Neointimal hyperplasia on a cell-seeded polytetrafluoroethylene graft is promoted by transfer of tissue plasminogen activator gene and inhibited by transfer of nitric oxide synthase gene

Hong Yu, PhD,[a,b] Wangde Dai, MD, PhD,[d] Zhe Yang, MD, PhD,[b] Rita L. Romaguera, MD,[c] Paul Kirkman,[d] and **Vincent L. Rowe, MD,[d]** *Miami, Fla, and Los Angeles, Calif*

Objective: **The objective of this study was to examine the effect of tissue plasminogen activator (tPA) and endothelial nitric oxide synthase (eNOS) on thrombosis and neointimal hyperplasia on a polytetrafluoroethylene (PTFE) graft seeded with smooth muscle cells (SMCs).**

Methods: **SMCs retrovirally transduced with tPA and eNOS genes were seeded on PTFE grafts and then implanted into the infrarenal rabbit aorta. Thrombosis and neointimal hyperplasia on the grafts were examined after 30 and 100 days of implantation.**

Results: **At 30 days of implantation, thrombus was observed on the luminal surface of both unseeded and SMC seeded control grafts, whereas grafts seeded with SMCs secreting tPA were nearly free of thrombus. At 100 days, the neointima on grafts seeded with tPA transduced SMCs was significantly thicker (925 ± 150 μm, n = 5) than neointima on the other grafts (range, 132 to 374 μm; $P < .001$). Neointima thickness on grafts seeded with eNOS transduced SMCs (154 ± 27 μm) was similar to that of unseeded grafts (132 ± 16 μm, $P > .05$); both were thinner than those on grafts seeded with SMCs transduced with only lacZ gene (287 ± 35 μm). The ratio of seeded cells in the neointima was significantly higher on SMC/tPA grafts (46% ± 8%) than SMC/NOS grafts (21% ± 6%, $P < .05$), indicating tPA transduced cells proliferated more than eNOS transduced cells.**

Conclusions: **Engineered tPA expression in seeded SMCs causes significantly more neointimal hyperplasia, despite the favorable inhibition of luminal thrombus. eNOS expression in the seeded cells inhibits neointimal hyperplasia. (J Vasc Surg 2005;41:122-9.)**

Clinical relevance: **Vascular prosthetic grafts are vulnerable to thrombosis and restenosis resulting in high incidence of limb loss when autogenous vein is not available. Modification of vascular prosthetic grafts to more closely mimic natural conditions may protect against clot formation and may have an immediate and prolonged effect on vascular surgical results. This study examined the patency of a prosthetic vascular graft by seeding the grafts with genetically engineered cells to prevent thrombosis and restenosis.**

Most vascular interventions fail as a result of thrombosis and restenosis. Small diameter vascular prosthetic grafts are particularly vulnerable. Seeding a polytetrafluoroethylene (PTFE) graft luminal surface with endothelial cells (ECs) has been advocated as a means to reduce surface thrombogenicity and improve patency.[1,2] However, the outcomes of EC seeding were not consistent in humans.[3-6] The major problem encountered is that ECs adhere poorly to prosthetic graft materials and are easily stripped away when exposed to blood flow.

Smooth muscle cells (SMCs) secrete larger amounts of extracellular matrix (ECM) that enable cells to adhere more firmly to the graft than ECs. We have shown that SMCs seeded beneath ECs on PTFE grafts improved EC retention both in vitro and in vivo.[7,8] In addition to enhancement of cell retention, seeded SMCs can serve as a candidate for delivery of a therapeutic protein because the multilayer formation of SMCs allows more "exogenous gene product forming" units per unit volume than ECs, which grow as a monolayer. Seeding of SMCs on grafts has been studied by a few groups as a potential vehicle for gene replacement therapy.[9-12]

Engineered overexpression of tissue plasminogen activator (tPA) in seeded cells was proposed as a means to enhance fibrinolytic capability and therefore inhibit thrombosis and restenosis after vascular interventions. tPA is a thrombolytic protease that converts inactive plasminogen into active plasmin, which then degrades fibrin complexes, a major component of thrombus. tPA expression has been engineered into ECs and SMCs by viral transfer of the tPA gene.[12-14] Adenoviral-mediated tPA gene transfer has been reported to prevent arterial thrombus formation.[15] The direct benefit of tPA secretion would be to alter the kinetics of fibrin cross-linking, diminishing local thrombosis. An

From the Department of Surgery,[a] Vascular Biology Institute,[b] and Department of Pathology,[c] University of Miami School of Medicine, and Department of Surgery, University of Southern California Keck School of Medicine[d].

Competition of interest: none.

