Antithrombotic Effect of Antisense Factor XI Oligonucleotide Treatment in Primates

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Objective—During coagulation, factor IX (FIX) is activated by 2 distinct mechanisms mediated by the active proteases of either FVIIa or FXIa. Both coagulation factors may contribute to thrombosis; FXI, however, plays only a limited role in the arrest of bleeding. Therefore, therapeutic targeting of FXI may produce an antithrombotic effect with relatively low hemostatic risk.

Approach and Results—We have reported that reducing FXI levels with FXI antisense oligonucleotides produces antithrombotic activity in mice, and that administration of FXI antisense oligonucleotides to primates decreases circulating FXI levels and activity in a dose-dependent and time-dependent manner. Here, we evaluated the relationship between FXI plasma levels and thrombogenicity in an established baboon model of thrombosis and hemostasis. In previous studies with this model, antibody-induced inhibition of FXI produced potent antithrombotic effects. In the present article, antisense oligonucleotides–mediated reduction of FXI plasma levels by ≥50% resulted in a demonstrable and sustained antithrombotic effect without an increased risk of bleeding.

Conclusions—These results indicate that reducing FXI levels using antisense oligonucleotides is a promising alternative to direct FXI inhibition, and that targeting FXI may be potentially safer than conventional antithrombotic therapies that can markedly impair primary hemostasis. (Arterioscler Thromb Vasc Biol. 2013;33:1670-1678.)

Key Words: antisense oligonucleotide ■ factor XI ■ platelets ■ thrombus formation ■ vascular graft

Thrombin generation is required for hemostasis at sites of vascular injury. This process is typically initiated when extravascular and subendothelial proteins, including tissue factor (TF) and collagen, are exposed to flowing blood, triggering platelet activation and formation of the TF/factor VIIa (FVIIa) complex. TF/FVIIa, in turn, activates FIX and FX that catalyze the production of thrombin. Thrombin clots fibrinogen and activates platelets and FXIII, contributing to the formation of a stable hemostatic plug.1,2 In contrast to hemostatic mechanisms that cause the arrest of bleeding, thrombosis that can restrict or block normal blood flow occurs within the lumen of blood vessels in response to vascular injury, altered blood flow patterns (eg, stenoses), and predisposing functional abnormalities of platelets and coagulation pathways. The coagulation protein FXI plays a limited role compared with FVIIa in hemostasis.3-6 Humans lacking FXI have a relatively mild disorder characterized by excessive trauma induced hemorrhage in tissues with high-fibrinolytic activity.3 However, several recent studies in rodents and nonhuman primates suggest that FXI contributes significantly to thrombosis.7,8 Furthermore, humans with increased levels of FXI are at an increased risk for venous thrombosis, myocardial infarction,9,10 and stroke,9,11 whereas patients with severe FXI deficiency have a reduced incidence of ischemic stroke12 and deep-vein thrombosis.13,14 The observation that FXI plays a significant role in thrombosis, with only a modest contribution to hemostasis, makes it an attractive target for pharmacological anticoagulant therapy.3,15,16

Several studies have supported the concept that inhibition of FXI might reduce thrombus formation without a significant risk of increased bleeding. FXI-deficient mice are viable without obvious hemostatic abnormalities.7 FXI deficiency protects mice from experimental thrombosis, including ferric chloride (FeCl3)–induced vessel occlusion in both carotid artery7,17 and inferior vena cava thrombosis models.18 Antibodies that inhibit FXIa activity, or prevent FXI activation by FXII, potently inhibit experimental thrombosis in rodent, rabbit, and primate models.3,15,16,19 More recently, we described a novel therapeutic approach to targeting FXI in which antisense oligonucleotides (ASOs) were used to selectively inhibit FXI mRNA expression, leading to a corresponding reduction of plasma FXI protein and activity. Treatment of mice with FXI ASOs produced potent, dose-dependent antithrombotic effects in arterial and venous thrombosis models without increased bleeding.20 Furthermore, combining FXI inhibition with conventional anticoagulants and antiplatelet therapies (enoxaparin and

