Antithrombotic Efficacy of a Recombinant Nematode Anticoagulant Peptide (rNAP5) in Canine Models of Thrombosis after Single Subcutaneous Administration

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ABSTRACT
We describe the antithrombotic effects of recombinant nematode anticoagulant peptide (rNAP5), a selective and direct factor Xa inhibitor, after a single s.c. administration in canine models of arterial and venous thrombosis. The systemic anticoagulant effects of rNAP5 were evaluated initially in conscious dogs after s.c. dosing (0.03, 0.1 and 0.3 mg/kg) that resulted in a dose-dependent increase in the activated clotting time and the activated partial thromboplastin time. The antithrombotic effects of rNAP5 were evaluated in anesthetized dogs where saline or rNAP5 (0.03, 0.1 and 0.3 mg/kg s.c.) was administered 1 hr before the left circumflex coronary artery was subjected to electrolytic injury. In the saline group (n = 10), the left circumflex artery occluded in 79 ± 9 min, and 5 of 10 animals progressed to sudden death due to ventricular fibrillation. rNAP5 significantly prolonged the time to occlusion in the 0.03 mg/kg (163 ± 62 min) and 0.1 mg/kg (327 ± 62) treatment groups (n = 6). In the 0.3 mg/kg group (n = 5), all of the injured vessels remained patent for 8 hr. There was a dose-dependent reduction in the thrombus mass in the rNAP5-treated animals as compared with controls, as well as a lower mortality rate. rNAP5, in the doses of 0.03 and 0.1 mg/kg, did not alter the bleeding time, whereas 0.3 mg/kg produced a 5-fold increase. In a separate study, we evaluated the efficacy of rNAP5 (0.1 mg/kg) in the prevention of carotid artery and jugular vein thrombosis. In response to endothelial injury, the carotid artery and jugular vein in the saline group (n = 6) occluded in 142 ± 16 and 100 ± 11 min, respectively, compared with rNAP5, which maintained vessel patency in the carotid artery (6/6) and jugular vein (5/6) and significantly decreased the thrombus weights. The results demonstrate that rNAP5 has antithrombotic efficacy in canine models of arterial and venous thrombosis after a single s.c. administration.

The response to vascular injury results in the sequential formation of the serine protease FXa and α-thrombin. The formation of α-thrombin results from the proteolytic activation of the zymogen prothrombin mediated by the catalytic prothrombinase complex composed of FXa and nonenzymatic cofactor factor Vα assembled with prothrombin, primarily on the surface of activated platelets that have adhered to the site of vascular injury. Thrombin is the principal mediator of the thrombotic response through its role as the primary catalytic agonist of platelet activation and subsequent aggregation, and through the proteolytic conversion of soluble fibrinogen to insoluble fibrin, both of which result in the formation of an intravascular thrombus. The penultimate position of FXa in the coagulation response makes it an attractive pharmacological target for preventing α-thrombin-mediated thrombus formation. This may be particularly relevant in settings where there is a locally high level of prothrombinase-mediated thrombin generation because of the presence of an extensive membranous surface resulting from the accumulation of adhered and activated platelets, as is observed in the damaged arterial wall.

Several natural proteins originally isolated from hematophagous organisms have been shown to be potent and selective direct inhibitors of FXa. ATS, isolated from salivary gland extracts of the Mexican leech Haementeria officinalis, was the first protein recognized as a natural inhibitor of FXa (Tuszynski et al., 1987; Nutt et al., 1988). It is a 119-amino acid protein that is a tight-binding, reversible inhibitor of FXa (Kᵢ = 0.31–0.62 nM) (Dunwiddie et al., 1989). TAP is a 60-amino acid protein isolated from the tick Ornithodoros moubata (Waxman et al., 1990). TAP is a slow-binding, stoi-
chiometric, reversible inhibitor of free ($K_i = 0.5 \text{nM}$) and prothrombinase-assembled $\text{FXa}$ ($K_i = 5.3 \text{pM}$). Both ATS and TAP were more effective than heparin in the prevention of high-shear, platelet-dependent arterial thrombosis (Schaffer et al., 1991; Sitko et al., 1992) and were as effective as heparin in the prevention of venous thrombosis in several experimental models (Vlasuk et al., 1991; Fioravanti et al., 1993; Schaffer et al., 1992) after continuous i.v. infusion. The antithrombotic efficacy of both TAP and ATS was associated with minimal elevation of the cutaneous bleeding time, and in case of TAP, there was minimal change in the ex vivo aPTT.

Recently, a family of potent anticoagulants termed NAP, for nematode anticoagulant protein, was isolated from a canine hookworm, *Ancylostoma caninum* (Cappello et al., 1995). Molecular cloning revealed a family of NAP anticoagulants that contribute to the anticoagulant activity observed in hookworm extracts. A recombinant version of one of these forms (rNAP5) was characterized as a 77-amino acid direct inhibitor of $\text{FXa}$ as determined by inhibition of free, uncomplexed $\text{FXa}$ ($K_i \sim \text{43 pM}$) and of $\text{FXa}$ complexed in the prothrombinase complex ($K_i \sim \text{144 pM}$) (Stanssens et al., 1996). The amino acid sequence of rNAP5 is shown in figure 1.

