Local treatment with recombinant tissue factor pathway inhibitor reduces the development of intimal hyperplasia in experimental vein grafts

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Background: **Tissue factor (TF)–initiated thrombin generation has been implicated in the development of intimal hyperplasia after arterial injury. An increase in intimal TF expression has been shown to precede the development of intimal hyperplasia in vein grafts. This study examines the effects of local treatment with recombinant human tissue factor pathway inhibitor (rTFPI) in experimental vein grafts.**

Methods: **Thirty-six male New Zealand white rabbits underwent bypass grafting of the carotid artery by use of the reversed ipsilateral jugular vein and were divided into four groups. Twenty animals had ex vivo incubation with rTFPI treatment (50 $\mu g \cdot mL^{-1}$; n = 10) or placebo vehicle (control; n = 10). Sixteen animals received both ex vivo incubation and in vivo gel treatment with rTFPI (50 $\mu g \cdot mL^{-1}$; n = 8) or without rTFPI (gel-control; n = 8). After operation, vein grafts were harvested at 3 days for immunohistochemical and Western analyses and at 28 days for histomorphologic study.**

Results: **Western analysis demonstrated a 6.2-fold reduction in the expression of TF protein with rTFPI treatment in comparison to without rTFPI treatment. CD-18 leukocyte staining was diminished, whereas Tie-2 endothelial staining was increased in all rTFPI-treated vein grafts, compared with control and gel-control vein grafts. Intimal thickness was reduced by 21% with ex vivo rTFPI treatment compared with placebo (69 ± 4 versus 87 ± 5 μm; $P < .05$) and by 30% with the addition of rTFPI in vivo compared with gel-control (60 ± 4 versus 86 ± 5 μm; $P < .01$).**

Conclusion: **Local administration of rTFPI exerts early beneficial effects and limits the development of intimal hyperplasia in vein grafts. Therefore blocking TF-mediated pathway may offer new therapeutic options to reduce vein graft failure. (J Vasc Surg 2001;33:400-7.)**

Each year more than 600,000 arterial bypass grafts are performed in the United States with autologous vein grafts. The development of intimal hyperplasia remains the major limitation to the long-term outcome of vein grafts.[1-3] Endothelial injury is a recognized precursor to the formation of intimal hyperplasia.[4-6] Many factors are believed to contribute to the formation of intimal hyperplasia in vein grafts, including hemodynamic forces, neutrophil-endothelial interaction, platelet activation, growth and coagulation factors, and cytokines.[6,7]

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Tissue factor (TF), a transmembrane glycoprotein, functions as a cell surface receptor for the serine protease–activated factor VII (VIIa).[8] The assembly of the TF and VIIa results in the activation of factors IX and X, leading to the generation of thrombin and formation of fibrin in the extrinsic coagulation pathway.[8,9] Thrombin, fibrinogen, and activated factor X (Xa) have been shown to stimulate the migration and proliferation of vascular smooth muscle cells.[6,10-12] In addition, thrombin and fibrinogen are potent platelet activators and promoters of leukocyte chemotaxis.[6,7,12] In balloon-injured arteries, there is a rapid and short-lasting (less than 12-24 hours) increase in TF expression.[13,14] TF-mediated thrombin generation has been shown to play an important role in the pathogenesis of intimal hyperplasia in several arterial injury models.[15-18]

The response of veins to arterial bypass graft surgery is different from the well-described responses of arteries to balloon injury.[7,13,19-21] In a well-established model of vein grafts implanted into the arterial circulation, we have demonstrated that an inflammatory response, consisting predominantly of polymorphonuclear leukocytes, begins within 6 hours and persists for 5 to 7 days after bypass

