety of prothrombotic substances, including TF, it is important to study the process of clot formation in ruptured plaques. We know from in vitro and in vivo studies that thrombus formation is probably less dependent on factor XI when there is a high concentration of TF(17;18). Furthermore, several other thrombogenic substances like oxidized low-density lipoproteins and macrophages are exposed to the bloodstream after plaque rupture(15). Despite these pro-thrombotic conditions, factor XI inhibition resulted in smaller thrombi, which suggests that factor XI antisense may be used in patients suffering from atherosclerosis related disorders like myocardial infarction and stroke. As the plaque rupture model is known to be dependent on tissue factor, these data indicate that, at least in mice, TF is a limiting factor in the in vivo coagulant activity, making the whole system dependent on factor XI. This observation regarding factor XI provides excellent possibilities for the secondary prevention of cardiovascular patients. Furthermore, previous experiments with this model have shown that this model relies on collagen exposure and that thrombus formation is inhibited by Glycoprotein VI inhibition and ADP receptor blockage as well as inhibition of thrombin(15;20). Hence we are able to conclude that thrombus formation on these ruptured plaques relies on both platelet activation (via Glycoprotein VI and P2Y12) and coagulation.

We observed no differences in bleeding time between factor XI antisense and nonsense oligonucleotide treated animals. This does not imply that (complete) factor XI inhibition in humans is safe. Patients with a severe factor XI deficiency may have a bleeding diathesis, while factor XI knockout mice do not have an increased bleeding tendency. This indicates that there is a discrepancy between mice and humans regarding factor XI levels and bleeding(21).
Due to the half-life of factor XI (approximately 48 hours) it takes 2 to 3 days before an anti-thrombotic effect is observed in mice and men. In humans, due to multiple factors, it will probably take 1-3 weeks before antithrombotic protection can be achieved. These factors include the time needed for the factor XI antisense oligonucleotides to distribute effectively to hepatocytes and clearance of the existing factor XI in circulation, which is dependent on its half-life. Nevertheless, this is a potential disadvantage of the drug. However, a treatment strategy incorporating heparins to bridge these first weeks before factor XI levels are reduced is probably a good solution. Most likely, the primary therapeutic use of factor XI antisense oligonucleotides will be for prevention and treatment of thrombosis in conditions such as atrial fibrillation and venous thromboembolism. Obviously, this has to be established in clinical trials.

Interestingly, clots were not only smaller, but also more unstable in factor XI antisense treated animals. Since we observed limited amounts of fibrin, this would suggest that the instability is due to insufficient thrombin generation and fibrin formation, rather than to the disintegration of preformed rigid fibrin clots. Reduced thrombus stability in factor XI deficient mice has been described before(22), however the explanation for this is not entirely clear. One possibility is that there is reduced activation of thrombin activatable fibrinolysis inhibitor, which protects the clot from degradation by plasmin. This might cause enhanced fibrinolysis with less stable clots. An argument against this theory is that D-dimer levels were not significantly different between factor XI antisense and nonsense treated animals.

Since mice with an atherosclerotic phenotype do not develop spontaneous thrombosis, an artificial trigger is necessary(23). Common methods like ferric chloride or Rose Bengal produce large thrombi which fully occlude the lumen of the vessel(24). However, thrombi formed on ruptured plaques in humans hardly ever cause full occlusion of the vessel(25;26). Additionally, these methods do not expose the content of the plaque core with all its thrombogenic substances to the bloodstream. In our model, plaque rupture was induced with an ultrasound wave, which induced thrombus formation without affecting blood flow(15). The formed thrombi never fully occluded the vessel, but did expose the plaque core to the bloodstream. Therefore, we think that this is an excellent model to study the dynamic process of thrombus formation on ruptured plaques. However, it is still a mouse model with an artificial trigger, so our model has to be translated to humans with some caution.
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Inhibition of factor XI activation attenuates inflammation(27) and indeed we found that macrophage infiltration was lower in thrombi from factor XI antisense treated animals. This was only observed in thrombi formed on the ruptured plaques and not in the underlying plaque itself. Since factor XI activity might be involved in pathways that affect leukocyte function and trafficking, this observation could merely reflect a decrease in circulating leukocytes(28). At present, there is no known mechanistic link between factor XI activation and leukocyte function, although there is evidence that factor XIa has direct cellular signaling functions through various receptors(28). Furthermore, factor XI is, together with factor XII and prekallikrein, part of the contact system which constitutes a link between coagulation and inflammation(29), and inflammatory cells like activated neutrophils are proposed to trigger coagulation(19). Therefore, inhibiting factor XI might suppress inflammatory pathways which are responsible for pathological thrombus formation on atherosclerotic plaques.

We conclude that inhibition of factor XI prevents thrombosis on atherosclerotic plaques. The question remains whether factor XI inhibition influences the process of atherogenesis. Several clotting factors, including thrombin, contribute to atherosclerotic plaque formation(30). Therefore, it would be interesting to study the role of factor XI during this long term process. Our current observations that inhibition of factor XI prevents thrombosis on acutely ruptured atherosclerotic plaques without impairment of normal clot formation and indicates that targeting factor XI could be a safe alternative for the prevention of atherothrombotic disease.
Chapter 5

References

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