Factor XIIa Inhibitor Recombinant Human Albumin Infestin-4 Abolishes Occlusive Arterial Thrombus Formation Without Affecting Bleeding

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Background—Blood coagulation is a tightly regulated process of sequentially activated serine proteases culminating in fibrin formation, which is critical for limiting posttraumatic blood loss but also may contribute to acute thrombotic diseases, most notably myocardial infarction and stroke. Recent studies with factor XII–deficient mice revealed that the factor XII–induced intrinsic coagulation pathway is essential for pathological thrombus formation but dispensable for hemostasis. Consequently, these findings led to the hypothesis that factor XII could be a promising pharmacological target for safe antithrombotic therapy.

Methods and Results—The complementary DNA of the previously described factor XIIa inhibitor Infestin-4, cloned from the midgut of *Triatoma infestans*, was fused to recombinant human albumin (rHA) and analyzed in vitro. The resulting protein rHA-Infestin-4 specifically inhibits factor XIIa and causes prolonged activated partial thromboplastin time in human, mouse, and rat plasma. To assess its inhibitory potency in vivo, mice and rats were injected with rHA-Infestin-4 and challenged in pathological thrombus formation models. In addition, bleeding assays were performed. rHA-Infestin-4 completely abolished occlusive arterial thrombus formation in mice and rats while leaving hemostasis fully intact. Furthermore, rHA-Infestin-4 was highly protective in a murine model of ischemic stroke.

Conclusion—These results identify rHA-Infestin-4 as a promising agent to achieve powerful protection from ischemic cardiovascular and cerebrovascular events without affecting hemostasis. (Circulation. 2010;121:1510-1517.)

Key Words: coagulation ■ factor XII ■ inhibitors ■ stroke ■ thrombosis

A t sites of vascular injury, both blood platelets and the plasma coagulation system are rapidly activated and act in concert to induce the formation of a fibrin- and platelet-rich clot. This process is essential for limiting excessive blood loss, but it can also cause complete vessel occlusion, resulting in myocardial and cerebral infarction, which are among the leading causes of mortality in industrialized countries.1

Clinical Perspective on p 1517

In the classic cascade or waterfall model, blood coagulation is described as a process that can be initiated by 2 distinct pathways, the extrinsic and the intrinsic pathway. Both cascades comprise a tightly regulated sequential activation of plasma serine proteases, culminating in the formation of thrombin, which in turn converts fibrinogen to fibrin.2,3 Clot formation through the extrinsic pathway occurs when tissue factor comes into contact with activated coagulation factor VII (FVIIa).4 According to the original assumption, the intrinsic pathway is initiated when coagulation factor XII (FXII) becomes activated on a charged surface,5 followed by successive activation of factor XI (FXI) and factor IX. This contact activation process serves as the basis of the activated partial thromboplastin time (aPTT), a method widely used to determine overall plasma coagulation in clinical practice. Although the relevance of the intrinsic pathway for blood coagulation in vitro is obvious, its importance for physiological clot formation has been elusive. A long-standing model suggested that fibrin formation is mainly, if not exclusively, mediated by the FVIIa–tissue factor pathway, whereas FXII-induced processes were considered not to be relevant for blood clotting. This hypothesis was based mainly on the observation that hereditary deficiency of FXII is not associated with bleeding, contrary to deficiencies of components of the extrinsic cascade such as FVII and tissue factor.6,7
This model was recently challenged by studies showing that FXII-deficient mice are profoundly protected in different models of arterial thrombosis and ischemic stroke and, like FXII-deficient humans, do not display any detectable alteration of hemostasis.8,9 These observations led to the hypothesis that inhibitors of FXII-dependent coagulation might represent powerful antithrombotic agents that do not induce an increased bleeding risk, the major clinical complication associated with current anticoagulative therapies, notably heparins and vitamin K antagonists.

One promising approach for the identification of new possible inhibitors of the plasma coagulation system is the analysis of substances produced by blood feeders. These insects have developed efficient mechanisms to overcome the host’s hemostatic barrier and to keep blood in a fluid state during acquisition and digestion. In 2002, Campos et al10 isolated a specific thrombin inhibitor, called Infestin, from the midgut of the hematophagus insect Triatoma infestans. Infestin belongs to the nonclassic Kazal-type serine protease inhibitor family and is composed of 2 nonclassic Kazal-type domains, although its gene encodes 4 domains, most probably because of unknown posttranscriptional mechanisms. Subsequent analysis of recombinant proteins expressing different nonclassic Kazal-type domains of the Infestin gene revealed that domains 1 to 2 show strong thrombin inhibition, whereas the fourth domain encodes a protein (Infestin-4) with strong inhibitory potential against activated FXII (FXIIa).11

In the present study, we show that Infestin-4 fused to recombinant human albumin (rHA) is a highly specific FXIIa inhibitor in human, mouse, and rat plasma that virtually abolished arterial thrombus formation in mice and rats and was highly protective in a murine model of ischemic stroke without altering hemostasis.

