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Novel Approach for Endothelializing Vascular Devices: Understanding and Exploiting Elastin–Endothelial Interactions

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Abstract

Elastin is an essential component of arteries which provides structural integrity and instructs smooth muscle cells to adopt a quiescent state. Despite interaction of endothelial cells with elastin in the internal elastic lamina, the potential for exploiting this interaction therapeutically has not been explored in detail. In this study, we show that tropoelastin (a precursor of elastin) stimulates endothelial cell migration and adhesion more than smooth muscle cells. The biological activity of tropoelastin on endothelial cells is contained in the VGVAPG domain and in the carboxy-terminal 17-amino acids. We show that the effects of the carboxy-terminal 17 amino acids, but not those of VGVAPG, are mediated by integrin $\alpha_v\beta_3$. We demonstrate that tropoelastin covalently linked to stainless steel disks promotes adhesion of endothelial progenitor cells and endothelial cells to the metal surfaces. The adherent cells on the tropoelastin-coated metal surfaces form monolayers that can withstand and respond to arterial shear stress. Because of the unique effects of tropoelastin on endothelial and smooth muscle cells, coating intravascular devices with tropoelastin may stimulate their endothelialization, inhibit smooth muscle hyperplasia, and improve device performance.

Keywords

Arteries; Endothelium; Receptors; Stents; Restenosis

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ELECTRONIC SUPPLEMENTARY MATERIAL

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INTRODUCTION

Complications of intracoronary stent placement include thromboembolic events and neointimal hyperplasia due to smooth muscle cell hyperproliferation, leading to in-stent restenosis. The central role of the vascular endothelium in preventing thrombosis and regulating neointimal hyperplasia has led to restenosis prevention strategies that focus on enhancing endothelialization.^{2,14,17,23} Novel stent coating strategies include delivery of vascular endothelial growth factor (VEGF) (e.g., VEGF-eluting stents³⁷) and the use of antibodies that recognize epitopes specific to endothelial cells (e.g., anti-CD34-coated stents²⁴). However, these noncytotoxic approaches are still at the investigational stage and their long-term outcome is unknown. Drug-eluting stents currently on the market, in contrast, release cytotoxic drugs to inhibit neointimal hyperplasia at the expense of delaying endothelialization.²⁵

Understanding the molecular details of cell–matrix interactions that are involved in the development and maintenance of patent vessels may facilitate the discovery of novel approaches to improve intracoronary stents. This study aimed to better understand elastin–endothelial interactions and explore the potential of elastin as a coating material for intravascular devices. Elastin is a predominant vascular extracellular matrix protein and plays an important structural and signaling role in the development of patent arteries.³² Elastin is synthesized from soluble tropoelastin which is organized into elastic fibers with contributions from fibrillin microfibrils³⁰ and glycoproteins such as fibulin-5.^{27,34,39} These fibers form concentric elastic lamellae around vascular lumens, providing the elasticity necessary to absorb and distribute mechanical stress during the cardiac cycle.

Despite the close proximity of endothelial cells to the internal elastic lamina, there have been only a few conflicting studies investigating the molecular details of elastin–endothelial interactions. Coacervated alpha-elastin has been shown to have a dose-dependent effect on porcine aortic endothelial cell proliferation and migration,^{18,19} while studies using human umbilical vein endothelial cells reported that adsorbed tropoelastin on plastic promoted cell adhesion but not cell spreading⁴⁰ and covalent immobilization of tropoelastin on plasma-treated metal surfaces promoted cell adhesion and proliferation.⁴¹ The variability in elastin effect in these studies may be due to specific domains of tropoelastin and their corresponding endothelial cell surface receptors, which remain to be explored.¹⁰ At least two cell adhesive sites have been identified on tropoelastin: the hexapeptide VGVAPG sequence, which is located in the middle of tropoelastin, and the carboxyl terminus of tropoelastin. Multiple elastin-binding proteins targeting the VGVAPG sequence have been identified and include a 59-kDa protein,⁵ a 67-kDa galactoside-binding protein,¹⁵ a 120-kDa protein called elastonection,¹⁶ and an unidentified G_i-linked G protein-coupled receptor (GPCR).²⁰ The carboxyl terminus of tropoelastin binds to cell surface glycosaminoglycans⁷ and the heterodimeric integrin $\alpha_v\beta_3$.^{31,34} Detailed studies of elastin–cell interaction studies have focused on vascular smooth muscle cells, fibroblasts, and cancer cells, but not endothelial cells.