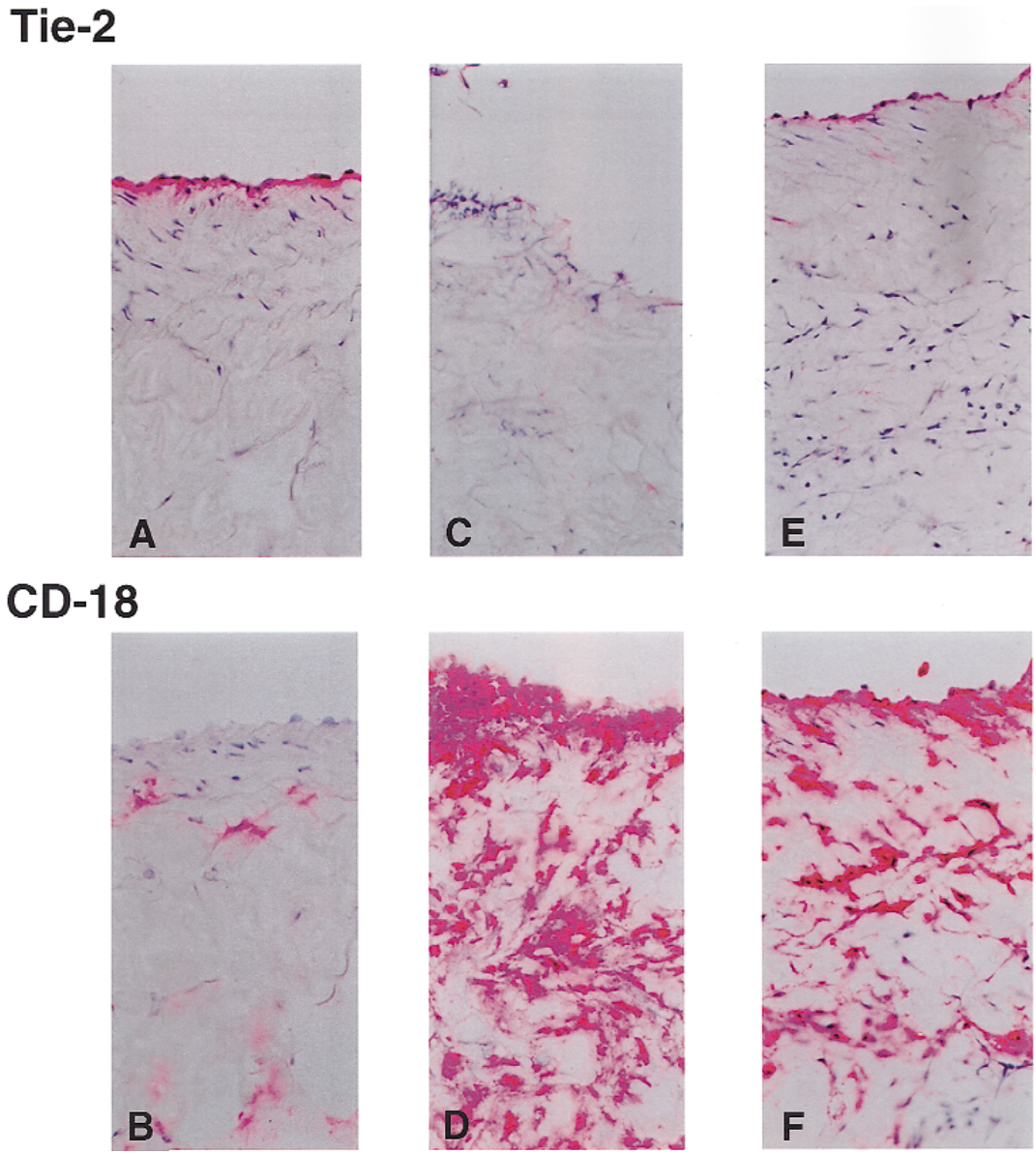

Fig 1. Immunohistochemistry of representative sections from ungrafted contralateral jugular vein **(A** and **B)**, 3-day gel-control vein graft **(C** and **D),** and 3-day rTFPI-in-gel vein graft **(E** and **F)**. Endothelial cell staining *(red)* was assessed by use of murine antibody raised against human Tie-2, endothelial cell–specific tyrosine kinase receptor. Murine monoclonal anti-rabbit CD-18, which detects β_2 chain of leukocyte adherence complex in activated neutrophils and monocytes *(red)*, was used to determine inflammatory infiltrate. In jugular veins, Tie-2 staining was uniform in endothelial cells **(A)** and CD-18–positive leukocytes were infrequently present **(B)**. At 3 days after operation, Tie-2 staining was virtually absent **(C)** and CD-18–positive leukocytes were markedly increased **(D)** in gel-control vein grafts. Treatment with rTFPI-in-gel preserved endothelial Tie-2 staining **(E)** and decreased the presence of CD-18 positive leukocytes **(F)** in vein grafts at 3 days. Note that Tie-2 staining in rTFPI-in-gel vein grafts was similar to that in ungrafted jugular veins **(E** versus **A,** respectively). Vessel lumen is at top in all sections. Original magnification × 50 in all panels.

grafting; the development of intimal hyperplasia in vein grafts starts microscopically at 5 to 7 days, accelerates, and reaches a plateau by 28 days.[5,7] We recently reported spatial and temporal changes in TF protein expression in veins after bypass grafting in the same model.[22] The major findings in this study were an increase in intimal TF protein for at least 3 days and colocalization of TF protein with CD-18 leukocyte positive immunostaining in the intima.[22] The alterations in TF protein expression in vein grafts differ from the changes seen in balloon-injured arteries. The changes in TF protein precede the development of intimal hyperplasia in both vein grafts and balloon-injured arter-