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clopidogrel) improved antithrombotic efficacy without an increase in bleeding tendency. These studies demonstrate that a significant antithrombotic benefit can be achieved through FXI inhibition, and that complete depletion of FXI is not required for this benefit in rodent models of thrombosis. The concept of selective inhibition of FXI with antisense technology has recently been extended to humans, where subcutaneous (weekly) administration of the FXI ASO ISIS-FXIe3 resulted in dose-dependent reductions in circulating FXI protein and activity, with a corresponding elevation in activated partial thromboplastin time (aPTT), but without any effect on prothrombin times.21 Because a reduced risk of thrombosis in humans has only been demonstrated in patients with severe FXI deficiency, the present study in primates was undertaken to define FXI ASO dose–response relationships for levels of anticoagulation and antithrombotic activity that might ultimately be achieved in clinical applications in humans. Accordingly, we assessed the antithrombotic activity and safety of titrated inhibition of FXI in a well-characterized baboon model of thrombosis and hemostasis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Inhibition of FXI With aXIMab: Effects on Thrombosis in the Baboon Model

Previously, a monoclonal antibody targeting FXI (aXIMab), given at a dose of 2 mg/kg, was shown to reduce levels of plasma FXI activity in baboons by 99%.13 To estimate the minimum level of FXI inhibition necessary to achieve measurable antithrombotic activity in the baboon thrombosis model, aXIMab was administered intravenously to baboons at increasing doses that achieved graded levels of FXI inhibition. At the various levels of FXI, which were observed after aXIMab treatment, propagated thrombus formation was assessed in each case as the accumulation of 111In platelets within the 10-cm long region of the arteriovenous shunt that was immediately distal to the 2-cm long collagen-coated graft segment (designated the tail of the graft thrombus; Figure 1A). As shown in Figure 2A, 50% inhibition of FXI plasma activity resulted in ≈50% inhibition of thrombus propagation after 60 minutes of blood flow. Measurable platelet accumulation was detected as early as 20 minutes after graft blood exposure, and a significant difference between the control and aXIMab-treated groups was apparent by 30 minutes (Figure 2B). In the control group not given aXIMab, and the group in which aXIMab produced <40% inhibition of FXI, measurements of platelet accumulation in propagated thrombus were similar and averaged ≈4×10⁹ platelets deposited by 60 minutes (Figure 2B). Inhibition of FXI levels to 45% to 75% of baseline values resulted in ≥50% inhibition of platelet deposition (≈1.4×10⁸ platelets deposited by 60 minutes), and >80% FXI inhibition resulted in almost complete abolition of thrombosis (≈0.2×10⁸ platelets deposited by 60 minutes) compared with the control results (Figure 2B). These effects were specific for FXI because FXII activity was not affected when FXI levels were reduced with the aXIMab antibody (data not shown).

Selective Inhibition of Baboon FXI With ASOs

The cynomolgus monkey/human cross-reactive FXI ASOs contained 2 mismatched bases compared with the baboon FXI mRNA sequence and would, therefore, not be expected to be active in this species. These mismatches were converted to the baboon sequences, and 3 baboon-specific FXI ASOs were evaluated for in vivo activity in baboons. The most potent of these baboon-specific FXI ASOs was then used to characterize the relationship between FXI plasma levels and antithrombotic activity in the baboon thrombosis model. The ASO was administered to a cohort of 4 baboons at a dose of 25 mg/kg, given 3x per week. The dosing intervals in each case are indicated by the shaded regions in Figure 3. FXI protein and activity levels were measured over the course of each experiment. After treatment, both FXI levels and FXI plasma activities were reduced in the 4 study animals, with similar kinetics. Inhibition of FXI plasma activity by 50% was achieved in all animals by day 25, reaching maximum inhibition (≈70%) toward the end of each infusion period (Figure 3A). FXI protein levels were similarly reduced (≈50%) during the infusions. After dosing was discontinued, both FXI protein and activity levels gradually increased over several months time. The prolonged reduction of FXI activity/protein is a function of the long tissue half-life of ASO in liver. FXI ASO treatment in cynomolgus monkey22 produced a substantial reduction in FXI mRNA in liver after cessation of ASO dosing, which correlated with the long tissue half-life of the ASO (≈3 weeks) and with FXI antigen reduction and eventual recovery. Because baboons were not euthanized in this study, we do not have data on RNA reduction. However, we expect results to be similar to our published results in cynomolgus monkey.