In this study, we compared the stimulating effect of recombinant tropoelastin on migration and adhesion of endothelial and smooth muscle cells. We also investigated whether the biological activity of tropoelastin on endothelial cells is contained in the VGVAPG domain and the carboxy-terminal 17-amino acids, as well as determining the type of receptors which bind each of these domains. Finally, we examined endothelial functionality (reaching confluence; adhesion and retention under flow) on metal surfaces coated with covalently immobilized tropoelastin. Our findings suggest that tropoelastin coating is a promising approach for enhancing endothelialization of intravascular device.

MATERIALS AND METHODS

Materials

A 48-well Boyden chamber apparatus and 8 μ m-pore polycarbonate membranes for migration experiments were from NeuroProbe (Cabin John, MD, USA). Type I collagen (CN) and fibronectin (FN) were from Biomedical Technologies (Stoughton, MA, USA). Monoclonal anti-integrin antibodies [mouse anti-human $\alpha_v\beta_3$ (clone LM109) and mouse-anti-human $\alpha_2\beta_1$ (clone BHA2.1)] were from Chemicon International (Temecula, CA, USA). Purified whole mouse IgG was purchased from Jackson Immuno-Research Laboratories (West Grove, PA, USA). Oregon Green 488-conjugated phalloidin and DAPI nuclear counterstain were purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA) and Vector Labs (Burlingame, CA, USA), respectively. Bovine serum albumin (BSA) and acetylated 1% gelatin from porcine skin was purchased from Sigma (St. Louis, MO, USA). Other chemical reagents and cell culture ware/reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Lonza (Walkersville, MD, USA), respectively, unless otherwise mentioned.

Recombinant Human Tropoelastin and Synthesized Elastin Peptides

The endogenous signal sequence of human tropoelastin (tELN) was removed and replaced with a 10 $\times$ -histidine tag. This cDNA was cloned into an isopropyl- β -D-thiogalactoside (IPTG)-inducible construct using the pET System and then expressed in the *Escherichia coli* host strain Rosetta-2 (DE3) pLysS (Novagen/EMD Biosciences, San Diego, CA, USA). LB growth media containing 50 μ g/mL ampicillin and 34 μ g/mL chloramphenicol were inoculated with Rosetta-2 (DE3) pLysS bacteria containing the recombinant tropoelastin plasmid, and the culture was grown at 37 °C until OD at 600 nm = 0.4–0.6. Induction was accomplished by adding IPTG to a final concentration of 1 mM. The culture was incubated at 37 °C for 4 h. The bacterial pellet was harvested by compounding centrifugation and weighed. The pellet was processed in Bugbuster HT + lysozyme (Novagen), and inclusion bodies were purified according to Novagen's protocol. Following the last wash, the inclusion bodies were pelleted and dissolved overnight at 4 °C in 1 $\times$ Bind Buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole) containing 6 M urea, pH 7.9. Insoluble material was then removed by centrifugation. The supernatant was filtered through a 0.45- μ m syringe filter. The sample was then added to His-Mag beads (Novagen) that had been pre-equilibrated to 1 $\times$ Bind Buffer with 6 M urea. Beads were collected using a Magnatight stand (Novagen) and washed four times with wash buffer (500 mM NaCl, 60 mM imidazole, 20 mM Tris-HCl) containing 6 M urea, pH 7.9. The recombinant tELN protein was eluted from the beads with 500 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 6 M urea, pH 7.9. The sample was dialyzed against HBSS using Slide-A-Lyzer Dialysis Cassettes with 10,000 MWCO (Pierce). Purified protein was analyzed by SDS-PAGE analysis by standard techniques and stored at –80 °C. Elastin-derived peptides [VGVAPG and IFPGGACLGKACGRKRK (Cterm peptide)] were synthesized at the DNA/Peptide Facility, University of Utah.

