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DEVELOPMENT AND DISEASE

A critical role for elastin signaling in vascular morphogenesis and disease

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SUMMARY

Vascular proliferative diseases such as atherosclerosis and coronary restenosis are leading causes of morbidity and mortality in developed nations. Common features associated with these heterogeneous disorders involve phenotypic modulation and subsequent abnormal proliferation and migration of vascular smooth muscle cells into the arterial lumen, leading to neointimal formation and vascular stenosis. This fibrocellular response has largely been attributed to the release of multiple cytokines and growth factors by inflammatory cells. Previously, we demonstrated that the disruption of the elastin matrix leads to defective arterial morphogenesis. Here, we propose that elastin is a potent autocrine regulator of vascular smooth muscle cell activity and that this regulation is important for preventing fibrocellular pathology. Using vascular smooth muscle cells from mice lacking elastin (*Eln*$^{-/-}$), we show that elastin induces actin stress fiber organization, inhibits proliferation, regulates migration and signals via a non-integrin, heterotrimeric G-protein-coupled pathway. In a porcine coronary model of restenosis, the therapeutic delivery of exogenous elastin to injured vessels in vivo significantly reduces neointimal formation. These findings indicate that elastin stabilizes the arterial structure by inducing a quiescent contractile state in vascular smooth muscle cells. Together, this work demonstrates that signaling pathways crucial for arterial morphogenesis can play an important role in the pathogenesis and treatment of vascular disease.

Key words: Elastin matrix, Vascular smooth muscle, Morphogenesis, G-protein signaling, Vascular proliferative diseases

INTRODUCTION

Vascular proliferative diseases are a heterogeneous group of disorders that include atherosclerosis, coronary restenosis, bypass-graft failure and transplant arteriopathy that lead to arterial narrowing, restriction of blood flow and tissue ischemia. These diseases arise from a complex pathophysiological response to injury by multiple factors, including hypercholesterolemia, diabetes, smoking, hypertension and mechanical injury (Ross, 1995; Lusis, 2000). Although their etiologies are diverse, these disorders all share a common pathological feature: the accumulation of vascular smooth muscle cells and fibrous connective tissue within the intima between the endothelium and medial layer of the vessel wall (Lusis, 2000; Raines and Ross, 1993). This fibrocellular pathology, known as neointimal formation, results from the activation, proliferation and migration of vascular smooth muscle cells (Raines and Ross, 1993; Schwartz, 1997). Understanding the molecular mechanisms that underlie vascular smooth muscle cell regulation is crucial for developing strategies to prevent and treat vascular proliferative disorders.

Vascular smooth muscle cells are not terminally differentiated and can alternate between a quiescent, contractile state and a proliferative non-contractile state (Raines and Ross, 1993; Owens, 1998; Thyberg, 1998). In a healthy artery, vascular smooth muscle cells are quiescent and largely comprise a contractile apparatus that functions to dilate and constrict the lumen as required by physiological demands. Actin stress fibers serve as the scaffold for the contractile apparatus, and are a hallmark of mature and quiescent vascular smooth muscle cells (Burridge and Chrzanowska-Wodnicka, 1996; Small and Gimona, 1998). Under circumstances of injury, repair and regeneration, vascular smooth muscle cells lose their contractile apparatus and dedifferentiate into an immature phenotype capable of proliferating and depositing

extracellular matrix (Owens, 1998; Thyberg, 1998). This fibrocellular response plays an important role in all forms of vascular proliferative diseases. In atherosclerotic lesions, the major components of the fibrous plaque are vascular smooth muscle cells, the matrix products deposited by these cells, and extracellular cholesterol (Ross, 1995; Lusis, 2000; Raines and Ross, 1993). In restenosis, transplant arteriopathy and vascular graft disease; smooth muscle cells are the predominant component of the occlusive lesion (Raines and Ross, 1993). The phenotypic modulation of vascular smooth muscle cells offers a tempting target for preventing vascular proliferative diseases.

