Topically applied tissue factor pathway inhibitor reduced intimal thickness of small arterial autografts in rabbits

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Purpose: **The purpose of this study was to investigate whether topically applied tissue factor pathway inhibitor (TFPI) reduces intimal thickness and increases long-term patency of small arterial autografts in rabbits.**
Methods: **An entire 10-mm long section of the left femoral artery was harvested and immersed in saline solution (control group, n = 10), 100 IU/mL of heparin (heparin group, n = 15), or 40 μg/mL of TFPI (TFPI group, n = 15) for 15 minutes. Then the graft was interposed to the right femoral artery. Patency rates were determined by flow measurements throughout the time course of the study, and the grafts were analyzed for measurement of intimal thickness at 3 months after operation. Immunohistochemical analysis was performed to examine whether topically applied TFPI binds to endothelial cells of the grafts.**
Results: **Three-month postoperative patency rates were 10% in the control group, 47% in the heparin group, and 73% in the TFPI group. The TFPI group had a significantly higher patency rate than that of the control group ($P < .005$). Compared with the heparin group, the TFPI group had a significant reduction in intimal area (0.19 ± 0.05 mm^2 vs 0.30 ± 0.09 mm^2, $P = .0051$), in percentage of stenosis (35.7% ± 7.7% vs 61.4% ± 15.8%, $P < .0001$), and in intimal/media areas ratio (0.64 ± 0.24 vs 1.04 ± 0.33, $P = .0051$). Immunohistologic analyses confirmed that topically applied TFPI bound to endothelial cells.**
Conclusion: **These results indicate that topically applied TFPI reduces intimal thickness and increases long-term patency of small arterial autografts in rabbits. (J Vasc Surg 2001;34:151-5.)**

Thrombosis and intimal hyperplasia have been implicated as major causes of graft failure in small arterial reconstructive procedures. Vessel dissection and anastomosis produce iatrogenic endothelial trauma, resulting in the exposure of subendothelial tissue, which initiates thrombotic processes.[1] Intimal hyperplasia is a complex process involving smooth muscle cells, platelets, leukocytes, endothelial cells, growth factors, mechanical injury, hemodynamic factors, and other unknown factors.[2,3] The relation between thrombus formation and intimal hyperplasia is complex and only partially understood.

Antithrombotic agents, such as heparin and hirudin, reduce intimal hyperplasia after artery angioplasty in minipig models.[4,5] Clinical studies have shown that these agents fail to prevent restenosis after coronary artery balloon angioplasty.[6,7] The high dosage levels used in animal experiments cause bleeding complications in human beings, which is clinically unacceptable.[8] The ideal antithrombotic agent should have high site-specific activity for the thrombogenic area on the vessel and low systemic effects on coagulation.[1]

Tissue factor pathway inhibitor (TFPI) is an endogenous protease inhibitor of the extrinsic pathway of the coagulation cascade, which is initiated by tissue factor. TFPI directly inhibits the tissue factor VIIa complex and factor Xa.[9,10] Animal experiments have documented the antithrombotic effects of topically applied TFPI.[11-13] Administration of TFPI after balloon angioplasty has been shown to reduce restenosis and intimal hyperplasia.[14-16]

The effect of TFPI on small arterial grafts has rarely been studied. We previously showed that topically applied TFPI prevents thrombotic occlusion of traumatized arterial autografts in the early postoperative period (7 days).[17] This study investigated whether topically applied TFPI reduces intimal thickness and increases long-term patency of small arterial autografts in rabbits. We also examined whether topically applied TFPI binds to endothelial cells of the grafts.

METHODS

Animal study. Forty-nine male New Zealand white rabbits weighing 2.5 to 3.5 kg were used in this study. All animal care complied with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, revised 1985). The study protocol was approved by the Kumamoto University Animal Care Committee. The full

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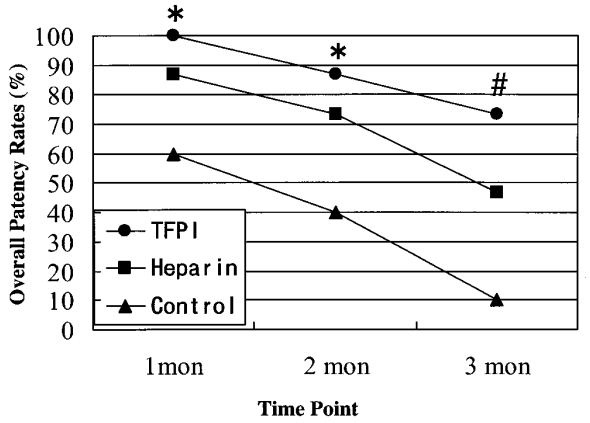

Fig 1. Overall patency rates. TFPI group had significantly higher patency rate than that of control group at 1 month, 2 months, and 3 months after operation. *Asterisk*, $P < .05$ versus control; *pound sign*, $P < .005$ versus control. *TFPI,* Tissue factor pathway inhibitor.

length of recombinant TFPI used in this study was a gift from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan.