ASO inhibition of FXI activity correlated with effects on a functional coagulation parameter, the aPTT. The aPTT measurements increased over the course of ASO treatment, and corresponded well with the decrease in FXI protein and activity levels (Figure 3B). When ASO administration was discontinued, elevated aPTT values gradually returned toward normal levels within several months. As expected for an inhibitor of the intrinsic pathway, no changes in prothrombin times values were observed after administration of FXI ASOs (data not shown).

Protection of FXI ASO–Treated Baboons From Collagen-Initiated Thrombus Propagation

Because administration of FXI ASOs resulted in time-dependent reductions in both FXI protein and FXI activity in the blood of treated baboons (Figure 3A), the capacity of ASO-mediated inhibition of FXI to reduce thrombus formation was subsequently evaluated in the baboon thrombosis model. In this model, it has been shown that thrombus that forms on the collagen-coated graft segment is platelet rich and relatively insensitive to inhibition by conventional anticoagulants, such as heparin. Although potent coagulation protease inhibitors, such as D-phenylalanyl-L-prolyl-L-arginine chloromethylketone and hirudin, can block thrombus formation on collagen in this model, these and other inhibitors of thrombin activity, or thrombin formation, can also produce...
severe bleeding. Consistent with these findings, little effect on thrombus accumulation on the collagen-coated grafts was seen after either administration of aXIMab at a dose that inhibited plasma FXI activity by <80%, or administration of FXI ASOs that produced marked reductions in plasma FXI levels (Figure 4A). When the 60-minute end point results (Figure 4A) were combined with results taken in 6 additional control animals, the levels of platelet accumulation in the treated group (2.77±0.11×10⁹ platelets deposited at 60 minutes) were not different versus the results found in the control group (2.67±0.24×10⁹ platelets deposited; P>0.5).

In contrast, the formation of fibrin-rich thrombus that propagates downstream from the collagen-graft segment (ie, the thrombus tail in Figure 1) is triggered by upstream thrombin generation and is sensitively inhibited by anticoagulants, such as heparin, low-molecular weight heparin (enoxaparin, 1 mg/kg IV), or by a saturating dose of aXIMab (≥0.1 mg/kg IV) that inhibits >99% of FXI procoagulant activity. In the present study, inhibition of FXI activity by >80% using aXIMab produced near complete inhibition of propagated thrombus (Figure 2). In accord with this finding, FXI ASO treatment also reduced platelet accumulation in propagated thrombus significantly (Figure 4B). As shown in Figure 5, when the end point results for the 4 treated animals (Figure 4B) were combined with the results from the 6 additional control studies, propagated thrombus accumulation after 60 minutes averaged 2.40±0.33×10⁹ platelets deposited in the ASO-treated animals, a value that was reduced by 41% compared with the control group results (4.06±0.83×10⁹ platelets deposited; P=0.037 versus the ASO-treated group). Interestingly, although a clear reduction of propagated thrombus formation was observed in 3 baboons (Figure 4B), little effect was seen in the fourth study animal (baboon no. 1). This result is most likely explained by the unusually low control values for platelet deposition on both collagen-induced and propagated thrombus, as discussed subsequently. Nonetheless, the overall results document a significant benefit of ASO therapy for inhibition of propagated thrombus formation, and are consistent with earlier studies, and the present results with the anti-FXI monoclonal antibody (aXIMab), showing that propagated thrombus (ie, venous-type, fibrin-rich thrombus) is sensitively inhibited by anticoagulants, whereas collagen-induced thrombus (ie, platelet-rich, arterial-type thrombus) is resistant to inhibition by anticoagulants (but may be inhibited by antiplatelet agents).

The measurements of platelet deposition in propagated thrombus, taken over a wide range of FXI levels, showed a good correlation between antithrombotic efficacy and reduced FXI levels (Figure 6A). These data suggest that titrated inhibition of FXI can achieve graded and potent antithrombotic activity in nonhuman primates, and that the minimally efficacious level of FXI inhibition is ≈50% versus baseline values as shown by results with both the FXI antibody (aXIMab) and FXI ASO (Figure 6A).