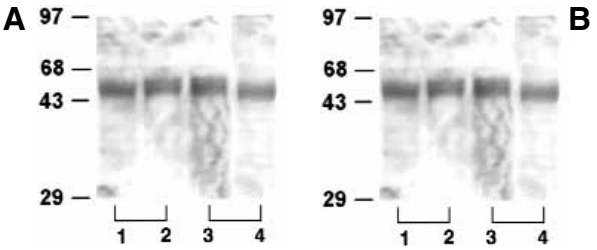

Fig 2. Representative Western blot analysis of total TF protein in ungrafted jugular veins and 3-day vein grafts. TF protein was detected as band just above 43-kDa molecular weight marker, by use of AP-1, antibody that has been shown to block TF-mediated thrombosis both in vitro and in vivo.[28,29] Equal amounts (10 μg) of protein were loaded in each lane. **A,** Compared with their respective contralateral ungrafted jugular veins (*lanes 1 and 3*), total TF proteins were similar in 3-day control vein grafts (*lane 2*) and 3-day gel-control vein grafts (*lane 4*). **B,** In contrast, there is marked reduction in total TF protein in 3-day rTFPI-in-gel vein grafts (*lanes 2 and 4*), compared with corresponding contralateral ungrafted jugular veins (*lanes 1 and 3*).

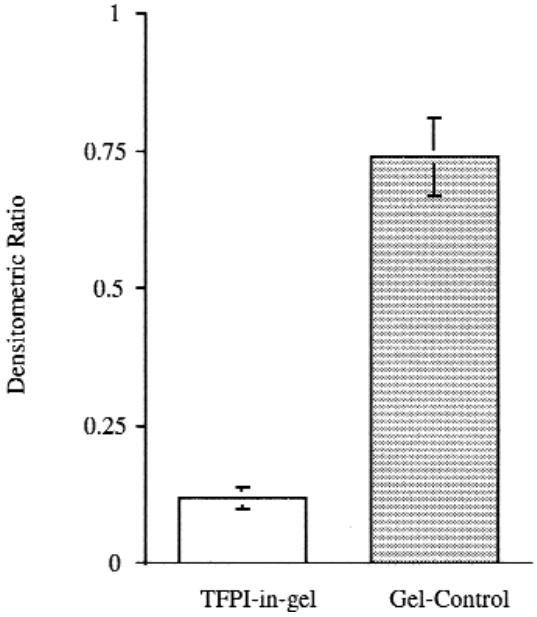

Fig 3. Densitometric analysis of total TF protein expression in 3-day rTFPI-in-gel and gel-control vein grafts. For each vein graft, total TF protein was expressed as densitometric ratio (densitometric ratio = integrated density of TF band in vein graft/integrated density of TF band in corresponding contralateral jugular vein). Combined ex vivo and in vivo treatment with rTFPI reduced total TF protein by 6.2-fold as compared with gel-control (densitometric ratio: 0.12 ± 0.02 versus 0.74 ± 0.07, respectively; $P < .05$). Values are means ± SEM (n = 3 animals per group). Statistical differences between two groups were compared by use of Mann-Whitney rank sum test.

ies, but the precise role of TF in the hyperplastic response seen in vein grafts is still unknown.

Tissue factor pathway inhibitor (TFPI), a Kunitz-type protease inhibitor produced predominantly by endothelial cells, provides negative feedback on the TF-initiated coagulation pathway in two steps: first, by binding to TF/VIIa complex and second, by binding to Xa, forming a quaternary inhibitory complex consisting of TF/VIIa/Xa/TFPI.[23,24] Our hypothesis was that TF-initiated coagulation pathway may be involved in the early inflammatory response and endothelial alterations, both of which precede the development of intimal hyperplasia in vein grafts. The aim of this study was to assess whether TF-initiated coagulation pathway plays a role in the hyperplastic development of intimal hyperplasia in vein grafts. We therefore treated experimental vein grafts with rTFPI, a human recombinant protein, and examined the short- and long-term effects of blocking TF-initiated coagulation pathway.