Elastin is the dominant extracellular matrix protein deposited in the arterial wall and can contribute up to 50% of its dry weight (Parks et al., 1993). The protein product of the elastin gene is synthesized by vascular smooth muscle cells and secreted as a monomer, tropoelastin. After post-translational modification, tropoelastin is crosslinked and organized into elastin polymers that form concentric rings of elastic lamellae around the arterial lumen. Each elastic lamella alternates with a ring of smooth muscle, and provides the compliance that arteries need to absorb and transmit hemodynamic force (Wolinski et al., 1967). There is a growing body of evidence that implicates elastin in vascular development and disease. We previously demonstrated that loss-of-function mutations of one elastin allele cause supravalvular aortic stenosis (SVAS) and Williams-Beuren Syndrome (Li et al., 1997; Curran et al., 1993; Ewart et al., 1993). These disorders are characterized by discrete fibrocellular stenoses in the aorta, coronary arteries, carotid arteries, pulmonary arteries and other peripheral arteries. Often, individuals with these diseases are young children who are susceptible to peripheral vascular disease, myocardial infarctions or stroke in the absence of other risk factors such as high serum cholesterol, diabetes and cigarette smoking (van Son et al., 1994). In subsequent experiments, we showed that the deposition of elastin matrix in the arterial wall during late fetal development is essential to arterial morphogenesis (Li et al., 1998). Mice that lack elastin ($Eln^{-/-}$) died from occlusive fibrocellular pathology caused by subendothelial proliferation and accumulation of vascular smooth muscle cells in early neonatal life. In $Eln^{-/-}$ vessels, there is no evidence of abnormal endothelial structure, activation or proliferation. Furthermore, the occlusive vascular phenotype occurred in the absence of hemodynamic stress and inflammation. This work demonstrated that elastin is required for arterial development. However, because the absence of elastin undoubtedly distorts the presentation and stability of other important elements of the matrix architecture, these studies failed to define the specific role for elastin in establishing and maintaining a mature artery.

Here we show that elastin regulates the phenotypic modulation, proliferation and migration of vascular smooth muscle cells in culture. We confirm a direct signaling effect by demonstrating that elastin regulates vascular smooth muscle cells via a G-protein-coupled signaling pathway. Finally, we establish that in vivo, exogenous elastin reduces the vascular proliferative response of an injured artery. These results demonstrate that elastin is a crucial signaling molecule that directly controls vascular smooth muscle biology and stabilizes arterial structure.

MATERIALS AND METHODS

Isolation of vascular smooth muscle cells

Newborn (postnatal day 0.5) $Eln^{+/+}$ and $Eln^{-/-}$ pups were sacrificed by CO_2 asphyxiation. Their ascending aortas were dissected free, endothelium removed and placed into individual wells of a plastic 12-well culture dish containing Amniomax C-100 growth medium (Gibco-BRL) supplemented with penicillin/streptomycin. Cultures of smooth muscle cells that formed around the tissue were trypsinized (0.05% trypsin, 0.53 mM EDTA), passaged, expanded and genotyped to confirm their identity. Cells were stained with SM α-actin (Clone 1A4, Sigma) and checked for classic 'hill-and-valley' morphology to confirm their smooth muscle status. In situ hybridization, immunostaining and RT/PCR analysis established that these cells expressed the vascular smooth muscle cell markers SM α-actin, SM γ-actin, SM-22, calponin, desmin, ang-1/ang-2, and did not express the endothelial markers Flk-1 or Flt-1.

Cellular assays

Cellular experiments of proliferation, actin polymerization and migration described below were performed on at least three independently isolated primary cell lines. Proliferation was assayed by cell count and [^{3}H]thymidine incorporation. Confluent cultures of $Eln^{+/+}$ and $Eln^{-/-}$ vascular smooth muscle cells from the fourth passage were seeded at a density of 2×10^3 cells/well on a plastic 24-well plate (Corning Costar, Corning, NY), and stimulated to proliferate in AmnioMAX C-100 growth medium (Gibco-BRL) treated with 100 μg/ml recombinant tropoelastin or left untreated. Cell numbers for each culture were assayed by hemocytometer after 24, 48 and 72 hours of incubation. For [^{3}H]thymidine incorporation, $Eln^{+/+}$ and $Eln^{-/-}$ cells were seeded at a density of 20,000 cells/well on a 24-well plate. After attachment, cells were starved in 0.1% BSA (Fisher) in Amniomax Basal Medium (Gibco) for 24 hours. Cells were then grown in whole Amniomax medium treated/untreated with 100 μg/ml tropoelastin (Grosso et al., 1991a). Cells were assayed for [^{3}H]thymidine incorporation using a scintillation counter after 24 hours by precipitating with 5% TCA, followed by NaOH solubilization. Data were calculated as mean±s.d. (n=6).

Subconfluent cultures of vascular smooth muscle cells were evaluated and scored for the presence of actin stress fibers in the cytoplasm following immunofluorescent staining for SM α-actin (Clone 1A4, Sigma), vinculin (Clone hVin-1, Sigma), desmin (Sigma) and tubulin (Clone DM-1A, Calbiochem). Cells were treated with tropoelastin (1 μg/ml) (Grosso et al., 1991a) or not, and assays were performed in serum-free media. Cells were scored as contractile if they had distinct actin stress fibers continuous throughout the cytoplasm, or at least 10 well-defined focal adhesions distributed throughout the cytoplasm and cell periphery. Scoring was performed by three separate observers blinded to the cell genotypes, and their numbers averaged. At least 100 cells were scored in three separate cultures for each genotype. Additionally, identical results were obtained for cells cultured on glass slides, 24 plastic tissue culture plates (Corning) coated with matrigel, and another brand of plasticware (Falcon).

