Adenovirus-Mediated Local Expression of Human Tissue Factor Pathway Inhibitor Eliminates Shear Stress–Induced Recurrent Thrombosis in the Injured Carotid Artery of the Rabbit

Takahiro Nishida, Hikaru Ueno, Nobuhiko Atsuchi, Ryuji Kawano, Yujiro Asada, Yo Nakahara, Yu-ichi Kamikubo, Akira Takeshita, Hisataka Yasui

Abstract—The main cause of acute coronary syndrome may be recurrent thrombosis, which is initiated by the activation of the extrinsic coagulation pathway. Tissue factor (TF) pathway inhibitor (TFPI) efficiently inhibits an early step in this pathway by the formation of a complex with factor VIIa, TF, and factor Xa. We determined whether local TFPI gene transfer can inhibit thrombosis in an injured artery without inducing systemic side effects. Balloon-injured rabbit carotid arteries were infected with an adenoviral vector that expressed either human TFPI (AdCATFPI) or bacterial β-galactosidase (AdCALacZ). Two to 6 days after gene transfer, thrombosis was induced by the production of constant stenosis of the artery, and blood flow was measured continuously with an electromagnetic flow probe. A cyclic flow variation, which is thought to reflect the recurrent formation and dislodgment of mural thrombi, was observed in all AdCALacZ-infected arteries as well as in saline-infused arteries. In contrast, no cyclic flow variation was detectable in AdCATFPI-transfected arteries, even in the presence of epinephrine (1 μg · kg⁻¹ · min⁻¹ infusion). Prothrombin time, activated partial thromboplastin time, and the ex vivo platelet aggregation induced by either adenosine diphosphate or collagen were unaltered in AdCATFPI-infected rabbits. We found that in vivo TFPI gene transfer into an injured artery completely inhibits the recurrent thrombosis induced by shear stress even in the presence of catecholamine, without affecting systemic coagulation status. Adenovirus-mediated local expression of TFPI may have the potential for the treatment of human thrombosis. (Circ Res. 1999;84:1446-1452.)

Key Words: inhibitor ■ thrombosis ■ blood flow ■ gene transfer ■ adenovirus vector

A direct cause of acute coronary syndrome is believed to be thrombotic occlusion of an atherosclerotic coronary artery.1 After balloon angioplasty and stent insertion, occlusion by thrombosis still occurs occasionally, despite the use of antithrombotic agents.2 Thus, inhibition of thrombus formation in the damaged artery is vital for the prevention of a lethal outcome. Current approaches against intravascular thrombosis rely on the systemic administration of drugs such as aspirin, heparin, hirudin, tissue plasminogen activator, and a chimeric antibody to the platelet glycoprotein IIb/IIIa integrin.3 Unfortunately, these agents can produce systemic side effects, most notably the induction of bleeding at locations distant from their intended site of action.4,5 Also, the antithrombotic effects of these drugs, such as aspirin, tend to weaken in the presence of catecholamines.5

Activation of the extrinsic coagulation pathway triggered by the binding of plasma protease factor VIIa/VIIa to the transmembrane protein tissue factor (TF) is considered to be the event that initiates blood coagulation.6 When bound with factor VIIa, TF efficiently converts factors X and IX to their active forms and leads to the generation of thrombin. Intact endothelial cells produce an anti-TF molecule, known as tissue factor pathway inhibitor (TFPI). TFPI directly inhibits the factor Xa and induces a feedback inhibition of the factor VIIa/TF catalytic complex.7,8 Thus, TFPI efficiently inhibits the upstream events in the coagulation pathway at multiple stages and suppresses thrombin formation.7,8

It has been shown repeatedly that adenovirus-mediated gene transfer into the cells of the vascular wall can elicit site-specific production of recombinant protein for a prolonged period of time.9-11 If an antithrombotic agent, such as TFPI, could be locally produced in damaged arteries as a result of gene transfer, this could prove to be both more potent and less harmful as a treatment for intravascular thrombosis than the systemic administration of antithrombotic or fibrinolytic drugs.

In the current study with balloon-injured rabbit carotid arteries, we tested whether local expression of TFPI in an
injured artery by adenovirus-mediated in vivo gene transfer would effectively inhibit thrombosis. We observed that local expression of TFPI completely eliminated thrombus formation without the induction of any apparent systemic side effects. The inhibition was not affected by the presence of epinephrine. Our results suggest a therapeutic potential for adenovirus-mediated transfer of TFPI in cases of acute coronary syndrome in humans.