**Reduced Thrombin Generation in FXI ASO–Treated Baboons**

Conceptually, FXI inhibition could reduce thrombus formation in vivo by limiting thrombin production (and

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**Figure 1.** Diagram of the baboon arteriovenous shunt thrombosis model showing interposition of a 2.0-cm long, 4.0 mm internal diameter (i.d.), thrombogenic vascular graft segment (A), and representative γ camera images of an acutely developing thrombus within the thrombogenic segment, measured in a baboon with normal factor XI levels (B). Circulating ¹¹¹Indium-labeled platelets accumulate at sites of thrombus formation. Platelet deposition on the collagen segment (ie, accumulated ¹¹¹Indium activity that is measurable above circulating background levels) appears within 10 minutes after restoring blood flow through the shunt. Images obtained at later times (30 minutes, 60 minutes) show propagation of the thrombus into the distal shunt tubing. Images were obtained in real time and acquired at 5-minute intervals. The blood flow rate was 100 mL/min (initial wall shear rate, 265/s).
thrombin-mediated platelet activation) through the FXI-dependent amplification pathway, and by increasing thrombolysis by limiting FXI-dependent thrombin activatable fibrinolysis inhibitor activation. Previously, we showed that the antithrombotic activity of aXIMab was associated with potent inhibition of thrombin-antithrombin complex generation and fibrin accumulation, but not with reduced D-dimer levels, suggesting that the antithrombotic effects of FXI inhibition were principally because of reduced platelet activation and fibrin formation. To assess the effects on thrombin generation of ASO-mediated reduction of FXI in baboons, an ex vivo thrombin generation assay was used. Samples taken from FXI ASO–treated animals were evaluated for their ability to generate thrombin on stimulation with small amounts of TF (Figure 6B). To exclude interference attributable to contact system activation, samples were pretreated with a 10-fold molar excess of corn trypsin inhibitor with respect to FXII levels. At normal levels of FXI, thrombin generation (PTG) reached a maximum of about 35 mmol/L. After 2 weeks of treatment with ASO, FXI plasma levels were reduced by 30%, and there was a significant delay in thrombin generation as well as a dramatic reduction in PTG (60%–75%). When FXI plasma levels were reduced to <50% of normal, thrombin generation was virtually abolished (Figure 6C). Thus, when limiting amounts of TF are used to trigger clotting, thrombin generation is sensitive to the plasma levels of FXI. These findings indicate that the potent antithrombotic effect in baboons of FXI depletion by ASO is mediated through an initial inhibition of thrombin generation, with subsequent reductions in platelet activation and fibrin mesh formation. In addition, these data demonstrate that even partial inhibition of FXI (=50%) can have a dramatic impact on thrombin generation in primates.

Treatment With FXI ASO Does Not Prolong the Bleeding Time in Baboons

Previously, it was demonstrated that reducing functional FXI levels with aXIMab did not prolong the bleeding time in baboons, whereas pretreatment with a single dose of aspirin nearly doubled the bleeding time. Various other antplatelet agents and anticoagulants have also been demonstrated to increase the bleeding time in baboons. When the safety of FXI ASOs with respect to bleeding was investigated in cynomolgus monkeys, the bleeding time in both naïve and FXI ASO–treated animals was =2 minutes. In an enoxaparin-treated group (2 mg/kg), the bleeding time was =10 minutes. In the present study, the safety of FXI inhibition with ASO treatment was also measured using the same method (duration of bleeding after a standardized skin incision). The results are given in Figure 7. Multiple bleeding time measurements were taken in each of the 4 baboons studied, beginning at least 2 weeks after administration of the ASOs, at which time the levels of FXI antigen and activity were reduced, and aPTT measurements were prolonged (Figure 3). Bleeding time measurements in the control group and in the 4 ASO-treated animals were similar (≈4 minutes; Figure 7). Overall, 25 measurements were performed in 9 naïve baboons and averaged 3.9±0.3 minutes. In the 4 ASO-treated animals, a total of 21 measurements were performed after at least 2 weeks of ASO administration and averaged 4.0±0.3 minutes (P=0.5 versus controls).