**Methods**

**Animals**

Experiments and animal studies were approved by the Bezirksregierung Unterfranken and the Regierungspräsidium Gießen. As genetically defined wild-type animals, NMRI mice, CD rats (Charles River, Sulzfeld, Germany), and C57Bl/6 mice (Harlan Winkelmann GmbH, Borchern, Germany) were used for experiments.

**Cloning of Albumin-Fused Infestin-4**

The Infestin-4 complementary DNA sequence11 was synthesized and extended with a coding sequence for a linker (Gly-Gly-Ser)3 in its 5′ position and inserted into BamHI and NotI sites of pRSETpuro3 (BD Biosciences, Heidelberg, Germany). Albumin complementary DNA was amplified by polymerase chain reaction with the forward primer 5′-GGGCTGACATGAAATGGGTACCCCTTTATITCCC-3′ and the reverse primer 5′-GGGGATCCGCCCCGACACGTTGACTTG-3′. The amplicon was digested with Nhel and BamHI and inserted into the Nhel/BamHI sites of the Infestin-4 vector.

The resulting vector, capable of expressing a fusion protein consisting of albumin-linker Infestin-4 (rHA-Infestin-4), was grown in Escherichia coli TOP10 (Invitrogen, Karlsruhe, Germany) and purified using standard protocols (Qiagen, Hilden, Germany). HEK-293 cells were transfected with Lipofectamine 2000 reagent (Invitrogen) and grown in serum-free medium (Invitrogen 293 Express) in the presence of 4 μg/mL puromycin. Transfected cell populations were grown in fermenters. Supernatant was harvested for purification of the produced fusion protein.

**Purification of (His)6-Tagged Infestin-4 and rHA-Infestin-4**

The (His)6-tagged Infestin-4 was purified by copper metal chelate chromatography on POROS MC 20. Fermentation supernatant was applied on a copper sulfate–loaded and phosphate–sodium chloride buffer (pH 7.7) – equilibrated column. (His)6-tagged Infestin-4 was subsequently eluted in an imidazole gradient.

rHA-Infestin-4 was purified by immune affinity chromatography. Fermentation supernatant was applied to an equilibrated antialbumin column. The product was eluted with a glycine buffer (pH 2.5).

**Pharmacokinetic Assay**

Mice were injected intravenously with 200 mg/kg rHA-Infestin-4 or 20 mg/kg (His)6-tagged Infestin-4. The dose level was selected to administer an approximately equimolar dose (2.7 μmol/kg) of both proteins. Blood samples were drawn retroorbitally under short-term anesthesia at different time points. The plasma level of Infestin-4 was assessed by a standard ELISA method.

**SDS-PAGE of Purified rHA-Infestin-4**

SDS-PAGE was performed with a Tris-glycine precast gel 8% to 16% according to the manufacturer’s (Invitrogen) standard application protocol.

**Quantification of aPTT, Prothrombin Time, and Chromogenic Assays**

aPTT and prothrombin time (PT) of standard human, mouse, and rat plasma were determined by standard methods (Siemens Healthcare, Eschborn, Germany). rHA-Infestin-4 was incubated with human FXIIa, FXIa, FXIIa, and thrombin (all Kordia, Leiden, the Netherlands) for 5 minutes at 37°C (pH 7.8). A specific chromogenic substrate (S-2303, S-2765, S-2238) for the respective protease was added, and the reaction was stopped by acetic acid. Absorption was measured at 405 nm and calculated against a standard curve. FXIIa and FXIa were also incubated with different concentrations of rHA-Infestin-4 and the macromolecular chromogenic substrate casein-resorufin, respectively, for 3 hours at 37°C. The reaction was stopped by trichloroacetic acid, and absorption was measured at 574 nm. Protease activity was calculated according to a standard curve.