Preliminary studies demonstrated that the s.c. administration of rNAP5 resulted in a favorable pharmacokinetic profile and prevented arterial thrombosis (Rote and Vlasuk, 1995; Vlasuk et al., 1995). The aim of the present study was to examine whether a single s.c. administration of a direct $\text{FXa}$ inhibitor, rNAP5, could provide adequate antithrombotic efficacy in the prevention of acute, platelet-dependent arterial thrombosis and venous thrombus formation.

**Materials and Methods**

**Drugs and reagents.** rNAP5 was prepared and characterized as described earlier (Stanssens et al., 1996). For all the studies described here, rNAP5 was dissolved in saline for s.c. administration. All other reagents used in this study were obtained from commercial sources.

**Guidelines for animal research.** The procedures followed in this study were in accordance with the guidelines of the University of Michigan (Ann Arbor) University Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in *The Guide for Care and Use of Laboratory Animals*, Department of Health, Education and Welfare publication no. NIH 78-23.

**Surgical Preparation**

Catheterization of the jugular vein for determination of plasma concentration and coagulation parameters in conscious dogs. Twelve purpose-bred, beagle dogs were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated and ventilated with room air. Using aseptic technique, the left jugular vein was exposed and cannulated. The catheter was tunneled under the skin and exteriorized on the dorsal surface of the neck. A surgical neck collar was placed, and the animals were returned to their quarters and administered daily injections of ampicillin suspension (200 mg s.c.). The animals were allowed 3 to 5 days to recover from the surgical procedure before returning to the laboratory.

**Model of coronary artery thrombosis.** Purpose-bred dogs weighing 10 to 15 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated and ventilated with room air (Harvard Apparatus, South Natick, MA). The heart was exposed by a left thoracotomy in the fifth intercostal space and suspended in a pericardial cradle. A 2-cm segment of the LCX was isolated by blunt dissection. A flow probe (Model 1.5SRB24, Transonic Systems Inc., Ithaca, NY) was placed around the artery. An external stenosis was produced by securing a suture-ligature around the artery and an adjacent 18-gauge hypodermic needle and then removing the needle. An intracoronary electrode was fashioned from the tip of a 25-gauge hypodermic needle attached to a 30-gauge Teflon-insulated, silver-coated copper wire. The needle tip electrode was inserted through the arterial wall so that the uninsulated portion was positioned against the endothelial surface. The external portion of the electrode was secured to the skin with a suture.

After a stabilization period of 2 hr, anodal direct current was applied to the endothelial surface of the LCX via the previously implanted electrode. Electrolytic injury was induced by connecting the intravascular electrode to the positive pole (anode) of a dual-channel square-wave generator (a Grass S88 stimulator and a Grass Constant Current Unit, Model CCU1A; Grass Instrument Co., Quincy, MA). The cathode was connected to a distant s.c. site. The current delivered to the vessel was monitored continuously on an ammeter and maintained at 150 $\mu$A for 3 hr. Thrombotic occlusion of the vessel in this experimental model occurs within the first 2 hr after the application of anodal current. The standard limb lead II of the ECG and the coronary artery blood flow were recorded and monitored continuously to determine the time to occlusion. At the end of the 8-hr protocol, all animals were euthanized by an overdose of anesthetic agent (pentobarbital sodium), and the heart was removed. The LCX was removed along its entire length by careful dissection. The vessel was opened longitudinally to confirm the proper placement of the anodal electrode and the presence of an electrolytic lesion in the arterial wall. Any existing thrombus mass was removed intact and weighed.

**Determination of myocardial infarct size.** The heart was cut from apex to base in six 1.0-cm-thick sections that were incubated in triphenyltetrazolium chloride for 5 min at 37°C. The transverse sections were weighed and traced onto clear acetate sheets. The red-pigmented tissue containing the precipitated formazan complex was considered to represent viable tissue, whereas tissue that remained pallid was considered to be infarcted. The demarcated areas were scanned on a flatbed scanner and digitized on a Macintosh II computer (Cupertino, CA), and the respective regions were quanti-

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**Fig. 1. Structure of rNAP5.**
Infarct size was quantified as a percentage of total left ventricular area.

