A Monoclonal Antibody Against Rabbit Tissue Factor Inhibits Thrombus Formation in Stenotic Injured Rabbit Carotid Arteries

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Tissue factor (TF) is a transmembrane protein that binds factor VII/VIIa, thus activating the extrinsic blood coagulation pathway. Since this pathway appears to be involved in the formation of intravascular thrombi, the anti-rabbit TF monoclonal antibody, AP-1, was produced and tested as an antithrombotic agent in a rabbit model of recurrent intravascular thrombosis. In this model, a plastic constrictor is positioned around the injured rabbit carotid arteries, and flow is monitored with a Doppler flow probe. This produces cyclic flow variation (CFV) in the carotid artery, which is caused by recurrent formation and dislodgment of thrombi at the site of the stenosis. After monitoring CFV pattern for 30 minutes, AP-1 was infused intravenously into nine rabbits at doses of 0.05 to 1.5 mg/kg body weight, and a control monoclonal antibody that does not react with rabbit TF was infused into four additional rabbits. In all rabbits receiving AP-1, CFV was abolished, and a steady normal blood flow was restored, indicating that thrombus formation had been blocked by AP-1. By contrast, in all rabbits that received the control monoclonal antibody, CFV continued unaltered. There was no change in the partial thromboplastin time and ex vivo platelet aggregation to several different agonists after infusion of AP-1, indicating an absence of systemic effects on the coagulation process. We conclude that activation of the extrinsic coagulation pathway has a key role in triggering intravascular thrombosis and that an anti-TF monoclonal antibody is an effective antithrombotic agent that could have therapeutic potential for humans. (Circ Res. 1994;74:56-63.)

Key Words: tissue factor • monoclonal antibody • rabbit model • intravascular thrombosis • cyclic flow variation

Blood coagulation proceeds by a cascade of sequential proteolytic reactions leading to the formation of a thrombus. The physiologically important reaction that initiates this cascade is the binding of the plasma protease factor VII/VIIa to the transmembrane protein tissue factor (TF). TF is normally present in blood vessel walls separated from factor VII/VIIa by an endothelial cell barrier. It has been shown in several animal studies that thrombus formation could be induced by disrupting the endothelial barrier. Similarly, thrombosis can be inhibited by blocking TF.

In the present study, a rabbit model of thrombosis based on the Folts canine model was used to test the hypothesis that activation of the extrinsic coagulation pathway is important in the initiation of intravascular thrombosis. This model was designed to provide conditions for thrombosis that closely resemble acute coronary syndromes as seen in humans. Thrombosis was induced by local injury to the carotid artery, followed by constriction of the artery. This procedure produces cyclic fluctuations of arterial blood flow (cyclic flow variation [CFV]) due to recurrent cycles of thrombus formation and subsequent dislodgment. The carotid blood flow velocity was monitored continuously by a Doppler flow probe positioned proximal to the site of injury. The importance of activation of the extrinsic coagulation pathway in mediating CFV was tested by infusing a monoclonal antibody (mAb) against rabbit TF (RTF). The results obtained demonstrate that low doses of the anti-RTF mAb can completely restore a steady normal blood flow and thus prevent intravascular thrombus formation, without incurring potentially harmful systemic effects.

Materials and Methods

Production of RTF mAb

Purification of RTF. RTF was purified from rabbit brain acetone powder (Pel-Freeze Biologicals, Rogers, Ark) by affinity chromatography using anti-RTF polyclonal antibody coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif) as previously described. Protein concentration was determined by a dye-binding assay and purity of the protein was assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Functional assay for TF. The coagulant activity of the crude acetone extract was estimated using a two-stage clotting assay. Purified RTF was first reconstituted in phospholipid...
vesicles and then assayed for procoagulant activity. TF activity was calculated using the standard semilog plot of clotting time versus concentration of human TF. One unit of TF activity is defined as the time in seconds required for 1 pg of reconstituted human TF to clot factor VII–deficient plasma in the standard assay conditions.

