Combination of a Brief Irrigation With Tissue Factor Pathway Inhibitor (TFPI) and Adenovirus-Mediated Local TFPI Gene Transfer Additively Reduces Neointima Formation in Balloon-Injured Rabbit Carotid Arteries

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Background—Tissue factor pathway inhibitor (TFPI) is a physiological antagonist of TF. We tested whether a brief irrigation with TFPI protein (rTFPI) or TFPI gene transfer into injured arteries would suppress TF activity and reduce fibroproliferative changes and investigated whether a combination of these methods would show an additive effect.

Methods and Results—We prepared adenoviruses expressing either TFPI (AdTFPI) or bacterial β-galactosidase (AdLacZ). Rabbit carotid arteries were balloon-injured and either infected with AdTFPI (or AdLacZ) or irrigated briefly with rTFPI (or saline). After injury, TF activity in arteries increased and was sustained; however, it was suppressed during the initial 24 hours by rTFPI irrigation (but not by gene transfer) and for a substantial period of time by TFPI gene transfer (but not by rTFPI irrigation). Four weeks later, the ratio of the intimal to medial areas was 34.3±8.7% (mean±SD, n=14) in saline-treated arteries and 33.3±4.2% in AdLacZ-infected arteries (P=NS versus saline). However, it was reduced to 25.5±8.5% in rTFPI-irrigated arteries (P<0.01 versus saline) and to 20.7±5.3% in AdTFPI-infected arteries (P<0.01 versus AdLacZ). With a combination of irrigation and gene transfer, the ratio was further reduced to 12.6±4.7% (P<0.01 versus rTFPI, P<0.05 versus AdTFPI). Systemic coagulation status was not affected in these animals.

Conclusions—A combination of rTFPI irrigation and TFPI gene transfer overcomes the shortcomings shown by each method when used alone and achieves a full coverage of TF activity suppression, thereby enhancing their therapeutic effects without systemic side effects. This combination may be an effective strategy for the prevention of thrombosis and proliferative changes after angioplasty in humans. (Circulation. 2001;103:570-575.)

Key Words: anticoagulants ■ gene therapy ■ platelets ■ thrombin ■ thrombus

Tissue factor (TF), a 47-kDa transmembrane glycoprotein, binds and activates factor VII, which then activates factors IX and X, thus initiating the blood coagulation pathway, leading to generation of thrombin. Thrombin is an inducer of fibrin formation and also a potent mitogen for the cells of the arterial wall. In normal arteries, TF is expressed only in the adventitia. In atherosclerotic arteries, however, it is expressed ubiquitously in the plaques. Furthermore, once injury occurs, TF becomes expressed within hours in almost all layers of the arterial wall. Accumulating evidence suggests that TF may have a variety of actions in addition to initiating thrombin formation. For example, TF VIIa induces the activation of mitogen-activated protein kinase and evokes intracellular Ca²⁺ mobilization, which may lead to cell proliferation. The finding that TF expression in the neointima is sustained for 8 weeks after injury and a report that thrombin activity in arteries is detectable for 2 weeks after balloon injury both suggest that TF and thrombin may be involved in the proliferative response after arterial injury and that anti-TF treatment could be effective not only in inhibiting thrombus formation but also in suppressing neointima formation.

Intact endothelial cells produce an anti-TF molecule, known as tissue factor pathway inhibitor (TFPI). TFPI, a Kunitz-type protease inhibitor, directly inhibits the factor Xa and the factor VIIa/TF catalytic complex. Because TF is an initiator of the coagulation cascade, we expected that TFPI would be a more effective molecule against thrombin than a direct thrombin antagonist such as hirudin. It has been shown repeatedly that adenovirus-mediated gene transfer into arteries can evoke site-specific production...
of recombinant protein for a prolonged period of time.\textsuperscript{15–17} In fact, we recently observed that adenovirus-mediated local expression of TFPI eliminates shear-stress–induced recurrent thrombosis in injured rabbit carotid arteries for 2 weeks, even in the presence of catecholamine, without inducing any apparent systemic side effects.\textsuperscript{18} One inherent limitation of gene therapy in any acute setting, such as vascular injury during angioplasty, however, is the lag time for adequate expression of the vector-encoded protein. An irrigation with recombinant TFPI protein (rTFPI) may be able to cover this lag phase, and a combination of rTFPI and gene transfer may show an additive therapeutic effect. The aim of this study was to test this hypothesis.

