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# Inhibition of transforming growth factor-β restores endothelial thromboresistance in vein grafts

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***Background:*** **Thrombosis is a major cause of the early failure of vein grafts (VGs) implanted during peripheral and coronary arterial bypass surgeries. Endothelial expression of thrombomodulin (TM), a key constituent of the protein C anticoagulant pathway, is markedly suppressed in VGs after implantation and contributes to local thrombus formation. While stretch-induced paracrine release of transforming growth factor-β (TGF-β) is known to negatively regulate TM expression in heart tissue, its role in regulating TM expression in VGs remains unknown.**

***Methods:*** **Changes in relative mRNA expression of major TGF-β isoforms were measured by quantitative polymerase chain reaction (qPCR) in cultured human saphenous vein smooth muscle cells (HSVSMCs) subjected to cyclic stretch. To determine the effects of paracrine release of TGF-β on endothelial TM mRNA expression, human saphenous vein endothelial cells (HSVECs) were co-cultured with stretched HSVSMCs in the presence of 1D11, a pan-neutralizing TGF-β antibody, or 13C4, an isotype-control antibody. Groups of rabbits were then administered 1D11 or 13C4 and underwent interpositional grafting of jugular vein segments into the carotid circulation. The effect of TGF-β inhibition on TM gene expression was measured by qPCR; protein C activating capacity and local thrombus formation were measured by in situ chromogenic substrate assays; and VG remodeling was assessed by digital morphometry.**

***Results:*** **Cyclic stretch induced TGF-$\beta_1$ expression in HSVSMCs by 1.9 ± 0.2-fold ($P < .001$) without significant change in the expressions of TGF-$\beta_2$ and TGF-$\beta_3$. Paracrine release of TGF-$\beta_1$ by stretched HSVSMCs inhibited TM expression in stationary HSVECs placed in co-culture by 57 ± 12% ($P = .03$), an effect that was abolished in the presence of 1D11. Similarly, TGF-$\beta_1$ was the predominant isoform induced in rabbit VGs 7 days after implantation (3.5 ± 0.4-fold induction; $P < .001$). TGF-$\beta_1$ protein expression localized predominantly to the developing neointima and coincided with marked suppression of endothelial TM expression (16% ± 2% of vein controls; $P < .03$), a reduction in situ activated protein C (APC)-generating capacity (53% ± 9% of vein controls; $P = .001$) and increased local thrombus formation (3.7 ± 0.8-fold increase over vein controls; $P < .01$). External stenting of VGs to limit vessel distension significantly reduced TGF-$\beta_1$ induction and TM downregulation. Systemic administration of 1D11 also effectively prevented TM downregulation, preserved APC-generating capacity, and reduced local thrombus in rabbit VGs without observable effect on neointima formation and other morphometric parameters 6 weeks after implantation.**

***Conclusion:*** **TM downregulation in VGs is mediated by paracrine release of TGF-$\beta_1$ caused by pressure-induced vessel stretch. Systemic administration of an anti-TGF-β antibody effectively prevented TM downregulation and preserved local thromboresistance without negative effect on VG remodeling. (J Vasc Surg 2011;54:1117-23.)**

***Clinical Relevance:*** **Vein grafts (VGs) are commonly used conduits for coronary and peripheral arterial bypass surgeries. Thrombosis is a major cause of early VG failure. Trombomodulin (TM), a key component of the anticoagulant protein C pathway, is downregulated early after VG implantation and facilitates local thrombus formation. We found that paracrine release of transforming growth factor-$\beta_1$ (TGF-$\beta_1$), caused by pressure-induced stretch, was a potent negative regulator of TM in rabbit VGs. Administration of a neutralizing anti-TGF-β antibody effectively prevented TM downregulation and reduced local thrombus generation without adversely affecting long-term VG remodeling. This may represent a novel strategy to improve patency in patients undergoing arterial bypass procedures.**

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Supported by National Institutes of Health grants HL080142 (to J.J.R.) and HL94909 (N.K.K.).

Competition of interest: Material support from the Genzyme Corporation, Cambridge, Mass.

Additional material for this article may be found online at www.jvascsurg.org.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00

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doi:10.1016/j.jvs.2011.04.037

Autologous saphenous vein grafts (VGs) remain widely used conduits for both peripheral and coronary arterial bypass graft surgeries. Compared with arterial conduits, VGs are particularly susceptible to thrombotic occlusion, which limits the clinical efficacy of these procedures.[1] Despite the routine use of antiplatelet agents, occlusion rates as high as 20% to 30% within the first postoperative year continue to be observed for VGs implanted into both the peripheral and coronary arterial circulations.[2-4]

Thrombomodulin (TM) is a transmembrane endothelial cell protein essential to maintaining vascular thromboresistance.[5] It binds and alters the active site specificity of thrombin, preventing both thrombin-mediated cleavage of fibrinogen and activation of cellular thrombin receptors while enabling thrombin-mediated enzymatic activation of circulating protein C. Activated protein C (APC) degrades

factors Va and VIIIa of the coagulation cascade, thereby inhibiting further thrombin formation.

We previously found that TM expression was profoundly reduced in rabbit VGs in the weeks following implantation, resulting in a reduced ability of the graft endothelium to generate APC that directly resulted in increased local thrombus formation.[6] Subsequent studies identified pressure-induced stretch as the critical hemodynamic stimulus negatively regulating TM gene expression in VGs.[7] The ability of stretch to negatively regulate endothelial TM expression and function is not confined only to VGs but appears to be a more general biological response. In a rat model of acute heart failure, we similarly found that TM expression by atrial endocardial endothelial cells was downregulated by pressure overload-induced stretch.[8] In the heart, stretch-induced paracrine release of transforming growth factor-β (TGF-β) by cardiac connective tissue was identified as the primary mediator of TM gene downregulation and impaired APC generating capacity in the overlying endocardial endothelial cells.

