Selective Factor Xa Inhibition by Recombinant Antistasin Prevents Vascular Graft Thrombosis in Baboons

Linda W. Schaffer, John T. Davidson, George P. Vlasuk, Christopher T. Dunwiddie, and Peter K.S. Siegl

A baboon model of high-shear, platelet-dependent vascular graft thrombosis was used to assess the antithrombotic effect of recombinant antistasin (rATS), a 119-amino acid protein with selective, subnanomolar inhibitory potency against coagulation factor Xa. In this model, a Dacron vascular graft segment of a femoral arteriovenous (AV) shunt provided the thrombogenic stimulus. Antithrombotic efficacy of rATS was assessed by continuous monitoring of $^{111}$In-labeled platelet and $^{125}$I-labeled fibrin(ogen) deposition onto the graft surface and blood flow through the vascular shunt. Systemic intravenous administration of rATS (2 or 4 $\mu$g/kg · min$^{-1}$) dose dependently decreased both platelet and fibrin(ogen) deposition onto the graft. Vascular graft thrombus formation was completely inhibited at a systemic dose of rATS of 4 $\mu$g/kg · min$^{-1}$. None of the AV shunts in animals receiving rATS at either dose occluded, and blood flow was maintained at 81 ± 4% (2 $\mu$g/kg · min$^{-1}$ rATS) or 96 ± 3% (4 $\mu$g/kg · min$^{-1}$ rATS) of basal flow. Systemic fibrinopeptide A elevations in response to exposure to the Dacron graft segment were completely suppressed by both doses of rATS. The ex vivo activated partial thromboplastin times were extended to greater than 150 seconds during infusion of both doses of rATS; however, even at fully antithrombotic doses, template bleeding times were not significantly increased. Thus, in this baboon model, rATS is a potent antithrombotic agent that inhibits both platelet and fibrin(ogen) deposition onto a Dacron vascular graft segment. Furthermore, these results demonstrate that selective inhibition of coagulation factor Xa by rATS can completely prevent vascular graft thrombus formation without significantly compromising primary hemostasis as measured by template bleeding time.

KEY WORDS • vascular graft thrombosis • factor Xa inhibition • antistasin

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Received November 4, 1991; revision accepted April 23, 1992.

Disruption of the delicate balance between the procoagulant and anticoagulant activities of the blood constituents and the vessel wall leads to occlusive thrombus formation. After damage to the subendothelium, arterial thrombosis is initiated by localized adhesion, degranulation, and aggregation of platelets.1-4 During this process, the coagulation cascade is also activated, with the subsequent generation of thrombin. Thrombin has been implicated as the primary mediator of platelet-dependent arterial thrombosis in a variety of experimental models, presumably by acting as a potent agonist for platelet aggregation. Furthermore, thrombin contributes to the growth and stabilization of a thrombus by catalyzing the formation of fibrin and by activating factor XIII, which causes fibrin cross-linking. Thrombin can accelerate its own formation and potentiate the aforementioned reactions5-9 through a positive-feedback mechanism involving the activation of coagulation factors V and VIII. Consequently, the evaluation of thrombin inhibitors as potential therapeutic agents for the treatment of arterial thrombotic disorders has been aggressively pursued.

The poor efficacy of heparin in animal models of platelet-rich arterial thrombosis has prompted the development of small, direct antithrombin III (ATIII)-independent and highly selective thrombin inhibitors.5-7,9,12 The improved antithrombotic efficacy of these molecules over that of heparin confirms the pivotal role of thrombin in arterial thrombosis. The antithrombotic benefits achieved with direct thrombin inhibitors, however, typically result in significant alterations in hemostatic function, as assessed by increased template bleeding times as well as elevations in activated partial thromboplastin time (APTT).

Recently, plasma and recombinant DNA–derived forms of activated protein C (APC) have been shown to inhibit platelet deposition in a baboon model of arterial thrombosis without an associated elevation in template bleeding time.13,14 These results suggest that decreasing the rate of thrombin formation by APC-mediated inactivation of the nonenzymatic cofactors Va and VIIIa may result in a more favorable antithrombotic/antihemostatic profile compared with direct inhibition of thrombin.

