Published in final edited form as:

Transl Stroke Res. 2012 September 1; 3(3): 381–389. doi:10.1007/s12975-012-0186-5.

Inhibition of Factor XII-Mediated Activation of Factor XI Provides Protection Against Experimental Acute Ischemic Stroke in Mice

Philberta Y. Leung,

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA; Aronora, LLC, Beaverton, OR 97006, USA

Sawan Hurst,

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA

Michelle A. Berny-Lang,

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA

Norah G. Verbout,

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA; Aronora, LLC, Beaverton, OR 97006, USA

David Gailani,

Departments of Pathology and Medicine, Vanderbilt University, Nashville, TN 37232, USA

Erik I. Tucker,

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA; Aronora, LLC, Beaverton, OR 97006, USA

Ruikang K. Wang,

Department of Bioengineering, University of Washington, Seattle, WA 98195, USA

Owen J. T. McCarty, and

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA; Department of Cell and Developmental Biology, Oregon Health & Science University, Portland, OR 97239, USA

András Gruber

Department of Biomedical Engineering, Oregon Health & Science University, 3303 SW Bond Ave, Portland, OR 97239, USA; Department of Medicine, Oregon Health & Science University, Portland, OR 97239, USA

Abstract

Blood coagulation factor XI (FXI) is an established risk factor for acute ischemic stroke (AIS) and thrombosis, but is also needed for normal hemostasis. Contact factor XII (FXII), an upstream activator of FXI, also contributes to experimental stroke, but is not required for hemostasis. We investigated whether selectively inhibiting FXII-mediated FXI activation, while leaving other FXI

© Springer Science+Business Media, LLC 2012

Correspondence to: Owen J. T. McCarty; András Gruber.

mccartyo@ohsu.edu, gruber@ohsu.edu.

Conflicts of Interest Oregon Health & Science University (OHSU) and Vanderbilt University have been seeking patent protection for the monoclonal antibody 14E11 that has been out-licensed to Aronora, LLC, an Oregon company, and these institutions. The authors AG, DG, PYL, EIT, and NGV have a potential financial interest in its commercial development.

and FXII functions intact, could improve the outcome of experimental AIS in mice. Twenty-four hours before induction of AIS by placement of a filament into the internal carotid artery for 60 min, mice were anticoagulated with an antibody directed against the apple 2 domain of FXI. This antibody selectively reduces the prothrombotic activation of FXI by FXIIa but does not affect activated FXI or hemostatic activation of FXI by thrombin, thus leaving hemostasis intact in mice and primates. In this model of AIS/reperfusion injury, mice that received the antibody before AIS displayed less ischemic damage, manifested as reduced cerebral infarction and fibrin deposition (thrombosis), increased cortical reperfusion, and improved neurological behavior. Further, the antibody-anticoagulated mice had no detectable hemostasis impairment. Consistent with the neuroprotective phenotype of FXII-deficient mice, our data suggest that a single molecular event, FXII-mediated FXI activation, contributes to the development of experimental AIS.

Keywords

Factor XI; Ischemia; Neuroprotection; Thrombolysis; Hemostasis

Introduction

Acute ischemic stroke (AIS), the clinical manifestation of focal cerebral ischemia, is the leading cause of disability and the third leading cause of death in the USA [1] and is usually caused by the progressive thrombotic or thromboembolic obstruction of cerebral blood vessels. Elective cardiovascular procedures, including angioplasty, stenting, cardioversion, and others, also carry the risk of thrombosis, embolism, and AIS [2, 3]. Thrombi form under flow inside blood vessels only under pathological conditions and are characterized by up to 50-fold increase in the local density of specific blood components (leukocytes, platelets, and fibrin). The Atherosclerosis Risk in Communities Study, encompassing nearly 16,000 individuals, found that higher coagulation factor XI (FXI) levels were associated with an elevated risk of ischemic stroke [4]. Consistent with this, FXI deficiency reduces the incidence of AIS in humans [5].

Recent observations suggest that thrombotic occlusions in larger blood vessels are supported by the pathological activation of the contact pathway, including activation of factors XII (FXII) and its downstream substrate, FXI [6, 7]. Of these two enzymes, FXI also contributes to normal hemostasis, while FXII has no known role in bleeding control, suggesting a separation of processes that contribute to thrombosis and hemostasis at this molecular level.