The aim of the present study was to investigate the relationship between TGF-β induction and TM regulation in VGs. We first characterized TGF-β isoform induction by vascular stretch in vitro and in vivo. We then explored the utility of systemic administration of a neutralizing anti-TGF-β antibody to restore TM expression and APC generating capacity and reduce local thrombus formation. Finally, we investigated the effect of TGF-β inhibition on the development of VG remodeling and neointimal hyperplasia.

## METHODS

**Cell culture.** Surplus saphenous vein segments were obtained from patients at the time of cardiac bypass surgery with approval of the Johns Hopkins School of Medicine and Tufts Medical Center offices of Human Subjects Research. Human saphenous vein endothelial cells (HSVECs) were isolated by brushing the luminal surface of divided vein segments with a sterile cotton swab that was then used to inoculate tissue culture plates containing EGM-2 culture medium (Cambrex, Walkersville, Md). Once established in culture, endothelial cells were subjected to fluorescence activated cell sorting to >99.9% purity with an anti-CD31 monoclonal antibody (#555445; BD Biosciences, San Diego, Calif) as previously described.[8] Human saphenous vein smooth muscle cells (HSVSMCs) were isolated using the outgrowth technique,[9] cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, Calif) supplemented with 1% L-glutamine, 10% bovine growth serum (HyClone, Logan, Utah), and antibiotics then characterized by positive staining for smooth muscle cell actin.

**In vitro stretch experiments.** Cells were cultured in their respective media to near-confluence on type-I collagen-coated 6-well Bioflex plates (Flexcell International, Hillsborough, NC), then subjected to cyclic stretch at 1 Hz at 37°C under 5% $CO_2$ for the indicated time with an FX-4000T Tension Plus System (Flexcell International). To determine the effects of paracrine release of TGF-β on endothelial TM expression in human vascular endothelial cells, a previously described coculture system was employed,[8] whereby HSVECs were first cultured on 24-mm diameter Transwell inserts with a 0.4-μm pore size (Corning, Corning, NY) in endothelial cell growth medium-2 (EGM-2) medium. When confluent, the inserts were suspended in the individual wells of Bioflex plates containing confluent HSVSMCs in endothelial basal medium (EBM) medium (Cambrex) containing 0.5 μg/mL of either 1D11, a TGF-β pan-neutralizing antibody, or 13C4, an isotype control antibody (both generously provided by the Genzyme Corporation, Cambridge, Mass). HSVSMCs were then subjected to 10% stretch at 1 Hz for 16 hours and TM mRNA expression in stationary HSVEC determined by quantitative polymerase chain reaction (PCR).

**Rabbit vein graft model.** All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Interpositional grafting of jugular vein segments into the carotid circulation was performed in male New Zealand White rabbits (Robinson Services, Clemmons, NC) as previously described.[6,7] Immediately before surgery, 3 mg/kg gentamicin and 250 mg cephazolin were administered intravenously for antibiotic prophylaxis. In a group of rabbits, the surgical protocol was modified to prevent pressure-induced vessel distension by externally stenting VGs with a nondistensible polyethylene 23-gauge needle cover as previously described.[7] Stents had a measured lumenal diameter of 2.0 ± 0.0 mm and reduced vessel diameter by over 60% compared with unstented vein grafts. For experiments to determine the effects of TGF-β neutralization, rabbits were intravenously administered 10 mg/kg of either the 1D11 or the 13C4 control antibody in normal saline biweekly for the indicated times.

**Quantitive PCR.** mRNA was extracted from cultured cells and freshly harvested rabbit VGs using Trizol Reagent (Life Technologies). Aliquots of mRNA were reverse-transcribed into cDNA using random hexamer primers (Superscript First-Strand Synthesis System for RT-PCR; Invitrogen) and real-time quantitative PCR (RT-PCR) was performed in triplicate on a 7900HT Sequence Detector (Applied Biosystems) using TaqMan Universal PCR Mastermix (Applied Biosystems) and primers and labeled probes to the appropriate target gene (see Supplementary Methods [online only] for a full list of primer/probe sequences).

The mean threshold cycle (CT) values for each sample were compared with those of a standard curve derived from serial dilutions of control jugular vein RNA. Gene expression for endothelial markers were normalized to 18S rRNA and CD31.

**Western blot and immunohistochemical analyses.** Western blotting was performed using anti-TM antibodies (#236 and #2380; American Diagnostica), anti-CD31 antibody (M0823; Dako, Carpinteria, Calif), and an anti-TGF-β1 antibody (MAB240; R&D Systems, Minneapolis, Minn) as previously described.[6] Bands were detected by autoradiography and quantified densitometrically using

UN-SCAN-IT software (Silk Scientific, Orem, Utah). Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded sections of rabbit vein grafts using an antibodies against human TGF-$\beta_1$ latent associated peptide (AF-246-NA; R&D Systems), rabbit TM (# 236; American Diagnostica), and human PAI-1 (# 395G; American Diagnostica) with an appropriate biotinylated secondary antibody and horseradish peroxidase-labeled streptavidin (Vector Laboratories; Burlingame, CA). Peroxidase activity was revealed by either aminoethylcarbazole (Dako) or diaminobenzidine (Vector).

