Thromboresistance of Balloon-Injured Porcine Carotid Arteries After Local Gene Transfer of Human Tissue Factor Pathway Inhibitor

Pierre Zoldhelyi, MD; Janice McNatt; Harnath S. Shelat, MS; Yasutaka Yamamoto, MD, PhD; Zhi-Qiang Chen, MD; James T. Willerson, MD

Background—Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of factor Xa and the coagulant initiator complex tissue factor/factor VIIa.

Methods and Results—To study the effects of TFPI gene transfer on thrombus formation, balloon-injured porcine carotid arteries were treated locally with an adenovirus encoding human TFPI (Ad-TFPI) or control virus. Gene transfer of TFPI was confirmed by detection of human TFPI in the conditioned medium of porcine carotid arteries kept in culture after in vivo transduction. When carotid flow was measured with Doppler probe 5 days after surgery, cyclic flow variations (CFVs) developed in 7 of 8 control pigs after constriction of the injured carotid artery by 40%, and all control-treated arteries occluded after 70% constriction. In contrast, CFVs were observed in only 1 of 8 Ad-TFPI–treated pigs after 40% constriction, and only 3 of 8 occluded after constriction by 70% (P=0.0027 and P=0.007, respectively). None of the 5 TFPI-transduced arteries open after 70% constriction developed CFVs during an incremental epinephrine infusion.

Conclusions—Compared with baseline, systemic hemostatic variables and platelet aggregation were unimpaired, suggesting that TFPI gene transfer can prevent arterial thrombosis in the presence of severe shear stress and without detectable hemostatic impairment. (Circulation. 2000;101:289-295.)

Key Words: angioplasty ■ cerebrovascular disorders ■ coagulation ■ genes ■ thrombosis

Tissue factor, a transmembrane protein receptor, is the cellular initiator of thrombin generation and blood coagulation in hemostasis and thrombosis. After its exposure following vessel injury or cytokine activation, tissue factor binds to circulating factor VIIa and, in the extrinsic pathway of blood coagulation, activates factor X, which in the prothrombinase complex converts prothrombin to thrombin.1 Tissue factor–driven thrombin generation plays a pivotal role in thrombosis, possibly in restenosis after percutaneous revascularization, and contributes to the thrombogenicity of the atherosclerotic plaque.2,3

Recently, recombinant tissue factor pathway inhibitor (rTFPI) has been studied in animal models as an approach to the prevention of thrombosis and restenosis after arterial injury.4–6 At physiological concentrations, TFPI binds to factor Xa, and this complex associates with and inhibits tissue factor/factor VIIa. Higher concentrations of TFPI can inhibit tissue factor/factor VIIa in the absence of factor Xa.7 Although attractive in principle, it is uncertain whether short-term administration of rTFPI will achieve lasting vasoprotection after percutaneous revascularization, particularly at sites of increased tissue factor burden.4,8 Moreover, the systemic doses of recombinant TFPI capable of preventing arterial thrombosis and potentially restenosis are substantial (100 μg · kg⁻¹ · min⁻¹ in Reference 6) and may entail significant hemorrhagic risks.

Here, we investigated in a porcine model whether local gene transfer of human TFPI can prevent platelet-driven thrombosis and flow reduction at sites of severe carotid injury and increased shear stress and whether antithrombotic protection would occur without systemic hemostatic impairment.

Methods

Preparation of Recombinant Adenovirus

The cDNA encoding human TFPI was ligated into the shuttle plasmid pACCMVpLpA9 and cotransfected into 293 cells with the rescue plasmid pJM17 (Microbix Biosystems). The recombinant adenovirus Ad-TFPI was plaque purified, as established by polymerase chain reaction of viral plaques and by an ELISA for human TFPI in the conditioned medium of the 293 cells infected with individual clones of Ad-TFPI or Ad-RR, a viral construct identical to Ad-TFPI but lacking a foreign gene. High-titer adenovirus was purified from 911 cells (IntroGene) with modification of a previously described procedure,10 including a 30-minute digestion with benzonase (100 U/mL, American International Chemical) and the addition of 2 CsCl (density 1.34 mg/mL) equilibrium centrifugation steps at 180 000g...
for 6 hours at 4°C. Purified virions were suspended in sucrose (2% wt/vol) and MgCl₂ (2 mmol/L) in PBS, desalted by sepharose CL-4B chromatography (Pharmacia), supplemented with 5% glycerol, and stored at −80°C. The concentration of infectious viral particles was determined as described elsewhere. Ad-RR was prepared and titrated in identical fashion. Viral preparations used were endotoxin free (<0.10 EU/mL) in a limulus amoebocyte lysate assay (BioWhittaker).