Specific inhibition of coagulation factor Xa (FXa) in the prothrombinase complex represents another approach to curtailing thrombin formation. We have re-
ently shown that tick anticoagulant peptide, a specific and potent inhibitor of blood coagulation FXa, can fully inhibit thrombus formation in this baboon model.15 In contrast, we and others have shown that thrombus formation in this model is resistant to heparin and aspirin.15–17

Antistasin is a 119–amino acid protein originally isolated from the Mexican leech Haementeria officinalis.18,19 Antistasin exhibits potent anticoagulant properties because of the stoichiometric and highly selective inhibition of FXa.20 In this study, we used recombinant DNA–derived antistasin (rATS) as a tool to further investigate the antithrombotic and antihemostatic effects of specific FXa inhibition in a baboon model of platelet-dependent, heparin-resistant vascular graft thrombosis.

Methods

Test Compounds

Purified rATS was prepared as described from cultures of baculovirus-infected Sf9 cells.20 The preparation was >98% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, reverse-phase high-performance liquid chromatography, and amino acid composition analysis. All experiments were completed by using a single lot of rATS.

Experimental Protocol

Ten normal male baboons (Papio anubis) weighing between 12 and 19.7 kg were used in this study. They were fasted overnight, sedated with ketamine hydrochloride (10 mg/kg i.m.) and acepromazine maleate (0.1 mg/kg i.m.), intubated, and maintained on 2% isoflurane (Anaquest, Madison, Wis.) for the duration of the experiment. A Silastic femoral arteriovenous (AV) shunt was aseptically placed at least 2 hours before the study commenced. A 5-cm Dacron vascular graft segment (Bard Cardiosurgery, Billerica, Mass.) was inserted into the shunt to provide the thrombogenic stimulus. The shunt and vascular graft were prepared as described by Hanson et al.21

Autologous platelets were labeled with 111In-oxine (Amersham). Briefly, the platelets were isolated from whole blood by differential centrifugation. The platelet-poor plasma was decanted and the platelet pellet resuspended in 0.9% sterile saline. 111In-oxine was added to the platelet suspension and incubated for 5 minutes at 37°C. Free 111In was removed by gentle washing with saline.22 The labeled platelets were then resuspended in autologous plasma and injected intravenously 1 hour before insertion of the first Dacron graft. Labeling efficiency was >90% in all studies. Deposition of 111In-labeled fibrinogen onto the graft segment was determined by using the 35-keV photopeak of the 125I radionuclide and correcting for the 111In photopeak spillover in each acquisition. The fold increase in 111In-labeled fibrinogen deposition over baseline was calculated by dividing the subsequent 125I activity level by the baseline 125I activity at each time point. Baseline values were determined by using a 1-minute acquisition of 125I activity in a blood volume–matched segment of the Silastic AV shunt before insertion of the Dacron graft.

Blood flow through the AV shunt was monitored continuously during each experiment with a Doppler ultrasonic flowmeter (Baylor College of Medicine, Houston, Tex.) and a transducer probe positioned on the Silastic tubing of the shunt. Flow was displayed on a Hewlett-Packard recorder (Chelmsford, Mass.). Initial blood flow through the shunt ranged from 150 to 210 ml/min. Blood flow was reported as the percentage of baseline flow.

The baboons were randomized to the following treatment groups: saline control (n=4); rATS (2.0 μg/kg·min⁻¹, n=4); or rATS (4.0 μg/kg·min⁻¹, n=4). The test compounds were administered systemically through a cephalic vein catheter. In each study, two Dacron vascular grafts were sequentially inserted into the AV shunt of each baboon. In all experiments, saline was infused during exposure to the first graft; thus, the thrombotic response to the first graft segment served as an internal control for each baboon. After occlusion of the initial Dacron graft, the segment was removed and the AV shunt reestablished. The appropriate therapy corresponding to each treatment group was infused for 1 hour before and during the 60-minute exposure to the second graft. After occlusion or 60 minutes after insertion of the second graft, the Dacron graft did not occlude, the Silastic shunt was removed and the baboon was allowed to recover. Each baboon received only one treatment. All procedures were reviewed and approved by the Institutional Committee for the Care and Use of Laboratory Animals and complied with federal regulations.