**D-Dimer Concentration Measurements**

Pooled mouse plasma was incubated with vehicle or rHA-Infestin-4 for 2 minutes at 37°C. Clot formation was induced with Thromborel S (Dade-Behring, Marburg, Germany) according to the manufacturer’s instructions. After 1 hour, the D-dimer concentration in the supernatant was determined with an ELISA kit (Asserachrom, Roche, Basel, Switzerland).

**FeCl3-Induced Occlusive Thrombus Formation in Mesenteric Arterioles**

The 3- to 4-week-old mice were anesthetized with ketamine/xylazine (100:5 mg/kg; Parke-Davis, Berlin, Germany, and Bayer, Leverkusen, Germany). After a midline abdominal incision was made, 35- to 60-μm-diameter arterioles were exteriorized and visualized at ×10 magnification with an inverted microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany) equipped with a 100-W mercury lamp (HBO) and a CoolSNAP-EZ camera (Visitron Systems, Munich, Germany). Endothelial damage was induced by application of a 3-mm2 filter paper saturated with 20% FeCl3 at defined time points after injection of either rHA-Infestin-4 (200 mg/kg body weight) or PBS as control. Metavue software was used to record adhesion and thrombus formation of fluorescently labeled platelets (DyLight 488-conjugated anti-GPIbX Ig derivative) for 40 minutes or until complete occlusion of the vessel (blood flow stopped for >1 minute).

**Carotid Artery Thrombosis Model**

Mice or rats were anesthetized and the left carotid artery was exposed through a midline incision in the neck. Thrombus formation...
was induced by a filter paper saturated with 10% (mice) or 35% (rats) FeCl₃ and placed on the carotid artery for 3 minutes. Blood flow was monitored with an ultrasonic flow probe (Transonic System, Ithaca, NY) until complete vessel occlusion occurred or for 60 minutes. Animals were injected intravenously with PBS or rHA-Infestin-4 (186 mg/kg body weight for mice, 100 mg/kg body weight for rats) 10 minutes before injury.

Tail Bleeding Assay
Anesthetized mice or rats were injected with rHA-Infestin-4 (190 and 400 mg/kg body weight for mice and 100 mg/kg for rats) intravenously 10 minutes before tail tip cut. Tail bleeding assay was performed as described.⁸

Transient Occlusion Model of the Middle Cerebral Artery
Analyses were conducted according to the published recommendations for research in mechanism-driven basic stroke studies.¹² Transient middle cerebral artery occlusion (tMCAO) was induced under inhalation anesthesia with the intraluminal filament (6021PK10, Doccol Co, Redlands, Calif) technique.¹³ After 60 minutes, the filament was withdrawn to allow reperfusion. For determination of ischemic brain volume, mice were euthanized 24 hours after induction of tMCAO, and brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Steinheim, Germany). Brain infarct volume was calculated and corrected for edema as described. rHA-Infestin-4 (200 mg/kg body weight) was injected 10 minutes before tMCAO.

Neurological Testing
Twenty-four hours after tMCAO, neurological function was analyzed by 2 independent and blinded investigators. Global neurological status was scored according to Bederson et al¹⁴ (Bederson score). Motor function and coordination were graded with the grip test.¹⁵

Statistics
Statistical evaluation was performed with the Welch test. Results are given as mean±SD. Bederson score and grip test data were analyzed with the Mann-Whitney U test. A value of P<0.05 was considered statistically significant.

Results
Characterization of rHA-Infestin-4
The aim of our study was to develop a new antithrombotic agent that targets FXII and exhibits clinically suitable pharmacokinetics. Thus, the previously described FXIIa inhibitor Infestin-4, cloned from the hematophagus insect Triatoma infestans, was expressed in HEK 293 cells and extended by an (His)₆-tag for successful purification by metal chelate chromatography. After purification, SDS-PAGE analysis confirmed the isolation of the 7-kDa protein and indicated a purity of >90% (data not shown).

To assess the pharmacokinetic characteristics of (His)₆-Infestin-4, mice were injected intravenously with 20 mg/kg body weight (2.7 μmol/kg) of the protein, and Infestin-4 antigen concentration was determined. Unfortunately, these measurements revealed that (His)₆-Infestin-4 lead to a low recovery of only 8% of the protein in mice and a short half-life of 0.3 hour (Figure 1A), which ruled out the application of (His)₆-Infestin-4 in further studies using a reasonably efficient treatment schedule.