**In situ protein C and thrombin activity assays.** APC-generating capacity and local thrombin generation were measured in situ on freshly harvested rabbit vein grafts 7 days after implantation using specific chromogenic substrate assays as previously described.[6,8] The rate of substrate conversion was determined spectrophotometrically using a $Z_{max}$ Kinetic Microplate Reader (Molecular Devices, Sunnyvale, Calif), and the amounts of APC and thrombin generated per luminal surface area were calculated by comparison to human APC (American Diagnostica) and $\alpha$-human thrombin (Sigma-Aldrich, St. Louis, Mo) standard curves.

**Morphometric analysis.** Rabbit vein grafts were harvested, formalin perfusion-fixed, and paraffin imbedded 6 weeks after implantation. Three to four Movat-stained transverse sections per graft were digitally imaged with distance calibration using a Spot RT digital camera (Diagnostic Instruments Inc, Sterling Heights, Mich) mounted on an Olympus BX60 microscope (Olympus, Optical Co, Tokyo, Japan). The lumen, neointima, and media areas were masked and measured using SigmaScan Pro 5.0 software (SYSTAT Software, Inc, Richmond, Calif), and average neointima thickness was calculated as previously described.[6] Mean values for each graft were determined from the separate sections and used to calculate group means.

**Statistical analysis.** All data are presented as mean ± SEM. Comparison between two groups is by two-tailed *t* tests and between multiple groups by one-way analysis of variance (ANOVA). Statistically, relationships not otherwise indicated are to be assumed nonsignificant ($P > .05$).

## RESULTS

**Paracrine effect of stretch-induced release of TGF-ß on endothelial TM expression.** As vein segments are subjected to pressure-induced stretch when implanted into the arterial circulation, we first characterized the effects of direct mechanical stretch on TM expression in endothelial cells and TGF-$\beta$ isoform expression in vascular smooth muscle cells in vitro. HSVECs and smooth muscle cells (HSVSMCs) were cultured on collagen-coated silastic membranes and subjected to increasing amounts of cyclic stretch for 16 hours. Direct stretch caused a dose-dependent induction of TM expression in endothelial cells (Fig 1, *A*) as well as of TGF-$\beta_1$ expression in smooth muscle cells (Fig 1, *B*). We previously showed that stretch of cardiac fibroblasts in vitro and of atrial tissue in vivo causes downregulation of endocardial TM expression via paracrine release of TGF-$\beta$.[8] To determine if a similar phenomenon might occur in vein grafts, HSVECs were cultured on stationary filters suspended in the media of wells containing HSVSMCs subjected to 10% cyclic stretch for 16 hours. In contrast to the direct effects of stretch, TM mRNA expression was significantly reduced in stationary cocultured HSVECs while that of the TGF-$\beta$-responsive PAI-1 mRNA expression was markedly increased (Fig 1, *C*). To confirm that endothelial TM downregulation was due to the paracrine effects of TGF-$\beta$ released by the stretched smooth muscle cells, 1D11, an antibody that neutralizes all three TGF-$\beta$ isoforms, was added to the culture medium. The 1D11 antibody, but not the isotype control 13C4 antibody that possesses no TGF-$\beta$ inhibitory properties, effectively prevented TM downregulation and PAI-1 upregulation in the stationary co-cultured endothelial cells (Fig 1, *D*).

**Expression of TGF-ß isoforms in vein grafts.** TGF-$\beta_1$ is known to be induced in rabbit VGs within days of implantation and persist for at least 6 months.[10] Because little is known about induction of other TGF-$\beta$ species, the relative mRNA expression of the three TGF-$\beta$ isoforms was determined in rabbit vein grafts 7 days after implantation into the arterial circulation, at the time of the known nadir in TM mRNA expression.[7] Similar to that observed in vitro, TGF-$\beta_1$ mRNA expression was increased in rabbit vein grafts 3.5-fold ($P < .001$) compared with ungrafted vein controls, while TGF-$\beta_2$ and TGF-$\beta_3$ expression was unchanged (Fig 2, *A*). Immunostaining revealed that TGF-$\beta_1$ and PAI-1 protein expression colocalized predominantly to the developing neointima 7 days after implantation and was associated with loss of TM protein expression in the overlying endothelial cells (Fig 2, *B*, second row panels). In contrast, TGF-$\beta_1$ and PAI-1 protein expression was minimal in the neointima but localized predominantly in the adventitia 6 weeks after implantation when TM protein expression by the luminal endothelium had returned (Fig 2, *B*, third row panels). To differentiate the effects of vascular stretch from those of pressure on TGF-$\beta_1$ induction, a group of VGs was externally stented at the time of implantation. Compared with unstented grafts, TGF-$\beta_1$ mRNA expression was significantly reduced by stenting, while TM expression was preserved (Fig 3), indicating that stretch, and not the direct effects of pressure, is the critical hemodynamic stimulus responsible for TGF-$\beta_1$ upregulation.