### Sensitivity of Porcine Plasma Clotting to Human rTFPI

The sensitivity of porcine plasma clotting to human rTFPI was studied in a prothrombin time (PT) assay. Because we do not have porcine tissue factor (rTFPI) and human (Dako, Mississauga, Ont, Tex) thromboplastin was used as clotting initiator. rTFPI was diluted in PBS, and 1 µL different dilutions was incubated for 1 minute at 37°C with 100 µL porcine or pooled human plasma, respectively. PT was determined in a fibrometer (BBL FibroSystem) by addition of 200 µL thromboplastin to 100 µL plasma. Simultaneously, the fibrrometer was started, and the time to clot formation was recorded. PBS 1 µL without added rTFPI was used as negative control.

### Gene Transfer of TFPI in a Porcine Carotid Balloon Injury Model

Repeated attempts to demonstrate human TFPI with immunohistochemistry in Ad-TFPI–infected porcine carotid arteries failed because of unresolved background problems. To evaluate TFPI production by the transduced artery, we used an ex vivo arterial culture technique. Initially, we determined whether the TFPI ELISA used (American Diagnostica) detected porcine TFPI. Because TFPI circulates in the blood, we analyzed citrated plasma from 5 pigs for the presence of endogenous TFPI. Heparin (200 U/kg), which promotes the endothelial release of TFPI, was administered to 3 pigs before blood was drawn. Plasma samples from 5 volunteers were tested as control.

A viral titer of 6×10⁶ pfu/mL has previously been shown to yield robust transgene expression in balloon-injured porcine carotid arteries. Therefore, after balloon angioplasty in the carotid arteries of 2 Yorkshire pigs, Ad-TFPI 6×10⁶ pfu/mL was delivered by 30-minute dwell to the injured vessel. The arteries were harvested after 24 hours, cut into 3-mm rings, and cultured ex vivo for an additional 4 days in DMEM supplemented with 10% FBS. Five days after angioplasty, the concentration of human TFPI in the conditioned medium from individual arterial rings was determined by ELISA. Ad-RR was prepared and titrated in identical fashion. Viral preparations used were endotoxin free (<0.10 EU/mL) in a limulus amoebocyte lysate assay (BioWhittaker).

Under institutionally approved protocols, TFPI gene transfer was studied in an established porcine carotid balloon injury model. Sixteen male Yorkshire pigs were sedated, intubated, and anesthetized with isoflurane, followed by performance of a femoral cutdown. Blood was drawn into 3.8% sodium citrate (1:9 vol/vol) for aggre-

### Assessment of Antithrombotic Effects of Local TFPI Gene Transfer

Five days after balloon injury, the pigs were reanesthetized with isoflurane. Femoral vein blood was collected for assessment of platelet aggregation and hemostatic variables. The damaged carotid artery was exposed, and 2-0 silk suture was applied as external loop around the center of the injured carotid artery. After CBV was recorded for 30 minutes with no tension on the silk loop, the loop was tightened until CBV decreased by 40% and 70% relative to values before angioplasty. At each stage, CBV was recorded for 30 minutes, and the number and severity of cyclic flow variations (CFVs) were recorded. CFVs were considered mild if CBV was reduced to 70% to 95% of the preangioplasty baseline, moderate if between 25% and 70%, and severe if <25%.