CD34+ Cell Purification

Human CD34+ cells were purified from a peripheral blood mononuclear cell preparation collected by an apheresis procedure. The donor was mobilized with 10 μ g/kg granulocyte colony-stimulating factor for 4 days prior to the collection procedure, as approved by the Institutional Review Board of the University of Utah. Purification of CD34+ cells was accomplished in a closed and sterile system using an automated cell selection device, CliniMACS (Miltenyi Biotec, Auburn, CA, USA), following manufacturer's instructions. 98% CD34+ cells were obtained by flow cytometry using a FACScan analyzer (Becton-Dickinson, San Jose, CA, USA) and ISHAGE protocol.³⁶

Endothelial Cells, Routine Cell Culture, and Preparation for Experiments

Human aortic endothelial cells (HAEC), human coronary artery endothelial cells (HCAEC), human microvascular endothelial cells (HMVEC), and human aortic smooth muscle cells (HASMC) were purchased from Lonza and cultured according to manufacturer's specifications in Endothelial Growth Medium-2 (HAEC), Endothelial Growth Medium-2MV (HCAEC, HMVEC), or Smooth Muscle Growth Medium-2 (HASMC). Low passage cells (2–7) were used in all experiments. Sixteen hours prior to experiments, cultures were serum-starved using basal medium [Endothelial Basal Medium-2 (EBM-2) for EC; Smooth Muscle Basal Medium-2 (SbBM-2) for SMC] and 0.5% fetal calf serum.

Migration Assays

Migration assays were performed as described.^{3,28,35} In brief, HAEC were lifted, diluted in buffer (0.5% BSA in EBM-2) to 1.5×10^6 cells/mL, and recovered in suspension at 37 °C and 5% CO₂ for 1 h. For inhibition assays, 25 µg/mL anti- $\alpha_v\beta_3$ antibodies or control IgG were added to the cell suspension during the recovery period. All migration assays were performed in the 48-well Boyden chamber apparatus. 30 µL of recovered cells (4.5×10^4 cells total) was added to the bottom chambers, and the wells were overlaid with an 8 µm-pore polycarbonate membrane coated with 1% acetylated gelatin. The apparatus was assembled and inverted for a 2-h period at 37 °C and 5% CO₂. The apparatus was then re-inverted, and 52 µL of 0.1% BSA in DMEM (diluent, random migration control) or chemoattractants [10 ng/mL fibronectin (positive control), 10–200 ng/mL tropoelastin, 10 µM VGVAPG, and 10 µM IFPGGACLGKACGRKRC term peptide] were added to the upper chambers. Migration was allowed to proceed for 2 h at 37 °C and 5% CO₂ in an incubator. Membranes were then removed, fixed in methanol, and stained with Hema 3. Non-migrated cells were removed from the membrane with a moistened swab, and membranes were mounted migrated-side down on glass slides. The number of cells migrating through the membrane per high power field (HPF) on a Zeiss Axiovert 200 inverted microscope equipped with a Zeiss Axiocam digital camera was recorded. Two HPFs were observed for each well and averaged for each independent sex-tuplicate-well set. Final results were expressed as the average number of cells per HPF from three or more independent experiments.

Adhesion Assays on 96-Well Plates

Adhesion assays were performed as described^{8,22} with minor modifications. 96-well plates were coated overnight at 4 °C with 10 µg/mL collagen type I, fibronectin, tropoelastin, or 0.1% BSA, and then blocked for 1 h at 25 °C in 5% BSA. HAEC were lifted from T-75 flasks, diluted in buffer (0.5% BSA in EBM-2), and recovered in suspension at working density (5×10^5 cells/mL) at 37 °C and 5% CO₂ in an incubator for 1 h. For inhibition assays, 25 µg/mL anti- $\alpha_2\beta_1$ antibodies, anti- $\alpha_v\beta_3$ antibodies, or control IgG were added to the cell suspension during the recovery period. 100 µL of recovered cells (5×10^4 cells total) was added to the coated wells, and adhesion was allowed to proceed for 30 min at 37 °C and 5% CO₂ in an incubator. Following this incubation, the plates were inverted and washed twice with phosphate buffered saline (PBS) to remove non-adherent cells. Wells were then fixed in Zamboni fixative, stained with Gill 1 hematoxylin, overlaid with 80% glycerol, and imaged at 100× magnification on a Zeiss inverted microscope. The number of adherent cells was averaged for two wells (duplicate) in each independent experiment. Final results were expressed as the average number of cells per well from three or more independent experiments.