Model of primary carotid artery and jugular vein thrombosis. The model used in this study is a modification of one described above for the study of experimentally induced coronary artery thrombosis (Romson et al., 1980). The experimental procedure resulted in the formation of a platelet-rich intravascular arterial thrombus or non-platelet-dependent venous thrombus at the site of an electrolytically induced endothelial lesion. The surgical preparation of animals was carried out essentially as described above. LCA and the right jugular vein were exposed with care in order not to injure the vessels. Flow probes (Model 2RB907, Transonic Systems Inc., Ithaca, NY) were placed on the carotid artery and jugular vein. Blood flow in each vessel was monitored continuously. The point of insertion of the intravascular electrode and the position of the external constrictor were downstream with respect to each of the flow probes. The external constrictor on the carotid artery was constructed of stainless steel, shaped to fit around the vessel. A nylon screw (2 mm in diameter) threaded through the C-shaped metal band was adjusted to decrease the circumference of the vessel and to produce a regional stenosis. The vessel was constricted to a point where the pulsatile flow pattern was reduced by 50% without altering the mean arterial blood flow. The stenosis placed on the jugular vein was accomplished with the use of a ligature. A PE240-silastic tube was placed parallel to the jugular vein, and a ligature was tied around the vessel and the tube. The tube then was removed, which resulted in a narrowing of the vessel lumen. The pulsatile flow pattern of the jugular vein was reduced by approximately 50% as a result of the constriction imposed by the surrounding ligature. The current delivered to each vessel was maintained at 300 μA. In all experiments, the anodal current was applied for a maximum of 3 hr.

Experimental protocols. Twelve conscious dogs with previously implanted jugular catheters were randomized to receive one of three doses of rNAP5 (0.03, 0.1 and 0.3 mg/kg; n = 4 for each dose). The drug was administered s.c. in a total volume of 1 to 2 ml. Blood (15 ml) was drawn at T = 0 (predrug) and at 1, 2, 4, 6, 8 and 24 hr after s.c. injection. The blood samples were analyzed for drug concentrations and hematologic parameters (ACT, aPTT and PT).

The experimental protocol for coronary artery thrombosis is outlined in figure 2. Twenty-seven dogs were randomized to one of four treatment protocols in which the control group received saline (1 ml s.c.) and the three drug-treated groups were administered rNAP5 (0.03, 0.1 or 0.3 mg/kg s.c.) in a total volume of 1 to 2 ml. All injections were performed at multiple sites in the previously shaven abdominal area of the anesthetized dogs.

Upon stabilization from the surgical interventions, blood was drawn from the femoral vein for base-line hematological tests. Dogs were then administered either saline or rNAP5. One hour later, an anodal current was applied to the LCX. The duration of anodal current application was 180 min. Periodic blood samples were collected to assess the coagulation parameters. The BP, HR and blood flow were monitored for 8 hr. At the end of the protocol, the animals were euthanized by an anesthetic overdose, and the injured vessel segment proximal and distal to the point of injury was removed without disturbing the intravascular thrombus. The vessel segments were opened longitudinally to allow removal of the intact thrombus. The weight of each thrombus was determined using an analytical balance.

The experimental protocol for carotid artery and jugular vein thrombosis was similar to that described above. Twelve dogs were randomized to receive either saline or rNAP5 (0.1 mg/kg s.c.). One hour later, anodal current was applied simultaneously to the carotid artery and the jugular vein. All the animals in this protocol were monitored for 6 hr. At the end of the protocol, thrombi were removed from both vessel segments and weighed.

Inclusion criteria. Animals that were included in the final protocol satisfied the following pre-established criteria: 1) a circulating platelet count of not less than 100,000 per μl; 2) demonstrated ability of epinephrine-primed platelets to aggregate in response to ADP (20 μM), arachidonic acid (0.65 mM) and γ-thrombin (70 nM) before administration of saline or rNAP5; 3) thrombotic occlusion of the blood vessels within 3 hr from the onset of vessel wall injury and 4) absence of heart worms upon final post-mortem examination.

Platelet studies and coagulation measurements. Whole blood (20 ml) was withdrawn from the jugular vein for assessing the hematologic parameters. The blood was collected in plastic syringes containing 3.7% sodium citrate as the anticoagulant (1:10 citrate/blood vol/vol) at base line and at 1, 2, 4, 6 and 8 hr. The platelet count was determined with an H-10 cell counter (Texas International Laboratories, Houston, TX). PRP, the supernatant present after centrifugation of anticoagulated whole blood at 140 g for 10 min, was used for aggregation studies. PPP was prepared after the PRP was removed by centrifuging the remaining blood at 2000 × g for 10 min and discarding the bottom cellular layer. Ex vivo platelet aggregation was assessed with a four-channel aggregometer (BioData-PAP-4, Bio Data, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP (adjusted to 200 × 10^3 platelets/μl) maintained at 37°C. Platelet aggregation was induced with arachidonic acid (0.65 mM), ADP (20 μM) or γ-thrombin (70 nM). A subaggregatory dose of epinephrine (550 nM) was used to prime the platelets before the agonists were introduced. Values are expressed as percent aggregation, which is represented by the fraction of light transmission standardized to PPP samples that yield 100% light transmission.