Discussion

In mouse models of thrombosis, antithrombotic activity was observed when plasma FXI levels were reduced by =80% or more. In cynomolgus monkeys, a 25% to 30% reduction in plasma FXI activity by ASOs resulted in significant elevations of the aPTT (by 10%–17%), suggesting that even modest reductions in FXI may be therapeutically relevant. Because appropriate cynomolgus monkey models were not available to test this hypothesis, a well-characterized baboon model of thrombosis and hemostasis was used. Previously, significant antithrombotic activity was demonstrated with the FXI monoclonal antibody aXIMab; however, the single dose level of aXIMab that was evaluated produced nearly complete elimination of FXI activity from plasma, making it difficult to ascertain the antithrombotic threshold for FXI inhibition. Therefore, the current study was performed to determine the minimum level of plasma FXI inhibition necessary to document an antithrombotic effect in primates, thereby helping guide dose selection for possible FXI inhibition.
therapies in humans. Dose titration of aXIMab demonstrated that a reduction of ≈50% in FXI protein levels resulted in an ≈50% inhibition of fibrin-rich thrombus that propagated distal to a collagen-coated segment of vascular graft, whereas >80% reduction of FXI nearly abolished propagated thrombus formation. Similarly, after ASO-mediated inhibition of FXI, a reasonably good correlation was found between reduced FXI plasma levels and inhibition of propagated thrombus formation (Figure 6A). Similar to the results with aXIMab, ASO administrations that reduced FXI levels by ≈50% produced 40% to 50% inhibition of thrombus propagation. Because the ASO dose regimen used here (25 mg/kg, 3× per week) reduced FXI levels by ≈50%, it is likely that even greater inhibition of FXI-dependent thrombus propagation (>50%) could be achieved by ASO regimens that would further reduce FXI levels, a possibility consistent with an earlier study in which near complete inhibition of FXI activity by aXIMab produced potent antithrombotic effects.15

Antisense technology was used to selectively reduce the level of the plasma coagulation protein FXI and to evaluate this strategy for the treatment of thromboembolic disease. Potent second generation ASOs (2’-methoxy ethyl modified, MOE Gapmer ASOs)26 were used in the study. Antisense technology takes advantage of base-pair hybridization of the oligonucleotide with its complementary sequence in the target mRNA,20 binding results in the selective and catalytic degradation of the targeted mRNA by a mechanism involving the nuclease RNAse H26–28 and leads to a corresponding selective reduction in target protein level.20–27

Second generation ASOs are an attractive drug class to target coagulation factors for several reasons. The technology allows for the rapid identification of highly selective inhibitors based on the linear sequence information of the targeted mRNA sequence, which are well characterized for coagulation factors. Because of prolonged tissue elimination half-lives, second generation ASOs can be administered by relatively infrequent subcutaneous injections (once weekly or even less often), regimens that are convenient for patients.28 Compared with other anticoagulant modalities, including small molecule inhibitors and the natural product anticoagulants, such as

Figure 3. Factor XI (FXI) protein and activity measurements. Four baboons were given FXI antisense oligonucleotides subcutaneously, 3× per week, at a dose of 25 mg/kg. After dosing for various lengths of time (shaded areas, days 39, 49, 60, 53), FXI plasma protein levels and activity (A) and activated partial thromboplastin time (aPTT) levels (B) were measured. Inhibition of FXI plasma activity by 50% was achieved in all animals by day 25, and reached maximum inhibition (≈80%) by day 35.
warfarin, antisense inhibitors offer a high degree of target selectivity, which should confer an additional measure of safety versus less selective pharmacological agents. Finally, FXI is synthesized primarily in the liver, which is one of the most sensitive tissues for targeting with ASO therapy.

One potential issue with ASOs is their relatively slow onset of action that arises because of the need for accumulation in target tissues (liver). However, some clinical conditions requiring anticoagulant therapy are chronic, and therefore the onset of action is of less concern. In such cases, ASOs can potentially be self-administered at home by weekly to twice monthly subcutaneous injection. Although less convenient than oral drug administration, for serious chronic diseases, such a regimen seems quite acceptable. Several second generation ASOs are currently in clinical development and have demonstrated significant therapeutic activity and safety in multiple disease indications. With FXI ASO having a slow onset of action but being long acting, this drug may be ideal for prevention purposes, including prevention of stroke in patients with atrial fibrillation, and prevention of adverse cardiovascular events in patients with coronary artery disease. In addition, this drug may be useful for preventing clots in patients at risk for venous thromboembolic disease. Toward this end, the human FXI antisense drug, FXI ASO ISIS-FXIRx, has recently demonstrated robust activity in humans. FXI antigen and activity levels were safely reduced by >95% in a phase I study involving healthy volunteers, with no evidence of bleeding.