Hemostasis is a vital process that relies on an orchestrated series of molecular and cellular events that prevent blood from escaping the vasculature upon injury via the rapid clotting of blood at wound sites. When vessel walls are damaged, tissue factor and exposed extracellular matrix proteins trigger the formation of hemostatic plugs through thrombin generation via the extrinsic pathway of blood coagulation, platelet activation, and fibrin formation [8]. The feedback activation of FXI by thrombin helps sustain thrombin generation and the formation of a more stable, lysis-resistant hemostatic plug structure [9]. Polyanions, collagen, laminin, bacteria, and other foreign blood-exposed surfaces trigger the activation of FXII to FXIIa, which then cleaves its natural substrates, most notably FXI and plasma prekallikrein (PK; [9, 10]). Contact activation of blood thus activates FXI and PK, inducing blood coagulation through prothrombotic thrombin generation and promoting inflammation through kallikrein-mediated liberation of bradykinin from high molecular weight kininogen by kallikrein [9, 10]. The catalytic activation of FXI by FXIIa appears to be a response to a pathological event that, at this time, has no known physiological function in vivo. This process is partially replicated during the extreme conditions of the well-

established activated partial thromboplastin time (aPTT) test, commonly used in clinical laboratories.

The pathological contribution of FXI to thrombosis has now been established. Men in the upper quintile of the normal distribution for plasma FXI levels have a ~2-fold increased risk of myocardial infarction compared to those in the lowest quintile [5]. Further, elevated levels of FXI are a risk factor for AIS, while severe FXI deficiency decreases the risk of AIS but is associated with mild bleeding tendencies in humans [5, 11]. In terms of stroke outcome, two recent clinical studies reported that the presence of circulating FXIa is associated with worse prognosis and more severe neurological deficits both acutely (within 72 h) and chronically (average, 36 months) following the cerebrovascular ischemic event [12, 13]. The worse clinical outcomes in FXIa-positive patients were hypothesized to be caused by the reduced elimination of thrombi in blood vessels due to increased clot stability and decreased fibrinolysis.

We have shown that the inhibition of FXI activity or activation is antithrombotic in primates and mice [6, 14] and subsequently demonstrated that administration of a monoclonal antibody, 14E11, which inhibits prothrombotic FXI activation by FXIIa but not hemostatic FXI activation by thrombin, reduces experimental thrombus formation in mice and baboons [15]. Both FXII and FXI contribute to the development of experimental AIS in mice, but it is not known whether the progression of thrombotic AIS is supported by the enzymatic activation of FXI by FXIIa, or whether the two proenzymes independently contribute to the development of AIS.

Currently, the only proven effective pharmacological treatment for AIS—thrombolysis with recombinant tissue plasminogen activator (tPA, Alteplase®, Genentech)—carries with it a risk of fatal intracranial hemorrhage, limiting the number of stroke patients who receive tPA and achieve reperfusion [16, 17]. Antithrombotic prophylaxis with antiplatelet drugs such as aspirin and clopidogrel, and anticoagulants such as heparin, warfarin, or rivaroxaban can reduce the risk of AIS [18-21], but these agents also carry a risk of potentially fatal hemorrhagic side effects that constrain their therapeutic potential. If the enzymatic step of FXI activation by FXIIa, which reduced experimental thrombus formation, contributes to the pathogenesis of AIS, it would provide a theoretically safe target for antithrombotic pharmacological interventions for prevention. Thus, we have utilized 14E11, an anticoagulant antibody, to test this hypothesis. This antibody binds to the A2 domain of FXI and inhibits the association between FXI and high molecular weight kininogen (HK), thus limiting the activation of FXI by FXIIa while leaving the thrombin-dependent enzymatic (hemostatic, etc.) functions of FXIa intact [14, 15].

In the present study, we test the hypothesis that pretreatment with 14E11 will improve neurological outcomes in a mouse model of AIS. We found that the 14E11-pretreated mice exhibited improved cortical reperfusion, which was associated with reduced neurological deficits, infarct size, and fibrin accumulation in the infarcted hemisphere. Our results suggest that contact activation of FXI through FXII has a pathogenetic role in the progression of cerebral ischemia during AIS.

Methods

Treatments

All animal experiments were approved by the Institutional Animal Care and Use Committee. The anticoagulant murine monoclonal anti-factor XI antibody, 14E11, was prepared at Aronora, LLC (Beaverton, OR) as described previously [14, 15]. Twenty-four hours prior to the induction of experimental AIS, isoflurane-anesthetized male C57Bl/6 mice (3 months

old; 21–26 g; Charles River Laboratories, Madison, WI) were randomly administered either vehicle [phosphate-buffered saline (PBS)] or 14E11 (2 mg/kg) as a single 185- μ L bolus injection through the isolated right femoral vein ($N=10$ in each treatment group). The 14E11 dose was selected based on previous studies that demonstrated the prolongation of the aPTT for several days without affecting the prothrombin time (PT).