We first investigated whether local delivery of TFPI, either by a single brief irrigation with rTFPI or by adenovirus-mediated gene transfer, would suppress TF activation and neointima formation in balloon-injured arteries. Furthermore, we examined whether a combination of these 2 methods could achieve a full coverage of TF activity suppression and show any enhanced inhibitory effect on neointima formation.

Methods

Preparation of Adenoviruses

Replication-defective E1\textsuperscript{+} and E3\textsuperscript{+} adenoviral vectors expressing human TFPI (AdTFPI)\textsuperscript{19} or bacterial β-galactosidase (AdLacZ)\textsuperscript{19,20} under a CA promoter\textsuperscript{21} were prepared as previously described.\textsuperscript{18,22}

In Vivo Gene Transfer Into Injured Arteries

All animals were treated under protocols approved by the animal care committee of Kyushu University. The experiment was carried out 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. Balloon injury and in vivo gene transfer into carotid arteries of Japanese White rabbits (male, weighing 3200±180 g) were performed as previously described.\textsuperscript{18} After heparinization (1500 U/kg), the isolated space within injured arteries was filled with saline (≈0.2 mL), AdTFPI, or AdLacZ (7.5×10\textsuperscript{9} pfu/mL) for 20 minutes. Then, arteries were irrigated with either rTFPI (300 ng) or saline for 20 minutes. The titer of adenovirus used in this study was below the inflammatory threshold (1.6×10\textsuperscript{9} pfu/mL), as recently reported.\textsuperscript{23} We did not notice any significant or consistent histological differences between arteries subjected to balloon injury and arteries given an injury followed by adenoviral infection, as previously reported.\textsuperscript{18,19}

After various treatments, some segments were harvested, then placed in DMEM with 10% serum for 24 hours at 37°C. The amount of TFPI protein in the medium was measured by a 1-step sandwich ELISA method with human recombinant TFPI as a standard, as previously described.\textsuperscript{18,22} The minimum amount of TFPI detectable by this method is 6.3 ng/mL.

The vessels were harvested 4 weeks later, and 4 sections (5 μm thick) were cut from the middle portion of each vessel. The cross-sectional areas of neointima and media were measured morphometrically with an automated computer-based image analyzer (DKC-5000, SONY) by a technician blinded to the treatment regimen. The ratio of intimal to medial area (I/M ratio) was calculated. The mean value from 4 sections was counted as the 1 value for the rabbit. Fourteen rabbits for each group were analyzed.

In Vivo TF Activity

Segments of injured arteries (10 mm long) were harvested, and the adventitia was carefully stripped off. TF activity was measured and expressed in arbitrary units, as previously described.\textsuperscript{11} The protein concentration was determined with Bradford reagent (Bio-Rad).

Figure 1. TFPI production in arteries infected with AdTFPI. Rabbit carotid arteries were balloon-injured and infected with either AdTFPI or AdLacZ. Three days later, arteries were subjected to immunohistostaining with a specific antibody against human TFPI. Preimmune serum did not stain AdTFPI-infected artery (not shown).

Immunohistochemistry

Some carotid arteries were perfusion-fixed with 4% paraformaldehyde and embedded in paraffin, then sections were examined immunohistochemically with the standard streptavidin-biotin-immunoperoxidase staining method (Histofine SAB-PO kit, Nichirei). Monoclonal antibodies against either human TFPI (American Diagnostica Inc) or the Ki-67 antigen (Dako) were used. Preimmune serum was used as a negative control.

Electron Microscopic Analysis

Seven days after the gene transfer, shear stress was applied by constriction for 30 minutes as previously reported,\textsuperscript{18} then arteries (n=4 in each group) were resected and subjected to a scanning electron microscopic analysis as previously described.\textsuperscript{23} A central area of injury (≈10 mm long) in each artery was examined (S-800 microscope, Hitachi).

Statistical Analysis

One-way ANOVA followed by the post hoc test was used to determine significant differences in multiple comparison among groups. A level of P<0.05 was considered statistically significant.

Results

TFPI Protein in rTFPI-Irrigated and AdTFPI-Infected Arteries

After TFPI gene transfer, TFPI protein was immunohistostained in a broad area of the arterial wall, whereas no staining was observed in the AdLacZ-infected artery (Figure 1). No signal was detectable in the AdTFPI-treated artery if
preimmune serum was used instead of the primary antibody (not shown). At that time, we did not know why TFPI protein was detectable in a wide area, even though it is a secreted protein.