Laboratory Studies

Whole-blood platelet, red, and white cell counts were determined with a Bakers system 9000 counter (Baker, Allentown, Pa.) with EDTA-anticoagulated blood. Ex vivo aggregation in response to ADP (20 μM) and collagen (10 μg/ml) was studied by using platelet-rich plasma adjusted to 2.5×10⁸ platelets/ml with timed-matched platelet-poor plasma. Responses were studied with an aggregometer and chart recorder (Chronolog, Havertown, Pa.). Fibrinogen concentrations and APTTs were determined by using a Coag-A-Mate XC (General Diagnostics, Durham, N.C.) and standard reagents (General Diagnostics). Fibrinopeptide A (FPA) values were determined by radioimmunoassay (ByK-Sangtec Diagnostica, Dietzenbach, FRG). To validate the cross-reactivity and sensitivity of the FPA
radioimmunoassay between human and baboon samples, FPA levels in plasma and serum samples from humans and baboons were determined. Plasma FPA levels were not different between human and baboon samples, indicating sensitivity of the kit to detect low FPA levels in both species. Serum samples from baboons and humans were diluted, and FPA levels were not different between the two species at similar fibrinogen concentrations. Template bleeding times were performed by using a Simplate device (Organon Teknika, Durham, N.C.) on the shaved volar surface of the forearm. Briefly, the shaved medial aspect of the forearm distal to the antecubital fossa was cleansed with alcohol and permitted to air dry for 30 seconds. A sphygmomanometer was positioned above the antecubital fossa, and the cuff was inflated to 40 mm Hg. After 30 seconds, the Simplate device was placed on the forearm about 5–10 cm distal to the antecubital fossa with the blade parallel to the antecubital crease. An incision was made by depressing the trigger, and the timer was simultaneously started. The flow of blood was carefully blotted at 30-second intervals until blood no longer stained the paper. Bleeding time was reported to the nearest 30 seconds.

Statistical Analysis
Data shown represent mean±SEM. The effect of rATS or saline on 111In-labeled platelet deposition and 125I-labeled fibrin(ogen) deposition was studied by using the fold increase from baseline. The fold increase of 111In activity or 125I activity was calculated by dividing subsequent 111In or 125I activity by the baseline 111In or 125I activity, respectively, for each time point. The effect of rATS on blood flow was determined by analyzing the absolute change. The statistical analyses were performed on changes in response during treatment infusion (graft 2) versus saline infusion (graft 1) in each animal with a repeated-measures analysis of variance. The significance of differences from the saline response at each time point was determined by using Dunnett’s test (one sided, p<0.05). Dose-related responses to rATS were determined by using linear contrasts of the time-response means. A two-sided paired Student’s t test was used to study differences between graft 1 and graft 2 on the following parameters: APTT, template bleeding time, thrombus weight, and FPA values.

Results
In each experiment, the hemostatic and thrombotic consequences due to the sequential placement of two Dacron graft segments were evaluated in each animal. The first graft segment, which served as the control, was studied during infusion of saline in all baboons. The response to the second Dacron segment, inserted into the AV shunt 60 minutes after starting the appropriate treatment (saline, or rATS infused at 2 or 4 µg/kg·min⁻¹) was compared with the response to the initial, saline control graft.

Effect of rATS on Thrombogenesis in Anesthetized Baboons
In saline control studies (n=8), 111In-labeled platelet deposition increased by 13.6±2.3-fold over the basal value (n=8). Simultaneously, 125I-labeled fibrin(ogen) deposition increased by only 2.0±0.3-fold over the basal value (n=8), reflecting the platelet-rich composition of the thrombus formed on the Dacron graft. This amount of platelets and fibrin(ogen) was accompanied by complete occlusion of the graft segment in all saline-treated animals.