If CFVs progressed to zero CBV, the pigs were killed and pressure perfusion fixed with 10% buffered formaldehyde, and the carotid arteries were harvested for histological analysis. If the pigs did not develop CFVs after 70% constriction, epinephrine was infused to increase local shear stress and platelet activation. Epinephrine was administered at doubling rates from 1.5 to 38 µg/min (10 minutes per dose). In the absence of irreversible zero flow, the infusion was continued for a total of 30 minutes at 38 µg/min before the pig was killed. The constricting suture was left in situ to demonstrate the degree of arterial constriction and folding. Arteries were histologically evaluated by an experimental cardiovascular pathologist (Dr Angela Hughes, Texas Heart Institute, Houston, Tex).

### Whole-Blood Aggregation Studies

Whole-blood aggregation was measured as electrical impedance (in ohms) in a Chronolog aggregometer (Chronolog) at the beginning of surgery (baseline) and the time of the thrombotic challenge (day 5). Blood was drawn into 3.8% sodium citrate (1:9 vol/vol) for aggregation to a/β thrombin (Fibrix, OrthoDiagnostic Systems) or sodium heparin (2 U/mL) for aggregation to adenosine diphosphate (Sigma), collagen (Hormon Chemie), and the thromboxane A₂ analogue U46619 (Cayman Chemical). Activated coagulation times (ACT), PT, and activated partial thromboplastin time (aPTT) were measured in a Hemochron-80 Dual Coagulation System (International Technidyne). Ear skin bleeding times (BTs) were measured before balloon angioplasty (day 0) and on day 5 by use of a Simplex II device (Organan Teknika).

### Statistical Analyses

Comparisons of the means of CBVF and the number of CFVs between Ad-TFPI– and control-treated pigs were performed by use of a 2-sided unpaired t test. P <0.05 was considered statistically significant. Values are given as mean ±SD.

### Results

Twenty-four hours after carotid balloon angioplasty and local delivery of Ad-TFPI at 6×10⁶ pfu/mL to 2 pigs, the injured and contralateral carotid arteries were harvested, cut in about 3-mm-long rings, and kept in individual wells in culture for an additional 4 days. Five days after delivery of Ad-TFPI, human TFPI was detected by ELISA in the conditioned medium of all but 1 arterial ring in each of the 2 arteries (Figure 1). No TFPI was detected in the conditioned medium of uninjured control arteries.
The ELISA was tested for its ability to detect endogenous TFPI in porcine blood. Whereas TFPI was detected in all 5 human plasma samples (49.4±21.7 ng/mL), none was detected in the porcine plasma samples drawn with (n=3) or without (n=2) prior heparin administration. The sensitivity of porcine plasma to rTFPI in a modified prothrombin assay (see Methods) was assessed by use of rabbit or human thromboplastin as clotting initiator. Clotting of porcine plasma with human rTFPI added ex vivo was prolonged more than human plasma, suggesting that porcine factors VII/Va and Xa were at least as sensitive to human rTFPI as the respective human factors (Figure 2).

No differences were observed in ACT, PT, aPTT, and BT in the pigs, which were alternatively assigned to treatment with Ad-TFPI (n=8; weight, 34.9±3.5 kg) and Ad-RR (n=8; weight, 37.9±4.4 kg) (Table 1). Compared with pigs assigned to Ad-RR, platelet aggregation to thrombin at baseline was significantly greater in pigs assigned to treatment with Ad-TFPI (Table 2). Aggregation to all other agonists was not significantly different. After completion of angioplasty, CBFV decreased to a similar extent in control vector– and Ad-TFPI–treated arteries, from 16.4±3.3 to 10.5±5 and 17.1±2.2 to 10.7±3.1 kHz, respectively.

Five days after surgery, Doppler flow probes were applied to the injured carotid artery segments (see Methods), and CBFVs were recorded continuously until the pigs were killed. CFVs were observed before external constriction of the artery in 4 of the 8 control vector–treated pigs and in 1 of the 8 pigs treated with Ad-TFPI. Constriction of the arteries by 40% precipitated severe CFVs in 7 of 8 Ad-RR–treated pigs but only 1 Ad-TFPI–treated pig (P<0.0027). After further constriction by 70%, all control vector–treated animals after constriction of the injured arteries. In contrast, blood flow was preserved in all TFPI-transduced vessels after constriction by 40% and in 5 of 8 Ad-TFPI–treated pigs after carotid artery constriction by 70%.