METHODS

Experimental protocol. Thirty-six New Zealand white rabbits underwent interposition bypass grafting of the right carotid artery with the reversed ipsilateral jugular vein as previously described.[5,22,25,26] The harvested jugular veins were incubated in Ringer's lactated buffer containing rTFPI 50 $\mu g \cdot mL^{-1}$ (Monsanto/Searle Co, St Louis, Mo, and Chiron Corp, Emoryville, Calif) for 15 minutes at 37° C before grafting to study the effects of ex vivo treatment. It was hypothesized that prolonged local delivery of rTFPI may provide greater effects. Combined ex vivo and in vivo rTFPI treatment was administered, with the addition of rTFPI (50 $\mu g \cdot mL^{-1}$) to 30% pluronic gel 2 mL (BASF, Washington, NJ), which was then applied in vivo, to coat the external surface of the vein graft before wound closure (rTFPI-in-gel). We have previously used pluronic gel to effectively deliver pharmacologic agents; the gel is in a liquid form at 4°C, semisolid at 37°C, and reabsorbed within 24 to 72 hours.[25] Control animals received only ex vivo placebo vehicle (control) or in vivo gel treatment without rTFPI (gel-control). Vein grafts and contralateral jugular veins were harvested on the third day after operation for Western blot and immunohistochemical analyses to study the short-term effects of rTFPI. Vein grafts were harvested for histomorphometric assessment on the 28th day after operation to examine the long-term effects of rTFPI.

On the day of harvest, animals were anesthetized and subsequently killed with an intravenous overdose of barbiturates.[5,22,25,26] All procedures performed on animals were approved by the Duke University Institutional Animal Care and Use Committee. Animal care and handling complied with the *Guide for the Care and Use of Laboratory Animals* issued by the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996).

Immunohistochemical analysis. The effects of rTFPI treatment on the inflammatory infiltration and on the endothelium of 3-day vein grafts were examined with

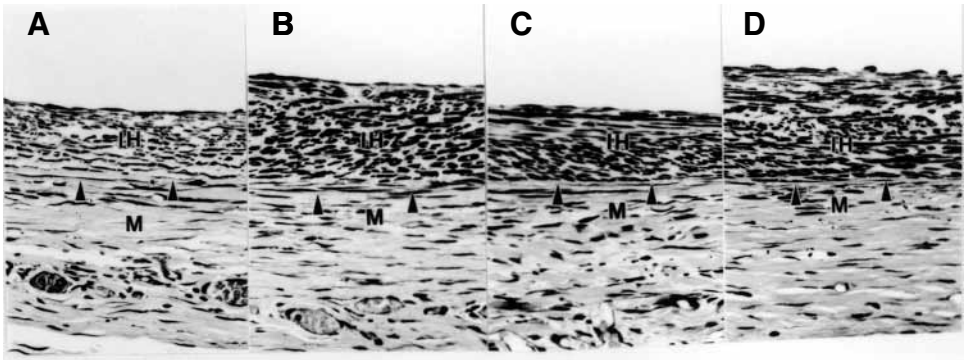

Fig 4. Composite photomicrograph of representative cross-sections from wall of 28-day rTFPI **(A)**, control **(B)**, rTFPI-in-gel **(C)**, and gel-control **(D)** vein grafts. *Arrowheads* delineate demarcation between intima and media. Vessel lumen is at top. *IH,* Intimal hyperplasia; *M,* media. Original magnification × 250.

Table I. Immunohistochemical analysis

	Veins	*rTFPI*	*Control*	*rTFPI-in-gel*	*Gel-control*
Tie-2	+++	++	+	++	+
CD-18	+	++	+++	++	+++

Qualitative scoring of Tie-2 and CD-18 staining patterns in the ungrafted jugular veins and 3-day vein grafts treated with ex vivo rTFPI, control, ex vivo and in vivo rTFPI and gel-control. A score of + was given for weak staining, ++ for intermediate staining, and +++ for intense staining, by an observer blinded to the experimental groups.

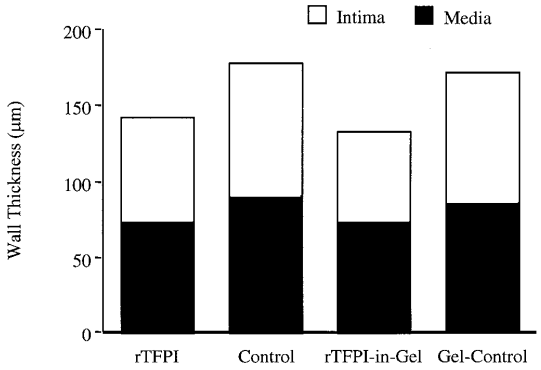

Fig 5. Cross-sectional thickness of intima and media of 28-day rTFPI, control, rTFPI-in-gel, and gel-control vein grafts. Ex vivo incubation treatment with rTFPI reduced intimal thickness by 21% in 28-day vein grafts, compared with control incubation (69 ± 4 versus 87 ± 5 μm, respectively; $P < .05$). Combined ex vivo and in vivo rTFPI treatment decreased intimal thickness by 30% (60 ± 4 vs 86 ± 5 μm, respectively; $P < .01$), compared with gel-controls. Medial thickness of 28-day rTFPI vein grafts was also reduced by 20%, compared with controls (73 ± 3 versus 91 ± 5 μm, respectively; $P > .05$), and by 15% in rTFPI-in-gel vein grafts compared with gel-controls (73 ± 4 versus 86 ± 4μm, respectively; $P > .05$), although these findings were not statistically significant.

