Dual cell seeding and the use of zymogen tissue plasminogen activator to improve cell retention on polytetrafluoroethylene grafts

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Objective: **The purpose of this study was to enhance the retention of seeded endothelial cells (EC) on prosthetic vascular grafts. Dual-layer EC and smooth muscle cell (SMC) seeding and gene transfer of a zymogen tissue plasminogen activator gene (tPA) into seeded EC were studied.**

Methods: **Polytetrafluoroethylene (PTFE) grafts were precoated with fibronectin, seeded with SMC followed by EC a day later, and then, 24 hours later, exposed to an in vitro flow system for 1 hour. Cell retention rates were determined for grafts seeded with EC only, a dual layer of EC on top of SMC, EC transduced with wild-type tPA, and EC transduced with zymogen tPA.**

Results: **Seeding efficiency of PTFE pretreated with fibronectin was 260 ± 8 cell/mm^2. After exposure to flow, only 39% ± 14% of the EC were retained when EC were seeded alone, whereas 73% ± 22% of EC remained on grafts when EC were seeded on top of SMC ($P < .001$, n = 10). The enzyme activity of a mutant zymogen tPA in absence of fibrin was 14 ± 1 IU/mL, which is 3.6-fold lower than that in the presence of fibrin (50 ± 19 IU/mL), whereas fibrin has no effect on the wild-type tPA activity. EC expressing a high level of wild-type tPA had a lower retention rate (37%) when compared with normal EC (45%). EC expressing the mutant zymogen tPA had an improved retention rate (54%, $P = .001$, n = 10) in absence of fibrin, whereas its retention rate was reduced to 43% when the cells were exposed to fibrin.**

Conclusion: **SMC seeded between EC and PTFE improves EC retention in vitro. Transduction of zymogen tPA increases thrombolytic ability of seeded cells with less adverse impact on cell retention than wild-type tPA. (J Vasc Surg 2001;34:337-43.)**

Seeding the prosthetic grafts with endothelial cells (EC) was proposed to reduce surface thrombogenicity of vascular grafts.[1,2] In the study by Deutsch et al,[3] a 9-year patency rate of EC-seeded polytetrafluoroethylene (PTFE) grafts was 65%, versus 16% for the unseeded control group, whereas Jensen et al[4] showed that the long-term outcome was not obviously influenced by EC seeding. The failure of cell seeding has been blamed for the poorly adherent EC that were stripped off the prosthetic surface when exposed to blood flow.

Extra cellular matrix (ECM) (collagens, proteoglycans, elastin, etc) secreted from cells contributes significantly to the adherence of cells to the prosthetic grafts. The precoating ECM on the surface of PTFE grafts has been shown to benefit cell seeding and adhesion.[5,6] We hypothesized that increasing and stabilizing the ECM secreted from cells may enhance cell adhesion and therefore improve cell retention on the prosthetic graft. Because smooth muscle cells (SMC) secrete a significantly larger amount of ECM than do EC, SMC could be used to improve cellular adhesion onto prosthetic materials. The seeding of SMC on grafts has been studied by other investigators,[7,8] but not in the context of enhancing EC seeding and retention. Another advantage of SMC is that the multilayer configuration of SMC provides more "exogenous gene product forming" units per unit volume than a monolayer of EC.

Tissue plasminogen activator (tPA), a thrombolytic protease, can be overexpressed in EC and SMC after gene transfer.[9,10] This enzyme converts inactive plasminogen into active plasmin, which then degrades fibrin complexes, a major component of a thrombus. The wild-type tPA has high protease activity when it is secreted from cells as a single chain.[11] A problem encountered with the seeding of wild-type tPA excreting cells is that overexpression of tPA will induce nonspecific proteolysis of the supporting extracellular matrix, thus decreasing the retention of seeded EC on prosthetic grafts.[12,13]

A zymogen tPA mutant (R275E, A292S, F305H),[14] which is secreted as an inactive precursor in single-chain form, with a 200-fold reduction in catalytic efficiency as compared with the wild-type tPA, retains full activity when cleaved into two-chain form after binding to fibrin. Its low

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Competition of interest: nil.

Supported by the American Heart Association, Pacific Vascular Research Foundation, May R. Wright Foundation, Culpepper Foundation.

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Published online May 21, 2001.