Mouse Model of Acute Ischemic Stroke

One day after 14E11 pretreatment, mice were anesthetized with 5 % and maintained with 1.5 % isoflurane in 37 % oxygen, and a transcranial laser Doppler flowmetry (LDF) probe (Moor Instruments Ltd, Oxford, UK) was secured over the right parietal bone to monitor focal changes in cortical perfusion during and after stroke induction [22]. Focal cerebral ischemia was induced by surgical deployment of a filament into the right internal carotid artery (ICA), described elsewhere as LDF-defined “middle cerebral artery occlusion” (MCAO) [22–24]. In brief, the common carotid artery (CCA) was clamped, and a heat-blunted and surface-coated (Xantopren Comfort Light and hardener mix, Heraeus, Hanau, Germany) 6–0 monofilament nylon surgical suture was advanced retrograde through the right external carotid artery (ECA) into the ICA until more than 75 % reduction in temporal LDF signal was observed. This reduction in relative cortical flow was defined as the start of MCAO. The ECA to MCAO phase of the procedure lasted approximately 5 min. The filament in the ECA was then secured and kept in place for the duration of the MCAO (60 min). If LDF-defined flow increased over 25 % of the baseline during occlusion, the experiment was terminated. In addition to significantly obstructing blood flow to the middle cerebral artery (MCA) region by blocking the bifurcation of the middle and anterior cerebral arteries, deployment of the intraluminal filament acts as a thrombogenic trigger due to vascular injury, flow disturbance, and stasis, as well as from the surface of the filament itself since it is a foreign material. These serve to induce progressive distal thrombogenesis in the MCA region and to expand the infarcted brain volume over time [23]. After allowing disease progression for 60 min, the filament was pulled back, the ECA was ligated, and the CCA clamp was released to repressurize the ICA. The LDF signal was monitored for an additional 15 min to quantify relative reperfusion, then the LDF probe was removed, isoflurane anesthesia was terminated, and the mouse was put back in his cage. The monitoring of the LDF signal during occlusion and at 15 min after the removal of the intraluminal filament (reperfusion) allowed us to determine whether anticoagulation with 14E11 could constrain the progression of distal thrombotic vascular occlusions during MCAO and therefore improve reperfusion immediately following occlusion.

Neurological Performance Evaluation

Following induction of AIS, mice were caged individually, and soft food and liquids were placed near the animals at the bottom of the cage for easy access. In addition, all animals were administered subcutaneous lactated Ringer’s solution with 10 % dextrose (1.5 mL total volume daily) for electrolyte/caloric supplement. Neurological performance scores were assessed every morning at the same time using a modified Hara scale (0, no neurological signs; 1, flexion of the contralateral torso; 2, circling to the contralateral side but normal posture at rest; 3, leaning to the contralateral side at rest; 4, responsive but no spontaneous motor activity; 4.5, moribund). Scores were ranked from 0 to 4.5 at intervals of 0.5; animals demonstrating neurological signs between two categories were given an intermediate score [22]. Animals that died were not included in the analysis as the time of death was not available and the cadavers could not be used for reliable histological evaluation. All analyses were performed by researchers blinded to treatment to avoid experimental bias.

Determination of Cerebral Infarct Size

Twenty-four hours following the induction of AIS and immediately following the evaluation of neurological function, animals were sacrificed, and their brains were dissected and sectioned into 2-mm coronal slices. After visually inspecting for macroscopic hemorrhage [22, 24], sections were stained with 1.5 % 2,3,5-triphenyl tetrazolium chloride (TTC) in PBS, and infarct volumes were evaluated using morphometric analyses. Sections were imaged, and the areas of hemispheres and infarcts were measured using ImageJ software. The measurements were multiplied by the section thickness and summed over the entire brain to yield infarct volumes. Ischemic damage data were calculated using the indirect method to minimize error introduced by edema and were presented as percent infarct=(volume of contralateral hemisphere–volume of non-infarcted tissue of the ipsilateral hemisphere)/(volume of contralateral hemisphere)× 100. Analyses were performed by researchers blinded to treatment to avoid experimental bias.

Detection of Fibrin Deposition in the Brain

To evaluate whether FXI activation contributes to the expansion of ischemic damage through contact pathway activation, fibrin accumulation in the harvested brains was determined by Western blot 24 h following MCAO. In brief, following TTC staining for the determination of infarct size, the 2-mm sections of brain tissue were separated into contralateral and ipsilateral hemispheres. Tissue fibrin deposits that serve as evidence of thrombogenesis were isolated [7], and the fibrin β -chain content was visualized by Western blotting using the fibrin-specific mAb 59D8 (generous gift from Drs. Marshall Runge, University of North Carolina and Charles Esmon, Oklahoma Medical Research Foundation). Blot films were digitally scanned, and band intensities were measured with ImageJ version 1.44. A ratio of the ipsilateral (infarcted hemisphere) fibrin band density relative to the band density of contralateral (control hemisphere) fibrin content served as a metric for relative fibrin deposition in vehicle-versus 14E11-treated animals.