Figure 4 shows Ad-RR– and Ad-TFPI–treated porcine carotid arteries. Because the sections analyzed include those obtained at the level of the constrictor and sections distal and proximal to it, arterial sections with both conserved and distorted architecture were observed. A section through the nonconstricted segment of the Ad-TFPI–treated artery illustrates the severity of balloon injury, resulting in disruption and effacement of the internal elastic membrane.

TABLE 1. Hemostatic Variables in Pigs Treated With Ad-RR and Ad-TFPI

<table>
<thead>
<tr>
<th></th>
<th>ACT, s</th>
<th>PT, s</th>
<th>aPTT, s</th>
<th>BT, s</th>
</tr>
</thead>
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<tr>
<td>Ad-RR, day 0</td>
<td>104±9</td>
<td>12±1</td>
<td>22±1</td>
<td>31±5</td>
</tr>
<tr>
<td>Ad-RR, day 5</td>
<td>109±9</td>
<td>12±1</td>
<td>22±1</td>
<td>41±34</td>
</tr>
<tr>
<td>Ad-TFPI, day 0</td>
<td>108±6</td>
<td>12±1</td>
<td>22±0</td>
<td>41±34</td>
</tr>
<tr>
<td>Ad-TFPI, day 5</td>
<td>101±11</td>
<td>12±1</td>
<td>22±0</td>
<td>33±18</td>
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</tbody>
</table>

To measure ACT, PT, and aPTT, blood was collected from the internal jugular vein (day 0) and femoral vein (day 5). BT was determined as ear skin bleeding time, using a Simplex II device as described in Methods. n=8 per group.
Representative CBFV tracings of Ad-TFPI– and Ad-RR–treated pigs are shown in Figure 5. Of the 5 TFPI-transduced carotid arteries without CFVs at 70% constriction, none developed CFVs during an incremental epinephrine infusion to 38 mg/min, equivalent to 1.14 ± 0.1 mg/kg21± min21 epinephrine.

Coagulation variables and BT were not significantly different between the treatment groups and between baseline (day 0) and day 5 (Table 1). Except for thrombin, whole-blood aggregation to agonists was unchanged at all concentrations (Table 2). Platelet aggregation to thrombin, which was higher before surgery in pigs assigned to Ad-TFPI, was not different between the 2 groups on day 5.

Discussion

We report here that local transduction of porcine balloon-injured carotid arteries with a single administration of the TFPI gene exerts protection against thrombosis even when epinephrine is infused during the thrombotic challenge. These observations confirm the important role of coagulant protease complexes upstream of thrombin in the pathogenesis of injury- and shear-dependent thrombosis and illustrate the antithrombotic efficacy of gene transfer techniques targeting tissue factor and factor Xa in the damaged vessel wall.

CFVs in stenosed arteries reflect the dynamic process of growth and embolization of platelet-rich thrombi, with the rate of flow reduction being proportional to the rate of platelet accumulation in the narrowed lumen,15,16 and have been observed in patients during the course of acute coronary syndromes.17,18 The nadir of flow during periods of cyclic flow reduction, often to zero flow, coincides with the presence of obstructive platelet-rich thrombi,15,16 as is also apparent in our study, in which obstructive thrombi were seen in all cases of zero flow. The platelet dependency of CFVs is stressed by their exquisite sensitivity to antiplatelet agents.18,19 In contrast, the spontaneous reduction in CBFV at the time of balloon injury is believed to represent vasoconstriction, to which platelet-derived mediators substantially contribute.20

Locally expressed TFPI was particularly effective against platelet-mediated thrombosis in the presence of increased shear stress and the additional challenge of an epinephrine infusion. These finding may have relevance given the strong