0741-5214/2001/$35.00 + 0 **24/1/114817**
doi:10.1067/mva.2001.114817

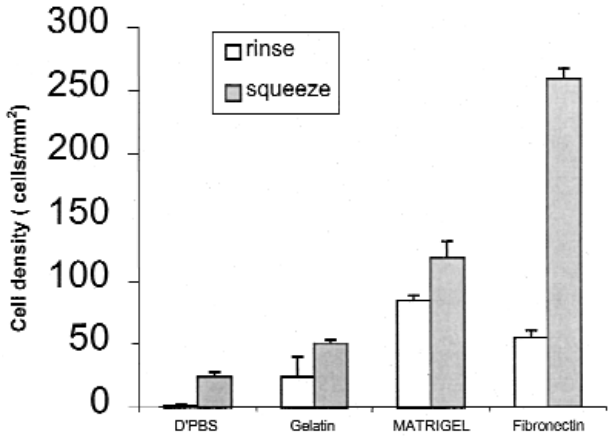

Fig 1. Cell seeding on PTFE grafts coated with different matrix. PTFE grafts (5-mm internal diameter) were either rinsed *(open bar)* or squeezed *(filled bar)* with matrix materials indicated. EC were grown on luminal surface of graft. Cell number was counted 1 day after cell seeding. Cell density on PTFE grafts was presented as mean ± SE from eight experiments with 10 to 36 seeded grafts.

protease activity, once secreted from the cell, should exhibit limited digestion of the ECM. The increased tPA expression should therefore have little adverse effect on cellular adhesion while maintaining its thrombolysis ability when it is bound to fibrin within the thrombus.

In this study, an SMC cellular layer between seeded EC and the graft surface was used to enhance EC retention on prosthetic grafts. The gene encoding the zymogen tPA mutant was transduced into seeded EC to avoid the adverse effects of wild-type tPA on cell adhesion while being able to enhance the antithrombotic activity of seeded cells.

MATERIAL AND METHODS

Cell culture. The 293T/17 cells (CRL11268)[15] were obtained from American Type Culture Collection and maintained in Dulbecco modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and glutamine 2 mmol/L (Gibco BRL). The 293/GPG cell line, a vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped murine leukemia viral vector (MuLV) packaging cell line, was provided by Ory et al[16] and maintained in DMEM described above with additional tetracycline 1 μg/mL (Sigma Chemical Co, St Louis, Mo), puromycin 2 μg/mL (Sigma Chemical Co), G418 0.3 mg/mL (Gibco BRL), and 1 mmol/L MEM sodium pyruvate (Gibco BRL) and has constantly expressed gag-pol genes and tet promotor-controlled VSV-G genes. All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Isolation of EC and SMC. Human saphenous vein EC and SMC were isolated as described previously[17] from human saphenous veins, which was approved by the Institutional Review Board (IRB 969-042). EC were cultured in MCDB 131 medium as described.[18] SMC were cultured in Williams' medium (Gibco BRL) supplemented with glutamine 2 mmol/L and 20% fetal bovine serum.

Plasmids. Plasmids containing the cDNA encoding for wild-type and zymogen tPA (tPA/R275E,F305H,A292S)[14] were provided by Dr Edwin Madison (Corvas International Inc, San Diego, Calif). The retroviral vector plasmids pG1wTSvNa and pG1zTSvNa carrying wild-type tPA and mutant zymogen tPA, respectively, were generated by inserting the Xba I fragments of ptPA and ptPA/R275E,F305H,A292S into G1X SvNa (obtained from Genetic Therapy, Inc/Novartis, Gaithersburg, Md). Retroviral vector, pLGFPSN,[19] was used for green fluorescence protein (GFP) expression.

Retroviral production and cell transduction. The replication-incompetent VSV-G pseudotyped MuLV vectors were first generated from a transient three-plasmid transfection system as described previously.[20] The viral supernatants from the transient transfection were used to transduce packaging cell line 293/GPG. The pools of the transduced 293/GPG were named as *293/GPG/X,* where *X* represents different vectors: G1nBgSvNa, G1wTSvNa, G1zTSvNa, and LGFPSN. The cell lines 293/GPG/X, without selection, were used as stable-vector producer cells because the transduction efficiency of VSV-G pseudotyped MLV vector is higher than 90%.[20] After the 293/GPG/X cells were cultured in DMEM with tetracycline to 90% confluence, the cells were washed with phosphate-buffered saline solution (PBS) and then further cultured in the fresh aforementioned DMEM but without tetracycline. At every 24 hours, retroviral supernatants were collected and replaced with fresh culture medium. The collected culture medium (up to 72 hours) was filtered through a 0.45-μm pore-size filter (Pall Gelman, Ann Arbor, Mich) and stored at –80°C for further use. The viral titers were between 10^6 to 10^7 colony formation units per milliliter as analyzed by neomycin resistance assay. All gene transfers were mediated with the VSV-G pseudotyped MuLV (VSV-G/MuLV) vectors generated from the producer cell lines 293/GPG/X. Transduction of EC and SMC with viral vectors was as described[21] by mixing the cells with viral supernatants and with 8 μg polybrene for 2 hours, followed by G418 (Gibco BRL) selection.