Evaluation of Hemostasis

The tail transection test was used for hemostasis assessment as described [22] with a separate group of mice than those used for the stroke studies. Anesthetized mice were administered 185 μ L of vehicle (PBS; $N=11$) or 14E11 (2 mg/kg; $N=12$) intravenously. Twenty-four hours later, tail transection (at 1.5-mm tail diameter) was performed, and the bleeding time (visual observation) was recorded.

Since 14E11 inhibits the activation of FXI by the up-stream contact factor FXIIa, we used the aPTT clotting test as a performance indicator for the efficacy of 14E11 to disrupt the FXII/FXI contact activation pathway. For clotting assays, mice were euthanized by CO₂ asphyxiation at 24 h following the administration of vehicle (PBS; $N=11$) or 14E11 ($N=12$), and blood was drawn immediately by cardiac puncture into 3.2 % sodium citrate (9:1, v/v) [22]. Plasma was prepared by centrifugation of blood for 3 min at 15,700×*g*. APTT (test for contact-initiated intrinsic pathway) measurements were performed using an APTT-ES kit (Helena Laboratories, Beaumont, TX), and PT (test for tissue factor-activated extrinsic pathway) measurements were performed using the Dade Innovin PT reagent. Both clotting tests were performed with a KC4 Coagulation Analyzer (Trinity Biotech; Bray, Co Wicklow, Ireland).

Statistical Analysis

Data are presented as means±SEM. Statistical significance between means was determined by the Student's *t* test. Survival data were analyzed by Kaplan–Meier analysis, and a

comparison between the treatment groups was done by the log-rank test. Significance for all statistical tests required $p < 0.05$.

Results

Improvement of Cortical Reperfusion in 14E11-Treated Mice

LDF-defined cortical reperfusion was increased in 14E11-treated mice following MCAO and re-pressurization of the MCA, with perfusion of the parietal cortex restored to $>60\%$ of pre-MCAO baseline values versus 41% of baseline in non-anticoagulated mice ($p < 0.05$; Table 1).

14E11 Pretreatment Improved Neurological Outcomes Following AIS in Mice

As assessed by neurological performance evaluation 24 h after MCAO, all surviving mice displayed signs and symptoms characteristic of AIS, whether treated with 14E11 or untreated. The neurological deficits ranged from circling to complete contralateral hemiparesis or non-responsiveness. Neurological performance scores (Fig. 1a) were significantly better for 14E11-treated mice than for vehicle-treated controls (2.65 ± 0.25 compared with vehicle controls at 4.20 ± 0.12 ; $p < 0.001$). To determine whether this subacute difference in neurological deficits between treatment groups was sustained over time, a subset of animals was monitored for 7 days following MCAO, with neurological performance assessed daily. The mean daily neurological score was significantly lower in 14E11-pretreated mice on days 2 through 5 post-MCAO (Fig. 2a, $p < 0.05$ on days 2 through 5). Pretreatment with 14E11 did not decrease mortality following AIS (Fig. 2b).

Reduction of Infarct Size and Fibrin Deposition Without Intracranial Bleeding in 14E11-Treated Mice

On autopsy and visual examination of the brains, there was little difference in macroscopic gross hemorrhage between the vehicle-treated (2/10) and 14E11-treated (1/10) mice. Morphometric analyses of the TTC-stained brain slices indicated that pretreatment of mice with 14E11 limited the expansion of the ischemic damage beyond the infarct core that was present in all animals that were sacrificed at 24 h post-MCAO (Fig. 1b). Infarct volumes of the 14E11-treated mice were $20.8 \pm 8\%$ compared with vehicle controls at $37.7 \pm 5\%$ ($p < 0.05$). There was significantly more relative fibrin deposition in the vehicle-treated mice (ratio of ipsilateral to contralateral hemisphere fibrin band density = 3.1 ± 0.41) versus the 14E11-treated mice (1.6 ± 0.22 ; $p < 0.05$).

Anticoagulation by 14E11 Without Hemostasis Impairment

Compared to vehicle-treated controls, the aPTT of plasma was prolonged by >2.5 -fold at 24 h following 14E11 administration (Fig. 3a). The aPTT of 14E11-treated mice remained prolonged for at least 2 days (data not shown). As expected, the antibody had no effect on the FXII-independent PT test (Fig. 3b). Tail bleeding times in 14E11-treated mice were comparable to vehicle-treated mice (Fig. 4), suggesting that the administration of 14E11 does not significantly impair primary hemostasis.