FXI is a component of the classic intrinsic pathway of blood coagulation, which has been shown to contribute to thrombin generation and clot formation in both arterial and venous models of thrombosis. We previously demonstrated that targeting FXI expression with ASO technology in mice resulted in a highly specific, dose-dependent reduction of FXI mRNA.

Figure 4. Effect of factor XI (FXI) antisense oligonucleotides (ASOs)–mediated inhibition on thrombus formation in individual study animals. Platelet deposition was quantitated within the components of the thrombogenic device that was inserted into arteriovenous shunts in baboons, including (A) the proximal, 2-cm long collagen-coated graft segment (designated the head of thrombus in Figure 1) and (B) the 10-cm long shunt segment containing thrombus that propagated immediately distal to the collagen segment (designated the tail of thrombus in Figure 1). FXI depletion with FXI ASO had little effect on thrombus formation on the collagen segment (A). In contrast, propagated thrombus, which is dependent on upstream thrombin generation, was reduced by ASO depletion of FXI (B).
in the liver and FXI protein in plasma. In several venous and arterial thrombosis models, the ASOs exhibited antithrombotic effects that were comparable in magnitude with those of warfarin and enoxaparin. However, unlike treatment with warfarin and enoxaparin, FXI ASO treatment did not result in increased bleeding. Combining FXI ASOs with conventional antiplatelet drugs such as clopidogrel, or anticoagulants such as enoxaparin, might further enhance antithrombotic potency without potentiating the bleeding risk commonly associated with such drugs.

In baboons, a single administration of aXIMab led to rapid inhibition of plasma FXI levels (≈40% by 60 minutes) and an increase in total FXI plasma protein levels circulating in complex with the antibody, reaching ≈300% of control values by day 8. Recovery of FXI procoagulant activity to baseline levels was delayed for >3 weeks. FXI protein levels continued to decrease over the period of ASO dosing and after cessation of ASO dosing. FXI protein levels and activity subsequently recovered over time but never surpassed 100% of baseline levels. Thus, no rebound effect occurred after FXI inhibition by ASOs. Similarly, FXI reduction by ASOs in mice was not associated with a rebound increase in FXI protein or activity levels. The absence of a rebound of functional FXI in multiple species after FXI inhibition is important because it suggests that the risk of an induced prothrombotic state after ASO cessation is minimal. It is not clear at this time whether the elevated FXI antigen levels seen in the antibody-treated baboons represent increased FXI synthesis, or whether the clearance of antibody-associated FXI is prolonged versus the normal plasma half-life of FXI (≈48 hours in humans).

Surprisingly, in 1 of 4 animals studied, ASO therapy did not reduce platelet thrombus deposition as expected (baboon no. 1; Figure 4B). In reviewing these data, it was noted that platelet deposition onto the proximal collagen–coated graft segment in this study was unusually low (Figure 4A); in fact, the apparent effect of ASO in this animal was to increase platelet thrombus deposition on collagen. Such an effect seems highly unlikely both conceptually, and in view of the other results reported here. This finding is also not in accord with previous studies documenting that anticoagulants have invariably inhibited thrombosis in this model, and that FXI inhibition by monoclonal antibodies (aXIMab) and FXI ASOs has consistently reduced thrombus formation in this and other thrombosis models. Moreover, FXI levels were markedly reduced in this study animal to levels comparable with those seen in the other 3 ASO-treated animals. It is more likely, therefore, that the control data for baboon no. 1 (Figure 4A and 4B) were artifactually reduced. Indeed, in this animal platelet deposition on collagen averaged only 1.7×10^9 platelets, a value
human volunteers, ISIS-FXIRX reduced FXI plasma activity with anti-FXI antibody or ASOs in the present study did not exhibit increased bleeding during surgical procedures for arteriovenous shunt placement or removal. Moreover, in healthy baboons treated with both aXIMab and FXI ASOs, an antithrombotic effect was achieved with only modest reductions in plasma FXI levels. These results suggest that FXI ASOs represent an attractive therapeutic strategy based on their potency, selectivity, and favorable risk/benefit profile.