Materials and Methods

Preparation of Adenoviral Vector

Replication-defective E1- and E3-adenoviral vectors that express human TFPI (AdCATFPI), bacterial β-galactosidase (AdCALacZ), or no exogenous gene (AdCA1w) under a CA promoter (composed of a cytomegalovirus enhancer and chicken β-actin promoter) were prepared by in vitro homologous recombination in 293 cells by the use of the adenovirus DNA-terminal protein complex as described previously. The titer of the virus stock was assessed by a plaque-formation assay with the use of 293 cells and expressed as plaque formation units (pfu).

In Vitro TFPI Secretion From Transfected COS Cells

COS cells were infected with AdCATFPI and incubated in growth medium for 24 hours. Then, the medium was replaced with serum-free medium and incubated for another 24 hours. The amount of TFPI protein in the medium was measured by a 1-step sandwich ELISA method with human recombinant TFPI (rhTFPI) as a standard as described previously. The total TFPI antigen level in rabbit plasma was also measured by this method.

TFPI activity was measured by a modified prothrombin time (PT) analysis, as described previously. The TFPI activity was expressed in terms of the amount of rhTFPI that would show the same activity. The minimum level detected by this assay is 265 ng/mL. The activity was also measured by a 2-stage chromogenic assay. The minimum amount of TFPI detected by this method is 5.3 ng/mL. Human recombinant TFPI was prepared from transfected Chinese hamster ovary cells as previously reported.

In Vivo Gene Transfer Into Injured Arteries

All animals were treated under protocols approved by the animal care committee of Kyushu University (Fukuoka, Japan). The experiment was performed in accordance with both the Guidelines for Animal Experiments in Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government. Japanese white rabbits (male, weight 3250 ± 350 g) were anesthetized with 25 mg/kg of sodium thiampylal and ventilated via a tracheal tube connected to a mechanical ventilator. After heparinization (1500 U/kg), a segment of the common carotid artery (~4 cm in length) was isolated with 2 small clips. Then, the common carotid artery was balloon injured 4 times by use of a balloon catheter (3F Fogarty, Baxter). After balloon injury, the isolated segment was filled with 0.2 mL of Ringer’s saline that contained either AdCATFPI or AdCALacZ (there was no fixed order for AdCATFPI- and AdCALacZ-infection; final titer 1.5 × 10^8 pfu) or with this saline alone. Incubation was allowed to proceed for 30 minutes, then the solution was retrieved, and blood circulation was restored. The contralateral common carotid artery was treated according to the same method described above.

We did not notice any significant or consistent histological differences between arteries subjected to balloon injury followed by infusion with saline and arteries given an injury followed by adenoviral infection as previously reported. It has recently been reported that no inflammation or vascular injury is induced in rabbit carotid arteries, if the titer of adenovirus used is below the inflammatory threshold, <1.6x10^7 pfu/mL. If the adenoviral β-galactosidase expression produces neither vascular injury nor infiltration of polynuclear monocytes (CD18-positive neutrophils), the titer we used in this study was 7.5x10^7 pfu/mL.

In Vivo TFPI Secretion

To measure in vivo TFPI secretion from the AdCATFPI-infected arteries, segments (2 cm in length) were harvested 3 days after gene transfer, rinsed, then placed in DMEM with 10% serum for 24 hours at 37°C. Media were assayed for TFPI antigen and activity as described above. Saline-infused and AdCALacZ-infected arteries were also assayed as controls.

Recurrent Thrombosis Model

Recurrent formation and dislodgment of thrombi in the stenotic artery was monitored by the measurement of carotid blood flow in a slightly-modified Fols model (the constriction was applied 2 to 6 days after endothelial denudation). Briefly, 2 or 6 days after gene transfer, a constrictor (0.7 mm in diameter) was placed around the carotid artery: an ~50% reduction in diameter, 75% reduction in area, and 55% reduction in blood flow was generated in both carotid arteries (cf Table 2). A flow probe (FG-020T, Nihon Koden) connected to a flowmeter was set downstream of the stenotic portion of the carotid artery, and cyclic blood flow variation (CFV) was monitored continuously. Heart rate and femoral artery pressure were also monitored. We first measured CFV in the right carotid artery then the left carotid artery. We confirmed that the order of measurement between right and left carotid arteries did not affect the outcome.

Determination of Ex Vivo Platelet Aggregation, PT, and Activated Partial Thromboplastin Time

To check platelet function, the platelet aggregation in response to either ADP or collagen was evaluated ex vivo. Blood (10 mL) mixed with sodium citrate was centrifuged at 120 g for 20 minutes at room temperature. Either ADP (1 to 20 mmol/L) or collagen (0.5 to 20 μg/mL) was added to this sample, and platelet aggregation was measured turbidimetrically by use of a chronolog aggregometer. PT and activated partial thromboplastin time (APTT) were measured with an amelung coagulometer (KC-10A, Baxter) before and after either an AdCATFPI-infection or an administration of rhTFPI (80 μg/kg).