Reprint requests: Hong Yu, PhD, Vascular Biology Institute, Department of Surgery, University of Miami School of Medicine, PO Box 019132 (R104), Miami, FL 33101.(e-mail: hyu@med.miami.edu)

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additional benefit, inhibition of intimal hyperplasia, would result from destabilization of the platelet coagulum on the luminal surface.

It is not clear whether seeding SMCs on PTFE will result in uncontrolled SMC proliferation leading to neointimal hyperplasia. Even with restoration of normal EC coverage, SMC hyperplasia takes longer to return to a homeostatic state.[16,17] Increased concentration of nitric oxide has been shown to inhibit SMC proliferation and mitogenesis responsible for intimal hyperplasia.[18,19] Gene transfer of NOS has also been successful in reducing intimal hyperplasia.[20-24] NOS catalyzes the production of nitric oxide (NO), a potent vasodilator that inhibits platelet aggregation and adhesion[25] as well as SMC proliferation and migration.[16,25,26]

Herein we report that grafts seeded with SMCs overexpressing tPA resulted in a thicker neointima than that in controls after implantation in a rabbit aorta, despite the favorable inhibition of laminated thrombus. Transduced eNOS in seeded cells diminished the neointimal hyperplasia.

MATERIALS AND METHODS

Isolation of SMCs. The New Zealand White rabbits were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and the experimental protocol was approved by the University of Southern California Animal Care and Use Committee. The isolation and culture of SMCs from the jugular veins of New Zealand White rabbits were described previously.[8] SMCs were characterized by cellular morphology under phase contrast microscopy, as well as by the presence of cytoplasmic smooth muscle α-actin. SMCs were used at passage 2-3 for gene transfer and at passage 3-5 for cell seeding.

Retroviral vectors and cell transduction. The replication-incompetent murine leukemia virus (MuLV) derived viral vectors, pseudotyped with the vesicular stomatitis virus G envelope glycoprotein (VSV-G), were used to mediate the gene transfer into SMCs. Vector plasmids pG1nBgSvNa, pG1wTSvNa,[7] and pLCNSN[21] carry genes encoding for nuclear-localized β-galactosidase (β-gal), wild-type human tPA, and human endothelium-specific constitutive nitric oxide synthase (eNOS), respectively. Viral vector supernatants were generated from 293/GPG-based producer cell lines as described previously.[7] The viral titers were between 10^6 to 10^7 colony-forming units (cfu) per milliliter analyzed by neomycin resistance assay. Transduction of SMCs with viral vectors was achieved[27] by overlaying viral supernatants containing 8 μg/mL polybrene on cells plated on culture dishes for 2 hours, followed by G418 (Gibco BRL, Gaithersburg, Md) selection.

All seeded cells were transduced with a retroviral vector carrying the β-gal reporter gene lacZ. This was necessary to distinguish these seeded cells from endogenous cells deposited onto PTFE grafts after implantation. The transduced cells were identified as the blue cells after x-gal staining.[12]

In addition to lacZ gene transduction, three groups of SMCs were further transduced with the vectors carrying genes coding for tPA, tPA and eNOS, or eNOS genes to generate SMC/tPA, SMC/tPA+NOS, and SMC/NOS, respectively. To transduce multi-genes into the same SMC, two or more vectors carrying the specified genes were mixed and overlayed on cells. Incubating cells with the two vectors effected double transduction. The co-transduction efficiency was reduced by approximately 10% from single transduction of 90% to double transduction of 80%, which was determined by using two marker vectors (G1nBgSvNa for β-gal and LGFPSN for green fluorescence protein).

Cell seeding and graft implantation. Expanded PTFE grafts (thin wall, internal diameter 4 mm), provided by IMPRA, Inc (Tempe, Ariz), were coated with fibronectin (0.1 mg/ml in phosphate-buffered saline; Gibco BRL) and seeded as described previously[7] with specified cells. Briefly, fibronectin coated grafts were filled with cell suspension (3×10^5 cells/mL), heat-crimped at both ends, and rotated at 1 rpm for 2 hours at 37°C. After the cell suspension was removed, the grafts were cultured in a culture dish with medium for 1 day before being implanted into animals. The cell coverage is approximately 500 cells/mm^2 with more than 80% confluent on the graft surface. Autologous cells were harvested from each rabbit and implanted back to the same rabbit after they were transduced and seeded on PTFE grafts. Five types of PTFE grafts were prepared. Type I grafts were not seeded with cells. Type II grafts were seeded with SMCs transduced with lacZ gene only. Type III grafts were seeded with SMC/tPA, type IV with SMC/tPA+NOS, and type V with SMC/NOS. Three rabbits were used in each group except that there were 10, 5, and 4 rabbits in types I, III, and V, respectively, at 100-day implantation. There were two groups within type I graft at 100 days, fibronectin coated or non-coated graft, with each group having five animals. No significant difference was found between these two groups. We combined them into n = 10. Operational failed (including paralyzed) animals that were killed at day 1 were excluded from data analysis.