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Disclosures

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References


Significance

Factor XI (FXI) is a coagulation protein, which may play a role in thrombosis with a more limited role in hemostasis. Finding new treatments to prevent thrombosis, while limiting bleeding, has important public health significance. This study demonstrates the successful use of antisense oligonucleotides to reduce FXI levels in a well-characterized baboon model of thrombosis and hemostasis. Our findings that reductions in FXI levels ≥50% result in a demonstrable and sustained antithrombotic effect without increasing the risk of bleeding will be useful for determining the minimal level of FXI inhibition necessary for clinical development of FXI-targeting agents.
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OHSU and Drs Tucker, Gruber and Hanson have a financial interest in Aronora, Inc., a company that may have a commercial interest in the results of this research and technology. This potential individual and institutional conflict of interest has been reviewed and managed by OHSU.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/33/7/1670.full.
Materials and methods

FXI antisense oligonucleotides

Single stranded antisense oligonucleotides were designed that were specific for the human mRNA transcript of FXI. Two lead compounds that were identified, each 20 nucleotides in length, were chemically modified with phosphorothioate in the backbone and 2-O-methoxyethyl on the wings with a central deoxy gap (“5-10-5” design). To identify a baboon ASO, one of the FXI lead compounds that was active in cynomolgus monkey studies was shortened to 18 nucleotides (with the “5-8-5” design). The baboon sequence had two nucleotide mismatches with the cynomolgus monkey sequence that were corrected for in the baboon ASO (FXI baboon ASO sequence, GCACGATTTCGTCAGGC). Oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (PerkinElmer Life and Analytical Sciences-Applied Biosystems) and purified. All ASOs were synthesized in our laboratory at Isis Pharmaceuticals (Carlsbad, CA) and were formulated in sterile pyrogen free phosphate buffered saline (PBS, pH 7-7.4).

Animals

Baboon studies were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Animals were individually housed in a controlled environment with constant temperature (21°C) and a 12-h light/dark cycle. Food and water were available ad libitum.

Antisense oligonucleotide treatment

Baboon studies were performed using male baboons (Papio anubis, 9-13kg). Four baboons were dosed subcutaneously with ASO, 25 mg/kg, 3 times weekly. The average dosing period was ~7 weeks (range: 39-60 days). Six additional untreated animals served as controls.

Baboon thrombosis model
Thrombus formation was initiated within chronic arteriovenous shunts that had been surgically placed in baboons by interposing a prosthetic vascular graft segment for 60 min, as described [8, 15, 22]. The 2.0 cm long vascular graft segments (ePTFE, 4.0 mm i.d.; WL Gore & Associates, Flagstaff, AZ) were coated with equine type I collagen (1 mg/ml; Chronolog Corp, Allentown, PA) for 15 min and then dried overnight under sterile airflow. These collagen-coated graft segments have been found to consistently trigger platelet-dependent thrombus formation in the baboon model. The collagen-coated grafts were then interposed between segments of silicon rubber tubing comprising the chronic AV shunts, and exposed to blood flow. The flow rate through the graft was restricted to 100 ml/min (Transonics Systems flowmeter, Ithaca, NY) by clamping the distal shunt segment, thereby producing an initial mean wall shear rate of 265 s⁻¹. The grafts remained patent without flow rate reduction for 60 min. Thrombus formation was assessed during the experiments by quantitative gamma camera imaging of radiolabeled platelet accumulation within the graft segment. Since thrombus was found to extend downstream from the collagen surface over time, platelet accumulation was also measured within a 10 cm-long region of the arteriovenous shunt immediately distal to the graft. This model of thrombus growth on a proximal collagen surface (at the thrombus “head”), with thrombus that propagates distal to the collagen segment (forming a thrombus “tail”), is illustrated in Figure 1A [22, 23]. Gamma camera images of ¹¹¹Indium-platelet deposition taken during the period of blood exposure, and photographs of thrombus taken at the 60 minute endpoint following blood exposure, showed that thrombus initially formed on the collagen segment, then quickly propagated distally (Figure 1B). This model of thrombus initiation by a thrombogenic substrate followed by distal thrombus propagation has been described previously [8, 22, 23].