**Effect of TGF-ß inhibition on TM expression and endothelial thromboresistance in vein grafts.** To determine if inhibition of TGF-$\beta_1$ could prevent stretch-induced downregulation of TM and improve VG thromboresistance, rabbits were administered 10 mg/kg intravenously of either the 1D11 or the 13C4 control antibody biweekly beginning prior to VG implantation and continuing for 7 days until graft harvest. Treatment with the TGF-$\beta$-neutralizing 1D11 antibody effectively prevented stretch-induced downregulation of TM gene and protein expression (Fig 4, *A* and *B*) and restored APC-generating capacity (Fig 4, *C*), which suppressed local thrombus formation as

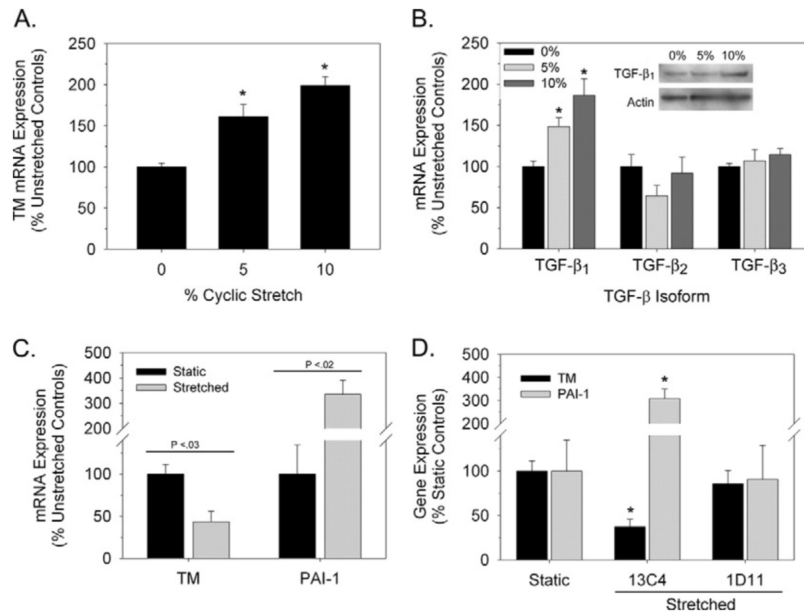


**Fig 1.** Effects of cyclic stretch on thrombomodulin (*TM*) and transforming growth factor-β (*TGF-β*) isoform expression. **A,** Human saphenous endothelial cells (HSVECs) were subjected to cyclic stretch for 16 hours and the change in TM mRNA expression was determined by quantitative polymerase chain reaction (PCR). Values shown are the mean ± SEM of n = 4/group. $^*P < .01$ versus unstretched controls. **B,** Human saphenous vein smooth muscle cells (HSVSMCs) were subjected to cyclic stretch for 16 hours and the change in TGF-β isoform mRNA expression was determined by quantitative PCR. Values shown are the mean ± SEM of n = 8 to 11/group. Panel insert is a Western blot showing stretch-induced change in TGF-$\beta_1$ protein expression. $^*P < .001$ versus unstretched controls. **C,** HSVECs were co-cultured on stationary filters suspended in the media of wells containing HSVSMCs subjected to 10% cyclic stretch for 16 hours and the changes in TM and PAI-1 mRNA expression were determined by quantitative PCR. Values shown are the mean ± SEM of n = 3/group. **D,** HSVEC were co-cultured on stationary filters suspended in the media of wells containing HSVSMCs subjected to 10% cyclic stretch for 16 hours in the presence of a pan-neutralizing anti-TGF-β antibody (1D11) or nonneutralizing isotype control antibody (13C4). Changes in TM and PAI-1 mRNA expression were determined by quantitative PCR. Values are the mean ± SEM of n = 3/group. $^*P = .02$ versus static controls.

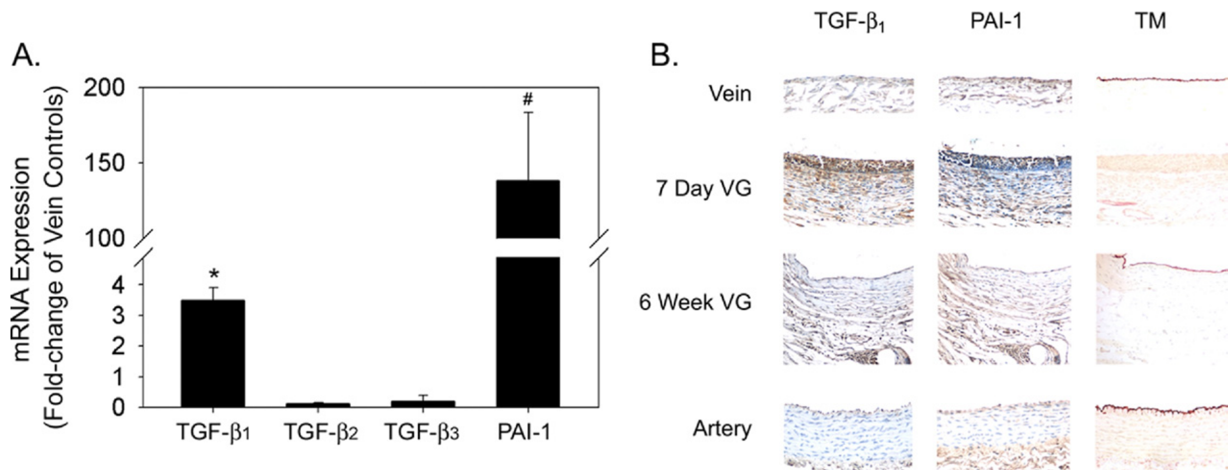


**Fig 2.** Transforming growth factor-β (*TGF-β*) expression in vein grafts. **A,** Rabbit vein grafts (*VGs*) were harvested 7 days after implantation, and relative changes in the mRNA expression of the three major TGF-β isoforms and PAI-1 were determined by quantitative polymerase chain reaction (PCR). Values are the mean ± SEM of n = 4 to 6/group. $^*P < .001$ and $^\#P = .02$ versus ungrafted vein controls. **B,** Immunostaining for TGF-$\beta_1$ (*left column, brown color*), PAI-1 (*middle column, brown color*) and thrombomodulin (*TM*) (*right column, red color*) in rabbit VGs harvested 7 days and 6 weeks after implantation. Staining in a control jugular vein and carotid artery are shown for comparison.