immunohistochemistry. Distal and proximal segments (5-10 mm) of 3-day vein grafts and contralateral jugular veins (n = 4 for rTFPI and control groups; n = 3 for rTFPI-in-gel and gel-control groups) were washed gently in ice-cold phosphate-buffered saline solution (PBS). The steps used in specimen processing and immunohistochemical detection of leukocytic infiltration (CD-18) and endothelial cells (Tie-2) were previously described.[22,27] Briefly, specimens were immediately placed in 30% sucrose in PBS at 4°C for 2 hours, embedded in OCT compound (Miles Inc, Elkhart, Ind), and frozen in liquid nitrogen. Representative sections were cut (5-6 μm), prepared on silane-coated microscope slides, and stored at –80°C. Slides were thawed at room temperature (5-10 minutes) and fixed in ice-cold acetone for 2 minutes. Blocking solution was applied to the sections for 30 minutes. This and all the following steps were performed at room temperature. A murine monoclonal antirabbit CD-18 (1:100 dilution; Serotec, Oxford, United Kingdom), which detects the β_2 chain of the leukocyte adherence complex in activated neutrophils and monocytes, was used to determine the extent of inflammatory infiltration.[22] The structural integrity of the endothelium was assessed by applying a murine monoclonal antibody (MoAb33, 1:100 dilution; provided generously by Dr K. G. Peters, Duke University) raised against the extracellular domain of human Tie-2 (also known as *TEK*), a receptor tyrosine kinase, expressed exclusively in the vascular endothelium.[26,27] After 1 hour of incubation with the respective primary antibody, a sequence of incubation with biotinylated alkaline phosphatase conjugated antimouse immunoglobulin G (IgG) and ABC reagent, according to the manufacturer's specifications (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif), was carried out. Levamisole was added to block endogenous alkaline phosphatase activity. Immune complexes were detected by use of the chromogenic alkaline phosphatase substrate vector red. Tissue sections were then counterstained with hematoxylin, dehydrated, and mounted. At least three cross sections per vein grafts and jugular veins in each experimental group were examined under light microscopy by an observer blinded to the experimental group and assigned a

Table II. Morphometric analysis of ex vivo rTFPI treatment

	rTFPI (n = 6)	*Control (n = 6)*	P *value*
Luminal area (mm^2)	26.7 ± 1.9	29.8 ± 2.8	.40
Intimal area (mm^2)	1.24 ± 0.09	1.75 ± 0.18	< .05
Medial area (mm^2)	1.30 ± 0.08	1.87 ± 0.16	< .05
Intimal ratio	0.47 ± 0.01	0.49 ± 0.01	.91
Luminal index	38.9 ± 2.1	33.4 ± 1.5	< .05

Wall dimensions of 28-day vein grafts treated with local ex vivo rTFPI incubation or placebo (control). Values are the mean ± SEM. Statistical differences between the two groups were compared by use of the Mann-Whitney rank sum test.
Intimal ratio, Intimal area/(intimal + medial areas); *luminal index,* luminal diameter/(intimal + medial thickness).

Table III. Morphometric analysis of ex vivo and in vivo rTFPI treatment

	rTFPI-in-gel (n = 5)	*Gel-control (n = 5)*	P *value*
Luminal area (mm^2)	21.3 ± 1.3	24.0 ± 2.4	.61
Intimal area (mm^2)	0.94 ± 0.05	1.55 ± 0.16	< .01
Medial area (mm^2)	1.20 ± 0.06	1.76 ± 0.16	< .05
Intimal ratio	0.44 ± 0.02	0.49 ± 0.01	< .05
Luminal index	38.7 ± 2.2	32.4 ± 2.0	< .05

Wall dimensions of 28-day vein grafts treated with both ex vivo incubation and in vivo gel containing rTFPI (rTFPI-in-gel) or without rTFPI (gel-control). Values are the mean ± SEM. Statistical differences between the two groups were compared with the Mann-Whitney rank sum test.
Intimal ratio, Intimal area/(intimal + medial areas); *luminal index,* luminal diameter/(intimal + medial thickness).

qualitative score for the staining pattern (weak, intermediate, or intense).