Determination of tPA concentration. EC, regardless of whether they were tPA gene transduced, were cultured in a 24-well plate to 90% confluence and then washed once with PBS. The culture medium was replaced with 1-mL serum-free EC medium and collected after 24-hour culture with the cells. The tPA fibrinolytic enzyme activity of the cell culture supernatant was determined by a chromogenic assay with the Chromolize tPA assay kit from Biopool (Ventura, Calif). The concentration of tPA in the supernatant was determined by measuring the tPA antigen concentration with an enzyme-linked immunosorbent assay kit (TintElize tPA) from Biopool. The enzyme-linked immunosorbent assay kits recognize both wild-type and

zymogen tPA. In the experiments of fibrin effect on tPA activity and cell retention, the fibrin (America Diagonostic, 25 μg/mL) was added to the reaction mixture or the medium in circuit system for in vitro flow study.

Cell seeding. Expanded PTFE grafts (regular wall thickness, internal diameter 5 mm) were provided by IMPRA, Inc (Tempe, Ariz). The grafts were coated with either fibronectin (0.1 mg/mL in PBS, Gibco BRL), Matrigel matrix (1:3 dilution, Becton Dickinson, Franklin Lake, NJ), gelatin (1%, Sigma Chemical Co), or PBS (control) for the seeding condition experiment. The coating treatment was either the grafts were rinsed with the solutions and air-dried, or the solution was squeezed through the graft wall by holding both ends of solution-filled graft and pressuring the agent into the graft wall.[6] Beyond the seeding condition experiment, the fibronectin-squeezed grafts were used for all further cell seeding experiments. After the coating, the grafts were filled with cells (3×10^5 cells/mL) dissociated from 100-mm plates, heat-crimped at both ends, and rotated at 1 rpm for 2 hours at 37°C. Then the ends of the graft were opened, and cell suspension was removed. The grafts were cultured in a 100-mm culture dish with EC medium for 1 day before being tested in flow circuit.

For single-cell seeding, both EC and SMC were transduced with retroviral vectors carrying the *lacZ* gene. The grafts were stained with either X-gal[20] or hematoxylin and eosin to visualize the seeded cells, and the cell numbers were counted under a microscope. For dual-layer seeding, EC and SMC were transduced with the *lacZ* gene and gene coding for GFP, respectively. EC and SMC were readily distinguishable by X-gal staining and fluorescent microscopy. SMC were seeded on PTFE grafts squeezed with fibronectin as described above. The grafts were cultured for 1 day before EC were seeded over the SMC. In the experiments of tPA effect on cell retention, only EC-seeded grafts were used.

In vitro flow studies. A pulsatile in vitro flow circuit housed in a tissue culture incubator was constructed to evaluate retention of seeded cells. A pulsatile blood pump (model 1421, Harvard Apparatus, South Natick, Mass) pumped MCDB 131 medium at 500 mL/min at 70 strokes/min with 100 mm Hg systolic pressure through a pair of PTFE grafts (5-mm internal diameter) in parallel, generating a shear stress of 6.1 dyne/cm^2 by use of the formula $4\eta Q/\pi r^3$ with η as the viscosity of the medium (0.009 g/cm × sec), Q as the flow rate in mL/sec, and r as the graft radius.[22]

Before connecting to the flow circuit, one fourth of the graft (1 cm) was cut as a preflow control. The cell-seeded grafts were placed in the flow circuit for 60 minutes. After this flow exposure, the graft segments were removed from the pump and rinsed with PBS. Both preflow and postflow grafts were fixed with 10% formaldehyde. The grafts were stained with X-gal to visualize the seeded cells. The number of residual cells were counted by use of a microscope and expressed as cell density in cells per millimeters squared. For dual-layer–seeded grafts, EC were quantitatively examined under bright-field microscopy, and the SMC were quantitatively examined under fluorescent microscopy.

Statistical analyses. All values are expressed as mean ± SEM. Mean values for continuous variables were compared with analysis of variance and paired 2-tailed Student *t* tests. Significance was attributed to a *P* value of less than .05.

RESULTS

Seeding cells on grafts. Squeezing the matrix through the PTFE wall significantly increases the cell seeding efficiency for every matrix tested ($P < .05$, Fig 1). The best seeding efficiency resulted from PTFE coated with fibronectin by the squeeze-through technique and was about fivefold higher than that of nonsqueezed PTFE coated with fibronectin (55 ± 6 cells/mm^2, n = 25). The cell density on the surface of PTFE reached 260 ± 8 cell/mm^2 (n = 36) after 1 day of culture, which was twofold higher than that of the squeeze through with Matrigel (118 ± 3 cell/mm^2, n = 26). Only a few cells (1 cell/mm^2) were successfully seeded on PBS-rinsed PTFE grafts.