As described earlier, bioavailability enhancement of peptides can be achieved by fusion to albumin. Therefore, rHA-Infestin-4 fusion protein was expressed in HEK 293 cells and purified by immune affinity chromatography. SDS-PAGE analysis demonstrated a high recovery of 92% and a half-life of 4.6 hours after intravenous injection (Figure 1A).

rHA-Infestin-4 Specifically Binds to FXIIa and Prolongs aPTT in Human and Mouse Plasma
To assess the effect of rHA-Infestin-4 on the integrity of plasma coagulation in vitro, we determined the clinical parameters aPTT and PT in standard human plasma. rHA-Infestin-4 prolonged aPTT in a dose-dependent manner but did not affect PT (Figure 1C and 1D). Similar results were obtained with mouse plasma in vitro (data not shown) and ex vivo for up to 6 hours after treatment with 200 mg/kg rHA-Infestin-4 (Table 1). These data indicated a highly specific inhibition of the intrinsic coagulation pathway. To test this in more detail, we analyzed the specificity of rHA-Infestin-4 for different human serine proteases of the intrinsic (FXIIa and FXIa), the extrinsic (FVIIa), and the
Inhibitor Molar Ratio Inhibition, %
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FXIIa:inhibitor 1:1, FXIIa:inhibitor >90
C1 inhibitor 1:16, FXIIa:inhibitor 85
FXIIa:inhibitor 1:90, FXIIa:inhibitor 0
C1 inhibitor 1:90, FXIIa:inhibitor 60
FVIIa:inhibitor 1:100, FVIIa:inhibitor 0
FXa:inhibitor 1:100, FXa:inhibitor 8
rHA-Infestin-4 1:100, thrombin:inhibitor 0

FXIIa, FXIa, FVIIa, FXa, and thrombin inhibition of rHA-Infestin-4 was assessed in a chromogenic assay. The inhibitor was incubated with the respective protease at the indicated molar ratio in presence of a specific protease substrate. The resulting protease activity was determined at 405 nm. The percentage of protease inhibition was calculated according to a standard curve. Results were obtained from 4 independent experiments. C1 inhibitor (plasmatic FXII inhibitor, CSL Behring) served as control inhibitor.

Table 2. Protease Specificity of rHA-Infestin-4

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Molar Ratio</th>
<th>Inhibition, %</th>
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</thead>
<tbody>
<tr>
<td>rHA-Infestin-4</td>
<td>1:1, FXIIa:inhibitor</td>
<td>&gt;90</td>
</tr>
<tr>
<td>C1 inhibitor</td>
<td>1:16, FXIIa:inhibitor</td>
<td>85</td>
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<tr>
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<tr>
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<td>1:90, FXIIa:inhibitor</td>
<td>60</td>
</tr>
<tr>
<td>rHA-Infestin-4</td>
<td>1:100, FVIIa:inhibitor</td>
<td>0</td>
</tr>
<tr>
<td>rHA-Infestin-4</td>
<td>1:100, FXa:inhibitor</td>
<td>8</td>
</tr>
<tr>
<td>rHA-Infestin-4</td>
<td>1:100, thrombin:inhibitor</td>
<td>0</td>
</tr>
</tbody>
</table>

FXIIa, FVIIa, FXa, and thrombin inhibition of rHA-Infestin-4 was assessed in a chromogenic assay. The inhibitor was incubated with the respective protease at the indicated molar ratio in presence of a specific protease substrate. The resulting protease activity was determined at 405 nm. The percentage of protease inhibition was calculated according to a standard curve. Results were obtained from 4 independent experiments. C1 inhibitor (plasmatic FXII inhibitor, CSL Behring) served as control inhibitor.

Table 1. Blood Coagulation in Mice Injected With rHA-Infestin-4 200 mg/kg

<table>
<thead>
<tr>
<th>Time, min</th>
<th>aPTT, s</th>
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<tr>
<td>0</td>
<td>20.9±3.8</td>
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<tr>
<td>5</td>
<td>61.4±13.2</td>
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<tr>
<td>15</td>
<td>46.2±0</td>
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<tr>
<td>240</td>
<td>59.5±15.6</td>
</tr>
<tr>
<td>360</td>
<td>48.2±8.3</td>
</tr>
</tbody>
</table>

aPTT measured at the indicated time points after rHA-Infestin-4 injection is shown. Values are mean±SD of 5 individual mice per time point.