Western blot analysis. The effects of rTFPI treatment on TF protein expression in 3-day vein grafts were assessed with Western blot analysis. Midsegments (1-2 cm) of the rTFPI-in-gel and gel-control vein grafts and of the contralateral jugular veins (n = 3; Table I) were washed in ice-cold PBS, blotted dry, weighed, and immediately snap-frozen in liquid nitrogen. Protein extraction steps were as described previously.[22] Briefly, tissues were ground into a fine powder in a mortar and pestle (in liquid nitrogen) then immediately sonicated in ice-cold Tris-saline buffer (1:4 weight/volume; 0.05 mol · L^{-1} Tris · HCl, 150 mmol · L^{-1} NaCl and pH 7.4). Insoluble debris was pelleted in a microcentrifuge at 14,000 *g* for 5 minutes at 4°C. The protein concentration in the supernatant (from total vessel extracts) was determined by use of the Bradford assay (Biorad Laboratories, Richmond, Calif).

Equal amounts of proteins from vessel extracts (10 μg) were separated by sodiumolodecyl sulfate–polyacrylamide (12%) gel electrophoresis and transferred to a nitrocellulose membrane. Nonspecific binding to the membrane was blocked with 5% nonfat dry milk overnight incubation at 4°C. The membrane was then probed with a previously characterized monoclonal murine antirabbit TF antibody (AP-1; 2 mg · mL^{-1})[28,29] for 1 hour at room temperature. Antibody binding was detected by incubating the membrane with a horseradish peroxidase–conjugated goat antimouse IgG (1:5000 dilution; Promega, Madison, Wis) at room temperature for 30 minutes. Protein bands were then visualized by use of an enhanced chemiluminescence kit (Amersham, Arlington Heights, Ill) and autoradiography. The autoradiographs were scanned and digitized, and the integrated density of visualized bands was measured (N.I.H. Image 2.1, National Institutes of Health, Washington, DC). TF protein expression in each vein graft was reported as a ratio of the integrated density of each vein graft to the integrated density of the corresponding contralateral jugular vein, which was used as the internal control for the individual animal. The TF ratio of the vein grafts were then compared between experimental groups.

Morphometric analysis. The long-term remodeling effects of rTFPI treatment were assessed by histomorphometric analysis of 28-day vein grafts. Twenty-eight–day vein grafts were perfusion fixed in situ at 80 mm Hg with 2% glutaraldehyde as previously described.[5,25] Briefly, cross sections (3 per graft) from the midportion of vein grafts were processed after standard histologic procedures, and specimens were stained with a modified Masson's trichrome and Verhoeff's elastin stain.[25] The morphologic condition of vein grafts was examined under light microscopy. Dimensional analysis was performed with computerized videomorphometry (Innovision 150; American Innovision, Inc, San Diego, Calif). The intimal, medial, and luminal areas and perimeter of each cross section were measured. The demarcation between the intima and media was defined as that interface between the "criss-cross" orientation of the smooth muscle cells in the former and the circular orientation of the smooth muscle cells in the latter. The outer limit of the media was delineated by the interface between the smooth muscle cells of the media and the connective tissue of the adventitia. The thickness of the intima and media was mathematically derived as were the intimal ratio [intimal area/(intimal + medial areas)] and luminal index [luminal diameter/(cross-sectional wall thickness)]. The luminal index is a measure of the changes in luminal diameter in relation to changes in wall thickness.

Data and statistical analysis. Values presented are the mean ± SEM. The two-tailed Mann-Whitney rank sum test was used for all statistical comparisons. A *P* value less than .05 was regarded as significant. Tie-2 and CD-18 immunostaining of 3-day vein grafts were qualitatively compared between experimental groups.

RESULTS

General effects of rTFPI. All animals survived, and all vein grafts were patent at harvest. There were no ill effects resulting from the surgery, control, or rTFPI treatment. In particular, no excessive bleeding was noted with rTFPI treatment.