As shown in Figure 1, administration of rATS resulted in a significant (p<0.05) dose-dependent decrease in platelet deposition onto the Dacron graft segment. 111In-labeled platelet deposition after 60 minutes of exposure to the Dacron graft increased over the basal value by 6.4±2.1-fold (rATS 2.0 µg/kg·min⁻¹, n=4) or 1.9±0.3-fold (rATS 4.0 µg/kg·min⁻¹, n=4) compared with 13.6±2.3-fold in saline-treated control grafts (n=8). Although the fibrin(ogen) content of the thrombus formed in this model was not extreme, rATS significantly (p<0.05) and dose dependently decreased 125I-labeled fibrin(ogen) deposition onto the Dacron surface (Figure 2). The deposition of 125I-labeled fibrin(ogen) maximally increased over the basal value by 1.3±0.1-fold during infusion of 2.0 µg/kg·min⁻¹ rATS (n=4) and by 1.1±0.0-fold during infusion of 4.0 µg/kg·min⁻¹ rATS (n=4) compared with an increase of 2.0±0.3-fold (n=8) during infusion of saline (Figure 2).

As expected from the effect of rATS on platelet and fibrin(ogen) deposition, rATS significantly (p=0.001) decreased thrombus weight during infusion of both doses of rATS (Figure 3). Thrombus weight after 60 minutes of exposure to the Dacron segment decreased from 769.8±21.2 mg (graft 1, saline control, n=4) to 214.6±12.7 mg (graft 2, rATS 4.0 µg/kg·min⁻¹, n=4) and from 771.5±30.5 mg (graft 1, saline control, n=4) to 328.6±36.9 mg (graft 2, rATS 2.0 µg/kg·min⁻¹, n=4). The poststudy weight of the Dacron graft segment after treatment with rATS at a dose of 4 µg/kg·min⁻¹ was equivalent to that of a blood-saturated graft.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Line plot showing effect of 2.0 µg/kg·min⁻¹ rATS (○, n=4), 4.0 µg/kg·min⁻¹ rATS (△, n=4), or saline (○, n=8) on ¹¹¹In-labeled platelet deposition during exposure to the Dacron graft segment. Values represent mean±SEM of the fold increase in ¹¹¹In activity over basal activity. rATS, recombinant antistasin.
Figure 2. Line plot showing effect of 2.0 µg/kg·min⁻¹ rATS (●, n=4), 4.0 µg/kg·min⁻¹ rATS (▲, n=4), or saline (○, n=8) on ¹²⁵I-labeled fibrinogen deposition during exposure to the Dacron graft segment. Values represent mean±SEM of the fold increase in ¹²⁵I activity over basal activity. rATS, recombinant antistasin.

(201.9±18.3 mg), confirming the lack of platelet and fibrinogen deposition at this dose.

All saline-treated baboons exhibited a progressive decline in blood flow through the AV shunt, which ultimately reached the point where there was no measurable flow, reflecting complete occlusion of the Dacron graft.

In contrast, none of the AV shunts in baboons treated with either dose of rATS became occluded in this study. Complete patency of the Dacron graft was illustrated by maintenance of blood flow at 81±4% or 96±3% of basal flow during the entire 60-minute infusion of 2.0 or 4.0 µg/kg·min⁻¹ rATS, respectively (Figure 4).

Effect of rATS on Hemostatic Parameters in Anesthetized Baboons

Insertion of the first Dacron vascular graft segment during infusion of saline resulted in a significant (p<0.05) increase in circulating FPA levels (Table 1) in all baboons studied. In contrast to the saline treatment group in which FPA levels increased significantly (p<0.05) during exposure to both sequentially placed graft segments, infusion of rATS at either dose completely prevented the systemic FPA elevation during exposure to the second graft.

Administration of rATS significantly (p<0.05) prolonged the APTT from 29.8±1.5 to 124±16.5 seconds in...
the lower-dose experiments and from 27.2±0.7 to >150 seconds in the higher-dose experiments after 60 minutes of infusion. Saline infusion had no effect on the ex vivo clotting time.

Administration of rATS had no significant (p<0.05) effect on template bleeding times in this study. Additionally, rATS administration did not affect platelet count, fibrinogen concentration, hematocrit, or red and white cell counts in this study. Ex vivo aggregation in response to ADP or collagen was not inhibited because of the infusion of rATS.