To assess the anticoagulation state of the animals, we determined the PTT and PT using a Hemochron (Technidyne, Edison, NJ) with reagents supplied by the manufacturer. Citrated whole blood was
used for these determinations. For ACT determination, 2 ml of uncitrate blood was collected separately.

Bleeding times were determined by means of a Simplate device (Simplate-R, Organon Teknika Corp., Durham, NC), which made a uniform incision 5 mm long and 1 mm deep on the upper surface of the tongue. The tongue lesion was blotted with filter paper every 30 sec until the transfer of blood to the filter paper ceased.

Quantification of plasma rNAP5 concentrations. Citrated plasma samples were frozen at −20°C and subsequently thawed at the time of the assay. The rNAP5 concentrations in the plasma were assayed by using a photometric determination (Coatest LMW Heparin/Heparin, Pharmacia Hepar Inc., Franklin, OH) with some modifications. In this assay, fXa catalyzes the hydrolysis of p-nitroaniline from the substrate N-ε-Cbo-D-Arg-Gly-Arg-pNA (S-2765), and the color produced is read photometrically. Pooled citrated plasma from untreated dogs was spiked with the LMWH standard (100 IU/ml) to concentrations of 0.05 to 2 IU/ml. Standards (10 μl) or samples (10 μl) were added in duplicates to a 96-well microtiter plate containing buffer (50 mM Tris, 7.5 mM EDTA, pH 8.4). Human fXa (75 μl) was added to achieve a final concentration of 3.3 nM, and the plate was incubated for 30 min at room temperature. After the addition of 75 μl of the substrate (S-2765, final concentration 360 μM), the plate was incubated for 8 min at room temperature. The enzymatic reaction of fXa was stopped by the addition of 75 μl of 20% acetic acid, and the color was read at 405 nm. The 25, 50 and 75 nM rNAP5 controls (n = 10) generated a specific activity of 263.3 ± 23.3 nM rNAP5/μM LMWH. The blank-subtracted values were read off on a four-parameter standard curve, and the values (expressed as IU/ml) were multiplied by the specific activity of rNAP5 in this assay determined from a standard curve (263.3 ± 23.3 nM rNAP5/μM LMWH) to obtain the plasma concentrations of rNAP5.

Statistical analyses. The data are expressed as mean ± S.E.M. and were analyzed by one-way analysis of variance for group comparisons and for repeated measures, followed by a Dunnett post-hoc t test to determine the level of significance. A paired t test (with a mathematical correction) was employed to assess the differences over time within a group. Values were considered to be statistically different at a level of P < .05.

Results

In the current study, 51 of the purpose-bred dogs that were initially selected met the inclusion criteria. Because all the animals conformed to the protocol, there was no need to exclude animals retrospectively.

Pharmacokinetic and pharmacodynamic profile of rNAP5 in conscious dogs. Before evaluating the antithrombotic efficacy of rNAP5 in experimental models of thrombosis, we evaluated the pharmacokinetic and pharmacodynamic profile of this agent after a single s.c. administration in conscious dogs. As shown in figure 3, there was rapid absorption of rNAP5 into the systemic circulation after a single s.c. administration, as evidenced by a dose-dependent increase in the plasma concentrations. The peak plasma concentrations after the 0.03, 0.1 and 0.3 mg/kg doses were 457 ± 105, 873 ± 133 and 1447 ± 126 ng/ml, respectively. The time to achieve the peak concentrations was approximately 4 hr, after which the plasma concentrations gradually declined to the base-line values (zero) at 24 hr in the 0.03 and 0.1 mg/kg treatment groups. In the 0.3 mg/kg treatment group, however, the plasma concentration of rNAP5 at 24 hr was 146 ± 85 ng/ml.

The effect of a single s.c. administration of rNAP5 on ex vivo blood coagulation parameters is shown in table 1. There was a dose-dependent increase in aPTT that maximally increased in the 0.1 and 0.3 mg/kg treatment groups at 4 hr and 1 hr after administration, respectively. At 24 hr, the aPTT in both these treatment groups was slightly above the base-line values (table 1). The PT was not affected by rNAP5 in the 0.03 and 0.1 mg/kg treatment groups, but there a 3-fold increase was observed in the 0.3 mg/kg treatment group. A dose-dependent increase in the ACT was observed in all three treatment groups, maximal increases of 2-fold and 12-fold being produced by the 0.03 mg/kg and 0.1 mg/kg doses, respectively. The ACT was maximally increased in the 0.3 mg/kg treatment group (1500 sec) and remained elevated significantly over the base-line value at 24 hr.

Ex vivo platelet aggregation in response to arachidonic acid (0.65 mM), ADP (20 μM), and γ-thrombin (70 nM) was not affected by rNAP5 over the dose range tested. Eight hours after rNAP5 administration, the percent platelet aggregation in response to all three agonists was 90% to 100% of the predrug values (data not shown). As with the two lower doses, there were no differences in the aggregation status throughout the protocol for any of the agonists employed.