Short-term effects of rTFPI on 3-day vein grafts. Three-day vein grafts were examined for the presence of

Tie-2 staining in endothelial cells and CD-18 positive staining leukocytes to determine whether rTFPI treatment would have short-term beneficial effects. In the contralateral jugular veins (ungrafted veins), Tie-2 staining was uniformly present in the luminal endothelial cells (Fig 1, *A*), whereas CD-18–positive leukocytes were sparsely present in the adventitia (Fig 1, *B*). Endothelial Tie-2 staining was decreased in both gel-control (Fig 1, *C*) and control vein grafts (not shown) when compared with ungrafted veins (Fig 1, *A*). On the other hand, CD-18–positive leukocytes were increased in both gel-control (Fig 1, *D*) and control vein grafts (not shown) when compared with ungrafted veins (Fig 1, *B*). Gel application alone did not seem to alter Tie-2 staining in endothelial cells or the staining pattern of CD-18 positive leukocytes (Table I). Local rTFPI treatment, ex vivo or combined ex vivo and in vivo, produced similar Tie-2 and CD-18 staining patterns (Table I). Endothelial Tie-2 staining was increased in rTFPI-in-gel vein grafts (Fig 1, *E*) when compared with gel-control vein grafts (Fig 1, *C*). Leukocytic CD-18 staining, in contrast, was reduced in rTFPI-in-gel–treated vein grafts (Fig 1, *F*) compared with gel-control vein grafts (Fig 1, *D*). The pattern of Tie-2 endothelial staining and CD-18–positive leukocyte staining in rTFPI-treated vein grafts was intermediate, between the staining pattern in ungrafted jugular veins at one end and the untreated controls at the other end (Table I).

TF protein expression was examined in 3-day vein grafts to verify the early effect of rTFPI treatment on TF-mediated pathway. Prior work from our laboratory has shown that TF protein is increased in the intima of vein grafts 3 days after bypass graft by immunohistochemistry.[22] The same study also demonstrated that total whole-wall (intima, media, and adventitia) TF protein in 3-day vein grafts is unchanged, as compared with the control contralateral (ungrafted) veins, by Western blot analysis.[22] Application of the pluronic gel in vivo without rTFPI did not alter TF protein expression (Fig 2, *A*). Local treatment with rTFPI-in-gel, however, reduced total TF protein as compared with control contralateral (ungrafted) veins (Fig 2, *B*). Furthermore, with the contralateral jugular vein used as an internal control, the total TF protein ratio in rTFPI-in-gel–treated vein grafts was reduced by 6.2-fold ($P < .05$) compared with the total TF protein ratio in gel-control vein grafts (Fig 3). TF protein expression in 3-day vein grafts treated with ex vivo rTFPI only was not significantly altered, when compared with 3-day controls (data not shown).

Long-term effects of rTFPI on 28-day vein grafts. Ex vivo incubation treatment with rTFPI reduced the cross-sectional thickness of the intima in 28-day vein grafts by 21%, compared with control incubation (69 ± 4 versus 87 ± 5 μm, respectively; $P < .05$, Figs 4 and 5). Combined ex vivo and in vivo rTFPI treatment decreased intimal thickness by 30% (60 ± 4 versus 86 ± 5 μm, respectively; $P < .01$, Figs 4 and 5). The intimal areas of 28-day rTFPI and rTFPI-in-gel vein grafts were also reduced by 29% and 39%, compared with Control and gel-control vein grafts, respectively (Tables II and III). The cross-sectional medial thickness of 28-day rTFPI vein grafts was also reduced by 20%, compared with controls (73 ± 3 versus 91 ± 5 μm, respectively; $P > .05$; Figs 4 and 5), and by 15% in rTFPI-in-gel vein grafts compared with gel-controls (73 ± 4 versus 86 ± 4 μm, respectively; $P > .05$; Figs 4 and 5), although these findings were not statistically significant. Similar reductions in the medial areas were observed in 28-day rTFPI (by 30%) and rTFPI-in-gel (by 32%) vein grafts, as compared with their respective controls (Tables II and III). Because of the greater decrease in the intimal area than in the medial area, the intimal ratio was decreased by only 10% in rTFPI-in-gel vein grafts, compared with gel-control vein grafts (Table III). There were no significant differences in luminal areas between rTFPI-treated vein grafts and the respective controls (Tables II and III). However, the luminal index was increased by 16% in 28-day rTFPI vein grafts as compared with controls and by 19% in 28-day rTFPI-in-gel vein grafts as compared with gel-controls (Tables II and III).

DISCUSSION

The long-term success of arterial bypass graft surgery remains limited by the development of intimal hyperplasia, which causes vein graft failure.[2,3,6,7] The formation of intimal hyperplasia is characterized by the migration and proliferation of smooth muscle cells and is the precursor to accelerated arteriosclerosis in human and experimental vein grafts.[3,4,6,7] Although a number of factors are considered to be involved in the hyperplastic response of vein grafts, events that trigger this response remain ill defined.[6,7] Our study is the first report to show that a pharmacologic agent, which blocks the extrinsic coagulation pathway, can modulate the response of veins to bypass. In the short-term, treatment with rTFPI reduced the inflammatory infiltrate, prevented the loss of endothelial staining, and attenuated TF protein expression. In the long-term, treatment with rTFPI decreased the hyperplastic response in vein grafts without altering luminal diameter.