To examine how long TFPI protein remained or when it was generated after rTFPI irrigation or TFPI gene transfer, respectively, treated arteries were resected 1 hour, 24 hours, or 3 or 25 days after injury and treatments and incubated ex vivo in culture medium for 24 hours. Then, TFPI antigen in the medium was measured by human TFPI-specific ELISA. A considerable amount of TFPI was still detectable in the medium from the rTFPI-irrigated artery resected 24 hours after irrigation, although no TFPI was detectable when harvested 3 days after irrigation (Table 1). From the AdTFPI-infected arteries, only a moderate amount of TFPI was generated during 1 to 2 days after infection, but the amount increased by 5-fold in arteries resected 3 days after infection. Notably, TFPI production was still detectable 25 days after gene transfer.

**TF Activity in Arteries Is Suppressed by rTFPI Irrigation and TFPI Gene Transfer**

To examine whether the TFPI present or produced in arteries after irrigation or gene transfer, respectively, possesses biological activity, we measured TF activity in the arteries. After injury, TF activity increased from 0.79 ± 0.18 AU/mg protein (mean ± SD, n=6) to 1.12 ± 0.26 AU/mg protein at 2 hours and to 1.67 ± 0.30 AU/mg protein at 4 hours (n=6, P<0.01 versus control). The increased activity of TF remained for ≥7 days; it was 1.62 ± 0.74 AU/mg protein (n=6) at 24 hours and 1.55 ± 0.35 AU/mg protein (n=4) on day 7 after injury. Interestingly, a single brief irrigation with rTFPI significantly reduced the TF activity at 4 hours (0.98 ± 0.28 AU/mg protein) and 24 hours (0.96 ± 0.1 AU/mg protein) after injury (Figure 2). The TF activity in the AdTFPI-infected arteries was not significantly reduced (1.2 ± 0.38, n=6) at 24 hours; however, it was significantly reduced on day 7 (0.65 ± 0.05 AU/mg protein, n=6) to a level even lower than that seen in intact arteries. These results indicate that in the 24 hours after injury, TF activation was largely suppressed after a single irrigation with rTFPI and that AdTFPI infection led to a sustained suppression of the TF activity. The data indicate that a combination of these 2 methods could be effective in inhibiting TF activity for a prolonged period of time starting immediately after injury.

**Absence of Fibrin Formation and Platelet Aggregation in the AdTFPI-Infected Arteries**

Seven days after injury and gene transfer, arteries under shear stress were subjected to an electron microscopic analysis. The luminal surface of the AdLacZ-infected artery was covered with a large number of aggregated platelets in which fibrin and many erythrocytes were entrapped (Figure 3, right). In contrast, in the AdTFPI-infected artery, only a monolayer of spread-out platelets was seen (Figure 3, left), with neither platelet aggregation nor fibrin formation being observed.

**Table 1. TFPI Protein in rTFPI-Irrigated or AdTFPI-Infected Arteries**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 Hour</th>
<th>1 Day</th>
<th>3 Days</th>
<th>25 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>rTFPI</td>
<td>3303±1240</td>
<td>33.5±17.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AdTFPI</td>
<td>ND</td>
<td>8.0±0.6</td>
<td>39.7±0.8</td>
<td>14.4±4.1</td>
</tr>
<tr>
<td>AdLacZ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates not detectable (mean±SD, n=6).

**Figure 2. TF activity in arteries after injury and various treatments.** Rabbit carotid arteries were balloon-injured and infused with saline (open columns), irrigated with rTFPI (solid columns), or infected with either AdTFPI (shaded columns) or AdLacZ (not shown), and TF activity in vivo was measured at various time points after injury. Intact arteries were also measured (Before injury). There was no significant difference in TF activity between saline-infused and AdLacZ-infected arteries. Values are mean±SD (n=6).

**Single Brief Irrigation With rTFPI and Local TFPI Gene Transfer Each Suppresses Neointima Formation in Balloon-Injured Arteries**

Next, we examined whether local application of TFPI, either by irrigation with rTFPI or by TFPI gene transfer, can suppress proliferative changes in injured arteries. Balloon-injured arteries were first dwelled with saline, then irrigated with either saline or rTFPI. Some injured arteries were infected with either AdLacZ or AdTFPI, then irrigated with saline. Four weeks later, neointima formation was examined histologically. The medial area did not vary significantly among the arteries examined (Figure 4A). A single rTFPI irrigation significantly reduced neointima formation (I/M ratio 25.5±8.5%, mean±SD, n=14, P<0.05) compared with that seen in saline-irrigated arteries (34.3±8.7%, n=14) (Figure 4B). AdTFPI-infected arteries also showed a significant inhibition (20.7±5.3%, n=14, P<0.05 versus AdLacZ) compared with that seen in AdLacZ-infected arteries (33.3±4.2%, n=14) (Figure 4B). There were no significant differences between the saline-infused and saline-irrigated arteries and the AdLacZ-infected and saline-irrigated arteries.