Grafts (5-cm long) were implanted into the infrarenal aorta in end-to-side fashion as described previously.[8] At the time of graft harvest after 30- or 100-day implantation, heparin (400 IU/rabbit) was injected intravenously to prevent fresh clot formation. The graft segments were removed, and the animals were killed with an overdose of sodium pentobarbitol (125 mg/kg).

tPA assays. The enzyme activity and antigen concentration of tPA in the medium that was conditioned with transduced cells were measured by using assay kits from Biopool (Ventura, Calif). Briefly, the transduced cells and the recovered grafts (1-cm long) were cultured in Dulbecco modified Eagle medium (Gibco) containing 0.1% fetal bovine serum for 24 hours. The conditioned medium was collected and centrifuged. The concentrations of tPA in the conditioned media were determined by measuring the tPA antigen concentration with TintElize tPA enzyme-linked immunosorbent assay kit (Biopool, Ventura, Calif). The

Neointima thickness (μm) on the recovered PTFE grafts*

Graft type (seeded cells)	Implantation period: 30 Days A	30 Days P	30 Days M	30 Days Average	100 Days A	100 Days P	100 Days M	100 Days Average	P value†
I (No cell)	48 ± 49	191 ± 220	134 ± 138	124 ± 72 (3)	126 ± 129	150 ± 129	121 ± 108	132 ± 16 (10)	>.05
II (SMC/lacZ)	109 ± 14	71 ± 33	101 ± 62	94 ± 20 (3)	318 ± 180	293 ± 159	249 ± 127	287 ± 35 (3)	<.05
III (SMC/tPA)	252 ± 140	351 ± 81	399 ± 68	334 ± 75 (3)	817 ± 328	1096 ± 141	861 ± 383	925 ± 150 (5)	<.001
IV (SMC/tPA+NOS)	222 ± 212	263 ± 243	317 ± 153	267 ± 48 (3)	404 ± 191	445 ± 192	272 ± 79	374 ± 90 (3)	>.05
V (SMC/NOS)	44 ± 22	158 ± 144	199 ± 86	133 ± 80 (3)	182 ± 156	128 ± 54	153 ± 37	154 ± 27 (4)	>.05

*Circumference of each PTFE section was measured at eight evenly distributed points, which were then averaged to give the thickness per position (*A*, proximal anastomosis; *P*, proximal one-third of graft; *M*, mid-graft) per rabbit. Average is the mean of three different positions. The values in this table represent the average ± standard deviation in μm from numbers of rabbits in parentheses.

†*P* values were from comparison between 30 days and 100 days of the corresponding groups (*t* test). *P* values for comparison among different types of grafts by one-way ANOVA at 30 days are I vs II > .05; I vs III < .05; I vs IV > .05; I vs V > .05; II vs III < .01; II vs IV < .05; II vs V > .05; III vs IV > .05; III vs V < .05; IV vs V > .05. *P* values for comparison among different types of grafts at 100 days are I vs II < .05; I vs III < .001; I vs IV < .05; I vs V > .05; II vs III < .001; II vs IV > .05; II vs V > .05; III vs IV < .001; III vs V < .001; IV vs V < .05.

tPA fibrinolytic enzyme activity was determined by chromogenic assay with Chromolize tPA assay kit (Biopool). The results were expressed as ng (Ag) or IU (activity) per 10^5 cells or per cm graft in 24 hours.

Neointimal measurement. The recovered grafts after 30- or 100-day implantation were treated as described previously[8] to measure thrombus formation and neointimal thickness. One part of the graft was opened longitudinally for luminal analysis for thrombosis. The second part (two thirds of graft) was assigned into 3 areas: (1) proximal anastomosis ("A"), (2) proximal one-third of graft ("P"), and (3) mid-graft ("M"). Grafts in each area were further cut into two segments; one segment was embedded in optimum cutting temperature compound for frozen section, and the other segment was fixed with 10% formaldehyde and paraffin embedded. Frozen sections (4-μm thick) were stained with X-gal and counterstained with eosin to visualize the seeded cells (blue) and endogenous cells (red), which were counted separately under a microscope. Two to three sections per segment were analyzed. The area of neointima was measured under the microscope by an individual blinded to sample source with a standard micro-ruler as a reference. The cell density, expressed in cells per mm^2, was determined by dividing the cell number per section by the area of neointima in that section.