Dual-layer cell seeding. EC and SMC, before being seeded, were transduced with the marker genes *lacZ* and GFP, respectively, to distinguish SMC from EC on the double-seeded graft. The two cell types were readily distinguishable by X-gal staining (for detection of EC, Fig 2, *A*) and fluorescent microscopy (for detection of SMC, Fig 2, *B*). The densities of EC seeded on PTFE, either single-cell EC seeding or dual-layer seeding, are similar, independent of prior SMC seeding (358 cells/mm^2 for single-cell seeding vs 330 cells/mm^2 for dual-layer seeding, Table I). SMC growing on a culture dish have a flattened stellate appearance (Fig 2, *C*), whereas the structure of SMC is elongated and spindle shaped while growing on PTFE (Fig 2, *D*).

Cell retention after in vitro flow. After the PTFE grafts were exposed to in vitro flow for 60 minutes, approximately 60% of EC were lost when the cells had been seeded on a graft alone (retention rate 39%, Table I, Fig 3, *A* and *D*). SMC seeded alone had a better retention (64%, Fig 3, *B* and *E*) than EC seeded alone (39%, $P < .001$, n = 10). The retention rate of EC seeded on top of SMC (73%) was significantly higher than that of EC seeded alone ($P < .001$, n = 10, Fig 3, *C* and *F*).

Effect of tPA on cell retention. After EC were transduced with retroviral vectors carrying genes encoding either the wild-type tPA or mutant zymogen tPA, the concentration and activity of tPA in the supernatants of the cell cultures were assayed in the presence and the absence of fibrin. The tPA production increased to 133 ± 38 ng/mL and 230 ± 64 ng/mL of EC transduced with wild-type tPA and zymogen tPA, respectively, from 10 ± 3 ng/mL of the nontransduced EC. Fibrin did not have a significant effect on the activity of wild-type tPA (Table II). However, the zymogen mutant tPA had lower enzyme activity without fibrin (14 ± 1 IU/mL) than with fibrin

JOURNAL OF VASCULAR SURGERY
August 2001

Table I. Retention of seeded cells on PTFE graft after exposed flow in vitro

	No. of cells on graft† (cells/mm²)		
*Cell seeded**	*Before flow*	*After flow*	*Retention rate‡ (%)*
EC only	358 ± 19 (10)	137 ± 14 (10)	39 ± 14
SMC only	201 ± 9 (13)	126 ± 12 (13)	64 ± 23
EC on top of SMC	330 ± 14 (10)	243 ± 27 (10)	73 ± 22

*PTFE (5-mm internal diameter) was seeded with EC alone, SMC alone, or EC on top of SMC 24 hours before exposure to flow. The grafts were exposed to a flow of culture medium at a rate of 500 mL/min (shear stress = 6.1 dyne/cm²) for 60 minutes in vitro.
†Cells on the graft were counted under a microscope in 10 random fields. Numbers represent the mean ± SE of the number of cells on the grafts examined (shown in parentheses).
‡Retention rate is the number of cells on a postflow graft expressed as a ratio of the number of cells on a preflow graft.

Table II. Activity of tPA and its effect on cell retention

			No. of cells on graft‡ (cells/mm²)		
*Cell seeded**	*Fibrin†*	*tPA activity (IU/mL)*	*Before flow*	*After flow*	*Retention rate‡ (%)*
EC	–	1.5 ± 0.1	334 ± 46	152 ± 25	45 ± 5 (10)
	+	1.0 ± 0.1	304 ± 127	130 ± 58	40 ± 8 (4)
EC/wild-type tPA	–	30 ± 1.4	229 ± 22	122 ± 19	39 ± 6 (10)
	+	29 ± 8.2	329 ± 49	111 ± 23	34 ± 18 (4)
EC/zg tPA	–	14 ± 1.0	250 ± 27	139 ± 50	54 ± 13 (10)
	+	50 ± 19	255 ± 19	113 ± 31	43 ± 8 (4)

*PTFE (5-mm internal diameter) was seeded with EC, EC transduced with wild-type tPA (EC/wt tPA), or EC transduced with zymogen tPA (EC/zg tPA) 24 hours before exposure to flow. The grafts were exposed to a flow as described in Table I.
†The fibrin (25 μg/mL) was added (+) or not added (–) to the medium in the flow circuit.
‡Cells on the graft were counted under a microscope in 10 random fields. Retention rate is the number of cells on a postflow graft expressed as a ratio of the number of cells on a preflow graft. Numbers represent the mean ± SE of the number of cells on the grafts examined (shown in parentheses).