Discussion

Here, we report for the first time that selectively inhibiting the FXII-dependent contact activation of FXI without blocking the hemostatic activation of FXI by thrombin limits damage from MCAO-initiated AIS. To target and prevent the activation of FXI by its upstream activator FXIIa, we used 14E11, an antithrombotic monoclonal antibody directed against the A2 domain of mammalian FXI. Mice that were pretreated with 14E11 exhibited improved LDF-defined cortical perfusion after removal of the filament from the MCA,

reduced fibrin deposition in blood vessels, smaller infarct sizes, and, importantly, reduced neurological deficits. Consistent with the known anticoagulant and antithrombotic effects of 14E11, fibrin accumulation was significantly decreased in the infarcted hemisphere of 14E11-pretreated animals compared to vehicle-treated controls. Direct causality between these observed differences cannot be determined at this juncture. However, we speculate that reduced fibrin deposition results, in theory, in better maintenance of tissue perfusion outside the margins of the ischemic core during MCAO, reduced spatial thrombotic progression of ischemia and infarction into the penumbra and therefore better outcomes. As expected, 14E11 treatment did not induce a detectable effect on hemostasis as defined by the tail bleeding test.

The use of antithrombotic agents, whether targeting platelets (e.g., aspirin and clopidogrel) or blood coagulation (e.g., heparin, warfarin, and rivaroxaban), is a rational causal approach to both the prevention and treatment of AIS. In prevention, they have been shown to produce overall benefit by reducing the incidence of AIS in high-risk patient populations. However, despite relentless efforts and numerous clinical trials with antithrombotic agents and protocols to treat AIS, many approaches have failed primarily due to serious bleeding side effects, including hemorrhagic transformation and intracerebral hemorrhage, that outweigh the antithrombotic benefit [18-21, 25]. The most recent rational strategy to inhibit thrombogenesis in stroke involves identifying and targeting those prothrombotic functions of platelets and coagulation factors that are not essential for hemostasis. In particular, experimental data have now confirmed that at least two components of the contact activation enzyme-cofactor complex (FXII and prekallikrein) contribute to pathological intravascular coagulation (thrombosis) without playing a demonstrable role in hemostasis. FXI is also prothrombotic in its contribution to hemostasis and has been shown to be associated with worse clinical outcomes acutely and chronically following ischemic stroke [12, 13].

Selective targeting of the FXI and/or FXII gene in mice and induction of experimental FXI or FXII deficiency reduce thrombogenic responses in various thrombosis models, including AIS [14, 26-30]. Importantly, FXI deficiency reduces the incidence of AIS in humans [5], and selective FXI inhibitors also reduce thrombus size in experimental thrombosis [6, 14, 31] in rabbit and nonhuman primate models, suggesting that blocking or downregulating FXI activity or expression could be an efficacious antithrombotic approach. Indeed, administration of anti-factor XI antibodies both intravenously and systemically produced a dramatic increase in endogenous thrombolysis in a rabbit thrombosis model [32]. However, severe FXI deficiency ("hemophilia C" FXI activity levels <15 U/dL) and/or acquired factor XI inhibitors are rare but well-established bleeding disorders that can be severe on occasion in humans [11]. Thus, pharmacologically blocking FXI activity inherently carries a risk of bleeding. In contrast, inherited FXII deficiency has no known negative hemostatic consequences in humans, and its inhibition or absence has been demonstrated to protect against pathological thrombosis in a mouse model of ischemic brain injury without affecting primary hemostasis. Therefore, selectively blocking a single enzymatic step, prothrombotic FXIIa-mediated FXI activation, without interfering with the prohemostatic, thrombin-mediated activation of FXI or interfering with other potential functions of FXIIa that are independent of FXI may represent the most specific, and presumably safest, antithrombotic strategy [33].

The data obtained in this study lend credence to the hypothesis that FXIIa-dependent FXI activation contributes to the pathogenesis of AIS since 14E11 administration led to significantly smaller infarcts and better neurological function for up to 7 days compared to vehicle controls after temporal MCAO. These improved outcomes were associated with increased cortical perfusion after a 60-min-long MCAO in 14E11-pretreated mice, suggesting that distal occlusions and the resulting expansion of ischemia during proximal

mechanical occlusion of the MCA were mediated at least in part by the activation of FXI by FXIIa and progressive blood clotting in the distal blood vessels of the ischemic hemisphere. As expected, 14E11 did not cause demonstrable impairment of hemostasis as measured by the tail bleeding test, which is in direct contrast with a neuroprotective dose of tPA in the same assay [22].