Adhesion Assays on Stainless Steel Disks

Adhesion assays using unmodified 6 mm stainless steel disks or disks modified with anti-CD34 antibody, tropoelastin, or polysaccharide base matrix as vehicle control (provided by OrbusNeich Medical, Fort Lauderdale, FL, USA) were performed as described above with the following modifications: 48-well plates were used, and twice the number/volume of cells (10^5 cells in 200 μ L) was added to each well. Adhesion was allowed to proceed for 30 min at 37 °C and 5% CO₂. Post-adhesion, the wells were washed in PBS, fixed in 4% neutral buffered formalin, permeabilized in 0.5% Triton X-100 (PBS), blocked in 5% non-fat milk (PBS), and stained for actin fibers with Oregon Green 488-conjugated phalloidin. Disks were mounted on modified microscope slides with Vectashield mounting medium + DAPI (Vector Labs) and imaged using a Zeiss fluorescent microscope. The numbers of adherent cells were counted and final results were expressed as the average number of cells per disk.

Spinning Disk Adhesion Strength Assay

22-mm round glass coverslips were coated with tELN, FN, anti-CD34 antibodies (Santa Cruz), or BSA for 2 h at room temperature in 6-well plates. 3 mL of 5×10^4 HAEC/mL in EGM2 was then added to each well and cells were allowed to adhere for 6 h at 37 °C and 5% CO₂. After 6 h, coverslips were inverted and submerged in HEPES-BSS on a custom holder at 37 °C and spun at 2100 RPM for 5 min as previously described.¹² After 5 min, coverslips were removed and immediately fixed and mounted on slides with Vectashield mounting medium + DAPI and imaged using a BD Pathway 855 microscope. The number of cells/HPF at various distances from the center of the coverslip was counted and expressed as the percentage of cells remaining compared to the center of the disk and normalized to static controls. The shear stress at each measurement location was calculated as described.¹²

Fluid Shear Stress Assays

HAEC were seeded onto 6-mm tropoelastin-coated stainless steel disks or controls (6 mm silicone disks coated with fibronectin) in 48-well plates as described above. Cells were allowed to grow to confluence on the disks for 48 h prior to subjection to flow. The disks were placed in flow chambers and exposed to 24 h of laminar arterial flow of 16 dyn/cm² or static conditions utilizing a recirculating flow system.^{11,33} The disks were fixed and stained for actin using phalloidin as previously described, then mounted with Vectashield mounting medium with DAPI and imaged. Images were processed for actin alignment using a custom Matlab program based on previously described image-processing methods.⁴²

Data Presentation and Statistical Analysis

Bar graphs represent the average of three or more independent experiments. Vertical error bars on data points represent the standard error of the mean. The Student *t*-test and analysis of variance were used to determine the significance of differences between two conditions and between more than two conditions, respectively. *p*-Value less than 0.05 was considered statistically significant.

RESULTS

Tropoelastin Stimulates Endothelial Cell Migration

The entire length of tropoelastin is well conserved among human, bovine, and murine proteins with 82.4% similarity across the length of the entire protein (Supplementary Fig. 1). Purified recombinant tropoelastin has a molecular weight of ~65 kDa and is free from significant contaminating proteins (Supplementary Fig. 2). Using a Boyden chamber apparatus, we performed migration assays with HAEC and various concentrations of full-length recombinant tropoelastin. Compared to the BSA control, there was a significant

increase (4.5-fold) in endothelial migration using tropoelastin doses as low as 10 ng/mL (Fig. 1a). At 200 ng/mL there was an eightfold increase that was comparable to the effect of 10 ng/mL VEGF. In order to differentiate chemokinesis versus chemotaxis, we also performed the experiment using equivalent tropoelastin concentrations in the upper and lower Boyden chambers and observed no increased migration compared to control, suggesting chemotaxis rather than chemokinesis (data not shown). Similar results were seen with other endothelial cell types, including HMVEC and HCAEC (data not shown).