The thickness of the neointima on a graft was measured with a microscopic ruler at eight evenly distributed locations on the graft circumference. The average of the eight values was used to represent the neointimal thickness in that graft segment. A final neointimal thickness of a graft was yielded from the average of the data on positions A, P, and M (Table).

Histology analysis. Paraffin embedded samples were cut into 4-μm thick sections and stained as described previously[21] with hematoxylin-eosin, Masson trichrome (DAKO, Carpinteria, Calif) for collagen, phosphotungstic acid (Sigma, St Louis, Mo) and hematoxylin (PTAH) for fibrin, Verhoeff's elastic stain (American MasterTech Scientific, Lodi, Calif) for elastin. The paraffin sections were also immunohistochemically stained for tPA, eNOS, smooth muscle α–actin (SMA), muscle specific actin (MSA), and anti-proliferating cell nuclear antigen (PCNA). Anti-human tPA mouse monoclonal immunoglobulin G1 (1:100 dilution) was purchased from Oncogene Research Products (Boston, Mass). Biotinylated anti-human eNOS goat immunoglobulin G (1:5 dilution) was from R & D Systems, Inc (Minneapolis, Minn). Monoclonal mouse antibodies of anti-human SMA (clone 1A4, 1:250 dilution), anti-human MSA (clone HHF35, 1:200), and anti-human PCNA (clone PC10, 1:100) were purchased from DAKO. A standard avidin-biotin peroxidase technique was used after antigen retrieval by steamer in citric buffer (DAKO).

Statistical analysis. Experimental values were obtained from at least three repeats and expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with GraphPad (San Diego, Calif) prism program was used to compare data from multiple groups in the Table and Fig 1. Other statistical comparisons were performed by using 2-tailed Student *t* test. Significance was attributed to a *P* value of less than .05.

RESULTS

Gene expression. After retroviral tPA gene transfer, production of tPA from SMCs increased from being undetectable in SMCs to detectable at 10.2 ± 5.4 ng/10^5 cells/24 h (n = 10). tPA activity increased accordingly to 0.57 ± 0.09 IU/10^5 cells/24 h. The tPA concentration in the conditioned medium incubated with the recovered grafts seeded with SMC/tPA (type III) or SMC/tPA+NOS (type IV) was similar (1.0 ± 0.4 ng/cm graft/24 h). Their tPA activity was 0.11 ± 0.08 IU/cm graft/24 h. There was no detectable tPA activity from the recovered types I, II, and V grafts. No significant difference of tPA production between 30-day and 100-day implantation was observed, which indicates retroviral transduced

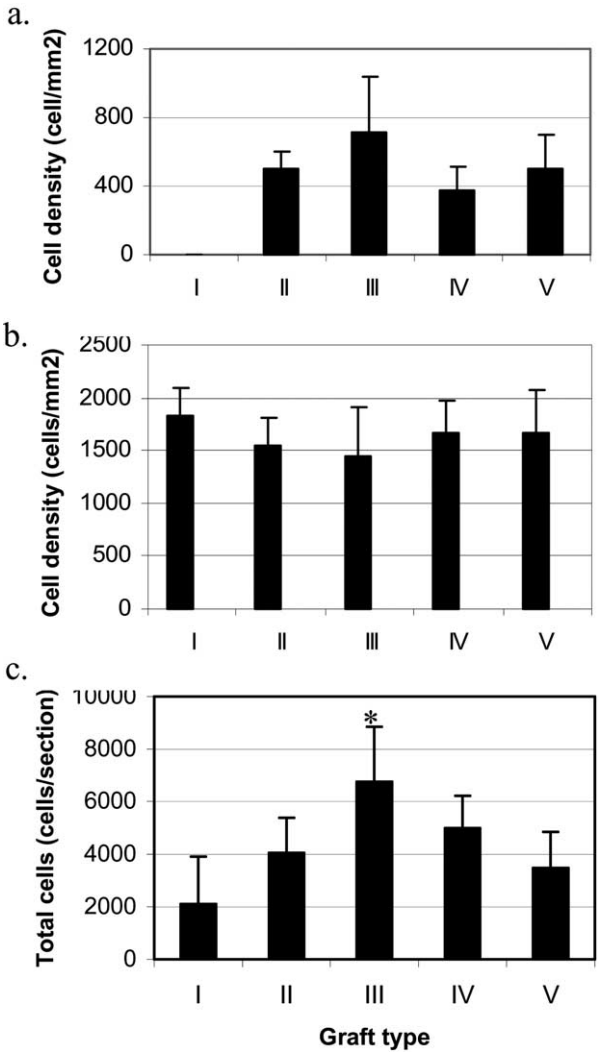

Fig. 1. Cell content in neointima. **a**, Density of seeded cells, which was calculated from lacZ positive cells in a measured area of neointima. **b**, Density of total cells, which was calculated from total cells counted in a measured area of neointima. $P > .05$ for all comparisons in (**a**) and (**b**) between different grafts (ANOVA). **c**, Total neointimal cells. Total cells in one section were calculated from the density of total cells multiplied by the area of neointima. The data are from 100-day explants. $*P < .05$ compared with type I graft. $P > .05$ for all other comparisons (ANOVA).