All rabbits were anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg body weight). An entire 10-mm long section of the left femoral artery was harvested and stored in either saline solution (control group, n = 10), saline solution with 100 IU/mL heparin (heparin group, n = 15), or saline solution with 40 μg/mL TFPI[12,17-20] (TFPI group, n = 15). Fifteen minutes later, the right femoral artery was exposed, and an autograft was implanted. A standard end-to-end anastomosis was performed with 10 stitches of 10-0 nylon suture. Before placement of the last two stitches, test solution 1 mL was injected into the lumen of the graft. Five minutes later, all clamps were removed, and blood flow was restored. After bleeding was controlled, the wound was closed. After surgery, animals were fed a standard diet without anticoagulants or antiplatelet agents. Animals were randomized to receive either TFPI or heparin or saline solution. Surgeries were performed by the same operator (L.B.S.) to minimize variability between groups.

Immunohistochemical analysis. The entire 10-mm-long section of the femoral artery was harvested and immersed at room temperature in either TFPI 40 μg/mL, heparin 100 IU/mL, or saline solution for 15, 30, or 45 minutes, respectively (n = 2 for each group and each time point). The grafts were rinsed with saline solution, immediately embedded in optimal cutting temperature compound (Miles Inc, Elkhart, Ind), and frozen in liquid nitrogen for frozen section analysis. Immunostaining was performed with a modified avidin-biotin complex method.[18] Sections 5 μm thick were mounted on polylysine-coated slides, fixed in acetone at –4° C for 10 minutes, and then preincubated in phosphate-buffered saline solution (PBS, pH 7.4) containing 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. After being washed with PBS, the sections were blocked with guinea pig serum, incubated with guinea pig antihuman TFPI polyclonal antibody (provided by Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), and diluted to 40 μg/mL in PBS for 1.5 hours at room temperature (PBS and guinea pig normal serum were used as negative controls). The sections were covered with biotinylated goat anti–guinea pig immunoglobulin G antibody solution (Sigma Chemical Co, St Louis, Mo) for 1 hour at room temperature. Antibody binding was visualized by treatment with 3,3′-diaminobenzidine. The sections were counterstained with Mayer's modified hematoxylin.

Results of histologic examination

	*Normal**	*Heparin*	*TFPI*
IEL diameter (mm)	0.77 ± 0.04	0.82 ± 0.05	0.84 ± 0.09
EEL diameter (mm)	1.02 ± 0.02	1.02 ± 0.09	1.05 ± 0.07
Luminal area (mm^2)	0.46 ± 0.03	0.19 ± 0.08†	0.34 ± 0.10
Percent stenosis (%)	—	64 ± 11	36 ± 7.7‡
Intimal area (mm^2)	—	0.30 ± 0.09	0.19 ± 0.05§
Medial area (mm^2)	0.34 ± 0.05	0.28 ± 0.10	0.31 ± 0.05
IA/MA ratio (%)	—	104 ± 33	64 ± 24§

*Normal femoral artery.
†$P < .001$ vs normal or TFPI.
‡$P < .001$ vs heparin.
§$P = .0051$ vs heparin.
IEL, Internal elastic lumina; *EEL,* external elastic lumina; *IA,* intimal area; *MA,* medial area.

Patency, histologic study, and morphometry. The patency of grafts was examined with an A-Model Doppler flowmeter (Smart Doppler ES-1000SP, Hayashi Denki Co Ltd, Japan) at 1 hour after operation and then weekly. After 3 months, the animals were anesthetized, and the patency of the grafts was assessed visually. Animals were then killed and perfusion fixed in situ at a pressure of 100 mm Hg with an initial infusion of heparinized saline solution (100 IU/L) followed by 10% buffered formalin. Then grafts were harvested, fixed in formalin, and embedded in paraffin. Five sections, which were taken from the middle region of the grafts, were stained with hematoxylin-eosin and elastin van Gieson to identify the elastic laminae. All sections were analyzed with computer image analysis software (NIH image 1.5) for morphometric measurement of intimal thickness and area, medial area, and luminal area. Data were evaluated with averaging measurements from the five sections.

Statistical analysis. Data are expressed as mean ± SD. Patency rates between the groups were compared by use of the Fisher exact test. Between groups, differences of intimal thickness and area, medial area, and luminal diameters were compared with the unpaired Student *t* test. Differences were considered significant at $P < .05$.