Discussion

Antistasin is a cysteine-rich, 119-amino acid peptide originally isolated from the Mexican leech Haementeria officinalis.19 Antistasin is an extremely potent and selective inhibitor of blood coagulation FXa.18 A recombinant version of antistasin (rATS) produced by an insect cell-baculovirus expression system has previously been shown to exhibit potent in vivo antithrombotic properties in a rabbit model of venous thrombosis25 and in a rhesus monkey model of thromboplastin-induced, mild disseminated intravascular coagulation.20 In the present study, we used rATS as a tool to evaluate the antithrombotic and antithrombotic effects of specific FXa inhibition in a well-characterized baboon model of high-shear, platelet-dependent thrombus formation.5-10,13,14,21,26-29 Because the experiments in this study were completed using anesthetized baboons, it should be mentioned that anesthesia may enhance antithrombotic effects because the anesthetic or the resultant decrease in blood pressure or flow through the shunt may alter the extent or rate of platelet deposition. Nonetheless, heparin has been evaluated in this model by us19 and others who have used unanesthetized baboons8,17 and has been shown to be ineffective in preventing platelet deposition and thrombus formation at doses that dramatically elevate the APTT despite differences in the use of anesthesia.

The results of the present study establish that specific FXa inhibition by rATS abolishes platelet and fibrinogen deposition onto a Dacron vascular graft and completely prevents occlusion of the graft segment in this heparin-resistant baboon model of vascular graft thrombosis. rATS displays dose-dependent antithrombotic effects, as illustrated by the inhibition of platelet deposition by 57% and 93% and inhibition of fibrinogen deposition by 70% and 90% at rATS doses of 2 and 4 µg/kg·min−1, respectively. Insertion of the thrombogenic Dacron graft segment during infusion of saline caused a significant increase in the thrombin-mediated cleavage of fibrinogen, as reflected by increased plasma levels of circulating FPA. The observed elevations of systemic FPA levels after insertion of the graft are completely suppressed by both doses of the FXa inhibitor despite significant platelet and fibrinogen deposition during infusion of the lower dose. This paradoxical result probably reflects the differences between the local and systemic thrombin concentrations generated at the lower dose. That is, the lower dose of rATS probably results in only partial inhibition of FXa, allowing thrombin to be generated at a rate sufficient to support platelet deposition but insufficient to elevate systemic FPA levels.

The role of thrombin as the primary mediator of platelet-dependent arterial thrombosis has been demonstrated in several animal models by use of direct (ATIII independent) low-molecular-weight inhibitors of thrombin.5-12 The results of the present study also support the contention that thrombin plays a central role in arterial thrombogenesis. By abolishing thrombin formation that is catalyzed by the prothrombinase complex through FXa inhibition, rATS effectively inhibited systemic and local thrombin activity. This hypothesis is further supported by the results of recent studies in animal models that use alternative methods to pharmacologically limit thrombin generation. For example, infusion of APc, which down-regulates thrombin formation through inactivation of the nonenzymatic coagulation cofactors Va and VIIa, inhibited platelet deposition in a similar baboon model of arterial thrombosis.13,14 Additionally, inhibition of the extrinsic tenase complex (factor VIIa/tissue factor) with the lipoprotein-associated coagulation inhibitor prevented thrombotic arterial reocclusion after thrombolysis in ves-

### Table 1. Effect of Recombinant Antistasin on Hemostatic Parameters in Anesthetized Baboons

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Graft 1 Time 0</th>
<th>Graft 1 20 Minutes</th>
<th>Graft 2 Time 0</th>
<th>Graft 2 20 Minutes</th>
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<td><strong>FPA (ng/ml)</strong></td>
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<td>0.88±0.28</td>
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<td>3.90±1.59</td>
<td>20.21±2.74*</td>
<td>2.01±0.41</td>
<td>1.94±0.31</td>
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<td><strong>APTT (seconds)</strong></td>
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<tr>
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<td>rATS 2.0</td>
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<td>28.0±0.8</td>
<td>124.6±16.5*</td>
<td>&gt;150*</td>
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<tr>
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<td>27.1±1.2</td>
<td>&gt;150*</td>
<td>&gt;150*</td>
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<td><strong>Bleeding time (minutes)</strong></td>
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<tr>
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<td>3.0±0.5</td>
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</table>

*Significantly (p<0.05) greater than time 0 value.