Cell proliferation activity in arteries 3 days after injury and gene transfer was semiquantified by immunohistostaining with a monoclonal antibody to the Ki-67 antigen, which detects proliferating cells. The Ki-67 labeling index (the proportion of all nuclei that stained positively with Ki-67) was calculated as a percentage for medial smooth muscle cells. Although many Ki-67–positive smooth muscle cell
nuclei were observed in the media of saline-treated (Ki-67 labeling index was 43.1±8.1%, mean±SD, 4 fields from each of 4 rabbits, total 16 fields for each group) and AdLacZ-infected (39.3±7.4%, P=NS versus saline) arteries, significantly low numbers of nuclei were positively stained in the media of both rTFPI-treated (22.8±6.8%, P<0.05 versus saline) and AdTFPI-infected (7.8±6.5%, P<0.01 versus AdLacZ [and saline]) arteries.

Combination of rTFPI Irrigation and TFPI Gene Transfer Additively Reduces Neointima Formation

Finally, we examined whether an enhanced reduction in neointima formation could be achieved by a combination strategy (a single irrigation with rTFPI plus TFPI gene transfer). This strategy produced the greatest reduction (I/M ratio 12.6±4.7%, n=14) among the arteries tested in this study (Figure 4B). The reduction was additive and significantly greater than those achieved either by rTFPI irrigation alone (preceded by saline incubation) (25.5±8.5%, P<0.05) or by TFPI gene transfer alone (followed by saline irrigation) (20.7±5.3%, P<0.05).

No Significant Changes in PT and aPTT in Plasma of rTFPI-Irrigated and AdTFPI-Infected Rabbits

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) in rabbits were measured with an Amelung coagulometer, as previously described. Neither PT nor aPTT was altered in AdTFPI-infected rabbits 7 days after gene transfer (Table 2), in which gene expression might be submaximal. Immediately after irrigation with rTFPI (300 μg), PT and aPTT became slightly longer than before treatment; however, the difference was not statistically significant. With heparin, PT and aPTT were greatly prolonged. In the presence of heparin, no significant further prolongation of PT and aPTT was observed after a subsequent irrigation with rTFPI (Table 2).

Discussion

It takes at least a few hours before a transferred gene becomes expressed as a protein, and it may take much more time for the protein to reach sufficient concentration to show biological effects (see Table 1). An irrigation with rTFPI should cover this lag phase until sufficient TFPI protein can be generated long-term by TFPI gene transfer. The aim of this study was to test this hypothesis. In fact, TF activity was increased and sustained after injury, but it was significantly suppressed by rTFPI irrigation in the early phase (the initial 24 hours) and by AdTFPI infection in the late phase (Figure 2). Each method led to significant reduction in neointima formation (Figure 4) without affecting the systemic coagulation status (Table 2). These results suggest that 2 periods of TF activation (and probably thrombin, fibrin, and thrombus formation, see Figure 3) (ie, in the early and late phases) may contribute independently to the fibroproliferative changes in injured arteries. Most importantly, we demonstrated that a combination of these 2 methods achieved an additive reduction in neointima formation (Figure 4). This is probably through a suppression of TF activity for a prolonged period of time starting immediately after injury (Figure 2). To the best of our knowledge, this is the first solid evidence showing the usefulness of a combination of recombinant protein and gene transfer, which overcame the shortcomings of each method by enhancing their therapeutic effects and avoiding systemic side effects.