(50 ± 19 IU/mL) because of its secretion in the inactive precursor form. The enzyme activity of zymogen was less than half the activity of wild-type tPA in the absence of fibrin, whereas it was higher than that of wild-type tPA in the presence of fibrin (Table II).

We then accessed the effect of tPA expressions on cell retention. PTFE grafts were seeded with EC or tPA-transduced EC and then exposed to flow in vitro for 60 minutes in the presence or absence of fibrin as described above. The retention rate of EC with wild-type tPA (39%) was lower than that without the tPA gene (45%). The presence of fibrin had no significant difference on the cell retention rate for grafts seeded with EC or EC transduced with wild-type tPA ($P > .2$, n = 4 to 10, Table II). Grafts seeded with cells carrying the gene for the zymogen mutant showed significant ($P = .001$, n = 10) improvement in cell retention (54%) in the absence of fibrin when compared with those with wild-type tPA (39%). In the presence of fibrin, the retention rate of cells with zymogen tPA was significantly ($P = .005$, n = 4) reduced to 43%, which was not significantly different than with wild-type tPA (34%, $P = .19$, n = 4).

DISCUSSION

There has been various enhancement of EC adhesion onto synthetic vascular graft surfaces reported: (1) coating the surface of the grafts with ECM protein ligands, such as fibronectin[23] or fibronectin together with integrin-independent (avidin-biotin) proteins[24]; (2) covalently attaching cell adhesion peptide sequences, such as Arg-Gly-Asp[25]; and (3) generating new molecular functional groups and changing surface properties by plasma discharge treatment.[26] However, the focus of these studies was on improvement of cell attachment on the graft surface and did not increase the cell retention rate significantly.

Fibronectin squeezed through PTFE has been reported to be the most effective technique to increase cell seeding efficiency.[6] Our data support these findings, and the seeding efficiency of grafts treated by means of the squeeze-through technique with fibronectin was significantly higher than grafts only rinsed with fibronectin (Fig 1), whereas the passive rinsing of PBS onto the hydrophobic surface of PTFE yielded a low seeding efficiency. The improved seeding efficiency with the squeeze-through technique could result from the reduced hydrophobic property of the PTFE surface after the fibronectin has penetrated the fine pores of the PTFE under the squeeze pressure. The passive rinsing matrix through the graft can do little to increase the hydrophilicity of the graft surface, which is essential for cells to attach.

To further improve retention of the seeded cells once they were exposed to the shear stress of the flowing

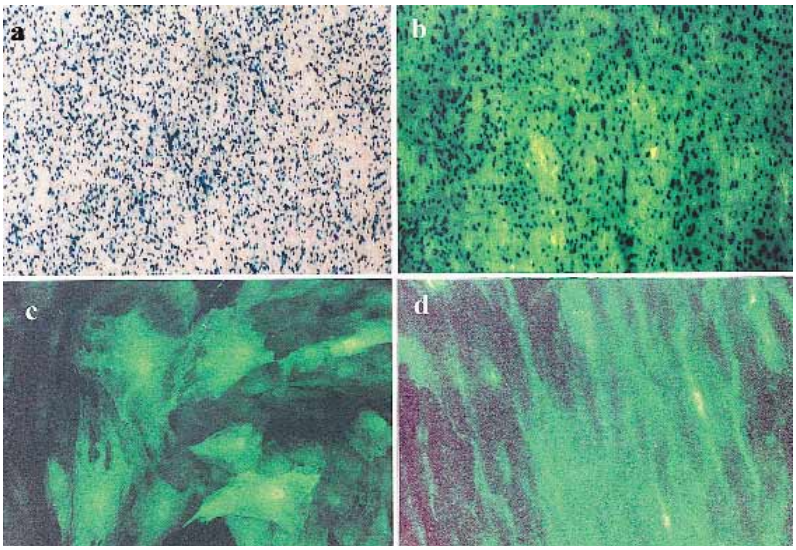

Fig 2. Distinguishing SMC from EC on dual-cell–seeded PTFE graft. SMC and EC were transduced with GFP and *lacZ* marker genes, respectively. GFP-transduced SMC were seeded on PTFE, and EC were seeded on top of SMC 1 day later. Dual-seeded graft was stained with X-gal. Under phase contract microscopy (**a,** Original magnification × 40), only EC in blue can be seen. Under fluorescent microscopy **(b)**, SMC (in *green*) and EC (in *dark spots*) can be distinguished. GFP-transduced SMC grown on a culture dish **(c)** was compared with SMC grown on PTFE **(d)**.