Statistical Analysis

Values of CFV (count) and carotid blood flow among arteries were statistically analyzed with ANOVA. One-way ANOVA with repeated measures was used for the analysis of PT and APTT. A value of P<0.05 was considered significant.

Results

TFPI Secretion From AdCATFPI-Infected Cells

In Vitro

We examined whether AdCATFPI infection can produce biologically active TFPI. COS cells were infected with AdCATFPI at various multiplicities of infection (MOI), then the TFPI in the medium was measured by ELISA. A large quantity of TFPI was detected in the medium from AdCATFPI-infected COS cells, which was the actual amount of MOI-dependent TFPI (Table 1). We also examined the TFPI activity by both a chromogenic method and a modified PT method. TFPI activity was increased in a MOI-dependent manner (Table 1). If the TFPI protein had been truncated, the activity measured by a modified PT method would be expected to be reduced. Thus, the fact that the values measured by the 2 methods were similar may suggest that most of the TFPI secreted was the full-length form. No TFPI antigen or its activity was detectable in medium prepared from AdCALacZ-infected COS cells. These results indicated that a large quantity of biologically active TFPI can
There was no significant difference in carotid blood flow before and immediately after the establishment of stenosis by use of the Folts model. 24 Six days after balloon injury and gene transfer, we measured CFV, which is thought to reflect recurrent cycles of thrombus formation and dislodgment. 25 In contrast, no CFV was detectable in the AdCATFPI-infected arteries (Figure 2). When epinephrine was systemically infused at a dose of 1.0 μg · kg⁻¹ · min⁻¹ for 30 minutes, a procedure which has been reported to abolish the inhibitory effect of aspirin on CFV, 27 systemic blood pressure and carotid blood flow were both increased, but no CFV appeared in the AdCATFPI-infected arteries (Figure 3). When we measured CFV 2 days after injury and gene transfer, similar results were obtained (data not shown).

No Significant Changes in PT and APTT in Plasma of AdCATFPI-Infected Rabbits
To examine the possible systemic effects that result from local infection with AdCATFPI, the coagulation indexes PT and APTT were measured before and 6 days after gene transfer, in which TFPI gene expression should be submaximal. Neither PT nor APTT was altered in the AdCATFPI-infected rabbits (Figure 4). On the other hand, in rabbits subjected to the intravenous injection of rhTFPI (80 μg/kg; with this amount of rhTFPI, CFV was temporarily attenuated to a large extent), both PT and APTT became significantly longer than before injection (Figure 4).

TFPI concentration in blood was monitored in AdCATFPI-infected rabbits. No TFPI was detectable by our assay system in intact rabbits or in rabbits that were injured and infected with AdLacZ (the antibody against human TFPI we used in the assay did not cross-react with rabbit TFPI). In the plasma of the AdCATFPI-infected rabbits (blood was collected 6 days after gene transfer), a small but significant amount of TFPI (8.2 ± 2.5 ng/mL, mean ± SD, n = 6) was detectable. Although the amounts of endogenous TFPI in normal rabbits are unknown due to lack of an appropriate assay system, note that the amounts of TFPI in the AdCATFPI-infected rabbits are considerably lower than those detected in normal healthy humans (52.4 ± 9.9 ng/mL, mean ± SD, n = 30). 17 In contrast, a

be generated and secreted into the medium from AdCATFPI-infected cells.

In Vivo TFPI Secretion
We next investigated whether active TFPI could be produced in AdCATFPI-infected rabbit carotid arteries. TFPI antigen and its activity were measured in 24-hour explant cultures by use of ELISA or a chromogenic assay, respectively. A considerable amount of bioactive TFPI was secreted from the AdCATFPI-infected arteries, although no secretion of TFPI was detectable in the AdCALacZ-infected arteries (Figure 1).