Systemic hemodynamics. The s.c. administration of rNAP5 was not associated with any changes in BP or HR (data not shown).

Antithrombotic efficacy of rNAP5 after coronary artery thrombosis in anesthetized dogs. The antithrombotic efficacy of rNAP5 (0.03, 0.1 and 0.3 mg/kg) was assessed after a single s.c. dose 1 hr before the initiation of electrolytic injury to the LCX. The consequence of electrolytic vessel wall injury and for 4 hr after termination of the LCX blood flow is shown in figure 4. Animals in the saline-treated group (n = 10) exhibited cyclic flow reductions within 30 to 45 min after the initiation of electrolytic current injury. The repetitive flow reductions were followed by total occlusion as evidenced by the loss of ultrasonic flow signal. Immediately after coronary artery occlusion, 5 of 10 control animals progressed to sudden death due to ventricular fibrillation.

The rNAP5-treated animals exhibited a dose-dependent maintenance of LCX patency during the 3-hr period of electrolytic vessel wall injury and for 4 hr after termination of the anodal current. The incidence of occlusion followed by death due to ventricular fibrillation was 3/6, 2/6 and 0/6 in the 0.03,
injection of rNAP5, 1 hr before the initiation of electrolytic injury. The mean time to coronary artery occlusion differed significantly for the duration of the protocol in the 0.1 and 0.3 mg/kg groups, respectively. One animal in the 0.03 mg/kg group exhibited cyclic flow variations throughout the protocol. However, a nonoscillatory flow pattern was observed for the duration of the protocol in the 0.1 and 0.3 mg/kg groups, and the coronary artery blood flow differed significantly (P < .05) when compared with that of the saline-treated animals. The mean time to coronary artery occlusion in the saline-treated group was 79 ± 9 min (table 2). The s.c. injection of rNAP5, 1 hr before the initiation of electrolytic injury, significantly prolonged the time to occlusion (163 ± 62 min for 0.03 mg/kg and 327 ± 62 min for 0.1 mg/kg) of the coronary artery. None of the coronary arteries in the 0.3 mg/kg group occluded over the time course of the experimental protocol. The time to occlusion for these animals was arbitrarily assigned as 420 min for the purpose of statistical comparisons.

Left ventricular infarct size, expressed as percent of the total left ventricle, was determined for animals that survived at the end of the experimental protocol. Of the 10 saline-treated animals that developed coronary artery occlusion, 5 did not progress to ventricular fibrillation and completed the entire protocol despite persistent ischemia (ST segment elevation in lead II) in the region of distribution for the LCX. The average myocardial infarct size in the five hearts subjected to regional ischemia for the duration of the protocol was 16% ± 2% of the total left ventricle. In the 0.03 mg/kg rNAP5 group, three dogs that occluded, but did not succumb to ventricular fibrillation, had an infarct size of 22% ± 3%.

### TABLE 1
Effect of rNAP5 on the hematologic parameters in conscious dogs

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time (hr)</th>
<th>aPTT (sec)</th>
<th>PT (sec)</th>
<th>ACT (sec)</th>
<th>Hb (%)</th>
<th>HCT (%)</th>
<th>Platelets (×10^12/L)</th>
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<td>0</td>
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<td>14 ± 0</td>
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<td>1</td>
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<td>60 ± 4</td>
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<td>91 ± 3</td>
<td>46 ± 3</td>
<td>548 ± 22</td>
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* 254 sec is the maximum readable value.
* 1500 sec is the maximum readable value.
Hb: hemoglobin.
HCT: hematocrit.
* P < .05 as compared with base line.

Fig. 4. Patency status of the LCX coronary artery during the administration of rNAP5 or saline. Electrolytic injury produced complete and persistent occlusion of LCX coronary artery in the saline-treated animals. However, administration of rNAP5 was associated with the maintenance of a sustained and nonoscillatory flow. Values are expressed as mean ± S.E.M.