**Blood sample collection**

Samples were collected proximal to the graft from the midstream of the shunt lumen into 3.8% citrate (1:9, v/v) before graft deployment, and then at 30 and 60 min. One sample was processed for platelet
poor plasma and used to assess FXI procoagulant activity levels (aPTT), while a second sample was used for determination of systemic coagulation markers (PT, FXI antigen, thrombin generation).

Hemostasis assessment

The effect of FXI inhibition by ASO treatment on hemostasis in baboons was assessed using the standard template skin bleeding time test (Surgicutt; International Technidyne, Piscataway, NJ). This standardized bleeding time measurement has been shown sensitive to the effects of therapeutic anticoagulants and antiplatelet agents in baboons [24]. All bleeding time measurements were performed by the same technician.

Pharmacological inhibition of FXI in vivo

The antihuman FXI monoclonal antibody (aXIMab, 1A6) that blocks both the activation of FXI and the activation of factor IX by activated FXI was generated in a bioreactor, purified, and formulated in sterile PBS for injection at Aronora, Inc. (Portland, OR), as described elsewhere [15]. In a pilot experiment, aXIMab was administered to 4 baboons at multiple doses ranging from 40 to 70 µg/kg in an escalating dose regimen, and blood samples were collected into 3.8% citrate anticoagulant to measure circulating FXI antigen (FXI:Ag) and FXI procoagulant activity. At various levels of FXI inhibition, as initially assessed by the prolongation of aPTT measurements, thrombus formation was measured after placement into AV shunts of a thrombogenic segment of collagen-coated vascular graft, as described above.

Coagulation tests aPTT and PT

Blood from baboons was collected into tubes containing 3.8% sodium citrate. Activated partial thromboplastin times (aPTT) and prothrombin times (PT) were measured using the ACL 1000 coagulation analyzer (IL Instrumentation; Beckman Coulter). The aPTT test with platelet-poor plasma was initiated using an ellagic acid mixture (APTT-XL, Pacific Hemostasis; Fisher Diagnostics) and calcium chloride (0.02M), whereas initiation of the PT assay in platelet-poor plasma was performed by
adding thromboplastin (Dade Thromboplastin C Plus, Dade Behring). aPTT and PT measurements made with saline-treated plasmas were pooled and served as baseline reference values. aPTT and PT ratios were calculated by dividing the values measured during the experiments by these baseline values.

**FXI and FXII activity**

To measure plasma FXI and FXII activities, baboon plasmas were diluted 40X in human FXI- or FXII-deficient plasmas (George King Bio-Medical Inc.), followed by aPTT measurements. Values from pooled normal human plasma were used as references.

**FXI antigen level**

FXI antigen concentrations were measured using a sandwich-style ELISA assay (FXI-EIA) that recognized human and primate FXI (Affinity Biologicals, Ontario Canada). Plasma samples were diluted 1:20 and the ELISA was carried out as recommended by the manufacturer.

**Thrombin generation assay**

Thrombin generation in plasma was measured by following cleavage of the fluorogenic substrate Z-Gly-Gly-Gly-Arg-AMC at 37°C on a Thrombinoscope (Thrombinoscope BV), with thrombin-α2-macroglobulin calibrators supplied by the manufacturer. Studies were performed in 96-well plates (Immuno 2HB, Thermo Fisher Scientific) coated with PEG 20,000. Plasma samples were supplemented with CTI (corn trypsin inhibitor, 4uM). Supplemented plasma (80 µL) was mixed with 20 µL Tyrode buffer, pH 7.4, containing phosphatidylcholine/phosphatidylserine vesicles (30 µM) and TF (0.05 pM). Final concentrations were 5 µM phosphatidylcholine/phosphatidylserine vesicles, 0.05 pM TF. For controls, 80-µL supplemented plasma was mixed with 20 µL calibrator. Reactions were initiated by adding 20 µL of 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.4, 100 mM CaCl₂, 6% BSA, and fluorescence was monitored (excitation λ 390 nM, emission λ 460 nM). Measurements under each set of conditions were performed at least 2 times in triplicate. Thrombin generation was determined using
Thrombinoscope Analysis Software, version 3.0. The area under the thrombin generation curves is referred to as the endogenous thrombin potential (ETP).

**Statistics**

Mean values are given ± 1 SEM (standard error of the mean). The two-tailed Student’s t-test was used for all other single pair comparisons. A p value ≤ 0.05 was considered significant.