genes were still expressed 100 days after implantation in rabbit.

Immunostaining of the recovered grafts with antibodies against human tPA and eNOS showed tPA and eNOS positive cells in the neointima of type III and IV grafts, respectively (Fig 2, *a* and *c*). Neither tPA nor eNOS was detected in the neointima of type II graft that was seeded with SMCs transduced with only lacZ gene. These data confirmed the long-term expression of the transduced genes.

Thrombus formation. No grafts were occluded at 30 days. Thrombus covered 40% ± 13% of the luminal surface

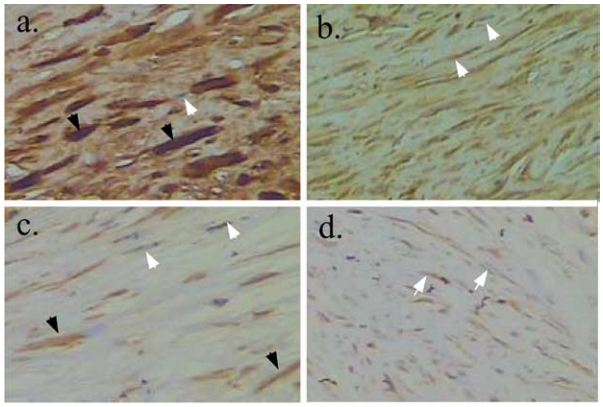

Fig. 2. Immunostaining of neointima. Paraffin sections of grafts seeded with SMC/tPA (**a**), SMC/tPA+NOS (**c**), or SMC (**b, d**) were stained with antibody against human tPA (**a, b**) or antibody against human eNOS (**c, d**). *Black arrows* indicate positively stained cells in *brown*. *White arrows* indicate negtively stained cells with nuclear stained in *blue*.

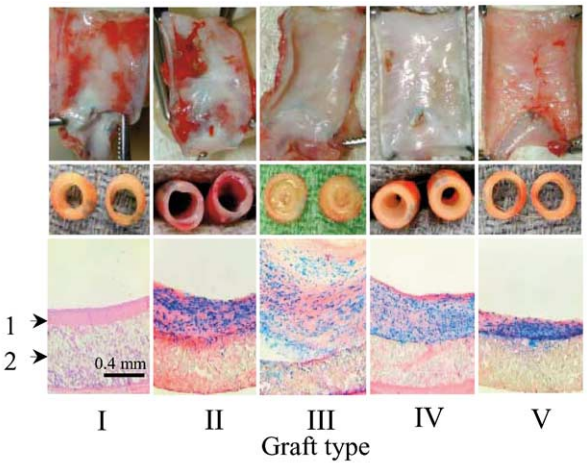

Fig. 3. Effect of tPA on thrombosis and neointima formation on PTFE graft. PTFE grafts (4 mm inside diameter) were seeded with no cell (*I*), SMC/lacZ (*II*), SMC/tPA (*III*), SMC/tPA+NOS (*IV*), or SMC/NOS (*V*) and then implanted into rabbit aorta as bypass grafts for 30 and 100 days. The recovered grafts were opened longitudinally (30 days, *top panel*). Thrombus (*red*) was observed on the luminal surface of type I and type II grafts. The grafts explanted at 100 days were patent except the graft seeded with SMC/tPA (*middle panel*). The sections of the grafts were stained with X-gal and hematoxylin-eosin to show the seeded cells (*blue*) and endogenous cells (*pink*) (*bottom panels*). *Arrow 1*, neointima; *arrow 2*, PTFE.

of type I grafts (n = 3) and 26% ± 4% of the luminal surface of type II grafts (n = 3) at 1 month after implantation (Fig 3, top panel). There was minimal thrombus seen on types III, IV, and V grafts (<5%) 30 days after implantation. The native arteries proximal and distal to the graft segment were free of thrombus in all rabbits.