Blocking FXIIa (or plasma kallikrein) directly could presumably reduce prothrombotic activity, bradykinin-mediated vasoregulatory, and potentially other enzymatic and proinflammatory activities of the contact system, which may or may not contribute to the pathology of thrombosis and hemostasis. However, by using FXII-deficient mice and an enzyme inhibitor that blocks FXIIa as well as kallikrein, among other proteases, Kleinschnitz et al. (2006) have demonstrated that one or more FXIIa activities positively contribute to cerebrovascular fibrin formation and cerebral ischemia in the intraluminal filament-induced AIS model. Our results show that 14E11-pretreated mice have significantly less fibrin accumulation in the infarcted hemisphere, indicating that the intravascular fibrin clot formation, which may be responsible in part for the progressive spatial expansion of ischemic damage in untreated controls, was limited by the inhibition of contact activation of FXI, leading to reduced thrombus formation and less extensive ischemia. Thus, we have demonstrated here that FXIIa-mediated FXI activation is important for the development of AIS and maintenance of prothrombotic thrombin generation during MCA occlusion in the MCAO model. Whether inhibiting FXII-mediated activation of FXI after the start of focal ischemia, before or after completion of the stroke, with or without subsequent reperfusion, would improve neurological and other outcomes remains to be evaluated. However, infarct size is time-dependent and expands in this model, likely due to continued occlusive thrombogenesis around the ischemic core caused by the initial mechanical blood flow restriction. Thus, inhibiting FXI activation at or shortly following stroke onset could still prove to be efficacious in limiting the growth of the thrombus since FXI is needed for the *maintenance* of thrombin generation. Thus, we anticipate that 14E11 or other compounds that selectively interfere with the association of FXI with HK could be safe anticoagulants that may have utility in both the prevention and treatment of AIS.

In summary, our current data demonstrate a critical role for FXIIa-mediated FXI activation in the progression of cerebral infarction in a mouse model of AIS. Thus, we propose that the targeted inhibition of FXIIa-catalyzed FXI activation is a potential antithrombotic approach in AIS that comes without secondary bleeding side effects.

Acknowledgments

Supportive discussions with Dr. Joseph Aslan and technical support from Jiaqing Pang are gratefully acknowledged.

Sources of Funding This work was supported in part by NIH grants HL 101972 (O.J.T.M. and A.G.), HL 095315 (A.G. and E.I.T.), and by an unrestricted grant from Bayer Healthcare (E.I.T. and A.G.).

References

1. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TN, Turner MB, Wong ND, Wylie-Rosett J. Heart disease and stroke statistics-2011 update: a report from the American Heart Association. *Circulation*. 2011; 123:e18–e209. [PubMed: 21160056]
2. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA. Neurological Outcome Research Group and the Cardiothoracic Anesthesiology

Research Endeavors Investigators. Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. *N Eng J Med*. 2001; 344:395–402.