The chemotactic activity of cells was assayed in a modification of the Boyden chamber method using 6.5 mm transwell polycarbonate chemotaxis filter inserts in a plastic 24-well tissue culture plate (Corning Costar, Corning, NY). Various concentrations of recombinant tropoelastin (Grosso et al., 1991a) were placed in both the upper or lower compartment and covered with a polycarbonate membrane filter (8 μm porosity). PDGF-BB (30 ng/ml) was added to the lower chamber and served as a positive control. Medium alone was used as the negative control. Cells to be tested were placed in the upper wells of the chamber and incubated at 37°C/5% CO_2 for 3 hours. After incubation, the cells adhering to the upper surface of the filter were scraped off and the cells that had migrated to the lower surface were fixed with 10% buffered formalin, stained with DAPI

(Molecular Probes, Eugene, OR) and viewed under a mercury lamp. Fifteen randomly selected high power (×200) fields were counted on each filter by observers blinded to their conditions. Chemotactic response was measured as the number of cells that had transversed the filter in response to tropoelastin, PDGF and collagen type I (10 μg/ml), and expressed as fold increase over baseline. Each condition was assayed in triplicate wells and each experiment was repeated at least three times. Actin polymerization and migration assays experiments were also performed in triplicate in the presence of 100 μM Actinomycin D (Calbiochem), 10 μg/ml Cycloheximide (Calbiochem), 20 μM Y27632 (Welfide), 20 μM EDTA, 100 ng/ml Ptxn (Calbiochem) or 100 ng/ml B-protomer (Calbiochem).

Measurement of cAMP and activated Rho A

All biochemical measurements were performed in cells that were serum starved overnight. cAMP assay levels were detected using an RIA kit (RPA538, Amersham Pharmacia). Briefly, cells were plated at a density of 80,000 to 100,000 cells on a plastic 12-well cell-culture plate, pretreated with 100 ng/ml cholera toxin (Calbiochem) for 3 hours, treated with 1 μg/ml recombinant tropoelastin for 20 minutes, and cAMP subsequently isolated. Experimental cAMP levels were quantified through use of a standard curve where known amounts of cAMP were added to radioimmunoassay.

RhoA-GTP was detected using a Rho Activation Kit (Upstate Biotechnology). Briefly, $Eln^{-/-}$ cells were grown to 70% confluence in a six-well plate, and pretreated with 100 ng/ml pertussis toxin or 100 ng/ml B-protomer for 3 hours. Control experiments with no pretreatment were also performed. Cells were then treated with 1 μg/ml recombinant tropoelastin for 3 hours and activated Rho A was isolated by immunoprecipitations with Rhotekin coated beads. RhoA-GTP was detected by western blot analysis after samples were run on a 10% acryllamide gel and transferred to nitrocellulose membranes. Equal amounts of total cellular lysate were run out for each sample to ensure that equivalent amounts of protein were used in all experiments.

Porcine coronary model of in-stent restenosis

Elastin sheaths were prepared from dissected common carotid arteries of adult domestic swine (40 to 60 kg) using a variation of a previously published protocol (Malone et al., 1984). Briefly, these vessels were sequentially treated with a 1% SDS solution supplemented with doxycycline (10 mg/l) and EDTA (5 mM), potassium hydroxide (5 N) at 60°C, collagenase solution (0.5 mg/ml, Collagenase D, Roche), autoclaved and γ irradiated. The product of these extractions, a tubular elastin matrix sheath, was cut to size and fitted over 14 mm long stainless steel expandable stents (Medtronic AVE, S670) mounted on 3.5 mm diameter angioplasty balloons and secured by a metal coil. Control stents deployed were identical to elastin covered stents except for the absence of elastin sheaths.

Nineteen domestic pigs (30 to 40 kg) on a normal diet were pretreated with oral aspirin (625 mg), ticlopidine (250 mg) and verapamil (120 mg), and placed under general anesthesia. Animals underwent placement of 32 stents in the left anterior descending, circumflex or right coronary artery. The methods of stent implantation have been previously described (Schwartz et al., 1994). Briefly, control stents or elastin sheath-covered stents were advanced under fluoroscopic guidance to an appropriate site in the coronary vasculature and deployed at a 1.2:1 to 1.4:1 stent-to-artery ratio compared with the baseline vessel diameter. After the procedure, the wounds were closed and the pigs were returned to their quarters on a normal diet. Pigs were sacrificed at day 3 (elastin-covered stent, n=2) and day 14 (elastin-covered stent, n=2) to assess biocompatibility of the elastin sheath. To study neointima formation, pigs were sacrificed at day 28 (control stents, n=15; elastin-covered stents, n=14) and instrumented arteries were removed. Coronary arteries were fixed by pressure perfusion (100 mm Hg) with 10% buffered formalin for 24 hours, dissected free, washed, dehydrated through graded alcohols and infiltrated with methylmethacrylate (MMA) at 4°C. After polymerization, embedded specimens were cross-sectioned at 5 μm thickness using a tungsten-carbide microtome blade. Each arterial segment was stained with Hematoxylin and Eosin and elastic Van Gieson stains. Three cross-sections taken from the two ends and the middle of each artery were used for histomorphometric analysis. Histological examination confirmed that the elastin sheaths were retained in the arterial wall of all specimens.