VGAPG and the Carboxy Terminus of Tropoelastin Stimulate Endothelial Migration Through Different Mechanisms

We next compared the effect of another known endothelial chemotaxis stimulant, fibronectin, with the effect of full-length recombinant tropoelastin and the elastin fragments VGAPG and the Cterm peptide. Because the carboxy terminus of elastin has been shown to bind the integrin $\alpha_v\beta_3$ in solid-phase assays³¹ and recently in fibroblasts,⁴ we also investigated the involvement of $\alpha_v\beta_3$ in this study using the $\alpha_v\beta_3$ blocking antibody LM609. The isotype-matched control anti-body IgG increased the baseline endothelial transwell-migration by twofold, perhaps by blocking the non-specific binding sites between the cells and the transwell membrane.²⁹ In the presence of IgG, each chemotaxis stimulants caused a 2 to 2.5-fold increase in endothelial migration when compared to the BSA control (Fig. 1b). The anti- $\alpha_v\beta_3$ antibody almost completely inhibited the stimulating effect of fibronectin, full-length tropoelastin, and the Cterm peptide, as would be expected for processes that involve signaling through $\alpha_v\beta_3$. However, endothelial migration to VGAPG (which may be mediated by an unknown GPCR²¹) was not affected by the anti- $\alpha_v\beta_3$ antibody. When the Cterm peptide was added to the upper and lower Boyden chambers (Fig. 1b, last bar), there was no increased migration, indicating that this peptide promotes chemotaxis. The anti- $\alpha_v\beta_3$ antibody had the same effect on tropoelastin and the Cterm peptide, suggesting that Cterm is sufficient for recapitulating the migratory response of endothelial cells to tropoelastin.

Tropoelastin Promotes Endothelial Cell Adhesion Through $\alpha_v\beta_3$

The adhesion of HAEC to wells pre-coated with 0.1% BSA or with 10 μ g/mL collagen (also an integrin-binding protein), fibronectin, or tropoelastin is quantified and shown in Fig. 2. To determine the involvement of integrin in cell adhesion, HAEC were incubated with either IgG control or anti- $\alpha_2\beta_1$ or anti- $\alpha_v\beta_3$ blocking antibodies prior to being exposed to the coated surfaces. In the presence of the IgG antibody, tropoelastin coating resulted in an approximately eightfold increase in endothelial adhesion compared to BSA coating; this amount of increased endothelial adhesion is similar in magnitude to the effect of collagen or fibronectin coating. Preincubation with anti- $\alpha_2\beta_1$ or anti- $\alpha_v\beta_3$ antibodies did not significantly decrease HAEC adhesion to fibronectin or collagen. This is likely explained by the fact that endothelial cells express multiple integrins that bind collagen or fibronectin.²⁹ However, endothelial adhesion to tropoelastin was blocked by more than 50% by the anti- $\alpha_v\beta_3$ blocking antibody. This suggests that binding of tropoelastin to the integrin $\alpha_v\beta_3$ is the predominant mechanism for endothelial adhesion to tropoelastin-coated surfaces.

Tropoelastin Promotes Adhesion and Migration of Endothelial and Smooth Muscle Cells to Different Degrees

Tropoelastin promotes the migration and spreading of mouse aortic smooth muscle cells through a non-integrin, G-protein-coupled signaling pathway.²⁰ In this study, we were interested in direct comparison of the stimulating effect of tropoelastin on the migration and adhesion of HAEC and HASMC. As shown in Fig. 3a, the baseline motility of HAEC is fivefold higher than that of HASMC. Both fibronectin and tropoelastin increased the motility of HAEC and HASMC, but the motility of HAEC remained significantly higher

than HASMC. As shown in Fig. 3b, the baseline adhesion of HAEC was comparable to that of HASMC. While collagen, fibronectin, and tropoelastin promoted the adhesion of both cell types, their stimulating effects for HAEC were significantly stronger than for HASMC.