3. Newman MF, Mathew JP, Grocott HP, Mackensen GB, Monk T, Welsh-Bohmer KA, Blumenthal JA, Laskowitz DT, Mark DB. Central nervous system injury associated with cardiac surgery. *Lancet*. 2006; 368:694–703. [PubMed: 16920475]
4. Suri MF, Yamagishi K, Aleksic N, Hannan PJ, Folsom AR. Novel hemostatic factor levels and risk of ischemic stroke: the Atherosclerosis Risk in Communities (ARIC) Study. *Cerebrovasc Dis*. 2010; 29:497–502. [PubMed: 20299790]
5. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood*. 2008; 111:4113–7. [PubMed: 18268095]
6. Gruber A, Hanson SR. Factor XI-dependence of surface-and tissue factor-initiated thrombus propagation in primates. *Blood*. 2003; 102:953–5. [PubMed: 12689935]
7. Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, Renne C, Gailani D, Nieswandt B, Renne T. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med*. 2006; 203:513–8. [PubMed: 16533887]
8. Mackman N, Taubman M. Tissue factor: past, present, and future. *Arterioscler Thromb Vasc Biol*. 2009; 29:1986–8. [PubMed: 19923557]
9. Gailani D, Renne T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *J Thromb Haemost*. 2007; 5:1106–12. [PubMed: 17388803]
10. Renne T, Oschatz C, Seifert S, Muller F, Antovic J, Karlman M, Benz PM. Factor XI deficiency in animal models. *J Thromb Haemost*. 2009; 7(Suppl 1):79–83. [PubMed: 19630774]
11. Seligsohn U. Factor XI deficiency in humans. *J Thromb Haemost*. 2009; 7(Suppl 1):84–7. [PubMed: 19630775]
12. Undas A, Slowik A, Gissel M, Mann KG, Butenas S. Circulating activated factor XI and active tissue factor as predictors of worse prognosis in patients following ischemic cerebrovascular events. *Thromb Res*. 2011; 128:e62–6. [PubMed: 21820158]
13. Undas A, Slowik A, Gissel M, Mann KG, Butenas S. Active tissue factor and activated XI in patients with acute ischemic cerebrovascular events. *Eur J Clin Invest*. 2012; 42(2):123–9. [PubMed: 21707613]
14. Tucker EI, Marzec UM, White TC, Hurst S, Rugonyi S, McCarty OJ, Gailani D, Gruber A, Hanson SR. Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI. *Blood*. 2009; 113:936–44. [PubMed: 18945968]
15. Cheng Q, Tucker EI, Pine MS, Sisler I, Matafonov A, Sun MF, White-Adams TC, Smith SA, Hanson SR, McCarty OJ, Renne T, Gruber A, Gailani D. A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. *Blood*. 2010; 116:3981–9. [PubMed: 20634381]
16. Hacke W, Kaste M, Fieschi C, Toni D, Lesaffre E, von Kummer R, Boysen G, Bluhmki E, Hoxter G, Mahagne MH. Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke. The European Cooperative Acute Stroke Study (ECASS). *JAMA*. 1995; 274:1017–25. [PubMed: 7563451]
17. Kilic E, Hermann DM, Hossmann KA. Recombinant tissue plasminogen activator reduces infarct size after reversible thread occlusion of middle cerebral artery in mice. *Neuroreport*. 1999; 10:107–11. [PubMed: 10094143]
18. Creager MA. Results of the CAPRIE trial: efficacy and safety of clopidogrel. Clopidogrel versus aspirin in patients at risk of ischaemic events. *Vasc Med*. 1998; 3:257–60. [PubMed: 9892520]
19. Gonzalez ER. Antiplatelet therapy in atherosclerotic cardiovascular disease. *Clin Ther*. 1998; 20(Suppl B):B18–41. [PubMed: 9589829]
20. Pfeilschifter W, Spitzer D, Czech-Zechmeister B, Steinmetz H, Foerch C. Increased risk of hemorrhagic transformation in ischemic stroke occurring during warfarin anticoagulation: an experimental study in mice. *Stroke*. 2011; 42(4):1116–21. [PubMed: 21330626]
21. Steffel J, Braunwald E. Novel oral anticoagulants: focus on stroke prevention and treatment of venous thrombo-embolism. *Eur Heart J*. 2011; 32(16):1968–76. [PubMed: 21421599]
22. Berny-Lang MA, Hurst S, Tucker EI, Pelc LA, Wang RK, Hurn PD, Di Cera E, McCarty OJ, Gruber A. Thrombin mutant W215A/E217A treatment improves neurological outcome and

- reduces cerebral infarct size in a mouse model of ischemic stroke. *Stroke*. 2011; 42(6):1736–41. [PubMed: 21512172]
23. Carmichael ST. Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx*. 2005; 2(3):396–409. [PubMed: 16389304]
 24. Stevens SL, Leung PY, Vartanian KB, Gopalan B, Yang T, Simon RP, Stenzel-Poore MP. Multiple preconditioning paradigms converge on interferon regulatory factor-dependent signaling to promote tolerance to ischemic brain injury. *J Neurosci*. 2011; 31:8456–63. [PubMed: 21653850]
 25. Abrams PJ, Emerson CR. Rivaroxaban: a novel, oral, direct factor Xa inhibitor. *Pharmacotherapy*. 2009; 29:167–81. [PubMed: 19170587]
 26. Rosen ED, Gailani D, Castellino FJ. FXI is essential for thrombus formation following FeCl₃-induced injury of the carotid artery in the mouse. *Thromb Haemost*. 2002; 87:774–6. [PubMed: 12008966]
 27. Wang X, Cheng Q, Xu L, Feuerstein GZ, Hsu MY, Smith PL, Seiffert DA, Schumacher WA, Ogletree ML, Gailani D. Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice. *J Thromb Haemost*. 2005; 3:695–702. [PubMed: 15733058]
 28. Kravtsov DV, Matafonov A, Tucker EI, Sun MF, Walsh PN, Gruber A, Gailani D. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009; 114:452–8. [PubMed: 19351955]
 29. Schumacher WA, Seiler SE, Steinbacher TE, Stewart AB, Bostwick JS, Hartl KS, Liu EC, Ogletree ML. Antithrombotic and hemostatic effects of a small molecule factor XIa inhibitor in rats. *Eur J Pharmacol*. 2007; 570:167–74. [PubMed: 17597608]
 30. Zhang H, Lowenberg EC, Crosby JR, MacLeod AR, Zhao C, Gao D, Black C, Revenko AS, Meijers JC, Strokes ES, Levi M, Monia BP. Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. *Blood*. 2010; 116:4684–92. [PubMed: 20807891]
 31. Yamashita A, Nishihira K, Kitazawa T, Yoshihashi K, Soeda T, Esaki K, Imamura T, Hattori K, Asada Y. Factor XI contributes to thrombus propagation on injured neointima of the rabbit iliac artery. *J Thromb Haemost*. 2006; 4:1496–501. [PubMed: 16839345]
 32. Minnema MC, Friederich PW, Levi M, von dem Borne PA, Mosnier LO, Meijers JC, Biemond BJ, Hack CE, Bouma BN, ten Cate H. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. *J Clin Invest*. 1998; 101(1):10–4. [PubMed: 9421460]
 33. Meijers JC. Feedback controversy stops here. *Blood*. 2009; 114:235. [PubMed: 19589932]