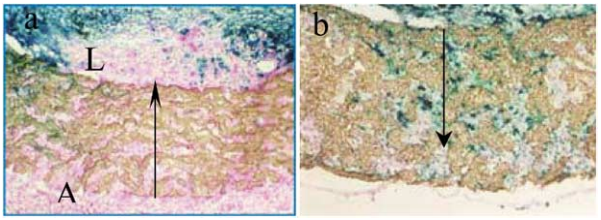

Fig. 4. Cell migration through the PTFE wall. PTFE grafts seeded with SMC/lacZ were implanted into rabbit aorta for 30 days. After recovery, the grafts were sectioned and stained with x-gal and hematoxylin-eosin. **a,** Cells in adventitial surface (*A*) (*red*) infiltrated into luminal surface (*L*) of PTFE graft with porous structure (in *brown*). **b**, Seeded cells (*blue*) migrated from luminal surface to adventitial surface.

All type III grafts (SMC/tPA, n = 5) were occluded by 100 days. Their lumen was filled with neointima except for a thin plug of thrombus in the center of the lumen (Fig 3, III, middle panel). No other type of grafts was occluded.

Neointima formation. Neointimal thickness at three positions of the grafts, the proximal anastomosis, the proximal one third of the graft, and the middle of graft, was not significantly different ($P > .1$) for all grafts implanted for 30 or 100 days (Table). The neointima on all type of grafts was thicker at 100 days compared to the same group 30 days after implantation. Neointimal thickness was not significantly different between type I and type II grafts 30 days after implantation ($P > .05$, Table) but was so at 100 days ($P < .05$). This indicates that SMCs seeded alone could incite more neointimal hyperplasia in the long-term.

On the other hand, the neointima in type III grafts was already significantly thicker than the other non-tPA types of grafts at 30 days of implantation ($P < .05$ for type III vs types I, II, and V) (Table). The neointimal thickness of type III grafts at 100 days of implantation was 925 ± 150 μm, which was significantly thicker than all other type of grafts. These data indicate that tPA expression in the seeded cells could stimulate neointimal hyperplasia.

Among the SMC seeded grafts, SMC/NOS grafts had the thinnest neointima (154 ± 27 μm), similar to that of grafts without cell seeding (Table). Comparing the effect of transduction of lacZ versus NOS gene and tPA versus tPA + NOS gene, adding the eNOS gene to SMCs resulted in a thinner neointima at 100-day implantation (II vs V and III vs IV, $P = .01$), although they were not significantly different at 30-day implantation ($P > .27$). These data indicate that eNOS expression in the seeded cells inhibits neointimal hyperplasia.

Neointimal cell density. The number of neointimal cells on each section of slides of 100-day explants was counted. There was no significant difference on either the density of seeded cells (Fig 1, *a*) or the density of total cells (Fig 1, *b*) in the neointima of all grafts recovered 100 days after implantation. However, the absolute cell number on neointima of type III grafts (6746 ± 2854 cells/section; Fig 1, *c*) was higher than that of other grafts. These data indicate that cells on type III grafts proliferate more and produce more ECM than cells on other types of grafts.

Origin of neointimal cells. The origin of cells in neointima could come from four sources: seeded cells, cells deposited from the circulation, cells migrating from the adjacent artery, and cells migrating from adventitial side of grafts. Because all seeded cells were transduced with the reporter lacZ gene, the seeded cells and their progeny can be differentiated from the endogenous cells populated on the neointima by the X-gal staining. Nearly half the cells (46% ± 8%) in the neointima of the grafts seeded with SMC/tPA were lacZ positive. This significantly ($P < .05$) exceeded the percentage of seeded cells on type II (38% ± 8%), type IV (22% ± 7%), and type V grafts (21% ± 5%). Because not 100% seeded cells had the lacZ gene due to lower co-transduction efficiency, and furthermore, lacZ gene expression could be inactivated *in vivo*, the number of seeded cells in the neointima could be underestimated. Higher percentage of seeded cells in SMC/tPA graft indicates more proliferation of tPA transduced cells. Lower percentage of seeded cells in types IV and V grafts indicates less proliferation of eNOS transduced cells. No cells were stained blue in control type I unseeded grafts.

Infiltration and migration of cells through the porous PTFE wall were observed (Fig 4). Cells on the periadventitial side infiltrated to the luminal side through the pores on the graft wall after 30-day implantation (Fig 4, *a*). This infiltration could also contribute to the intimal hyperplasia on the luminal surface. Similarly, seeded cells were observed to migrate from luminal surface of the graft to the periadventitial side of the graft through the pores on the graft wall (Fig 4, *b*).