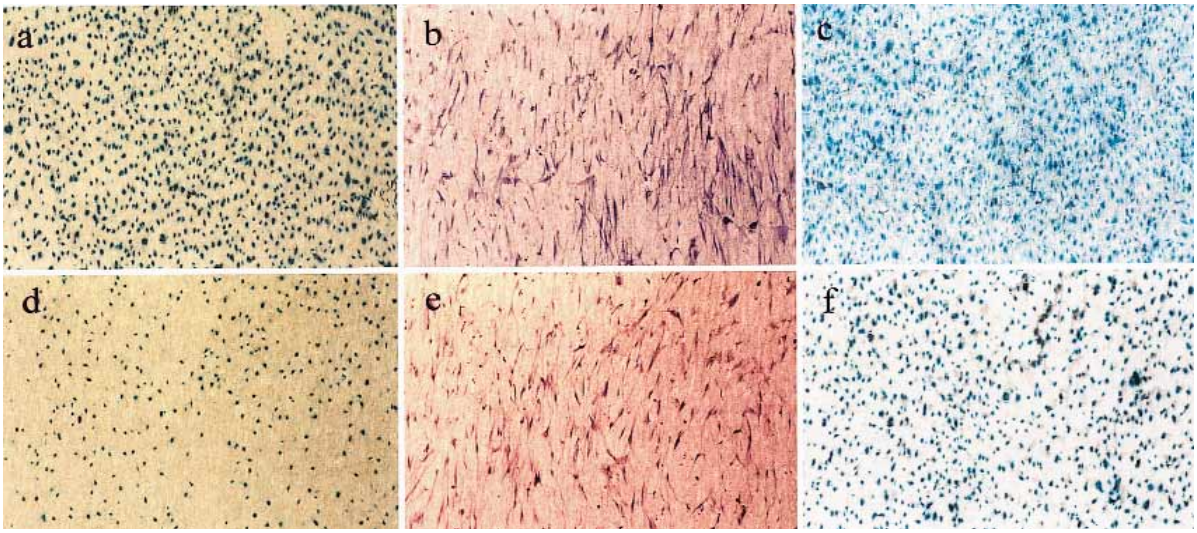

Fig 3. Cell retention on PTFE grafts. PTFE grafts seeded with EC alone **(a, d)**, SMC alone **(b, e)**, and EC on top of SMC **(c, f)** were exposed to flow in vitro for 60 minutes. EC were stained by X-gal, which shows *blue color*. SMC were stained with hematoxylin and eosin. *Upper panels* are cells before flow; *lower panels* are cells after flow. Dual seeding significantly improves surface retention of cells **(c, f)**.

medium, we added vascular SMC as an additional layer between the EC and the graft surface. Vascular SMC are a major cellular component of the vessel wall, secreting significant amounts of extracellular matrix components.[27] SMC seeded alone had a better retention rate (64%) than EC seeded alone (39%) (Table I), perhaps because of the expression of ECM on seeded cells to enhance adhesion. EC seeded on top of the SMC also resulted in a higher retention rate (73%) than EC seeded alone. Ono et al[28] have shown that EC-conditioned medium stimulated the adhesion and growth of SMC, suggesting EC produce and secrete accelerators for the adhesion of SMC. Therefore, the higher retention rate in dual seeding could be a result of the better adhesion of SMC on graft surface, or the

interaction between EC and SMC increases the cell adhesion. The 87% improvement of the retention rate for EC seeded on top of SMC compared with EC seeded alone suggests that the SMC provide a more effective way for retaining EC on artificial graft surfaces. The growth pattern and physiologic properties of SMC lend themselves to an excellent nature matrix for the attachment of EC. The dual-cell seeding on the surface of prosthetic grafts will best mimic the natural structure of vessels. In addition, SMC provide a multilayer network of cells, allowing for increased production of exogenous gene product when transduced with a selected therapeutic gene. This higher quantity of cell-producing unit per unit area can be used in the design of a graft that acts as an intravascular drug delivery unit.

A concern of use of SMC with this dual-layer model is the lack of control growth of the SMC, which may result in graft stenosis. However, studies have found that EC markedly inhibit the SMC growth in a coculture of EC and SMC.[29,30] Because there is an EC layer covering the SMC layer, the proliferation of SMC may be modulated and limited by the EC layer. The in vivo studies have also shown that the extensive neointimal proliferation after luminal injury was restricted by reendothelialization of vessel walls.[31] Furthermore, either seeded SMC or EC could be genetically modified by transferring a gene coding for nitric oxide synthase (NOS) to stimulate production of nitric oxide, which has been shown to inhibit the SMC proliferation and mitogenesis responsible for intimal hyperplasia.[32,33] Gene transfer of NOS into EC has also been successful in reducing SMC hyperplasia.[34,35] Therefore, the proliferation of seeded SMC could be controlled by both EC and NOS gene transduction.