**Immunization protocol.** RTF (30 μg) was emulsified in Freund’s complete adjuvant (GIBCO, Grand Island, NY) and injected intraperitoneally into the BALB/c mouse on day 1. Two weeks later, the animal received an intraperitoneal booster containing 10 μg RTF emulsified in Freund’s incomplete adjuvant, and a second booster was administered on day 31. On day 60, 2 μg RTF was administered intravenously into the tail vein. On day 64, the mouse was killed, and spleen cells were harvested and fused with AG8 myeloma cells. The fused cells were grown in selective medium to generate hybridoma lines.

**Screens for Anti-RTF Monoclonal Antibodies**

**Enzyme-linked immunosorbent assay.** Culture supernatants were screened for mAbs recognizing RTF by use of a multistep enzyme-linked immunosorbent assay similar to the one developed for use with human TF. Briefly, the wells of an Immulon II microtiter plate were sequentially incubated (inversely with washes) with (1) affinity-purified rabbit antibodies against mouse immunoglobulins (Collaborative Research Inc) in 50 mmol/L sodium bicarbonate buffer (pH 9.5), (2) 5% milk solids in 50 mmol/L Tris-saline (TBS), (3) hybridoma culture media, (4) biotinylated RTF in TBS containing 2% bovine serum albumin and 0.2% Tween 20, and (5) avidin–horseradish peroxidase conjugate (Collaborative Research Inc) in TBS containing 2% bovine serum albumin and 0.2% Tween 20. The bound peroxidase activity was detected using phenylenediamine.23

**Inhibitory activity.** The culture media were incubated with RTF (1 μL culture media mixed with 1 ng repurified purified RTF in 100 μL TBS) at 37°C for 30 minutes, and the inhibition of TF procoagulant activity was measured in a two-stage clotting assay.20 One inhibitory hybridoma, called AP-1, was further expanded, cloned, and stored frozen.

**Purification and Characterization of AP-1**

Larger quantities of antibody were obtained by production of intraperitoneal tumors in mice that had been primed at least 1 week previously with an intraperitoneal injection of 0.5 mL Pristane (Aldrich). The ascites fluid was centrifuged at 3000g for 15 minutes and filtered through a 0.4–μm filter (Amicon), and the AP-1 was affinity-purified using protein G Sepharose (Pharmacia) and desalted using a PD-10 column (Pharmacia). Protein concentration was determined by a dye-binding assay of Bradford,27 and purity of the protein was assayed by SDS-PAGE.18 IgG subtyping of this clone was carried out using a hybridoma Sub-Isotyping Kit (Calbiochem Corp, La Jolla, Calif).

Endotoxin levels were determined by the limulus amebocyte lysate method using the Etoxate Kit (Sigma Chemical Co, St Louis, Mo) and verified by testing (Associates of Cape Cod, Inc, Woods Hole, Mass). Endotoxin levels of purified AP-1 used in the present study were <0.03 EU/mg.

**Rabbit Model of Thrombosis**

The rabbit model has been described in detail elsewhere.8,26 Briefly, New Zealand White rabbits of either sex were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Anesthesia was maintained during the course of the experiment by an intravenous infusion of ketamine of sufficient strength to abolish the corneal reflex. Through a median incision of the neck, the left or right common carotid artery was exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were inserted into a jugular vein for drug administration and also into a femoral artery for continuous blood pressure monitoring. A segment of the exposed carotid artery was injured by gently squeezing the artery between a pair of rubber-covered forceps. An external plastic constrictor was placed around the damaged site. Carotid blood flow velocity...
was continuously measured by a Doppler flow probe positioned proximal to the constrictor (Fig 1). All animals developed CFV, which was characterized by a gradual decline of flow to almost zero values, followed by a restoration of flow that occurred spontaneously or after gentle tapping of the constrictor. In this model, CFV was related to recurrent thrombosis at the site of the stenosis followed by dislodgment of the thrombus. The CFV pattern was monitored in a group of nine rabbits for 30 minutes. CFV frequency (cycles per hour) and severity (carotid blood flow at its nadir as a percentage of baseline), heart rate, and arterial blood pressure were continuously measured throughout the experiment. An intravenous bolus of AP-1 was then administered at an initial dose of 0.05 mg/kg. This initial dose was determined from extrapolation of in vitro studies. Additional doses were administered to a maximum of 1.5 mg/kg only in those animals in which an effect was not seen within about 20 minutes. All animals were monitored for 2 hours after CFV pattern was abolished.