<table>
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<tr>
<th>Aggregation, %</th>
<th>Ad-RR Day 0</th>
<th>Ad-RR Day 5</th>
<th>Ad-TFPI Day 0</th>
<th>Ad-TFPI Day 5</th>
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</thead>
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<tr>
<td>ADP, μmol/L</td>
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<tr>
<td>50</td>
<td>82±17</td>
<td>90±11</td>
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<td>100</td>
<td>89±16</td>
<td>87±13</td>
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<td>93±8</td>
<td>96±7</td>
<td>87±5</td>
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<td>Collagen, μg/mL</td>
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<td>2</td>
<td>93±21</td>
<td>82±27</td>
<td>92±12</td>
<td>94±16</td>
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<td>5</td>
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<td>106±15</td>
<td>113±26</td>
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<td>115±10</td>
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<td>Thrombin, IU/mL</td>
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</tr>
<tr>
<td>2.5</td>
<td>1±3</td>
<td>34±30*</td>
<td>49±12§</td>
<td>44±33</td>
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<tr>
<td>5.0</td>
<td>26±20</td>
<td>73±19†</td>
<td>75±19†</td>
<td>93±15</td>
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<tr>
<td>7.5</td>
<td>59±20</td>
<td>78±27</td>
<td>83±9</td>
<td>90±15</td>
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<tr>
<td>U46619, ng/mL</td>
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<td></td>
</tr>
<tr>
<td>50</td>
<td>43±7</td>
<td>33±27</td>
<td>33±20</td>
<td>19±25</td>
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<tr>
<td>100</td>
<td>47±8</td>
<td>44±22</td>
<td>46±17</td>
<td>43±20</td>
</tr>
<tr>
<td>200</td>
<td>52±11</td>
<td>57±22</td>
<td>57±19</td>
<td>58±9</td>
</tr>
</tbody>
</table>

*P=0.03 and †P=0.0002 vs day 0; §P=0.05 and ¶P=0.004 vs pigs that received Ad-RR.

| Table 3. Number and Severity of CFVs and Incidence of Thrombus 5 Days After Balloon Injury |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| CFVs                           | Nonconstricted | 40% Stenosis    | 70% Stenosis    | Histologically |
|                                | Mild | Moderate | Severe | Mild | Moderate | Severe | Mild | Moderate | Severe | Confirmed |
| Ad-RR                          | 3   | 6       | 11     | 6   | 8       | 13     | 0   | 7       | 13     | 8/8       |
| Ad-TFPI                        | 4   | 3       | 0      | 0   | 0       | 2      | 4   | 2       | 11     | 3/8       |

40% and 70% stenosis denote external carotid constriction to reduce blood flow velocity by 40% and 70% relative to that observed before angioplasty. CFVs were considered mild if flow at nadir was reduced to 70% to 95% of the postangioplasty baseline, moderate if reduced to 70% to 25%, and severe if <25%.
induction of tissue factor by shear stress. Added to severe wall damage, constriction functions to increase shear stress and initiate CFVs. Epinephrine exerts additional shear-dependent and -independent actions on platelet aggregation. The antithrombotic efficacy of local TFPI gene transfer in the presence of heightened shear stress is not unexpected. Indeed, among all platelet agonists known, thrombin is the most potent, and systemic administration of the direct antithrombin hirudin has been effective in preventing platelet-rich thrombus formation in pigs, in other animal species, and in the course of acute coronary syndromes in patients. Because of the dual function of TFPI as a direct inhibitor of factor Xa and as an inhibitor of the catalytic tissue factor/VIIa complex, we are unable to distinguish between the relative role of factor Xa and tissue factor/factor VIIa as a target of TFPI gene therapy. Nonetheless, studies of specific factor Xa inhibitors and tissue factor–blocking antibodies have demonstrated that both factor Xa and tissue factor play essential roles in arterial thrombogenesis.

The timing for the testing the efficacy of TFPI gene therapy (ie, 5 days after injury) warrants discussion. Our previous observations in conscious pigs, in which carotid flow was continuously recorded for 10 days, indicate that in this model CFVs begin to fade 3 to 6 hours after their initiation by severe vascular injury. In unprotected vessels, CFVs recur after a period of quiescence of 2 to 3 days and rapidly increase in number and severity to peak 4 to 8 days after the initial insult. In agreement with these earlier observations, the incidence of total occlusion (zero flow) in our study was 100% in the control (Ad-RR)–treated pigs on day 5. Approximately 10 days after balloon injury, CFVs spontaneously fade, concurrent with endothelial healing at the site of injury.