Fig. 1.

Anticoagulant pretreatment with 14E11 reduces infarct size and neurological deficits after experimental stroke. **a** Mice were administered vehicle or 2 mg/kg of the anticoagulant antibody 14E11 intravenously 24 h prior to the initiation of experimental acute ischemic stroke (AIS) that was induced by deploying an intraluminal filament into the right middle cerebral artery for 1 h. Mice that were anticoagulated with 14E11 had better neurological performance scores 24 h after AIS than vehicle-treated mice, as assessed using the modified Hara scale of neurological deficit evaluation in mice [21]. **b** 14E11-treated mice exhibited significantly smaller infarcts than their vehicle-treated counterparts as assessed with TTC staining of the brains 1 day after AIS. **c** There was more fibrin trapped in the ischemic hemisphere in both vehicle- and 14E11-treated groups. Quantitative immunoblotting showed 48 % less fibrin accumulation in the ischemic hemisphere of 14E11-treated mice compared to vehicle controls. *Ipsi* ipsilateral (infarcted hemisphere), *Cont* contralateral hemisphere ($n=3$ to 4). Values are mean $\pm$ SEM, $n=10$ for each group, unless noted otherwise, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus vehicle controls

Fig. 2.

Benefits of anticoagulation on neurological outcomes are sustained for at least 7 days following AIS. **a** Neurological performance was scored daily for 7 days after AIS in one anticoagulated (14E11-treated) and one non-anticoagulated (control) groups of mice, beginning at 24 h after stroke initiation. The neurological performance scores of the first day after stroke appeared to be sustained in surviving mice. There was no trend for improvement or worsening of the neurological performance in those animals that survived for 7 days. **b** The 7-day survival benefit for 14E11 did not reach statistical significance (Kaplan–Meier analysis, log-rank test). Values are mean±SEM, $n=7-15$, * $p<0.05$ versus vehicle

Fig. 3.

Anticoagulation with 14E11 is sustained for at least 24 h in mice. Groups of naive, anesthetized mice were administered a bolus of vehicle or 14E11 (2 mg/kg, i.v.). Twenty-four hours later, the animals were euthanized with CO₂ inhalation, and cardiac blood was drawn from the left ventricle immediately into sodium citrate for verification of sustained anticoagulation. Plasma was prepared by centrifugation for APTT (test for contact-initiated intrinsic pathway) and PT (test for tissue factor-activated extrinsic pathway) measurements. APTT was significantly prolonged by 14E11 pretreatment (**a**), whereas PT measurements were not different between vehicle and 14E11 treatments (**b**). Values are mean±SEM, $n=11-12$, * $p<0.05$ versus vehicle

Fig. 4.

Anticoagulation with 14E11 does not prolong tail bleeding time. Anesthetized naive mice were administered a bolus of vehicle or 14E11 (2 mg/kg, i.v.), and tail transection bleeding time was determined 24 h later as described [21]. The time taken for bleeding to stop was not significantly different between vehicle- and 14E11-treated mice. Values are mean $\pm$ SEM, $n=11-12$

Table 1

14E11 pretreatment improves cortical reperfusion after MCAO

Treatment	<i>n</i>	During MCAO, % baseline LDF	Post-MCAO, % baseline LDF
Vehicle	16	11.2±1.1	41.1±5.3
14E11	16	9.7±1.1	61.7±6.2 *

Transcranial laser Doppler flowmetry (LDF) over the parietal lobe was continuously recorded during MCAO and reperfusion (15 min after removal of filament). Values are mean±SEM

*
 $p < 0.05$ versus vehicle