### TABLE 2
Effect of rNAP5 on the occlusion parameters during thrombosis

<table>
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<th>Groups</th>
<th>Thrombus Weight (mg)</th>
<th>Time to Occlusion (min)</th>
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<tr>
<td>Coronary Artery Thrombosis</td>
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<tr>
<td>Saline (n = 10)</td>
<td>7.69 ± 1.64</td>
<td>79 ± 9</td>
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<tr>
<td>rNAP5 (0.03 mg/kg, n = 6)</td>
<td>4.38 ± 0.72</td>
<td>163 ± 62</td>
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<td>rNAP5 (0.1 mg/kg, n = 6)</td>
<td>1.79 ± 0.57*</td>
<td>327 ± 62*</td>
</tr>
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<td>rNAP5 (0.3 mg/kg, n = 5)</td>
<td>0.8 ± 0.27*</td>
<td>&gt;420*</td>
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<tr>
<td>Carotid Artery Thrombosis</td>
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<tr>
<td>Saline (n = 6)</td>
<td>32.72 ± 5.01</td>
<td>142 ± 16</td>
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<tr>
<td>rNAP5 (0.1 mg/kg, n = 6)</td>
<td>8.93 ± 2.75*</td>
<td>&gt;300*</td>
</tr>
<tr>
<td>Jugular Vein Thrombosis</td>
<td></td>
<td></td>
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<tr>
<td>Saline (n = 6)</td>
<td>38.35 ± 6.2</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>rNAP5 (0.1 mg/kg, n = 5)</td>
<td>9.55 ± 1.41*</td>
<td>&gt;300*</td>
</tr>
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* P < .05 as compared with saline group.
* Absolute values were used to assess statistical difference.
The template bleeding time was assessed in anesthetized dogs during the coronary artery thrombosis protocol (figure 5). Bleeding times were unchanged by the lower doses. However, the 0.3 mg/kg dose produced a 5-fold (P < .05) increase in the bleeding time.

**Patency status of the carotid artery and jugular vein.** On the basis of the information gained from the effects of rNAP5 on the hematological parameters in conscious dogs and the antithrombotic efficacy in the setting of coronary artery thrombosis described above, we selected the 0.1 mg/kg dose of rNAP5 to examine its potential to inhibit simultaneous thrombus formation in the carotid artery and jugular vein in an anesthetized preparation. One hour after the s.c. administration of either saline or rNAP5, electrolytic injury was initiated. The mean base-line flows in the saline group (carotid artery = 146 ± 14 ml/min and jugular vein = 108 ± 12 ml/min) were similar to those in the rNAP5 group (carotid artery = 114 ± 7 ml/min and jugular vein = 95 ± 14 ml/min). All the animals in the saline group (n = 6) exhibited a progressive decrease in blood flow in both the carotid artery and the jugular vein in response to electrolytic injury, culminating in total occlusion (figure 6). The mean time to occlusion for the jugular vein in the saline group was 100 ± 11 min, whereas that for the carotid artery was 142 ± 16 min. Administration of rNAP5 significantly delayed the decrease in blood flow in both the artery and the vein over the 5-hr time course of the protocol. At the end of the protocol, 6 of 6 carotid arteries and 5 of 6 jugular veins were patent. Complete jugular vein occlusion developed in one animal, time to occlusion being 204 min. The data for both the carotid artery and the jugular vein times to occlusion are summarized in table 2.

**Thrombus weights.** At the conclusion of each experimental protocol, thrombi were removed from the injured vessels and weighed. In the model of coronary thrombosis, a dose-dependent and significant reduction in the thrombus mass in LCX was noted in the rNAP5-treated animals as compared with the saline-treated animals (table 2). In the model of arterial and venous thrombosis, a similar reduction in the thrombus mass was observed in the injured vessel segments in the presence of rNAP5 (table 2).

**Discussion**

Hookworms are hematophagous nematodes that infect a wide range of mammalian hosts, including the human. The organisms possess an anticoagulant substance(s) that can undermine host hemostasis to facilitate the acquisition of a blood meal. A new family of small anticoagulant proteins has been purified (Cappello et al., 1995) and the corresponding cDNAs cloned from the adult *Ancylostoma caninum* hookworm (Stanssens et al., 1996). Two of the recombinant forms of nematode anticoagulant peptide, rNAP5 (77 amino acids) and rNAP6 (75 amino acids), directly inhibit the catalytic activity of both the free IXa and the IXa in the prothrombinase complex, whereas the third form, rNAPc2 (84 amino acids) predominantly inhibits the catalytic activity of a complex composed of VIIa and tissue factor in a IXa-dependent fashion (Stanssens et al., 1996). We examined the antithrombotic efficacy resulting from IXa inhibition by a single s.c. administration of rNAP5 in the experimental settings of acute arterial and venous thrombosis induced by electrolytic vessel wall injury.