The mechanisms by which rTFPI reduces intimal hyperplasia are likely complex and may either involve the inhibition of TF-initiated coagulation pathway or be mediated directly by the rTFPI protein itself. We recently reported that local delivery of a TF antibody (AP-1) reduces early CD-18–positive leukocytic infiltration but fails to limit both TF protein expression and intimal hyperplasia in the rabbit vein grafts.[30] In this study we show that combined ex vivo and in vivo rTFPI treatment significantly attenuated TF protein expression, in addition to reducing CD-18 positive leukocytic infiltration and preserving endothelial Tie-2 staining. Because leukocyte infiltration can contribute to endothelial injury by producing oxygen-free radicals and promoting the release of cytokines and proteases, the overall early effect of rTFPI treatment would be to lessen the endothelial injury.[5,6,21,31] Taken together, these results further strengthen the role of TF-mediated pathway in the development of intimal hyperplasia. A

reduced TF expression could conceivably lead to less thrombin generation and consequently decrease thrombin-mediated effects. It is known that TF-mediated thrombin generation may exert damaging effects on vein grafts by promoting endothelial activation, stimulating platelet aggregation, and potentiating the release of cytokines and proteases.[10,12,32-35] rTFPI may eliminate the mitogenic effects of thrombin on vascular smooth muscle cells by blocking TF-mediated thrombin generation.[11]

Interestingly, although ex vivo rTFPI treatment alone failed to reduce TF protein expression significantly, it did promote preservation of endothelial Tie-2 staining, reduce CD-18–positive leukocytic infiltration, and decrease the hyperplastic response in treated vein grafts. These findings suggest that rTFPI may also exert effects that are, in part, thrombin independent. For instance, rTFPI can abrogate the migration of vascular smooth muscle cells induced by TF, which has been shown to have in vitro chemoattractant effects comparable to platelet-derived growth factor and basic fibroblast growth factor.[36] Furthermore, direct in vitro dose-dependent inhibition of human neonatal aortic smooth muscle cell proliferation by rTFPI has been shown by Kamikubo et al.[37] On the other hand, Hamuro et al[38] recently reported rTFPI-induced endothelial cell apoptosis in culture, which would seemingly contradict our present findings. Greeno et al,[32] however, demonstrated an association between increased TF activity on cell surface and apoptosis of endothelial cells. Although it is not clear whether the increased TF activity is the cause or effect of endothelial cell apoptosis, we postulate that reduced TF activity may be linked to the survival of endothelial cells. All in all, these findings suggest that rTFPI effects are complex and extend beyond its inhibitory effects on TF/VIIa complex and Xa.

The role of TF-initiated coagulation cascade is well established in acute thrombosis after either arterial injury or vascular anastomosis.[16,18,24,39-41] In a pig model of vein grafts, Rapp et al[41] reported that local administration of rTFPI significantly enhanced graft patency at 1 and 7 days. In their study, the concentration of rTFPI (90 $\mu g \cdot mL^{-1}$) was in similar range to the dose used in our study, but the delivery method was different (direct flush into the arteriotomy site), and intimal hyperplasia was not measured. Dumanian et al[40] showed that in vitro incubation of human placental arteries with rTFPI reduced platelet deposition at anastomotic sites by 42%. Rubin et al[42] demonstrated that preincubation with rTFPI reduces fibrinogen deposition in polyester grafts by 36%. In this study we show a marked reduction in TF protein in rTFPI-treated vein. Altogether these findings support a potential role for rTFPI in blocking TF-mediated events to favorably alter the remodeling response in vein grafts.

Although we have shown that rTFPI treatment may produce advantageous remodeling responses in vein grafts at 3 and 28 days after operation, interpretation of these results must be cautioned. The short-term effects of rTPFI examined by immunohistochemistry (Tie-2 and CD-18 staining) were analyzed qualitatively, and interpretation of these results is limited as such. Additionally, we have demonstrated an association between the short- and long-term effects of rTFPI but have not proved a causal relationship. The precise mechanisms by which rTFPI treatment modulates the hyperplastic response in vein grafts remain to be determined in future studies, such as measurements of factor VII and X and thrombin in the presence and absence of rTFPI.

In conclusion, local administration of rTFPI produces early beneficial effects on the endothelium and significantly limits the hyperplastic response in vein grafts. Moreover, the results of our study suggest that blocking the initiation of TF-mediated pathway with rTFPI may provide new therapeutic options to reduce vein graft failure.

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