RESULTS

Patency. All grafts were patent at 1 hour after operation. Overall patency rates are shown in Fig 1. After 1

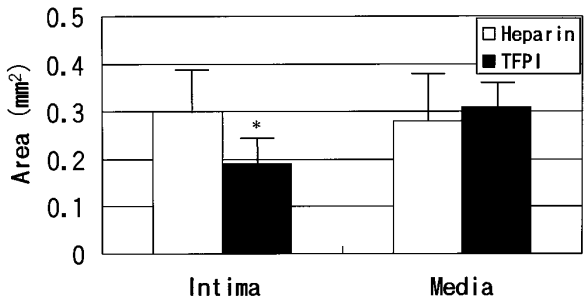

Fig 2. Comparison of changes in intimal and medial areas between TFPI-treated grafts and heparin-treated grafts after 3 months. **P* = .0051 versus heparin. *TFPI,* Tissue factor pathway inhibitor.

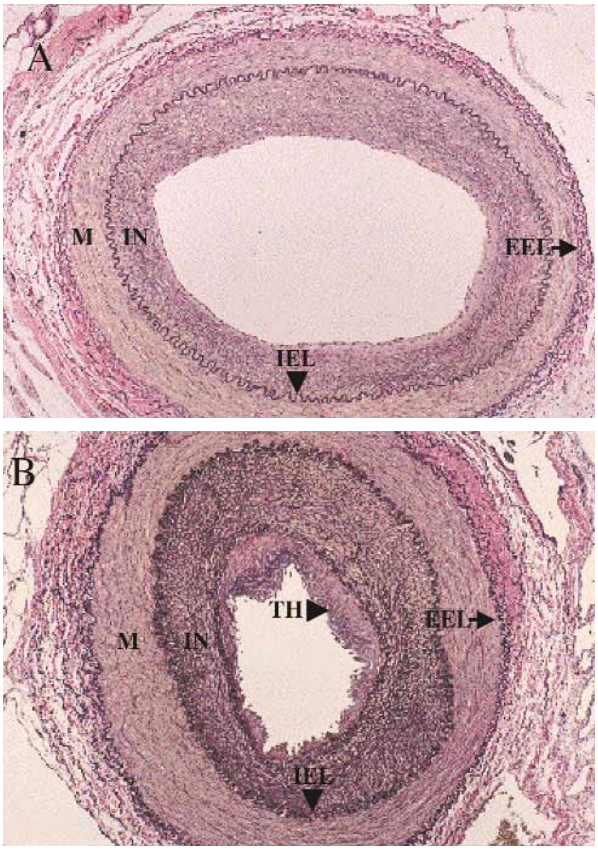

Fig 3. Cross sections of TFPI-treated graft **(A)** and heparin-treated graft **(B)** 3 months after implantation. Both sections were taken from middle region of grafts. Note differences in intima thickness and luminal surface between two groups. *IN,* Intima; *M,* media; *IEL,* internal elastic lumina; *EEL,* external elastic lumina. (Elastin van Gieson; original magnification ×100.)

month, the patency rates were 60% (6 of 10 grafts) in the control group and 87% (13 of 15 grafts) in the heparin group. No TFPI-treated graft was occluded during the same period ($P < .05$ vs control). At 2 months, patency rates were 40% in the control group (4 of 10 grafts), 73% in the heparin group (11 of 15 grafts), and 87% in the TFPI group (13 of 15 grafts) (TFPI vs control, $P < .05$). Three-month postoperative patency rates were 10% (1 of 10 grafts) in the control group, 47% (7 of 15 grafts) in the heparin group, and 73% (11 of 15 grafts) in the TFPI group. The TFPI group had a significantly higher patency rate than that of the control group ($P < .005$). The TFPI-treated grafts had a higher patency rate than that of the heparin-treated grafts (73% vs 47%), but the difference was not statistically significant ($P = .264$).

Histologic examinations. Results of histologic examinations are shown in the Table. Three preimplant grafts were studied as a normal control. In the TFPI group the luminal areas were significantly larger than that of the heparin group. Intimal areas of the TFPI group were significantly smaller than that of the heparin group (Fig 2). Medial areas were similar between the groups. The intimal/medial area ratio was significantly lower in the TFPI group than in the heparin group.

Representative photomicrographs of histologic cross sections are shown in Fig 3. In the TFPI-treated grafts (Fig 3, *A*), intima was thin, smooth, and covered by a layer of endothelial cells. In contrast, the heparin-treated grafts (Fig 3, *B*) had a thicker intima and a coarse surface.

Localization of TFPI in graft. Staining with an anti-human TFPI polyclonal antibody detected that TFPI was localized to endothelial cells and that adventitia of grafts occurred after 15 minutes immersion (Fig 4). TFPI was absent in heparin-treated grafts and saline solution–treated grafts. Increasing the immersion period to 45 minutes did not change the results.