As a control, in a second group of four animals, CFV pattern was established as described above. After 30 minutes, the effects of administering an unrelated antibody (anti-human fibrin mAb BTC 11-16, British Bio-technology Products, Oxon, UK) was tested intravenously at a dose of 2 mg/kg. After 2 hours, the animals were reinjected with AP-1 as described above.

**Ex Vivo Platelet Aggregation and Prothrombin Time Determination**

To determine whether AP-1 affected platelet function per se, the response to several aggregating agents was evaluated ex vivo both before and after administration of the last bolus of AP-1. Fourteen milliliters of blood was drawn into a syringe containing 1.5 mL of 3.8% sodium citrate and centrifuged at 120g for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). PRP was removed and recentrifuged at 1000g for 5 minutes to obtain platelet-poor plasma (PPP). Platelet aggregation was measured turbidimetrically on a Chronolog aggregometer and recorded on a linear recorder. The aggregometer was calibrated by the use of PRP, and the test was performed on 250 μL PRP in a siliconized cuvette with continuous stirring. The platelet count in the PRP was adjusted to 3×10^7/μL by dilution with PPP as needed. Aggregation was induced in PRP in response to collagen, ADP, ristocetin, the thromboxane analogue U46619, and arachidonate at various concentrations.

To determine the effect of AP-1 administration on prothrombin time, blood was collected using sodium citrate as an

<table>
<thead>
<tr>
<th>Table 1. Effect of AP-1 on Cyclic Flow Variation</th>
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CFV indicates cyclic flow variation.

*All rabbits received an initial dose of AP-1 of 0.05 mg/kg.
†Minutes from last dose of AP-1.
TABLE 2. Effect of a Control Antibody on Cyclic Flow Variation in the Rabbit Model

<table>
<thead>
<tr>
<th>Injured Rabbit</th>
<th>CFV Frequency, cycles/h</th>
<th>Flow Rate, % of the normal rate</th>
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<td>Baseline</td>
<td>After Injection of Control mAb</td>
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CFV indicates cyclic flow variation; mAb, monoclonal antibody.

All rabbits were initially injected with 2.0 mg/kg control mAb. After monitoring the effect of the control mAb, the rabbits were injected with AP-1. Rabbits 1 and 3 received a single injection of 0.05 mg/kg AP-1, and rabbits 2 and 4 received multiple injections up to cumulative doses of 0.15 and 0.2 mg/kg AP-1, respectively.

anticoagulant (3.8%) and centrifuged at 2000g for 10 minutes at 4°C to separate the plasma. Prothrombin time was measured before and after each injection of AP-1 using rabbit brain thromboplastin on the Cascade TM 480 (Helena Laboratories, Beaumont, Tex). All assays were performed in duplicate.

Immunostaining of Rabbit Vascular Tissues

Histological examination of the carotid arteries was performed as described by Wilcox et al3 in four additional animals, of which two did not receive any treatment and the other two were administered AP-1. The injured carotid arteries as well as the normal contralateral carotid arteries were dissected and cleaned by brief immersion in phosphate-buffered saline (PBS) and then maintained in 15% sucrose/isotonic PBS for 6 to 16 hours at 4°C. Tissues were embedded in OCT (methyl methacrylate) compound, quick-frozen in isopentane precooled in liquid nitrogen (−196°C), and stored at −70°C in airtight bags. Sections (10 μm) of the frozen tissues were mounted on polylysine-coated microscope slides and immediately frozen at −70°C.