Inhibiting the coagulation system at the level of IXa after a single s.c. administration of rNAP5 offered a favorable antithrombotic effect in the experimental model described here. Despite deep arterial wall injury, the administration of rNAP5 maintained coronary artery patency for 8 hr and led to a reduction in the size of the thrombus mass adherent to the site of vessel wall injury. Although different mechanisms
contribute to arterial vs. venous thrombus formation, rNAP5 was effective in preventing thrombotic occlusion in both injured carotid artery and injured jugular vein. This result is consistent with the ability of rNAP5 to limit thrombus formation by inhibiting continued thrombin generation locally at the site of vessel wall injury. Inhibition of thrombin generation would have the added benefit of limiting the thrombin-mediated positive-feedback loop responsible for activating factors V and VIII (Kane and Davie, 1988) as well as preventing FXIII-induced cross-linking and fibrin accretion. These effects of rNAP5 can result in reduced prothrombinase activity and suppress the rapid and amplified generation of thrombin that occurs in response to activation of the coagulation system after vascular injury (Lindhout et al., 1990). The platelet-vessel wall interaction is a major component of arterial thrombus formation, whereas stasis and hypercoagulability participate in venous thrombosis. Thus rNAP5, by interrupting thrombin generation, may abrogate the consolidation of platelets at the site of arterial injury. Also, by blocking coagulation at the level of FXa, rNAP5 would substantially decrease the formation of fibrin at the site of injury in the vein. These salutary effects have been observed with minimal alteration in primary hemostatic parameters as measured by template bleeding time. Indirect inhibition of thrombin by standard heparin has limited efficacy, in part because of its neutralization by endogenous inhibitors and restricted access of the heparin-antithrombin III inhibitory complex to fibrin-bound thrombin and FXa within the developing thrombus (Weitz et al., 1990; Lane et al., 1984; Jordan et al., 1982). The limited accessibility of heparin/antithrombin III also prevents the glycosaminoglycan from inhibiting FXa within the catalytic prothrombinase complex (Teitel and Rosenberg, 1983). Use of selective antithrombins such as r-hirudin can reduce the free, as well as the clot-bound, thrombin. However, an important limitation of direct antithrombins is their inability to inhibit thrombin-mediated thrombosis adequately in the absence of sufficient plasma levels of the drug because of the continued generation of thrombin mediated by prothrombinase complexes at the site of the vascular lesion. Therefore, there is a potential for continued thrombus formation or rapid rethrombosis of a newly recanalized vessel after the premature termination or insufficient dosing of a direct thrombin inhibitor. This is particularly relevant in cases where there is ongoing thrombin generation because of the accumulation of a large membrane-bound prothrombinase pool, as is observed in partially occluded vessels.

Our study indicates that there appears to be a dichotomy between the rNAP5-associated antithrombotic effects and alterations in coagulation parameters as assessed by the ex vivo clotting assays (aPTT and ACT). This may be due to the nature of the clotting assays, the potency and kinetics of rNAP5 in inhibiting FXa or a lack of platelet inhibition. The accessibility of soluble thrombin in the aPTT and ACT assays may provide a sensitive and reliable means to evaluate direct and indirect thrombin inhibitors. The evaluation of direct FXa inhibitors in the clotting assays is complicated by the fact that FXa catalyzes the formation of thrombin only after its assembly into the prothrombinase complex. Therefore, the kinetics of prothrombinase inhibition within the time course of the clotting assay may have a profound effect on the observed anticoagulant potency of a FXa inhibitor. This has been demonstrated with other FXa inhibitors such as TAP and ATS. At fully antithrombotic doses, TAP had minimal effect on the aPTT values (Schaffer et al., 1991; Sitko et al., 1992), whereas ATS produces a >5-fold increase (Schaffer et al., 1992). A similar difference was observed between rNAP5 and TAP in the aPTT clotting assay (Cappello et al., 1995). The effect of rNAP5 on the aPTT and ACT clotting assay may be related to the rapid rate of association of the inhibitor with the prothrombinase complex. Despite a >10-fold increase in the aPTT and ACT, there was no effect on the bleeding time after the administration of 0.1 mg/kg of rNAP5. These results agree with those reported for ATS (Schaffer et al., 1992), which increased aPTT with minimal effects on bleeding time. Moreover, it was noted that none of the doses of rNAP5 tested had a direct effect on the platelet aggregation profile, including aggregation in response to γ-thrombin. In general, bleeding time is considered a marker of the integrity of primary hemostatic mechanisms, which are platelet-dependent and possibly mediated by adhesion rather than fibrin. A steep dose-response relationship was observed with the rNAP5-associated increase in the template bleeding time. Whereas the 0.3 mg/kg dose produced a 5-fold increase in the template bleeding time, the 0.1 mg/kg dose had no significant effect. Furthermore, the data for rNAP5 in the model of coronary thrombosis (fig. 5) and the model of carotid/jugular vein thrombosis (fig. 6) indicated that 0.1 mg/kg was an effective antithrombotic dose. Therefore, the antithrombotic efficacy with rNAP5 (0.1 mg/kg) could be achieved without altering the hemostatic parameters.

We did not observe untoward effects (bleeding from the jugular catheter site, vomiting, diarrhea, agitation or the like in the conscious dogs treated with escalating doses of rNAP5. It is unlikely that the antithrombotic effects of rNAP5 are due to inhibition of coagulation factors other than FXa (Cappello et al., 1995; Vlasuk et al., 1995). Structurally, rNAP5 lacks significant amino acid homology to any known serine protease inhibitor or other naturally occurring anticoagulants. Concentrations of rNAP5 that inhibited 98% of FXa activity did not inhibit the amidolytic activity of trypsin, chymotrypsin, thrombin, FVIIa, FXIIa, kallikrein, plasmin, protein C, tPA or urokinase (Cappello et al., 1995). Given the limitations of conventional heparin therapy, such as hemorrhage, hypersensitivity, thrombocytopenia and the need for frequent drug monitoring, the search for an ideal FXa inhibitor continues. The present study suggests that FXa inhibitors may provide a favorable risk/benefit ratio and that further studies with rNAP5 are warranted.