The seeding of tPA-transduced cells onto grafts has been proposed to enhance thrombolytic activity of the grafts and thereby to improve graft patency. In addition to direct thrombolysis, inhibition of the fibrin cross-linked stabilization of the platelet coagulum by in situ overexpression of a thrombolytic agent may have the added benefit of slowing the development of myointimal hyperplasia and atherosclerosis within or in the outflow vasculature distally, hence, improving graft patency. However, overexpression of tPA has been shown to further decrease retention of seeded EC on prosthetic grafts because the wild-type tPA is secreted from cells as an active protease that induces nonspecific proteolysis of the supporting extracellular matrix, thus reducing the adhesion of EC to the graft surface.[12,13]

To avoid the tPA-induced reduction of cell retention, we have applied zymogen tPA in this study and shown that the tPA production of seeded cells can be increased without sacrificing their retention efficiency. Zymogen mutant tPA has been reported to display a reduction in catalytic efficiency by a factor of 200 in the single-chain form, while retaining full activity in its cleaved form, which is accomplished by binding to fibrin.[14] We observed that the retention rate of cells with the zymogen tPA was significantly higher in the absence of fibrin than that with wild-type tPA (Table II). In the presence of fibrin, the protease activity increased about fivefold (Table II), and the retention rate of cells with zymogen tPA was significantly reduced to a similar level as that with wild-type tPA. These results fit our hypothesis that lower protease activity associated with zymogen tPA will have limited digestion of ECM, resulting in better cell retention; increased tPA activity induced by fibrin leads to ECM digestion and lower cell retention.

The property of low enzymatic activity of mutant zymogen tPA in absence of fibrin is beneficial because the increase of zymogen tPA production in the cells will not increase protease activity significantly inside the cells when the zymogen tPA has not contacted with fibrin outside of the cells. Once the zymogen tPA is secreted and encountered fibrin on the lesion site, it will be converted into an active one. By this way, the nonspecific proteolysis of the supporting ECM can be minimized and the cell retention be increased, whereas the antithrombosis ability will be enhanced.

REFERENCES

1. Herring M, Gardner A, Glover J. A single-staged technique for seeding vascular grafts with autogenous endothelium. Surgery 1978; 84:498-504.
2. Eickhoff JH, Broome A, Ericsson BF, Buchardt Hansen HJ, Kordt KF, Mouritzen C, et al. Four years' results of a prospective, randomized clinical trial comparing polytetrafluoroethylene and modified human umbilical vein for below-knee femoropopliteal bypass. J Vasc Surg 1987;6:506-11.
3. Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. Surgery 1999;126:847-55.
4. Jensen N, Lindblad B, Bergqvist D. Endothelial cell seeded dacron aortobifurcated grafts: platelet deposition and long-term follow-up. J Cardiovasc Surg 1994;35:425-9.
5. Kaehler J, Zilla P, Fasol R, Deutsch M, Kadletz M. Precoating substrate and surface configuration determine adherence and spreading of seeded endothelial cells on polytetrafluoroethylene grafts. J Vasc Surg 1989;9:535-41.
6. Falk J, Townsend LE, Vogel M, Boyer M, Olt S, Wease GL, et al. Improved adherence of genetically modified endothelial cells to small-diameter expended polytetrafluoroethylene grafts in a canine model. J Vasc Surg 1998;27:902-9.
7. Clowes MM, Lynch CM, Miller AD, Miller DG, Osborne WR, Clowes AW. Long-term biological response of injured rat carotid artery seeded with smooth muscle cells expressing retrovirally introduced human genes. J Clin Invest 1994;93:644-51.
8. Geary RL, Clowes AW, Lau S, Vergel S, Dale DC, Osborne WR. Gene transfer in baboons using prosthetic vascular grafts seeded with retrovirally transduced smooth muscle cells: a model for local and systemic gene therapy. Hum Gene Ther 1994;5:1211-6.
9. Shayani V, Newman KD, Dichek DA. Optimization of recombinant t-PA secretion from seeded vascular grafts. J Surg Res 1994;57:495-504.
10. Ekhterae D, Stanley JC. Retroviral vector-mediated transfer and expression of human tissue plasminogen activator gene in human endothelial and vascular smooth muscle cells. J Vasc Surg 1995;21: 953-62.
11. Boose JA, Kuismanen E, Gerard R, Sambrook J, Gething MJ. The single-chain form of tissue-type plasminogen activator has catalytic activity: studies with a mutant enzyme that lacks the cleavage site. Biochemistry 1989;28:635-43.
12. Huber TS, Welling TH, Sarkar R, Messina LM, Stanley JC. Effects of retroviral-mediated tissue plasminogen activator gene transfer and expression on adherence and proliferation of canine endothelial cells