Immunohistochemistry was performed as follows. Briefly, frozen sections were air-dried for 10 minutes, acetone-treated for 2 minutes, blocked with 10% horse serum for 30 minutes at 37°C, incubated with AP-1 (1 μg/mL in 2% horse serum) for 1 hour at 37°C, washed with 3× PBS, incubated with biotinylated anti-mouse IgG (Vectastain ABC kit, Vector Laboratories, Inc, Burlingame, Calif), diluted in 2% horse and 2% rabbit serum for 30 minutes at room temperature, washed with 3× PBS, incubated with ABC reagent for 30 minutes at room temperature, and color-developed for peroxidase using 0.1% diaminobenzidine tetrahydrochloride in PBS containing 0.5% Triton X-100 and 0.02% H2O2. Sections were counterstained with hematoxylin, dehydrated in graded ethanol, displaced in xylenes, and mounted with Permoun (Fisher Scientific).

Immunohistochemistry of carotid vessels from animals injected with AP-1 was performed as described above except that the first incubation with the primary antibody, AP-1, was omitted.

Statistical Analysis

All values are expressed as mean±SEM. The rate of inhibition of CFV by AP-1 was evaluated by Fisher's extract test. Measurements of hemodynamic variables, platelet aggregation, and prothrombin time were compared before and after administration of AP-1 by one-way ANOVA with a design for repeated measurements, which was followed by Student's t-test for paired samples with Bonferroni's correction when an F value was found to be significant. A value of P<.05 defined significant differences between populations.

Fig 4. Graphs showing effect of AP-1 administration on ex vivo platelet aggregation. Maximum (MAX) platelet aggregation is shown in response to ADP (A), the thromboxane analogue U46619 (B), collagen (C), arachidonate (D), and ristocetin (E). No statistically significant differences were seen.
from a total of 900 hybridoma lines generated from mice immunized with the RTF, one was chosen. The antibody produced by this clone, AP-1, was selected because of its potent neutralizing activity against RTF: 8 ng/mL purified AP-1 gave 50% inhibition of RTF activity in a two-stage clotting assay (Fig 2).

Effect of AP-1 in Rabbit Model of Thrombosis

Recurrent thrombosis was induced in the carotid arteries of nine rabbits by injuring the vessel wall and applying a constrictor at the site of injury. CFV was monitored for 30 minutes to establish a stable baseline. AP-1 was then infused intravenously at an initial dose of 0.05 mg/kg body weight. In five of the nine rabbits, CFV was abolished within 20 minutes after a single injection of this dose of AP-1 (Fig 3). The other four rabbits, which did not respond to the initial dose of AP-1, received additional doses and also showed inhibition of CFV. The maximum cumulative dose of AP-1 required to restore normal blood flow in all nine rabbits was 1.5 mg/kg (Table 1). Normal blood flow continued for the duration of the experiment, which was terminated after 2 hours.

A control experiment was performed with an mAb that showed no inhibitory activity in the two-stage coagulation assay against RTF. Injection of this mAb into injured rabbits at the relatively high dose of 2 mg/kg body weight had no effect on CFV, whereas a subsequent injection of AP-1 abolished them (Table 2). Thus, the antithrombotic effect of AP-1 appears to result from its inhibitory activity against RTF rather than from nonspecific effects.

To test for possible systemic effects of AP-1, which may predispose the rabbit to an increased risk of bleeding, prothrombin times (in seconds) were measured on blood samples obtained before and after injection of AP-1 and were not statistically different (8.0±0 versus 8.4±4 seconds). Thus, the dose of AP-1 sufficient to eliminate CFV in injected rabbits produced no detectable systemic effect on coagulation.