common (FXa and thrombin) pathway using chromogenic assays. After incubation of rHA-Infestin-4 with the respective protease in the presence of a specific chromogenic substrate, the remaining protease activity was determined from absorption measurements at 405 nm. Interestingly, a 1:1 molar ratio of FXIIa and rHA-Infestin-4 resulted in efficient FXIIa inhibition of >90%, whereas even a molar ratio of 1:90 of FXIa and rHA-Infestin-4 showed no effect on FXIa activity (Table 2). Similar results were obtained when a more physiologically structured macromolecule (casein-resorufin) was used as chromogenic substrate. Under these conditions, a 1:1 ratio of FXIIa and rHA-Infestin-4 also led to a remarkable protease inhibition of ~85%, whereas it did not affect FXIa activity. Furthermore, rHA-Infestin-4 did not inhibit FVIIa or thrombin activity and only mildly influenced (8% inhibition) FXa activity when tested in a 100-fold molar excess (Table 2). These results clearly confirmed that rHA-Infestin-4 is a specific inhibitor of FXIIa.

To test a possible effect of rHA-Infestin-4 on fibrinolysis, pooled mouse plasma was incubated with either vehicle or different concentrations of rHA-Infestin-4 before clotting was triggered by thromboplastin (extrinsic pathway). The degree of subsequent lysis was determined as D-dimer concentration assessed by an ELISA system 1 hour after starting the reaction. An rHA-Infestin-4 concentration of 0.1 mg/mL had no influence on D-dimer concentration, whereas 1 mg/mL, which approximately mimics the inhibitor concentration used for the following in vivo experiments, moderately but significantly decreased D-dimer concentration compared with control by 23% (158.8±16.6 ng/mL for rHA-Infestin-4, 205.5±21.0 ng/mL for control; P=0.04). However, this reduction never exceeded 38% even when a 10-fold higher concentration of rHA-Infestin-4 (10 mg/mL) was used. These results showed that rHA-Infestin-4 exerts a moderate inhibitory effect on fibrinolysis.

**Prevention of Arterial Thrombosis in rHA-Infestin-4–Treated Mice**

At sites of atherosclerotic plaque rupture, platelets and the plasma coagulation system become activated and lead to arterial thrombosis as a result of the exposure of subendothelial extracellular matrix constituents. To determine the inhibitory effect of rHA-Infestin-4 on thrombus formation in vivo, we used an occlusive arterial thrombosis model in which injury was induced on small mesenteric arterioles by topical application of 20% FeCl3. FeCl3 destroys the vascular endothelium through the production of free radicals, which in turn leads to platelet adhesion to the exposed subendothelial extracellular matrix, followed by aggregation and occlusive thrombus formation. On the basis of results of previous dose-finding studies, 1 consistent and potent concentration of rHA-Infestin-4 was chosen for the in vivo experiments (200 mg/kg body weight). It is important to note that only ~10% of the molecular mass of rHA-Infestin-4 corresponds to the active inhibitor, Infestin-4 (∼7 kDa), and albumin (∼66 kDa) accounts for the rest. To additionally assess the time course of the effect of rHA-Infestin-4, arterioles of mice treated intravenously with the recombinant fusion protein or PBS as control were injured at different time points after the treatment and monitored by intravital fluorescence microscopy. In the first group of mice, vessels were subjected to injury 10 minutes after the injection. In all animals, platelets rapidly adhered to the injured vessel wall. Whereas in control mice, formation of thrombi and finally full occlusion of the vessel occurred in all cases (mean time to occlusion, 16.9±2.2 minutes), only platelet adhesion was detectable in all (7 of 7) of the rHA-Infestin-4–injected mice throughout the observation period of 40 minutes (Figure 2A and 2C and Movies I and II in the online-only Data Supplement). A similar inhibitory potency of the fusion protein was detectable when injury was induced 60 minutes after injection (data not shown). When the lesion was induced in vessels ~105 minutes after the application of rHA-Infestin-4, small platelet aggregates formed, but this formation was delayed in 71% of the arterioles (20.0±3.7 minutes for rHA-Infestin-4–treated mice and 6.3±0.7 minutes for control; P<0.001; Figure 2B).

Furthermore, all formed thrombi were unstable and in no case reached the size necessary for vessel occlusion. This was due mainly to the detachment of single platelets from the surface of the thrombi. Similar findings were obtained when the mice were challenged in a second in vivo model in which the carotid artery was injured and blood flow was monitored by an ultrasonic flow probe to assess thrombus formation. In all control animals (10 of 10), irreversible vessel occlusion...
deficient mice, rHA-Infestin-4–injected animals showed no avoidable. However, similar to the observations in FXII-vessels, small lesions of the circumjacent tissue were un-
inhibitor of arterial thrombus formation.