The antithrombotic efficacy of local TFPI gene therapy compares favorably with the dose requirement of systemically administered recombinant TFPI, which falls in the milligram-per-hour range. The relative inefficacy of circulating TFPI vis-à-vis severe arterial thrombosis is also reflected by the observation that unrelieved coronary thrombosis often occurs despite elevated plasma levels of endogenous TFPI. On the other hand, the presence of TFPI in the low picogram-per-milligram range in human carotid atherectomy specimens was shown to attenuate local tissue factor activity measured ex vivo in these specimens, consistent with the efficacy of TFPI expressed in situ.

Systemic hemostatic impairment after antithrombotic drug interventions, especially those targeting thrombin, has limited the dosage of antithrombotic interventions. Not surprisingly, Oltrona et al found that systemic administration of rTFPI led to markedly prolonged PT in pigs, suggesting that rTFPI at doses preventing arterial thrombosis is associated with substantial systemic bleeding risk. Lack of hemorrhagic complications and unimpaired platelet aggregation, BT,
plasma coagulation at the time of the thrombotic challenge in this study contrast those observations and extend our previous studies with COX-1 gene transfer, demonstrating that in a large animal species local antithrombotic gene therapy can be highly effective in reducing thrombosis at sites of deep arterial injury in the absence of detectable hemostatic perturbation.11

Although inflammation and thrombosis are in general associated phenomena, the influences of the adenoviral vectors on local thrombogenesis are difficult to assess. Directly related to this issue are reports suggesting that exposure to adenoviral vectors of uninjured vessels in rabbits promotes thrombus formation,28 arterial neointimal formation,29 and inflammation of experimental vein grafts.30 This issue is compounded by the uncertain purity of viral batches from different laboratories preparing non–clinical-grade vectors (eg, the concentration of mycoplasma and other contaminants) and the tissue and species dependency of viral-host interactions.

As in our previous study,11 no differences were observed in the degree of inflammation between Ad-TFPI–treated and Ad-RR–treated (control) porcine carotid arteries. Given the lack of a buffer-only control group in the present study, however, we are unable to assess the contribution of adenoviral vectors to the inflammatory response triggered by severe balloon injury, thrombosis, and external manipulation of the vessel. Nonetheless, in our previous study, the incidence of histological thrombosis, CFVs, and degree of inflammation were not different between control buffer–treated (mock) and control (null) vector–treated arteries.11 Thus, published data, including our own, suggest that adenoviral vectors may either aggravate thrombosis and neointimal formation or, in the porcine carotid injury model, have no discernable prothrombotic effect.

In conclusion, our study of local TFPI gene transfer to balloon-injured carotid arteries offers new support for the important role of tissue factor/factor VIIa and its downstream proteases in the pathogenesis of platelet-mediated thrombosis. We demonstrate that a single local administration of a vector encoding human TFPI inhibits platelet-dependent thrombosis at sites of severe vascular injury and increased shear stress and that vasoprotection occurs without detectable hemorrhagic risk. Potentially, this approach may serve as a strategy to reduce the thrombotic risk after percutaneous revascularization interventions without engendering hemostatic impairment and bleeding risk. Newer vectors and the use of species-specific TFPI may extend the duration of antithrombotic protection.

Acknowledgments
Supported in part by NIH grants HL-50179 and HL-54839; the Keith Acton Meadows Foundation, Dallas, Tex; and a Texas state grant. We thank Dr Tze-Chein Wun, Searle/Monsanto Corporation, Chesterfield, Mo, for the gift of rTFPI and the cDNA encoding human full-length TFPI; Dr Robert D. Gerard (University of Michigan, Ann Arbor) for the shuttle plasmid pACCMVpLpA and control vector Ad-RR; and IntroGene, Inc (Leyden, the Netherlands) for the gift of 911 cells.

References


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Circulation. 2000;101:289-295
doi: 10.1161/01.CIR.101.3.289

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

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