It is still controversial whether mural thrombosis persists for a long time after balloon injury. Some workers have reported that no thrombus can be found ≥48 hours after injury. Conversely, Gallo et al showed that thrombin activity in the plasma is detectable even 2 weeks after such injury. Hatakeyama et al showed that thrombin activity in the plasma is detectable even 2 weeks after such injury. In our study, an enhanced activity of TF persisted for ≥1 week after injury (Figure 2). Together, these findings suggest that TF activation and thrombin generation may be sustained for a prolonged period of time after injury. Although it is difficult to evaluate in a quantitative manner whether or not thrombus and fibrin formation actually occurred, our electron microscopic examination revealed that they were present in the AdLacZ-infected arteries, whereas only platelet adherence was seen in the AdTFPI-treated arteries, even under shear stress (Figure 3). TF and thrombin seem to have many biological effects in addition to...
initiating thrombosis, and thus, the sustained activation and/or generation of these molecules might be expected to be involved in fibroproliferative responses. Our finding that TFPI treatment leads to a reduction in neointima formation is consistent with this notion. However, the possibility that TFPI may have some direct inhibitory effects on cell proliferation and migration cannot be excluded.

An interesting point in this study is that a substantial amount of TFPI indeed remained in the arteries after a single brief irrigation with rTFPI (300 μg for 20 minutes) (Table 1) and that this irrigation with rTFPI achieved a significant suppression of TF activity, at least in the initial 24 hours (Figure 2), and a significant reduction in neointima formation (Figure 4). Oltrona et al. reported that only a continuous infusion for 24 hours of a relatively large amount of TFPI (0.5 mg/kg bolus plus 100 μg·kg⁻¹·min⁻¹ for 24 hours), but not an infusion for 3 hours, reduced neointima formation in balloon-injured pig carotid arteries. We do not know the explanation for this discrepancy. The method used for the administration (local irrigation versus intravenous infusion) and/or species differences (rabbits versus pigs) may be relevant factors. Other antithrombosis reagents, such as hirudin, also require a continuous infusion for as long as several days. Interestingly, it was reported that TFPI remained at the surface of the injured arterial wall for ≥3 days, although its activity was not examined. In this study, we also observed that a substantial amount of rTFPI indeed remained in arteries even 24 hours after irrigation, and furthermore, we confirmed that it had a biological effect. The detailed mechanism underlying this sustained survival of rTFPI needs to be clarified in future studies. TFPI inhibits an initial step in the coagulation pathway; thus, it should efficiently inhibit new thrombin formation. These features of TFPI (long survival and being an inhibitor at the initial step of the coagulation pathway) should be beneficial and of practical advantage in clinical practice. It must be admitted that the reduction in neointima formation after adenovirus-mediated TFPI gene transfer into rabbit carotid arteries (the present study) was similar to that after adenovirus-mediated hirudin gene transfer into rat carotid arteries. However, direct comparison in the same species needs to be done to determine which of these molecules is the more effective at suppressing thrombosis and proliferative changes in injured arteries.

**TABLE 2. PT and aPTT in Rabbits Under Various Treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PT, seconds</th>
<th>aPTT, seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment (n=6)</td>
<td>8.9±0.3</td>
<td>18.2±3.2</td>
</tr>
<tr>
<td>Immediately after irrigation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rTFPI without heparin (n=5)</td>
<td>10.3±0.9</td>
<td>20.1±5.5</td>
</tr>
<tr>
<td>Heparin only (n=5)</td>
<td>12.7±3.1*</td>
<td>&gt;70*</td>
</tr>
<tr>
<td>rTFPI with heparin (n=6)</td>
<td>12.5±1.8*</td>
<td>&gt;70*</td>
</tr>
<tr>
<td>Seven days after infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdTFPI (n=6)</td>
<td>8.3±2.6</td>
<td>18.3±4.0</td>
</tr>
<tr>
<td>AdLacZ (n=6)</td>
<td>8.8±2.1</td>
<td>19.0±3.2</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*P<0.01 vs before treatment.

In summary, our study demonstrated that a combination of rTFPI irrigation and TFPI gene transfer can suppress TF activation for a prolonged period of time starting immediately after injury and achieve an additive reduction in neointima formation without inducing systemic bleeding. This combined therapy may prove to be an effective and relatively safe strategy for the prevention of thrombosis and restenosis after angioplasty in humans.

**Acknowledgments**

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**References**


**Figure 4.** Intimal and medial areas and their ratio of injured arteries under various treatments. Balloon-injured rabbit carotid arteries were subjected to various treatments (infected with AdLacZ or AdTFPI, or infused with saline followed by irrigation with either saline or rTFPI, as indicated). Four weeks later, arteries were examined histologically. A, Cross-sectional areas of neointima (hatched columns) and media (open columns) (mm²). B, Intimal/medial area ratios. Values are mean±SD (n=14).
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