During the operations to gain access to the respective vessels, small lesions of the circumjacent tissue were unavoidable. However, similar to the observations in FXII-deficient mice, rHA-Infestin-4–injected animals showed no alterations in surgery-caused bleeding compared with controls. To corroborate this observation, we studied the effect of rHA-Infestin-4 on hemostasis in a tail bleeding model (Figure 3). All rHA-Infestin-4–treated mice (10 of 10) were able to arrest bleeding within the same time frame as the control animals, even when injected with an extremely high dose (400 mg/kg body weight) (114±77 seconds for mice treated with 190 mg/kg rHA-Infestin-4, P=0.38; 115±35 seconds for mice treated with 400 mg/kg rHA-Infestin-4, P=0.34; 153±146 seconds for control). These results indicate that rHA-Infestin-4 treatment does not interfere with normal hemostasis.

rHA-Infestin-4 Blocks Arterial Thrombus Formation in Rats

To exclude that the observed inhibitory potency of rHA-Infestin-4 was restricted to mice, we analyzed the effect of the inhibitor on blood coagulation in rats. After intravenous injection of rHA-Infestin-4 (100 mg/kg body weight), aPTT was prolonged in these animals compared with controls (38.7±10.6 seconds for rHA-Infestin-4–injected rats, 24.3±2.4 seconds for control; P=0.003), whereas PT was not altered (10.0±0.7 seconds for rHA-Infestin-4–injected rats, 9.9±0.8 seconds for control; P=0.74). To test the effect of rHA-Infestin-4 on occlusive thrombus formation, the animals were subjected to a carotid artery thrombosis model. Application of 35% FeCl₃ induced continuous decrease of blood flow in 8 of 9 control rats, resulting in full and stable vessel occlusion in 7 of 9 animals within 32 minutes (mean time to occlusion, 15.6±7.7 minutes). In contrast, blood flow slightly and only transiently decreased after injury in only 2 rHA-Infestin-4–treated rats, and all vessels (10 of 10) remained open during the whole observation period of 60 minutes (Figure 4A).

Although rHA-Infestin-4–treated rats displayed profound protection from arterial thrombosis, they had, in line with observations obtained from mice, normal bleeding times (298±165 seconds for rHA-Infestin-4–injected rats, 45±300 seconds for control; P=0.35) as assessed in a tail bleeding assay (Figure 4B). Together, these results revealed that rHA-Infestin-4 has a comparable antithrombotic effect in rats and mice.

**Figure 2.** rHA-Infestin-4 blocks arterial thrombus formation in mice. Thrombus formation after FeCl₃-induced injury of mesenteric arterioles was monitored with intravital fluorescence microscopy. Time to first thrombus formation >10 µm and time to full vessel occlusion after injury 10 minutes (A) and 105 minutes (B) before intravenous injection of rHA-Infestin-4 (n=7) and PBS (n=7) are illustrated. Each symbol represents 1 arteriole. C, Representative images taken at the indicated time points. Bar=50 µm. The asterisk indicates complete vessel occlusion. D, Time to occlusion after FeCl₃-induced thrombosis in the carotid artery (n=10). Each symbol represents 1 individual. Ctrl indicates control; Inf-4=rHA-Infestin-4 fusion protein.

**Figure 3.** Tail bleeding assay of control and rHA-Infestin-4–treated mice. The tail tip was removed 10 minutes after intravenous injection of PBS (n=15) or rHA-Infestin-4 (n=10). Each symbol represents 1 individual. Ctrl indicates control; Inf-4, rHA-Infestin-4 fusion protein.

**Figure 4.** rHA-Infestin-4 protects rats from thrombosis without influencing bleeding. A, FeCl₃-induced thrombus formation in the carotid artery. Blood flow was monitored with an ultrasonic flow probe. Time to full vessel occlusion is shown (n=9 for control, n=10 for rHA-Infestin-4–injected rats). B, Tail bleeding times. PBS (n=5) and rHA-Infestin-4 (100 mg/kg body weight) (n=5) were injected 10 minutes before respective injury. Each symbol represents 1 individual. Ctrl indicates control; Inf-4=rHA-Infestin-4 fusion protein.