DISCUSSION

In vascular surgery, thrombus formation and intimal hyperplasia are serious problems. Topically applied TFPI is reported to prevent thrombosis formation without any effects on systemic coagulation such as prothrombin time or activated partial thromboplastin time.[12] Our study showed that topically applied TFPI reduces intimal thickness of small arterial autografts in rabbits.

The exposure of subendothelial tissue during vessel dissection and anastomosis initiates thrombotic processes. One of the thrombogenic elements is tissue factor, which exists in subendothelial tissue and adventitia. Dumanian et al[20] showed that TFPI reduced vessel wall tissue factor activity. Other studies have shown that topically applied TFPI has antithrombogenic effects in microvascular anastomosis and in balloon-induced artery injury models.[11-13] In our study, a 100% patency rate was observed in the TFPI group at 1 month after operation; this might be due to the antithrombogenic effects of TFPI.

The mechanism by which topically applied TFPI reduces intimal hyperplasia is not specifically addressed in this study. Several hypotheses have been raised to explain the mechanism: (1) Thrombin promotes intimal hyperplasia through a direct mitogenic effect on smooth muscle cell proliferation.[21-23] Previous studies have reported that

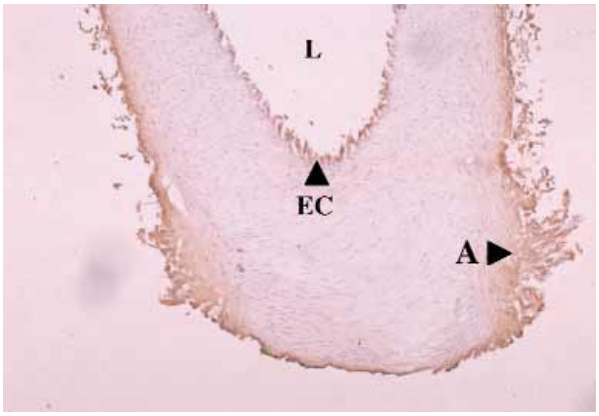

Fig 4. Immunohistochemical staining for TFPI-treated arterial graft. Section was taken from middle region of graft. TFPI is indicated by *brown color* and shows binding with endothelial cells and adventitia. *L,* Lumen; *EC,* endothelial cell; *A,* adventitia. (Original magnification ×100.)

significant thrombin activity is present on intact and anastomosed segments of arteries[24] and that TFPI can reduce thrombin activity.[20] (2) Platelets play an important role in intimal hyperplasia. Topically applied TFPI reduces platelet aggregation on the injured intimal surface after balloon angioplasty or intimectomy.[15,20] (3) Factor Xa stimulates mitogenesis in cultured blood vessels.[25,26] TFPI directly inhibits factor X.[9] Han et al[27] found that inhibiting factor VII/TF with TFPI has better effects on intimal hyperplasia than inhibiting factor Xa alone. (4) Smooth muscle cell proliferation is a key event in intimal hyperplasia. In an in vitro study, TFPI directly inhibits proliferations of cultured human aortic smooth muscle cells.[28] Obviously, more work needs to be done to understand the mechanism of reduction in intimal hyperplasia with TFPI.

The timing of the therapeutic intervention may be an important factor to prevent intimal thickness. Thrombogenicity of vascular anastomosis is maximal during the first 30 minutes after blood flow restoration.[29,30] Coating the injured surface with TFPI before exposure to circulating blood may have preventive effects. Heparin lacks anticoagulative effects without antithrombin III. Some studies have shown that thrombin becomes resistant to heparin/antithrombin III when it binds to the surface of the vessel wall.[31]

It is very important to determine whether TFPI could bind to graft by just "immersion." Lantieri et al[12] and Ornberg et al[32] have reported that topically applied TFPI binds to the injured surface. The results of our immunohistochemical analysis confirm that topically applied TFPI could bind to the endothelial cells and adventitia. We did not examine the duration of TFPI binding to the surface of arterial grafts. Lantieri et al[12] observed that with local irrigation, TFPI binds to the intimal arterial surface for at least 3 days after operation.

On the basis of our previous studies, we chose the TFPI concentration of 40 μg/mL.[17] Other studies have demonstrated that TFPI at 40 μg/mL completely inhibited tissue factor activity,[20] and patency was similar between low dose (10-40 μg/mL) and high dose (125-250 μg/mL).[12] We chose 15 minutes for incubating grafts because it is acceptable in clinical practice and is enough time for TFPI to bind to the endothelium of grafts.

These results indicate that topically applied TFPI reduces intimal thickness and increases long-term patency of small arterial autografts in rabbits. We think that inhibition of the initial step of thrombus formation at the early phase may reduce intimal proliferation at the late phase.

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