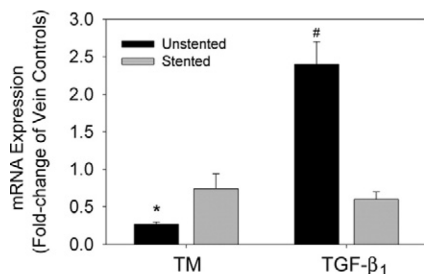


**Fig 3.** Effect of external stenting on transforming growth factor-$\beta_1$ (*TGF-*$\beta_1$) and thrombomodulin (*TM*) mRNA expression in vein grafts. TGF-$\beta_1$ and TM mRNA expression was measured in rabbit vein grafts (VGs) with and without external stents 3 days after implantation. Values are the mean ± SEM of n = 3 to 4/group. $^*P < .001$ and $^\#P = .02$ versus ungrafted vein controls.

measured by bound thrombin activity on the graft luminal surface (Fig 4, *D*). Administration of 1D11 also suppressed expression of PAI-1 mRNA, which is known to be induced by TGF-$\beta_1$ (Fig 4, *E*).

**Effect of TGF-β inhibition on vein graft neointima formation.** The induction of TGF-$\beta_1$ has been implicated in the remodeling of VGs as they adapt to the arterial circulation.[10,11] To determine if antibody-mediated TGF-β inhibition would alter VG remodeling, rabbits were intravenously administered 10 mg/kg of either the anti-TGF-β 1D11 or the13C4 control antibody biweekly beginning prior to VG implantation and continuing for 6 weeks until graft harvest. Despite having beneficial effects on endothelial thromboresistance, administration of the 1D11 antibody had no discernable effects on VG remodeling, including neointima formation (Table).

## DISCUSSION

The major findings of this study were (1) TGF-$\beta_1$ was the predominant TGF-β isoform induced in vitro by cyclic stretch of human vascular fibroblasts and by pressure-induced stretch of rabbit VG in vivo; (2) paracrine release of TGF-$\beta_1$ was a potent negative regulator of TM expression and function in rabbit VGs; (3) systemic administration of a pan-neutralizing anti-TGF-β antibody effectively prevented TM downregulation, restored APC-generating capacity, and reduced local thrombus formation in rabbit VGs without altering VG remodeling.

Early VG thrombosis is a multifactorial process resulting from a complex interplay of systemic factors, such as inflammation and hypercoagulation, with factors inherent to the graft, such as impaired blood flow and endothelial dysfunction or injury.[1] Of these factors, endothelial dysfunction caused by exposure of vein segments to arterial pressure and flow is the least well understood. We have previously shown that endothelial TM protein expression in VGs is reduced by ~95% within days of implantation and remains depressed for many weeks.[6] TM is a key component of the protein C anticoagulant pathway that is critical to maintaining vascular thromboresistance.[5] Acquired loss of TM with consequent reduced capacity to activate protein C has been observed in a number of thrombotic conditions such as transplant rejection, atherosclerosis, and especially sepsis where administration of recombinant APC is now an approved therapy.[12,13] While diminished nitric oxide generation and increased tissue factor expression are among other recognized hemostatic derangements that impair VG thromboresistance in the early postoperative period, the importance of dysfunction of the TM-protein C pathway is evidenced by the ability of targeted restoration of TM expression and APC-generating capacity to reduce local thrombus formation.[6,14,15]

Modifications to the standard rabbit VG model to alter various hemodynamic parameters performed in our laboratory revealed that TM expression was inversely proportional to wall tension caused by pressure-induced distension of the thin-walled veins.[7] Adaption of VGs to the arterial circulation is characterized by medial hypertrophy and neointimal hyperplasia, which increase wall thickness and thus reduces wall tension over time. With a decline in wall tension, a concurrent rise in TM expression was observed, though by 6 weeks, the level of TM expression remained less than that observed in native arteries. We build on those findings in the current study by demonstrating that pressure-induced stretch induced the expression and release of TGF-$\beta_1$, which acts in a paracrine fashion to suppress endothelial TM expression. This mechanism is similar to that observed in a rat model of acute heart failure, where paracrine release of TGF-β inhibited endocardial TM expression in pressure-overloaded left atria.[8]

The TGF-β family encompasses a wide array of pleiotropic growth factors intimately involved in inflammation, wound repair, angiogenesis, and tissue remodeling.[16,17] TGF-$\beta_1$ mRNA expression is known to be induced in VGs in the days after implantation in several different animal models.[18-21] In rabbit VGs, TGF-$\beta_1$ upregulation persists for at least 6 months and is temporally associated with myofibroblast recruitment and matrix deposition that occur during vessel remodeling.[10,11,19] Our findings add to these data by demonstrating that TGF-$\beta_1$ is the dominant TGF-β isoform induced in VGs, that the primary stimulus for induction is vessel stretch rather than the direct effects of pressure, and that TGF-$\beta_1$ protein localized to the developing neointima early after implantation, concurrent with maximal suppression of endothelial TM expression, but predominated in the adventitia at later time points when endothelial TM expression returned. Although we found that systemic administration of a neutralizing anti-TGF-β antibody effectively prevented the downregulation of TM expression in the VG endothelium, we did not observe any effects on VG remodeling, especially neointima formation. This is in contrast to a prior study where adenovirus-mediated expression of anti-sense RNA directed against TGF-$\beta_1$ had a modest net effect at reducing neointima formation in a rat VG model, although the adenovirus vector itself in this study appeared to stimulate neointima formation.[22]