Since it has been shown that inhibition of platelet function usually results in elimination of CFV in this model, so ex vivo platelet aggregation in response to various agonists was studied in plasma samples obtained from rabbits before and after infusion of AP-1. No differences were observed after the administration of AP-1 in platelet aggregation in response to ADP, collagen, arachidonate, U46619, or ristocetin (Fig 4). Thus, AP-1 at the dose used in the present study does not affect platelet function.

Immunohistochemical Localization of TF in Rabbit Carotid Vessel Using AP-1

Carotid vessels were studied by immunohistochemistry to map the distribution of RTF in the vessel wall. RTF was found predominantly in the adventitia in both the injured and noninjured blood vessels. TF was not detectable in the intima, including the endothelium, or media of the vessel (Fig 5). These results are consistent with earlier reports.5-7,12

In the two animals injected with AP-1 before death, binding of the mAb to TF in the adventitia was seen in both the normal and injured vessels, as demonstrated...
Discussion

The present study demonstrates that activation of the extrinsic coagulation pathway plays an important role in the formation of intravascular thrombi and that AP-1, an mAb against rabbit TF, is a potent antithrombotic intervention in this model.

The rabbit model of thrombosis used in the present study is based on a model developed by Folts et al.\(^1\) that was originally designed for dogs and later adapted for rabbits.\(^8\) This model allows a reproducible pattern of recurrent thrombosis to be established before testing potential antithrombotic agents. A key feature of the model is the provision of an internal control for each animal. The sequential cycles of thrombus formation and dislodgment at the site of induced arterial injury causes CFV, which can be monitored accurately with a Doppler flow probe positioned proximal to the injury. Histological studies on arterial tissue samples and studies with radiolabeled platelets have demonstrated that the CFV pattern is a reliable indicator of the presence of thrombus at the site of injury.

The agents tested for antithrombotic activity in the present study and in four other animal studies\(^1-14\) (Table 3) inhibit an early step of the extrinsic coagulation pathway involving the activation of factor X by the TF–factor VII/VIIa complex. Inhibition of this step offers two major advantages over blocking later steps in the coagulation pathway. One is that the TF–factor VII/VIIa complex is probably formed only at the site of arterial injury, where it remains membrane associated; another is that the coagulation pathway involves a rapidly amplified cascade of proteolytic reactions that would necessitate, at a late step in the cascade, the use of relatively high doses of an antithrombotic agent to exert a significant inhibitory effect.

![Image](https://example.com/image1.png)

**Fig 6.** Photomicrograph showing localization of anti-tissue factor monoclonal antibody AP-1 (arrowed) to the adventitia (A) in carotid vessels of animals injected with AP-1. Biotinylated anti-mouse IgG (Vector Laboratories) was used for localization of mouse antibody. M indicates media; L, lumen.

![Image](https://example.com/image2.png)

**Fig 7.** High-power photomicrograph of injured stenosed vessel stained with Verhoeff's stain for elastic tissue (arrowed ET). T indicates thrombus; A, adventitia. Note the severe disruption of the vessel wall. This photomicrography represents the most extreme form of injury in this model. It demonstrates exposure of tissue factor to the circulating blood.
In normal blood vessels, TF is synthesized by cells in the adventitia.\textsuperscript{3-7} In atherosclerotic vessels, TF is also produced by foam cells, monocytes, and mesenchymal-like cells within the plaque,\textsuperscript{5} suggesting that TF in the core of an atherosclerotic plaque might contribute to thrombosis associated with plaque rupture. A previous study has shown that administration of an mAb to TF had an antithrombotic effect in an everted rabbit femoral artery preparation.\textsuperscript{11} A second study reported that a recombinant lipoprotein-associated coagulation inhibitor resulted in prevention of rethrombosis after thrombolysis in dogs in which the thrombus was induced by electrical injury to the endothelium.\textsuperscript{12} These important studies have limitations because of the unphysiological experimental conditions used to induce intravascular thrombosis.