Elimination of Stenosis-Induced Thrombosis in AdCATFPI-Infected Arteries
We investigated whether the local expression of TFPI would effectively inhibit stress-induced thrombosis in injured arteries by use of the Folts model. 24 Six days after balloon injury and gene transfer, we measured CFV, which is thought to reflect recurrent cycles of thrombus formation and dislodgment. 25 There was no significant difference in carotid blood flow before and immediately after the establishment of stenosis among arteries infected with either AdCATFPI or AdCALacZ or infused with saline (Table 2). After stenosis, CFV was induced in the AdCALacZ-infected and in the saline-infused arteries. CFV was also induced in the AdCALacZ-infected arteries (data not shown). These results indicate that neither adeno viral infection itself nor β-galactosidase expression caused CFV. In contrast, no CFV was detectable in the AdCATFPI-infected arteries (Figure 2). When epinephrine was systemically infused at a dose of 1.0 μg · kg⁻¹ · min⁻¹ for 30 minutes, a procedure which has been reported to abolish the inhibitory effect of aspirin on CFV, 27 systemic blood pressure and carotid blood flow were both increased, but no CFV appeared in the AdCATFPI-infected arteries (Figure 3). When we measured CFV 2 days after injury and gene transfer, similar results were obtained (data not shown).

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### Table 1. TFPI Protein and Its Activity in the Medium From AdCATFPI-Infected COS Cells

<table>
<thead>
<tr>
<th>TFPI Secretion, ng/mL</th>
<th>TFPI Activity, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
</tr>
<tr>
<td>Control</td>
<td>nd</td>
</tr>
<tr>
<td>AdCATFPI, moi</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<tr>
<td></td>
<td>300</td>
</tr>
</tbody>
</table>

### Table 2. Blood Flow and Flow Variation Frequency in Injured Carotid Arteries

<table>
<thead>
<tr>
<th>TFPI Concentration</th>
<th>CFV Frequency, cycles/h</th>
<th>Before Ligation</th>
<th>After Ligation</th>
<th>% Reduction of CBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCALacZ</td>
<td>13.4±1.5*</td>
<td>48.6±6.8</td>
<td>26.2±3.0</td>
<td>54.3±4.6</td>
</tr>
<tr>
<td>AdCATFPI</td>
<td>0</td>
<td>56.0±8.4</td>
<td>31.2±8.2</td>
<td>55.0±6.2</td>
</tr>
<tr>
<td>Saline</td>
<td>14.8±1.6*</td>
<td>51.2±5.4</td>
<td>26.8±3.6</td>
<td>52.6±7.0</td>
</tr>
</tbody>
</table>

Balloon-injured carotid arteries were infected with either AdCATFPI or AdCALacZ or infused with saline. Six days after infection, carotid blood flow (CBF) was monitored by a flowmeter for 10 minutes before and immediately after the ligation used to produce a stenosis. CFV was monitored for 1 hour. Mean±SD (n=6) are shown.

*P<0.05.
high concentration of TFPI (330±21.8 ng/mL) was detected 1 minute after a bolus injection of rhTFPI (80 μg/kg).

**Preserved Platelet Function in AdCATFPI-Infected Rabbits**

Because an inhibition of platelet function can result in an elimination of CFV,24 platelet function was examined by use of an ex vivo platelet aggregation assay. The aggregation in response to either ADP or collagen at various concentrations was measured before and 6 days after gene transfer. Platelet-aggregation was not affected in AdCATFPI-infected rabbits nor in rabbits infused with rhTFPI (80 μg/kg; Figure 5).

**Discussion**

The main finding of this study is that local expression of TFPI via adenovirus-mediated single gene transfer achieves a potent (complete) inhibition of shear stress–induced mural thrombosis in injured arteries for a prolonged period of time without alternating the systemic state of coagulation. Thrombin generation and subsequent thrombus formation are critical events that lead to acute occlusion in injured arteries. These responses are initiated by an activation of the extrinsic pathway that results from an encounter between coagulation factors in the blood and TF in the endothelium-stripped...
arterial wall.\textsuperscript{28,29} It is known that TF expression in the arterial wall increases rapidly (within hours) after balloon injury.\textsuperscript{6,29} This event would be expected to be amplified in an atherosclerotic artery, because abundant TF is consistently expressed in the neointima in such vessels.\textsuperscript{28} It was reported that plasma levels of TF and TFPI were significantly elevated in patients with ischemic heart diseases versus control subjects.\textsuperscript{30} All this information suggests that an efficient method of inhibiting TF function may also be an efficient way of suppressing thrombosis. To support this hypothesis, an antithrombotic effect caused by systemic infusion of a monoclonal antibody against TF has been reported in stenotic rabbit carotid arteries.\textsuperscript{31} It has also been reported that an active-site mutated form of factor VIIa efficiently inhibits thrombosis.\textsuperscript{32} These reports further support the concept that TF exposure plays a critical role in the generation of thrombin and the thrombus and that inhibition of TF activity should therefore effectively suppress the resulting thrombosis. With this background in mind, in the current study, we locally expressed TFPI, a physiological suppresser of TF,\textsuperscript{7,8} in balloon-injured arteries through adenovirus-mediated in vivo gene transfer. This may have an advantage over the use of a thrombin antagonist, such as hirudin, because TFPI would be expected to suppress any fresh formation of thrombin by inhibition of the very first steps in the extrinsic\textsuperscript{8,32} and the common coagulation pathways (by a direct inhibition of factor Xa,\textsuperscript{7} although whether this is true in vivo needs further study).