While both TGF-$\beta_1$ and TGF-$\beta_2$ are known to be capable of downregulating TM expression in endothelial cells, ex-

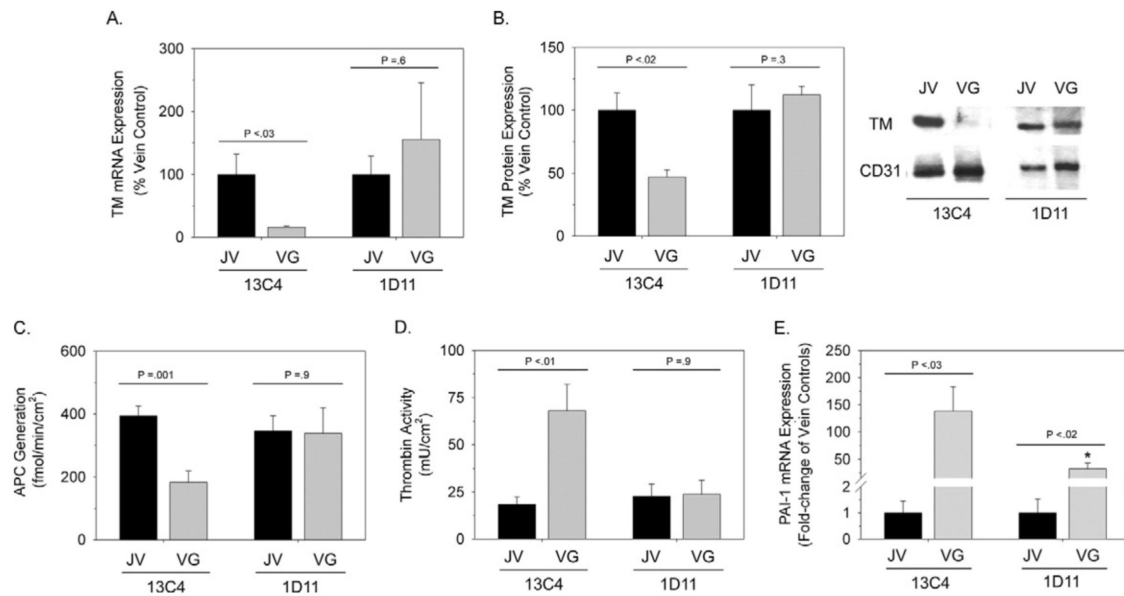


**Fig 4.** Effect of transforming growth factor-β (*TGF-β*) inhibition on vein graft (*VG*) thromboresistance. Rabbits were administered 10 mg/kg of the anti-TGF-β 1D11 antibody or the isotype control 13C4 antibody before VG implantation with two subsequent doses prior to graft harvest on day 7. **A,** Thrombomodulin (*TM*) mRNA expression was determined by quantitative polymerase chain reaction (PCR) analysis. **B,** TM protein expression was determined by densitometric analysis of Western blots (a representative Western blot shown in *right panel*). **C,** Activated protein C (*APC*) generating capacity and **D,** bound thrombin activity, an indicator of the degree of thrombus formation, were measured in situ using chromogenic assays. **E,** PAI-1 mRNA expression was determined by quantitative PCR analysis. Values shown are the mean ± SEM for n = 6/group. *$P < .05$ versus 13C4-treated VGs.

**Table.** Vein graft morphometry 6 weeks after implantation

| *Parameter* | *1D11 (n = 8)* | *13C4 (n = 8)* | *P value* |
|---|---|---|---|
| Lumen area (mm$^2$) | 36.3 ± 11.9 | 42.8 ± 10.4 | .65 |
| Neointima thickness (μm) | 101.5 ± 9.1 | 112.5 ± 10.2 | .39 |
| Neointima area (mm$^2$) | 2.1 ± 0.4 | 2.5 ± 0.4 | .36 |
| Medial thickness (μm) | 108.4 ± 4.6 | 114.8 ± 12.4 | .62 |
| Medial area (mm$^2$) | 2.3 ± 0.3 | 2.8 ± 0.6 | .39 |
| Lumen radius/wall thickness ratio | 15.7 ± 2.3 | 16.9 ± 2.3 | .67 |
| Neointima area/external elastic lamina area ratio | 0.07 ± 0.01 | 0.07 ± 0.01 | .98 |