Tropoelastin Covalently Linked to Stainless Steel Promotes Adhesion and Spreading of Endothelial Cells

The above results suggest that soluble tropoelastin promotes HAEC migration and that tropoelastin coating of plastic surfaces by adsorption promotes HAEC adhesion to those surfaces. *In vivo* endothelialization of intravascular devices presumably occurs through adhesion of circulating endothelial cells and/or their progenitors, and through invasion of endothelial cells from the borders of the coated surface and/or from the vasa vasorum.⁹ To examine the effects of tropoelastin on adhesion and spreading of endothelial cells on stainless steel, tropoelastin was covalently immobilized to 6-mm diameter stainless steel disks (Fig. 4a) that were pre-treated with a stable polysaccharide base matrix (Ssens BV, Hengelo, The Netherlands). Other disks were coated with anti-CD34 antibodies or with polysaccharide vehicle alone. Coronary stents coated with immobilized anti-CD34 antibodies (Genous Bio-engineered R stent™) accelerate endothelialization by promoting the capture of circulating endothelial cells and endothelial progenitor cells.¹ Adhesion experiments using human CD34+ endothelial progenitor cells, HAEC, and HMVEC (Fig. 4b) demonstrated a greater than ninefold increase in endothelial adhesion to disks coated with anti-CD34 antibodies or tropoelastin over the vehicle control. Additionally, the tropoelastin coating increased the number of adherent HAEC and HMVEC, but not CD34+ cells, compared to the CD34 antibody-coated disks.

We next examined cell spreading on the coated metal surfaces by staining adherent cells with phalloidin to highlight the actin cytoskeleton and DAPI to mark cell nuclei. Both CD34-antibody and tropoelastin coating enhanced the adhesion of human CD34+ cells and HAEC to the metal disks, though these cells did not spread well on either coating within 30 min of adhesion (Fig. 4c, left and middle panels). In contrast, HMVEC adherent to tropoelastin-coated metal disks were well spread and demonstrated an organized actin cytoskeleton within 30 min of adhesion (Fig. 4c, lower right panel). HAEC formed confluent monolayers on tropoelastin-coated metal disks when allowed to adhere for hours rather than minutes (Fig. 5b, left panel). Overall, these studies demonstrate that modifying foreign surfaces with tropoelastin may stimulate endothelial spreading in addition to adhesion.

Cells Remain Adherent to Tropoelastin-Coated Surfaces Under Fluid Shear Stress

Intravascular devices modified with tropoelastin would be exposed to physiological fluid shearing forces. To assess whether cells attaching to tropoelastin-modified surfaces would persist through such conditions, the effects of shear stress were examined. A majority of HAEC seeded onto tropoelastin-coated coverslips remained adhered through short (5 min) exposures to shear stresses up to 250 dyne/cm² (Fig. 5a). Our data show that all four types of coating (tropoelastin, fibronectin, BSA, and anti-CD34 antibodies) resulted in similar cell retention when shear stress was 150 dyn/cm² or lower. At higher shear stress, however, cell retention on tropoelastin and fibronectin was higher than on BSA and anti-CD34 antibodies. In particular, cell retention on tropoelastin was twofold higher than on anti-CD34 antibodies at the largest shear stress tested (250 dyn/cm²). These data suggest that tropoelastin coating may provide superior cell retention to anti-CD34 antibody coating in high shear stress conditions, as might be expected in and around the complex architecture of an intravascular device.²⁶ HAEC seeded onto covalently modified stainless steel disks and exposed to arterial shear stresses (16 dyne/cm²) showed continued adhesion after 24 h of flow (Fig. 5b, right panel). Further, HAEC actin filaments were more ordered and tended to align

unidirectionally under arterial flow, as compared to the random alignment of the static samples (Fig. 5c). The same effect was observed in the HAEC monolayers on fibronectin-coated silicone disks (data not shown). This suggests that cells that attach to tropoelastin-coated intravascular devices remodel in response to flow, in addition to withstanding physiological flow conditions.