Graft 1, control graft (saline); graft 2, treatment graft; FPA, fibrinopeptide A; rATS, recombinant antistasin; rATS 2.0, 2.0 µg/kg·min−1 recombinant antistasin; rATS 4.0, 4.0 µg/kg·min−1 rATS; APTT, activated partial thromboplastin time. Graft 2 time 0 values were obtained after completing 60 minutes of saline or rATS infusion. All values represent mean±SEM (n=4 animals per treatment group).

**TABLE 1.** Effect of Recombinant Antistasin on Hemostatic Parameters in Anesthetized Baboons

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sels subjected to extensive vascular injury in dogs. Therefore, direct inhibition of thrombin, as well as inhibition of thrombin generation, are effective approaches toward controlling platelet-dependent arterial thrombogenesis.

The potent antithrombotic effects of rATS in this model are consistent with the reported effects of recombinant tick anticoagulant peptide (rTAP), a novel peptide FXa inhibitor isolated from the soft tick Ornithodoros moubata. The striking antithrombotic efficacy of these specific inhibitors of FXa in this heparin-resistant primate model of vascular graft thrombosis further demonstrates that direct inhibition of thrombin generation catalyzed by FXa in the prothrombinase complex is an effective means of controlling thrombin activity at the site of high-shear thrombus formation.

Both doses of rATS used in this study caused a prolongation in the ex vivo APTT of >150 seconds, reflecting the rapid inactivation of FXa in the prothrombinase complex. In contrast, complete antithrombotic efficacy by rTAP in this model was achieved at doses of rTAP that elevated APTT by less than twofold over the basal value. Because both rTAP and rATS are potent and specific inhibitors of FXa, their antithrombotic benefit is achieved by the same mechanism. The contrasting effects of rTAP and rATS on APTT probably result from differences in their respective rates of association with FXa in this assay.

Template bleeding times were not significantly elevated at fully antithrombotic doses of rATS. These results concur with those recently reported by Kelly et al., who demonstrated that antithrombotic doses of rATS were not associated with elevations of template bleeding times as determined by the modified Ivy technique in conscious baboons. This finding is noteworthy because most agents that are antithrombotic in this model of thrombosis, including direct thrombin inhibitors and antiplatelet agents, are associated with impairment of primary hemostasis as reflected by protracted template bleeding times.

The prolongation of template bleeding times by antiplatelet compounds probably reflects the direct inhibitory effect of these agents on platelet function. The potent antithrombotic profile of rATS, rTAP, and APC does not result from a direct effect on platelets. It is currently unclear how these agents exert an antithrombotic effect without compromising primary hemostasis in this model. It might be inferred that suppressing the rate of thrombin formation by either direct inhibition of FXa in the prothrombinase complex or indirect inhibition of the prothrombinase complex or the tenase complex (via inactivation of cofactors Va and VIIIa) allows sufficient thrombin generation to support hemostatic plug formation in the peripheral circulation but insufficient thrombin generation to facilitate high-shear thrombus formation. In contrast, doses of direct thrombin inhibitors required to maintain antithrombotic efficacy at the local site of thrombus formation may be too high to allow sufficient systemic thrombin activity for hemostatic plug formation.

In conclusion, the potent and selective FXa inhibitor rATS effectively prevents high-shear, platelet-dependent thrombus formation without adversely affecting primary hemostasis in a primate model of vascular graft thrombosis. The striking antithrombotic efficacy of rATS in this model suggests that specific FXa inhibition represents a pharmacologically useful approach for the prevention of acute arterial thrombus formation in settings where heparin is ineffective.

Acknowledgments

The authors thank Joan Brooke for secretarial assistance; Edgar Brown, John Doyle, and George Morgan for technical assistance; and Barbara Francis for preparation of 125I-labeled fibrinogen. We also acknowledge George Hunt for providing conditioned baculovirus culture media containing rATS and Elka Nutt for her help in purifying and characterizing the recombinant protein. In addition, we thank Dr. Dana Abendschein for providing us his manuscript before publication.

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doi: 10.1161/01.ATV.12.8.879
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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