rHA-Infestin-4 Does Not Reduce Residual Thrombus Formation in FXII$^{-/-}$ Mice

Previous studies on FXII-deficient mice revealed that they are markedly protected from arterial thrombosis, but the formation of microaggregates and unstable thrombi was still observed after endothelial damage in those animals. In contrast, FXIIa inhibition in wild-type mice by rHA-Infestin-4 resulted in completely abolished thrombus formation, raising the question of whether the inhibitor, in addition to FXIIa, interfered with other components of the coagulation system. To test this directly, we compared thrombus formation in the mesenteric arteriole thrombosis model between wild-type and FXII$^{-/-}$ mice, in each case untreated or treated with 200 mg/kg rHA-Infestin-4. In all control animals (6 of 6), FeCl$_3$-induced injury led to initial thrombus formation within 7.3±0.9 minutes, resulting in complete vessel occlusion during the 40-minute observation period (mean time to occlusion, 17.7±3.0 minutes). As previously reported, the beginning of thrombus formation in FXII$^{-/-}$ mice was delayed (18.4±9.3 minutes; P=0.02) compared with control mice, and all formed thrombi were consistently unstable, degraded, and frequently detached from the vessel wall and consequently never occluded the vessel. Remarkably, virtually the same result was obtained when FXII$^{-/-}$ mice were treated with rHA-Infestin-4 10 minutes before injury. In these animals, platelets rapidly adhered to the vessel wall and formed little thrombi within a time frame similar to that of untreated FXII$^{-/-}$ mice (18.1±3.0 minutes; P=0.95). These thrombi were similarly unstable as those observed in untreated FXII$^{-/-}$ mice and never reached vessel occlusion (Figure 5A and 5B). Importantly, in wild-type mice injected with rHA-Infestin-4, no aggregates formed, and the vessel did not occlude during the observation period (data not shown). These observations demonstrated that rHA-Infestin-4 had no detectable effect on thrombus formation in FXII-deficient mice.

rHA-Infestin-4 Protects Mice From Ischemic Brain Infarction

Ischemic brain infarction is a thromboembolic disease that is highly associated with death and severe disability. As recently shown in knockout mice, FXII is an important player in the development of experimental ischemic stroke, making it a promising target for effective and safe therapy. To test this hypothesis directly, we assessed the protective potency of rHA-Infestin-4 in a murine model of ischemic stroke. To initiate transient cerebral ischemia, a thread was advanced through the carotid artery into the MCA and allowed to remain for 1 hour (tMCAO), reducing regional cerebral flow by >90%. In rHA-Infestin-4–treated animals, infarct volumes 24 hours after reperfusion, as assessed by TTC staining, were reduced to <17% of the infarct volumes in untreated animals (69.5±33.7 versus 11.2±5.3 mm$^3$; P<0.001; Figure 6A). Importantly, the observed protective effect of rHA-Infestin-4 was functionally relevant because the Bederson score, which assesses global neurological function (P<0.001;
inhibits human FXIIa (Table 2). The antithrombotic activity of rHA-Infestin-4 was also evident in a transient cerebral ischemia model in which mice displayed a profound protection from neurological damage (Figure 6A through 6C). Furthermore, rHA-Infestin-4 moderately inhibited fibrinolysis. Although the underlying mechanism is not clear at present, this inhibition does not explain the effective antithrombotic potency of the inhibitor. Together, these results indicate that rHA-Infestin-4 might be effective in the prophylaxis and treatment of thromboembolic diseases.

Unexpectedly, the marked protection of rHA-Infestin-4 treated mice from arterial thrombosis and ischemic stroke exceeded the defects previously found in FXII-deficient mice.8,9 Notably, we could show that rHA-Infestin-4 did not abrogate the residual thrombotic activity in FXII-deficient mice, which excludes an additional albeit subordinated influence of rHA-Infestin-4 on other proteins. From these findings, rather, we may hypothesize that the genetic loss of FXII in the mutant mice leads to compensatory upregulation of alternative mechanisms that account for the residual thrombotic activity (Figure 5A and 5B). Remarkably, the temporary and efficient inhibition of FXIIa in vivo was not associated with excessive bleeding (Figures 3 and 4B), even under conditions of major surgery, in accordance with observations in FXII-deficient mice and humans.8,18 These findings support the intriguing hypothesis that hemostasis and thrombosis are 2 mechanistically different processes8,16 and establish rHA-Infestin-4 as a promising candidate molecule for highly effective and safe antithrombotic therapy. Such a safe therapy might be particularly advantageous in the treatment of acute stroke because the conventional therapy of preventing lesion progression and recurrent thromboembolism with platelet aggregation inhibitors and anticoagulants is inherently associated with an increased risk of intracranial hemorrhages.19–21 Moreover, transient blockade of FXIIa activity may help to bridge the risk of vessel reocclusion after thrombolytic therapy. Lack of a sufficiently effective therapy for acute ischemic stroke makes it one of the leading causes of death and disability worldwide.22,23