The rabbit model of thrombosis used in the present study is based on a model developed by Folts et al.\textsuperscript{27} This model has been used by many investigators to evaluate clot formation in vivo (See Reference 24). CFVs were also observed in humans after angioplasty.\textsuperscript{33} Histological studies of arteries\textsuperscript{26} and studies with radiolabeled platelets\textsuperscript{34} have demonstrated that CFV is a reliable marker for recurrent thrombus formation. In our study, we measured CFV 2 to 6 days after balloon injury and gene transfer, although in the original Folts model, CFV is monitored immediately after endothelial denudation. Balloon injury evokes many responses including de novo TF production,\textsuperscript{35} proliferation and transformation of smooth muscle cells,\textsuperscript{36} and accumulation of extracellular matrix, which is a major source of TF expression.\textsuperscript{37} For that reason, the arteries we used might have been more prothrombotic and possibly more resistant to antithrombosis agents than the arteries used in the original model. We could argue that we

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Infusion of epinephrine did not induce CFV in AdCATFPI-infected arteries. Carotid arteries were balloon injured and infected with AdCATFPI. Six days later, carotid blood flow was monitored after partial ligation. Then, epinephrine (1 \(\mu g\cdot kg^{-1}\cdot min^{-1}\)) was infused intravenously. Systemic blood pressure monitored at the femoral artery increased in response to the epinephrine. Similar results were obtained in 3 rabbits.}
\end{figure}
tested the inhibitory effect of TFPI gene transfer under more complicated conditions than those created by the original model. In consideration of the above factors, the fact that CFV was completely abolished in the AdCATFPI-treated arteries seems to suggest a potential future clinical application for AdCATFPI. Finally, note that even in the presence of epinephrine, no CFV was generated in AdCATFPI-treated arteries. Because the inhibitory effect of aspirin, which depends on its suppression of platelet function, is lost with epinephrine, these results suggest that the inhibitory effect of TFPI does not depend on platelet function. Rather, TFPI may act on the arterial wall.

A major advantage of the local expression of TFPI through in vivo gene transfer is shown by the fact that no systemic side effects were seen in our rabbits (Figures 4 and 5). In the search for effective ways to prevent or treat intravascular thrombosis, many antithrombotic or profibrinolytic drugs have been administered systemically. Unfortunately, although some of these drugs exerted a significant inhibitory effect on thrombosis, such agents also tended to induce systemic bleeding. This is an important factor in the determination of clinical usefulness and indicates the potential advantage of local gene transfer into an injured artery. On the other hand, note that with gene transfer, some time (at least a few hours) will elapse before an amount of protein large enough to produce meaningful biological effects becomes available. Brown et al have shown that local irrigation with rTFPI, inhibited platelet aggregation 10 minutes after arterial intervention in the rabbit carotid arteries, although it was not demonstrated whether a single irrigation with rTFPI inhibits the thrombus formation for a long time, such as a week. Our study demonstrates that TFPI gene transfer into the injured arteries completely inhibits the shear stress–induced recurrent thrombosis ≥6 days after gene transfer even in the presence of catecholamine. Combined treatment with rhTFPI protein and TFPI gene transfer should have the advantage that the 2 effects would complement each other, thus leading to a more complete suppression of mural thrombosis.

In addition to hirudin gene transfer, gene transfer of cyclooxygenase has been efficacious in the augmentation of prostaglandin I₂ production, thereby inhibiting thrombosis in the injured porcine carotid artery, although a high titer of virus was required. Additional studies are required to determine which of the various antithrombotic molecules is likely to be the best candidate for future antithrombosis gene therapy.

In summary, our study shows that adenovirus-mediated site-specific TFPI gene transfer into injured arteries eliminates shear stress–induced mural thrombosis for a substantial period of time, even in the presence of catecholamine, without inducing apparent systemic side effects. The anti-thrombosis effect of AdCATFPI infection must be confirmed in animals fed cholesterol, in arteries in which neointima is already formed, and in coronary arteries of larger mammals before this technique is considered a suitable future approach for the treatment of human arterial thrombotic disorders.

Acknowledgments
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References
Gly212-Phe243, of the third Kunitz domain is a heparin-binding site.


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