tremely little is known about the molecular mechanism by which this occurs.[23,24] TGF-$\beta_1$ is initially secreted in an inactive form via association with one of several latency-associated peptides (LAPs) that anchor it to the extracellular matrix in the interstitial space.[25] Biologically active TGF-$\beta_1$ can be released from the latent complex through proteolytic cleavage by matrix metalloproteinases and plasmin or through a conformational change mediated by the binding of thrombospondin or $\alpha_v$ integrins to the LAP complex.[26] Active TGF-$\beta_1$ is able to bind specific serine/threonine kinase type I and type II receptors causing them to dimerize and phosphorylate a series of downstream effector proteins known as Smads. While most Smad proteins positively regulate TGF-β signal transduction, Smad6 and Smad7 are known to exert inhibitory effects. Only one prior study to date has explored the molecular mechanism by which TGF-β modulates endothelial TM expression. In a study by Sandusky et al, antisense inhibition of Smad7 potentiated the downregulation of TM by TGF-$\beta_1$ in endothelial cells, while antisense inhibition of a splice variant of Smad6, known as Smad6s, unexpectedly blunted TM downregulation.[27] Preliminary data in our laboratory indicate that TGF-$\beta_1$-induced TM downregulation in endothelial cells is predominantly mediated by activation of Smad3 and Smad4, with little observed role of canonical Smad6 (data not shown). It is currently not known what signaling pathways distal to Smad4 activation regulate TM gene transcription, although this remains an area of active investigation.

In summary, our study demonstrates that TM downregulation in VGs is mediated by stretch-induced paracrine release of TGF-$\beta_1$. Systemic administration of a neutralizing anti-TGF-β antibody effectively restored TM expression and APC-generating capacity, which consequently reduced local thrombus formation. Our findings may have implications for preventing VG thrombosis in patients after peripheral and coronary artery bypass surgeries without subjecting them to the bleeding risks of systemic anticoagulation.

The authors thank Kushal Sanghvi, MSc, for his technical help.

## AUTHOR CONTRIBUTIONS

Conception and design: NK, JS, JR
Analysis and interpretation: NK, CB, CD, JS, NK, JR
Data collection: NK,CB, EL, CD, JS, BH, NK, JR
Writing the article: NK, JR
Critical revision of the article: NK, JR
Final approval of the article: NK, CB, EL, CD, JS, BH, NK, JR
Statistical analysis: NK, JR
Obtained funding: NK, JR
Overall responsibility: JR

## REFERENCES

1. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. Circulation 1998;97: 916-31.
2. Conte MS, Bandyk DF, Clowes AW, Moneta GL, Seely L, Lorenz TJ, et al. Results of PREVENT III: a multicenter, randomized trial of edifoligide for the prevention of vein graft failure in lower extremity bypass surgery. J Vasc Surg 2006;43:742-51.
3. Alexander JH, Hafley G, Harrington RA, Peterson ED, Ferguson TB Jr, Lorenz TJ, et al. Efficacy and safety of edifoligide, an E2F transcription factor decoy, for prevention of vein graft failure following coronary artery bypass graft surgery: PREVENT IV: a randomized controlled trial. JAMA 2005;294:2446-54.
4. Gluckman TJ, Segal JB, Schulman SP, Shapiro EP, Kickler TS, Prechel MM, et al. Effect of anti-platelet factor-4/heparin antibody induction on early saphenous vein graft occlusion after coronary artery bypass surgery. J Thromb Haemost 2009;7:1457-64.
5. Esmon CT. The protein C anticoagulant pathway. Arterioscler Thromb Vasc Biol 1992;12:135-45.
6. Kim AY, Walinsky PL, Kolodgie FD, Bian C, Sperry JL, Deming CB, et al. Early loss of thrombomodulin expression impairs vein graft thromboresistance: implications for vein graft failure. Circ Res 2002; 90:205-12.
7. Sperry JL, Deming CB, Bian C, Walinsky PL, Kass DA, Kolodgie FD, et al. Wall tension is a potent negative regulator of in vivo thrombomodulin expression. Circ Res 2003;92:41-7.
8. Kapur NK, Deming CB, Kapur S, Bian C, Champion HC, Donahue JK, et al. Hemodynamic modulation of endocardial thromboresistance. Circulation 2007;115:67-75.
9. Ross R, Kariya B. Morphogenesis of vascular smooth muscle in atherosclerosis and cell structure. In: Bohr DF, Somlyo AP, Sparks HY (eds). Handbook of physiology: the cardiovascular system. Bethesda: American Physiology Society; 1980. p. 69-91.
10. Jiang Z, Yu P, Tao M, Fernandez C, Ifantides C, Moloye O, et al. TGF-beta- and CTGF-mediated fibroblast recruitment influences early outward vein graft remodeling. Am J Physiol Heart Circ Physiol 2007; 293:H482-8.
11. Jiang Z, Tao M, Omalley KA, Wang D, Ozaki CK, Berceli SA, et al. Established neointimal hyperplasia in vein grafts expands via TGF-beta-mediated progressive fibrosis. Am J Physiol Heart Circ Physiol 2009; 297:H1200-7.
12. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, et al. Dysfunction of endothelial protein C activation in severe meningococcal sepsis. N Engl J Med 2001;345:408-16.
13. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001;344:699-709.
14. Mann M, Gibbons GH, Tsao PS, von der Leyden HE, Cooke JP, Buitrago R, et al. Cell cycle inhibition preserves endothelial function in genetically engineered rabbit vein grafts. J Clin Invest 1997;99:1295-301.
15. Channon KM, Fulton GJ, Davies MG, Peters KG, Ezekowitz MD, Hagen PO, et al. Modulation of tissue factor protein expression in experimental venous bypass grafts. Arterioscler Thromb Vasc Biol 1997;17:1313-9.
16. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342:1350-8.
17. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. Chest 2005;128(6 Suppl):585S-90S.
18. Hoch JR, Stark VK, Turnipseed WD. The temporal relationship between the development of vein graft intimal hyperplasia and growth factor gene expression. J Vasc Surg 1995;22:51-8.
19. You WJ, Xiao MD, Yuan ZX. Significance of changes in transforming growth factor-beta mRNA levels in autogenous vein grafts. Chin Med J [Engl] 2004;117:1060-5.
20. Dattilo JB, Lust RM, Dattilo MP, Parker FM, Meadows WM Jr, Sun YS, et al. The temporal expression of transforming growth factor-beta 1 in early aortocoronary vein grafts. J Surg Res 1997;69:349-53.
21. Hu H, Feng Y, Wei C, Zhang Q, Duan Z. [Time course of TGF beta 1 and VEGF expression in experimental vein grafts]. Zhonghua Wai Ke Za Zhi 2000;38:861-4, 48.
22. Wolff RA, Ryomoto M, Stark VE, Malinowski R, Tomas JJ, Stinauer MA, et al. Antisense to transforming growth factor-beta1 messenger RNA reduces vein graft intimal hyperplasia and monocyte chemotactic protein 1. J Vasc Surg 2005;41:498-508.
23. Ohji T, Urano H, Shirahata A, Yamagishi M, Higashi K, Gotoh S, et al. Transforming growth factor beta 1 and beta 2 induce down-modulation of thrombomodulin in human umbilical vein endothelial cells. Thromb Haemost 1995;73:812-8.
24. Tran ND, Correale J, Schreiber SS, Fisher M. Transforming growth factor-beta mediates astrocyte-specific regulation of brain endothelial anticoagulant factors. Stroke 1999;30:1671-8.
25. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 2003;116:217-24.
26. Massagué J. TGF-beta signal transduction. Annu Rev Biochem 1998; 67:753-91.
27. Sandusky G, Berg DT, Richardson MA, Myers L, Grinnell BW. Modulation of thrombomodulin-dependent activation of human protein C through differential expression of endothelial Smads. J Biol Chem 2002;277:49815-9.