seeded onto expanded polytetrafluoroethylene. J Vasc Surg 1995;22: 795-803.
13. Dunn PF, Newman KD, Jones M, Yamada I, Shayani V, Virmani R, et al. Seeding of vascular grafts with genetically modified endothelial cells. Secretion of recombinant tPA results in decreased seeded cell retention in vitro and in vivo. Circulation 1996;93:1439-46.
14. Tachias K, Madison EL. Variants of tissue-type plasminogen activator which display substantially enhanced stimulation by fibrin. J Biol Chem 1995;270:18319-22.
15. DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, Calos MP. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 1987;7:379-87.
16. Ory DS, Neugeboren BA, Mulligan RC. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 1996;93: 11400-6.
17. Eton D, Terramani TT, Wang Y, Takahashi AM, Nigro JJ, Tang L, et al. Genetic engineering of stent grafts with a highly efficient pseudotyped retroviral vector. J Vasc Surg 1999;29:863-73.
18. Terramani TT, Eton D, Bui PA, Wang Y, Weaver FA, Yu H. Human macrovascular endothelial cells: optimization of culture conditions. In Vitro Cell Dev Biol Anim 2000;36:125-32.
19. Kwon YJ, Yu H, Peng CA. Enhanced retroviral transduction of 293 cells cultured on liquid-liquid interfaces. Biotechnol Bioeng 2001;72:331-8.
20. Yu H, Eton D, Wang Y, Kumar S, Tang L, Terramani T, et al. High efficiency in vitro gene transfer into vascular tissues using a pseudotyped retroviral vector without pseudotransduction. Gene Ther 1999; 6:1876-83.
21. Yu H, Wang Y, Eton D, Stins M, Wang L, Apuzzo ML, et al. Retroviral vector-mediated transfer and expression of human tissue plasminogen activator cDNA in bovine brain endothelial cells. Neurosurgery 1999;45:962-968; discussion 968-970.
22. Dewey CF Jr. Fluid mechanics of arterial flow. In: Werthessen NT, Wolf S, editors. Dynamics of arterial flow. New York: Plenum; 1979. p. 55-103.
23. Kesler KA, Herring MB, Arnold MP, Glover JL, Park HM, Helmus MN, et al. Enhanced strength of endothelial attachment on polyester elastomer and polytetrafluoroethylene graft surfaces with fibronectin substrate. J Vasc Surg 1986;3:58-64.
24. Bhat VD, Truskey GA, Reichert WM. Fibronectin and avidin-biotin as a heterogeneous ligand system for enhanced endothelial cell adhesion. J Biomed Mater Res 1998;41:377-85.
25. Massia SP, Hubbell JA. Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is mediated by integrin alpha 4 beta 1. J Biol Chem 1992;267:14019-26.
26. Pratt KJ, Williams SK, Jarrell BE. Enhanced adherence of human adult endothelial cells to plasma discharge modified polyethylene terephthalate. J Biomed Mater Res 1989;23:1131-47.
27. Glukhova MA, Koteliansky VE. Integrins, cytoskeletal and extracellular matrix proteins in developing smooth muscle cells of human aorta. In: Schwartz SM, Mecham RP, editors. The vascular smooth muscle cell. San Diego: Academic Press; 1995. p. 17-36.
28. Ono O, Ando J, Kamiya A, Kuboki Y, Yasuda H. Flow effects on cultured vascular endothelial and smooth muscle cell functions. Cell Struct Funct 1991;16:365-74.
29. Fillinger MF, O'Connor SE, Wagner RJ, Cronenwett JL. The effect of endothelial cell coculture on smooth muscle cell proliferation. J Vasc Surg 1993;17:1058-67; discussion 1067-8.
30. Powell RJ, Cronenwett JL, Fillinger MF, Wagner RJ, Sampson LN. Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. Ann Vasc Surg 1996;10:4-10.
31. Schwartz SM, R. OBE, Deblois D, Giachelli CM. Relevance of smooth muscle replication and development to vascular disease. In: Schwartz SM, Mecham RP, editors. The vascular smooth muscle cell. San Diego: Academic Press; 1995. p. 81-121.
32. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774-7.
33. Kariya K, Kawahara Y, Araki S, Fukuzaki H, Takai Y. Antiproliferative action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. Atherosclerosis 1989;80:143-7.
34. von der Leyen HE, Gibbons GH, Morishita R, Lewis NP, Zhang L, Nakajima M, et al. Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene. Proc Natl Acad Sci U S A 1995;92:1137-41.
35. Chen L, Daum G, Forough R, Clowes M, Walter U, Clowes AW. Overexpression of human endothelial nitric oxide synthase in rat vascular smooth muscle cells and in balloon-injured carotid artery. Circ Res 1998;82:862-70.

Submitted Aug 18, 2000; accepted Jan 18, 2001.