DISCUSSION

Here, we propose a biomimetic approach for enhancing endothelial recovery: mimic the internal elastic lamina by coating intravascular devices with tropoelastin. This approach capitalizes on the biological properties of tropoelastin and does not require faithfully recreating the complex physical properties of elastic fibers. Thus, the need for protein production in mammalian expression systems or the use of elastic fibers extracted from animals is avoided.

We confirm that the biological activity of tropoelastin on endothelial cells is contained in the VGVAPG domain and the carboxy-terminal 17-amino acids, and we provide evidence that the activity of the carboxy terminus is mediated via $\alpha_v\beta_3$ and appears distinct from the effect of the hexapeptide VGVAPG. We demonstrate that recombinant tropoelastin stimulates endothelial cell migration and adhesion, and the effect is stronger for endothelial cells than smooth muscle cells. Further, we show that tropoelastin coatings on metal surfaces (by covalent immobilization) enhances adhesion of endothelial progenitor cells and several endothelial cell types to the metal surfaces, and that endothelial cells can form confluent monolayers on the metal surface which resist and respond to arterial shear stresses. It is possible that the alignment of endothelial cells on tropoelastin may be mediated by $\alpha_v\beta_3$, which has been shown to mediate the effect of shear stress on endothelial cytoskeleton reorganization on fibronectin.^{13,38}

Tropoelastin has several potential advantages when compared to two other novel intravascular device coatings, VEGF and anti-CD34 antibodies. The endothelial chemotaxis stimulating effect of tropoelastin at 1.5 nM was comparable to the effect of VEGF at 0.5 nM. Thus, tropoelastin is similarly effective in attracting endothelial cells but does not have the potent vascular permeability enhancing effects of VEGF which result in several well-documented adverse effects.⁶ We also show that tropoelastin, while not as specific as anti-CD34 antibodies in its adhesive effects, has a strong pro-adhesive effect on endothelial cells, and preferentially promotes the adhesion of endothelial cells over non-endothelial cell types. Additionally, we show that the endothelial cells adherent to tropoelastin spread their cytoplasm and intracellular actin network to a greater degree than on anti-CD34 antibodies, as they might after adhesion to an intact internal elastic lamina *in vivo*. Cell spreading is critical as it is required for the effective coverage of the foreign surface and for the cells to withstand fluid shearing forces. Thus, a potential advantage of tropoelastin coating is enhanced cell spreading, which is not expected or observed in strategies that aim to capture endothelial cells using antibodies to cell surface markers. Indeed, our data show that cell retention under high fluid shear stress is better on tropoelastin than on anti-CD34 antibody-coated surfaces.

Stainless steel is a very common material in the construction of intravascular devices, and therefore is the focus of this study. It should be noted, however, that our experiments also included tissue culture plastic surfaces and silicone sheets, and tropoelastin performed similar to the results we described on stainless steel, suggesting that tropoelastin coating could be applicable as an intravascular device coating on any number of other device materials in use now or in the future. This study suggests that tropoelastin coating is a promising approach for enhancing endothelialization of intravascular device, but further

exploration is needed to determine the promise and practicality of accelerating endothelialization by immobilizing tropoelastin to the surfaces of vascular devices either alone or in combination with other drug eluting or coating strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Soluble recombinant tropoelastin stimulates migration of HAEC through different mechanisms. (a) HAEC migration toward 0.1% BSA, 10 ng/mL VEGF, or 10–200 ng/mL tELN for 2 h in a 48-well Boyden chamber apparatus. * $p < 0.05$ when compared to the BSA control; # $p < 0.05$ when compared to 10 ng/mL tELN. (b) HAEC pretreated with either nonspecific IgG or anti- $\alpha_v\beta_3$ antibodies were allowed to migrate toward 0.1% BSA, 10 ng/mL FN, 10 ng/mL tELN, 10 μ M VGVAPG, or 10 μ M IFPGGACLGKACGRK (Cterm) for 2 h. Migration with the Cterm peptide in the upper and lower chambers of the Boyden apparatus (C/C, last bar) was carried out to differentiate between chemokinesis and

chemotaxis. * $p < 0.05$ when compared to the BSA-IgG control; # $p < 0.05$ when compared to respective anti- $\alpha_V\beta_3$ treated samples; § $p < 0.05$ when compared to Cterm-IgG.