Submitted Sep 20, 2010; accepted Apr 14, 2011.

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**Supplementary Methods (online only).**

The sequences of primers/probes used for quantitative PCR included:

1) human TM (GenBank #NM_000361)
   Forward: 5′CCCAACACCCAGGC TAGCT 3′
   Reverse: 5′CGTCGATGTCCGTGCAGAT 3′
   6FAM-labeled probe: 5′TGCCCTGAAGGCTACATCCTGGACG3′)
2) human TGF-$\beta_1$ (GenBank #NM_000660)
   Forward: 5′CGAGCCTGAGGCCGACTAC3′
   Reverse: 5′TCGGAGCTCTGATGTGTTGAA3′
   6FAM-labeled probe: 5′CCAAGGAGGTCACCCGCGTGC 3′
3) human TGF-$\beta_2$ (GenBank #NM_000660)
   Forward: 5′TATTGCCCTCCTACAGACTTGAGA3′
   Reverse: 5′TGGACGCAGGCAGCAATT 3′
   6FAM-labeled probe: 5′CCAACCGGCGGAAGAAGCGTC3′)
4) human TGF-$\beta_3$ (GenBank #NM_000660)
   Forward: 5′CCTGAGCCAACGGTGATGA3′
   Reverse: 5′TCCCGGGTGCTGTTGTAAAG3′
   6FAM-labeled probe: 5′CCACGTCCCCTATCAGGTCCTGGC3′)
5) human PAI-1 (GenBank #NM_000602)
   Forward: 5′CGAGCCTGAGGCCGACTAC3′
   Reverse: 5′TCGGAGCTCTG ATGTGTTGAA3′
   6FAM-labeled probe: 5′CCAAGGAGGTCACCCGCGTGC3′
6) rabbit TM (GenBank #AY138902)
   Forward: 5′GATGAATGCGACAACGGCTAT3′
   Reverse: 5′CGCA AATGCACT CGTAGCT3′
   6FAM-labeled probe 5′CCAGGACGAGTGCCGAAACCTCCT3′
7) rabbit TGF-$\beta_1$ (GenBank #XM_002722312)
   Forward: 5′TGCGGCAGCTGTACATTGAC3′
   Reverse: 5′CAGGGCCAGGACCTTGCT3′
   6FAM-labeled probe: 5′CCCTACATCTGGAGGAGCCTGGACACCC3′
8) rabbit TGF-$\beta_2${Yoshino, 1995 2032/id}
   Forward: 5′GATTTCCATCTACAAGACCACGAGGGACTTGC3′
   Reverse: 5′CAGCATCAGTTACAT CGAAGGAGAGCCATTCG3′
   6FAM-labeled probe: 5′ GCAATGGAGAAGAATGCTTCC3′
9) rabbit TGF-$\beta_3${Kryger, 2007 2034/id}
   Forward: 5′CGGCTCAAGAAGCAGAAGGA3′
   Reverse: 5′CGGTGCGGTGGAATCATC3′
   6FAM-labeled probe: 5′ CACCACAACCCTCACCTCATCCTC3′
10) rabbit PAI-1 (GenBank #XM_002712504)
   Forward; 5′CTGCTGGTGAATGCCCTCTACT3′
   Reverse: 5′CCGTCCGACTTGTGGAAGA3′
   6FAM-labeled probe: 5′TTCTCCAAGTCTGGCACCCACCACCTA3′
11) rabbit CD31{889}
   Forward: 5′ AACTTCACCATCCAGAAGG 3′
   Reverse: 5′CACTGGTATTCCACGTCTT3′
   VIC-labeled probe: 5′ACACACGGCTATCTGGACCGCGT3′