Neointima content analysis. To identify the cellular and matrix property of neointima, recovered grafts at 100 days of implantation were analyzed with histologic stains. Cells within the neointima were long and spindle shaped (Fig 5, *a* and *e*). They were positively stained with antibody against SMA (Fig 5, *b* and *f*) and MSA (data not shown). Collagen-rich matrix in the neointima was illustrated by Masson trichrome stain (Fig 5, *c* and *g*). The fibrin stain by PTAH was negative in the neointima layer. Verhoeff-van Gieson stain for elastin fibers was negative in the neointima on all PTFE grafts (data not shown). The results indicate that the major composition of the neointima is SMC and ECM. There are more PCNA positive cells in the neointima of type III grafts (Fig 5, *h*) than other types of grafts (Fig 5, *d*), indicating that cells in the neointima of type III grafts proliferated faster than cells on the other types of grafts. Thrombus was observed at the center of the occluded type III grafts on hematoxylin-eosin stained sections. The PTAH stain and factor VIII immunostaining showed a fibrin-poor but platelet-rich thrombus (data not shown).

DISCUSSION

Seeding tPA-transduced cells onto grafts has been proposed as a means to enhance the thrombolytic activity of the graft, thereby increasing graft patency.[12-14] Indeed, we observed a thrombus-free lumen on grafts seeded with

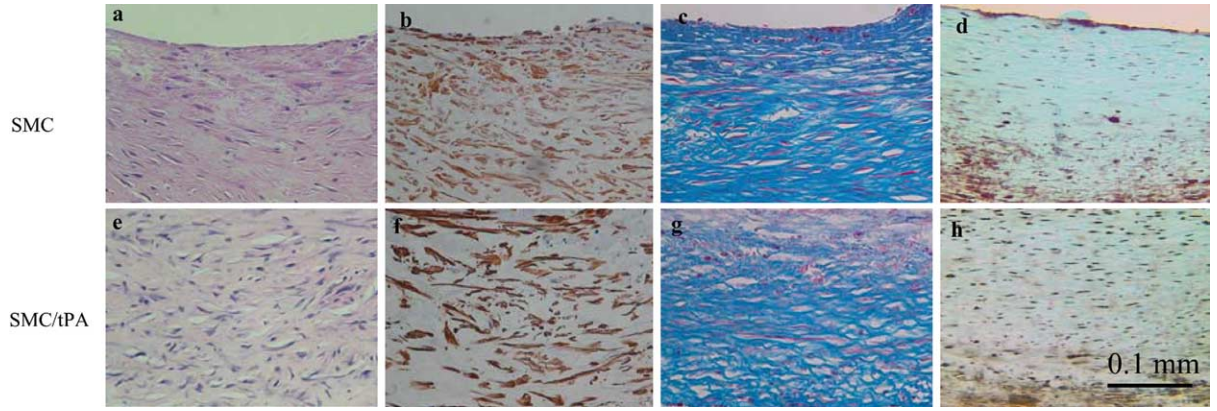

Fig. 5. Analysis of neointima. Paraffin sections of grafts seeded with SMC (*upper panel*) or SMC/tPA (*lower panel*) were stained with hematoxylin-eosin (**a, e**), antibody for smooth muscle α–actin (**b, f**), Masson trichome (**c, g**), and antibody for PCNA (**d, h**). Nuclear are *purple* in hematoxylin-eosin staining; SMCs are *brown* in SMA staining; collagen is *blue* and cells are *pink* in trichome staining; nuclear of proliferating cells are *dark blue* in PCNA staining.

SMC/tPA at 30 days of implantation (Fig 3, III, IV top panel). In comparison, there was thrombus on other control grafts that did not hypersecrete tPA (Fig 3, I and II top panel). This result agrees with the report that adenoviral-mediated tPA gene transfer prevents arterial thrombus formation.[15]

However, tPA expression from seeded SMCs stimulated intimal hyperplasia. Neointima on the grafts seeded with SMC hypersecreting tPA (type III and IV grafts) was significantly thicker than those not expressing tPA (Table). The neointima on the type III grafts was more than sevenfold and three-fold thicker than those on the type I and II grafts, respectively. The stenosis in type III grafts was so severe at 100 days that tPA expression could not inhibit thrombosis of the small remaining lumen, and the grafts were eventually occluded by thrombus in the middle of the grafts. This hypothesis was supported by histology analysis, which showed that thrombus in type III grafts was Factor VIII positive and PTAH negative. These data demonstrated that the thrombus is formed by platelets with little fibrin.