Discussion

Our results clearly show that rHA-Infestin-4 specifically inhibits FXIIa in vitro and in vivo regardless of the analyzed species and induces potent protection of mice and rats from thrombotic events without any detectable effects on hemostasis. Immediately after injection, rHA-Infestin-4 abolished platelet aggregation and thrombus formation at sites of vascular injury (Figure 2A and 2C and Movie II in the online-only Data Supplement), demonstrating rapid and highly efficient inhibition of FXIIa. This strong inhibitory effect was reversible and gradually declined when rHA-Infestin-4 was metabolized and cleared from the circulation as small but unstable thrombi (diameter, \( \approx 10 \) \( \mu \)m) formed on vessel injury when rHA-Infestin-4 was administered \( \approx100 \) minutes before injury. This effect of rHA-Infestin-4 on arterial thrombus formation was confirmed in 2 different arterial beds, small mesenteric arterioles, and the carotid artery (Figure 2A through 2D) and found to be similar in mice and rats (Figure 4A). This indicates that the inhibitor might be effective in different species, including humans, because it very potently

**Figure 6.** rHA-Infestin-4–injected mice are protected from ischemic stroke. Formation of cerebral ischemia and consequential neurological defects was investigated in a murine stroke model. A, Brain infarct volumes in controls (n=13) and rHA-Infestin-4–treated mice (n=10) presented as mean±SD. **P<0.001; Figure 6C), were both significantly better in rHA-Infestin-4–treated mice compared with control. These findings strongly indicate that inhibition of FXIIa by rHA-Infestin-4 could be a promising strategy to prevent ischemic brain infarction.

Conclusions

We have demonstrated that the newly developed selective FXIIa inhibitor rHA-Infestin-4 is highly active in human plasma and profoundly protects mice and rats from pathological thrombus formation while not affecting hemostasis. These findings indicate that rHA-Infestin-4 might be a powerful yet safe agent for
the prevention and treatment of acute ischemic cardiovascular and cerebrovascular events in humans.

**Source of Funding**
This work was supported by the Deutsche Forschungsgemeinschaft (SFB 688, TPA3, B1).

**Disclosures**
Drs Schmidbauer and Dickneite are employees of CSL-Behring GmbH, Marburg, Germany. The other authors report no conflicts.

**References**

**CLINICAL PERSPECTIVE**
Atrial fibrillation is a major cause of thromboembolism, inducing ischemic damage to numerous organs, including the brain. Anticoagulation with warfarin targets the synthesis of coagulation factors II, VII, IX, and X and provides an efficient therapy against atrial fibrillation–induced thromboembolism, but only at the expense of an increase in bleeding complications, which can be life-threatening. In contrast, recent studies in mice lacking coagulation factor XII, which was previously thought to be irrelevant for blood clotting, revealed that this factor is essential for occlusive arterial thrombus formation but dispensable for normal hemostasis. These results led to the proposal that inhibition of factor XII or factor XIa might be a suitable therapeutic strategy to prevent or treat acute ischemic cardiovascular and cerebrovascular diseases without bleeding complications. In the present study, we report the generation and antithrombotic potential of the highly specific factor XIIa inhibitor, recombinant human albumin (rHA)-Infestin-4. Mice or rats treated with rHA-Infestin-4 were profoundly protected from arterial thrombus formation in different models, whereas tail bleeding times were unaffected even when extremely high doses of the inhibitor were injected. In addition, rHA-Infestin-4–treated mice were protected from neuronal damage in a model of focal cerebral ischemia without an increased incidence of intracranial hemorrhages. These results indicate that specific factor XIIa inhibitors such as rHA-Infestin-4 may help to control and limit thromboembolic events with a better safety profile than coumarins or heparins.
Factor XIIa Inhibitor Recombinant Human Albumin Infestin-4 Abolishes Occlusive Arterial Thrombus Formation Without Affecting Bleeding

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Circulation. 2010;121:1510-1517; originally published online March 22, 2010; doi: 10.1161/CIRCULATIONAHA.109.924761

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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