Results similar to those in the present study have been obtained with other direct prothrombinase inhibitors. For example, ATS and TAP were considered superior to heparin in preventing platelet-rich thrombi in Dacron arteriovenous femoral grafts (Schaffer et al., 1991; Schaffer et al., 1992) and as effective as heparin in suppressing fibrinopeptide A formation in a rhesus monkey disseminated intravascular coagulation model (Dunwiddie et al., 1992; Neeper et al., 1990). In addition, TAP was more effective than heparin in preventing venous thrombus formation initiated by a local injection of a thromboplastin/blood mixture followed by stasis in rabbits (Vlasuk et al., 1991) and in preventing formation of heparin-resistant platelet thrombi in a primate model (Schaffer et al., 1991). In all of these and other studies ( Nicolini et al., 1996; Lynch et al., 1995; Lefkovits et al., 1996), the
inhibitor was administered as a continuous i.v. infusion to achieve efficacy. The unique feature of the present study is that rNAP5 was effective on an antithrombotic even when administered as a single s.c. injection. The data that have emerged thus far with fXa and thrombin inhibitors lead us to speculate that thrombus-bound fXa may be a more important pharmacologic target than thrombus-bound thrombin in terms of preventing procogulant activity.

The results of this study support the concept that inhibition of the coagulation system at a site(s) proximal to thrombin’s action can be an effective strategy to interrupt thrombogenesis. Such a “higher-tier” blockade of the coagulation system at a site(s) proximal to thrombin generation while having minimal effects on platelet-mediated hemostasis. Therefore, fXa inhibition can provide efficient anticoagulation in the prevention of arterial and/or venous thrombosis with a minimal potential for inducing bleeding.

**Limitations of the study.** This manuscript describes preliminary evaluation of rNAP5 in an animal model of thrombosis. The experimental model (Romson et al., 1980) used in this study has been exploited successfully for related studies by other investigators (Mickelson et al., 1989; Sudo et al., 1995; Nicolini et al., 1996; Shebuski et al., 1990; Lynch et al., 1995; Chen et al., 1995). Electrolytic injury to the intimal surface of the artery or vein invariably results in the formation of an arterial, platelet-rich, or a venous, fibrin-rich, occlusive intravascular thrombus. The coagulation system also is activated, as evidenced by an increase in thrombin-antithrombin complexes (Rebello et al., 1997). Studies employing the model have shown that the arterial thrombus is composed of platelet aggregates interspersed with erythrocytes (Nicolini et al., 1996; Sudo et al., 1995), whereas the venous thrombus consists mainly of erythrocytes with the incorporation of leukocytes, platelets and fibrin strands (Sudo et al., 1995). Formation of an occlusive lesion is prevented by inhibition of the platelet glycoprotein IIb/IIIa receptor (Mickelson et al., 1989) or, once formed, is susceptible to lysis by systemic thrombolytic therapy (Nicolini et al., 1996). Although electrolytic injury does not occur in humans, it is well established that activation of the coagulation system and platelet aggregation are two important components of thromboembolic diseases. The present model utilizes an electrolytic current to induce endothelial injury. Disruption of the endothelial surface initiates a sequence of events that culminates in occlusive vascular thrombosis. It is the latter biologic phenomena that are influenced by the pharmacologic agents under study. Although animal models may not mimic fully the clinical condition, they enable the investigator to test new pharmacologic entities before initiating clinical trials. Thus the relevance of the present animal model with respect to rNAP5 can be confirmed only by its efficacy in clinical trials.

**Clinical implications.** The use of LMWH in the treatment of venous thromboembolic conditions is on the rise because of their convenience of administration and ease of monitoring. Most studies have shown that they are at least as effective as unfractionated heparin (Green et al., 1994) in the prevention of deep vein thrombosis. Although their propensity for hemorrhagic complications and thrombocytopenia appear to be less, the risk still exists (Friedel and Balfour, 1994; Frampton and Faulds, 1994; Barradell and Buckley, 1992). To date, LMWHs have been approved only for management of venous thrombosis. The antithrombotic potential of rNAP5 in arterial and venous thrombosis may differentiate it from LMWHs. Furthermore, the platelet count was not altered by rNAP5 in this study, which indicates that it may be less likely to produce thrombocytopenia. Although the present study reveals the salutary effects of rNAP5 in the electrolytic injury models of thrombosis, more extensive preclinical studies are needed to investigate its advantages over LMWH. However, as with any new therapeutic intervention, final evaluation must rely on clinical testing based on sound preclinical data.

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**References**


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