The impact of the tPA gene transfer on intimal hyperplasia is controversial in the literature. It was reported that intimal hyperplasia in an allograft heart transplantation was reduced 64% by ex vivo transfer of human tPA gene into a rabbit heart.[28] However, tPA gene transfer in the injured artery has been reported to cause a thicker neointima compared to controls.[29] Re-endothelialization with endothelial progenitor cells that had been transduced with tPA gene aggravated intimal hyperplasia.[30]

A possible mechanism of tPA effect on neointimal hyperplasia is that tPA activates plasminogen into plasmin, which is a nonspecific serine protease and can activate matrix metalloproteinases, a family of proteolytic enzymes.[31] Both plasmin and matrix metalloproteinases degrade many components of the ECM. ECM degradation can cause vascular remodeling by inducing cell proliferation and migration,[32] as well as ECM deposition,[33] which are necessary steps leading to neointimal development.[34-36] The breakdown of ECM releases the embedded growth factors that stimulate cell proliferation.[37] We have demonstrated in vitro that overexpression of tPA in SMCs resulted in ECM degradation and promoted SMC proliferation and migration (manuscript in preparation).

There were more total cells on the type III grafts compared with other types of grafts (Fig 1, *c*). The ratio of seeded cells in the neointima was higher on SMC/tPA grafts (46%) than other cell-seeded grafts (21% to 38%). More PCNA positive cells were also observed on SMC/tPA grafts (Fig 5, *h*). These data indicate that cells on SMC/tPA grafts proliferate more than cells on other types of grafts.

Because the neointima is thicker and the total neointimal area is commensurately greater on the SMC/tPA grafts, this results in a cell density that is not significantly different from other grafts. The higher absolute number of cells but similar cell density indicates that accompanying the cellular proliferation, there must be more ECM produced, resulting in a thicker neointima but similar cell density. This eventuates in more rapid narrowing of the vessel and subsequent occlusion.

On the other hand, the tPA gene transduced into rabbit SMC is from human cDNA. Although there is a possibility that expression of human tPA in this rabbit model induced an immunoreaction that incites graft occlusion, both type III and type IV grafts secreted human tPA, but occlusion occurred only in type III grafts. This result makes immunoreaction less likely.

Neointimal thickness on the unseeded grafts progressed little from 30 days to 100 days, although their surface had more thrombus than grafts secreting tPA at 30 days (Table). However, the neointima of grafts seeded with SMCs progressed significantly between 30 and 100 days. This indicates that seeding a PTFE graft with SMCs results in a thicker neointima. SMCs were selected as the seeding cells in lieu of ECs because SMCs adhere better on the

grafts, and SMCs will generate multilayers of cells capable of delivering an engineered gene product.

Modulation of the SMC proliferation can be achieved by dual seeding an EC layer overlying SMC. ECs have been reported to inhibit SMC growth in a co-culture of ECs and SMCs.[38,39] In vivo studies have shown that extensive neointimal proliferation after luminal injury was restricted by re-endothelialization of vessel walls.[40] Our prior investigation found that the neointimal thickness for dual cell–seeded grafts (ECs on top of SMCs) was significantly thinner than those seeded with SMC/lacZ and was similar with that from grafts without cell seeding after 100-day implantation.[8]

Another way to modulate neointimal hyperplasia from SMC seeding is to modify SMCs in vitro by transducing them with genes that modulate SMC proliferation, such as NOS[20] or homeobox transcription factor Gax.[41] Higher NOS expression increases the production of NO. We, along with others, have reported that transfer of eNOS gene can reduce neointima formation.[20-24] In this study, we found that the neointima on type V grafts was the thinnest among the cell-seeded grafts and was similar to that of unseeded grafts. This indicates that eNOS can modulate the growth of seeded SMCs on the grafts (Table). The neointima of type IV grafts is significantly thinner than that of type III grafts, indicating that effect of eNOS on inhibition of SMC proliferation could overcome some of the effect of tPA on stimulation of SMC proliferation. NO also exerts antithrombotic effects in arterial endothelium. Early studies demonstrated that NO inhibits platelet activation and platelet recruitment to the growing thrombus.[42] The antithrombotic effects of NO on arterial endothelium are also attributable to inhibition of the expression of the prothrombotic protein plasminogen activator inhibitor-1.[43,44] The antithrombotic effect of NO explains our observation that minimal thrombus formed on type V grafts at 30 days.

A new strategy toward surface engineering of a thromboresistant graft without inducing excessive neointimal hyperplasia could be to replace the wild-type tPA with a zymogen tPA.[7,45] A zymogen tPA has a reduced protease activity after it is synthesized inside a cell. It is not activated until it binds to extracellular fibrin. This lower protease activity will result in less ECM degradation and less neointimal hyperplasia when tPA is used to develop a thromboresistant graft.

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