FIGURE 2.

Adhesion of HAEC to recombinant tropoelastin-coated plastic surface is mediated by $\alpha_v\beta_3$. Adhesion experiments were carried out using 96-well plates pretreated with 0.1% BSA, 10 $\mu\text{g}/\text{mL}$ collagen (CN), 10 $\mu\text{g}/\text{mL}$ fibronectin (FN), or 10 $\mu\text{g}/\text{mL}$ tELN. HAEC in suspension were pretreated with anti- $\alpha_2\beta_1$ or $\alpha_v\beta_3$ antibodies or non-specific IgG before being allowed to adhere to the protein-coated surfaces for 30 min. * $p < 0.05$ when compared to the BSA-IgG control; # $p < 0.05$ when compared to tELN-IgG; § $p < 0.05$ when compared to tELN- $\alpha_2\beta_1$.

FIGURE 3.

Effects of recombinant tropoelastin on stimulating migration and adhesion of HAEC and HASMC. (a) HAEC and HASMC were allowed to migrate toward 0.1% BSA, 10 ng/mL FN, or 10 ng/mL tELN for 2 h in a Boyden chamber apparatus. The number of migrated cells was normalized to the 0.1% BSA-EC control. * $p < 0.05$ when compared to the BSA-EC control; # $p < 0.05$ when compared to respective SMC samples; § $p < 0.05$ when compared to the BSA-SMC samples. (b) Adhesion experiments were carried out using 96-well plates that were pretreated with 0.1% BSA, 10 μ g/mL CN, 10 μ g/mL FN, or 10 μ g/mL tELN overnight. HAEC and HASMC in suspension were allowed to adhere to the protein-coated surfaces for 30 min. The number of adherent cells was normalized to the 0.1% BSA-

EC control. * $p < 0.05$ when compared to the BSA-EC control; # $p < 0.05$ when compared to respective SMC samples; § $p < 0.05$ when compared to the BSA-SMC samples.

FIGURE 4.

Adhesion of endothelial cells and CD34+ endothelial progenitor cells to stainless steel disks modified with anti-CD34 antibodies or tropoelastin. (a) 6-mm stainless steel disks were modified with base polysaccharide (vehicle control), anti-CD34 antibodies (CD34 Abs), or tELN. (b) CD34+ endothelial progenitor cells (CD34+ Cells), HAEC, and HMVEC were allowed to adhere to disks for 30 min. * $p < 0.05$ when compared to the vehicle controls regardless of cell types; # $p < 0.05$ when compared to the corresponding cell type on CD34 Abs-coated disks. (c) CD34+ cells, HAEC, and HMVEC adherent on modified disks were stained with phalloidin (green) and DAPI (blue). Each panel depicts a representative fluorescent microscopic image.

FIGURE 5.

Adherence of endothelial cells to coated surfaces under conditions of flow. (a) HAEC adhered to FN, tELN, BSA, or anti-CD34 antibodies (CD34 Abs) coated coverslips were exposed to shear stress. ⁺ $p < 0.05$ when comparing FN and BSA coatings; ^{\$} $p < 0.05$ when comparing tELN and BSA coatings; [#] $p < 0.05$ when comparing FN and anti-CD34 Abs coatings; ^{*} $p < 0.05$ when comparing tELN and anti-CD34 Abs coatings. (b) HAEC grown on tELN-coated disks were stained with phalloidin (green) and DAPI (blue). Each panel depicts a representative fluorescent microscopic image. (c) Upper panels: Each panel depicts a representative HPF fluorescent microscopic image of actin cytoskeleton (green) and nuclei (blue). Lower panels: The directional alignment of actin fibers is shown using a ColorMap

to represent the local angle of each fiber. Cells under arterial flow show an ordered and aligned cytoskeleton, as demonstrated by more uniform fiber coloration (higher levels of blue and purple). In contrast, cells under static conditions show similar levels of all colors, indicating random alignment.