In the present study, which we regard as an extension of those quoted above, conditions were produced to model situations seen clinically. Immunohistochemistry of the rabbit carotid vessels using AP-1 showed extensive staining of TF in the adventitia, consistent with an earlier report.\textsuperscript{11} Careful histological examination of samples from the damaged vessels revealed an injury extending to the adventitia in some sections (Figs 5 and 7). Therefore, it is possible that adventitial TF was responsible for triggering the extrinsic pathway in this model. Nevertheless, AP-1 was able to block TF-induced thrombosis irrespective of the site and quantity of TF present.

In separate experiments, immunohistochemistry was performed using anti-mouse IgG antibodies to detect AP-1 in injured and noninjured vessels. AP-1 was identified in the adventitia in both vessels of rabbits injected with AP-1 (Fig 6) but not in the rabbits injected with the control antibody. This result suggests that intravenously injected antibody not only blocks the TF in the damaged inner layers but also binds to the adventitial TF. Thus, the intact IgG molecule can infiltrate either through the vasculature or vasa vasorum and reach the target within minutes, thus possibly avoiding the need for local delivery.

The antithrombotic agent used for the present study was AP-1, an mAb that was generated against rabbit TF. AP-1 has a high specific affinity for RTF, and it blocks the extrinsic blood coagulation pathway in an in vitro assay system. It was possible to completely prevent thrombosis in our rabbit model by injecting doses of AP-1 ranging from 0.05 to 1.5 mg/kg, which are considerably lower concentrations than required in earlier studies with a polyclonal anti-RTF antibody\textsuperscript{13} or anti-human TF mAb\textsuperscript{11} (Tables 1 and 3). The high potency and specificity of AP-1 probably accounts for its antithrombotic effect in the absence of concomitant systemic effects, as demonstrated by the absence of changes in both prothrombin time and platelet aggregation. Thus, normal hemostasis and platelet function are maintained during the treatment with AP-1 in contrast to the systemic effects seen
with TF pathway inhibitor\textsuperscript{12} and other antiplatelet agents. Even within the low range of the antibody used, there was a 30-fold variation in the dose. This is probably due to the quantity of TF present at the site of injury. Larger quantities of TF require higher doses of the antibody for neutralization.

In previous studies,\textsuperscript{11,12} inhibitory reagents were infused over the duration of the study or before the thrombus had formed. In the present study, AP-1 was injected once a stable baseline thrombus had formed. This strategy of inhibiting thrombi once formed is important for potential clinical applications. The present model more closely mimics conditions seen in humans than the published studies.\textsuperscript{11,14} It was designed to simulate the spontaneous rupture of an atherosclerotic plaque as might occur in patients with unstable angina or myocardial infarction. It is our hypothesis that such vascular injury exposes TF in the subendothelium or even in the adventitia, which then initiates the coagulation cascade. In support of this hypothesis, we were able to block continuing thrombosis in the rabbit at the site of vascular injury by the infusion of the anti-TF mAb. Previous work by Weiss et al\textsuperscript{12} supports the hypothesis that subendothelial TF is the essential initiator of thrombus formation in injured blood vessels. Using human umbilical vein segments, they demonstrated that removal of the endothelium is necessary for clotting to occur on the vessel surface and that thrombus formation is blocked by preexposure of the damaged vessel surface to an anti-TF mAb. Although we were unable to detect TF in the subendothelium, intima, or media by immunohistochemical technique, we believe that this was probably due to the lower sensitivity of the detection technique.

To summarize, infusion of low doses of anti-RTF mAb completely inhibited thrombus formation in all rabbits tested without apparent systemic effects. Thus, the administration of a neutralizing anti-TF mAb promises to be an effective antithrombotic strategy in vivo and would be ideal for inhibiting thrombosis in patients with unstable angina. The present study confirms the role of TF in triggering thrombosis via the extrinsic coagulation pathway.

**Acknowledgments**

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