Histomorphometric analysis

Mean neointimal thickness, percent stenosis and mean injury score were measured on Van Gieson-stained sections using calibrated digital microscopic planimetry as previously described (Schwartz et al., 1992; Schwartz et al., 1994). Briefly, the mean neointimal thickness was measured by drawing a radial line from the lumen border to the point maximum penetration for each stent strut, and averaging the measurements. Percent area stenosis was calculated as % stenosis=100 × [1–(stenotic lumen area/original lumen area)]. The original lumen area was measured as the area subtended within the internal elastic lamina. The injury score measures the physical penetration of the stent strut into the vessel wall and was calculated using a graded scale from 0-3 as previously described (Schwartz et. al., 1992). Briefly, a score of 1 indicated that only the internal elastic lamina was lacerated; a score of 2 indicated laceration of the arterial media; and a score of 3 indicated that the external elastic lamina was lacerated. The mean injury score for each histological section was calculated as mean injury score=Σ injury scores for every strut/number of struts present per section.

Statistics

For all in vitro experiments, mean and standard deviations were calculated and statistical analysis were carried out by analysis of variance (ANOVA). For in vivo experiments, statistical analysis was performed on injury score, average neointimal thickness and percent lumen stenosis using regression modeling (Schwartz et al., 1994). Linear regression modeling accounts for injury (a strong covariate) and the injury-dependent neointimal thickening between control arteries and elastin treated arteries. Three models were used to establish whether there were: (1) differences in intercepts, (2) differences in slopes allowing any intercept or (3) differences in slopes when the intercepts are fixed. Differences between the elastin sheath-stent and control stent at each injury level were analyzed using the Tukey-Kramer multiple comparisons t test for all three regression models.

RESULTS

Elastin inhibits the proliferation of vascular smooth muscle cells

Investigating the role of elastin in regulating vascular smooth muscle cells is confounded by the fact that these cells synthesize and secrete elastin. Thus, to determine the role of elastin in regulating cellular proliferation, differentiation and migration, we isolated vascular smooth muscle cells from mice lacking elastin ($Eln^{-/-}$) and from wild-type sibling control animals ($Eln^{+/+}$). Immunofluorescent staining and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses established that $Eln^{+/+}$ vascular smooth muscle cells expressed and synthesized elastin (antibody to murine elastin kindly provided by Dr Robert Mecham, Washington University), whereas $Eln^{-/-}$ cells did not (Fig. 1A-C).

To evaluate the role of elastin in vascular smooth muscle cell proliferation, we compared the growth rate of $Eln^{+/+}$ and $Eln^{-/-}$ cells. Cells were seeded at the same density and growth

rates were assayed by counting cells after 24, 48 and 72 hours. The number of $Eln^{-/-}$ cells was greater than $Eln^{+/+}$ cells at all time points, and was increased over twofold at 72 hours (Fig. 1D). These data indicate that in the absence of elastin synthesis, vascular smooth muscle cells proliferate at an increased rate.

To prove the specificity of the effect of elastin on vascular smooth muscle cell growth, we tested whether adding exogenous recombinant tropoelastin to the culture media inhibits cellular proliferation. Recombinant tropoelastin was synthesized using a bacterial expression system (Grosso et al., 1991a) and determined to be pure by gel electrophoresis and amino acid composition. Tropoelastin had a dose-dependent inhibitory effect on proliferation, with a maximal effect at 100 μg/ml (Fig. 1D; data not shown). At this dose, the number of $Eln^{-/-}$ cells was nearly identical to $Eln^{+/+}$ cells at 72 hours. At each time point, more than 98% of all cells were viable as determined by Trypan Blue staining, indicating that tropoelastin was not cytotoxic. Similar results were observed when acid hydrolyzed elastic fibers, α elastin, were used for this assay. The responses of $Eln^{+/+}$ and $Eln^{-/-}$ cellular proliferation to tropoelastin (Fig. 1E) and α elastin (data not shown) were reproduced in [^{3}H]thymidine incorporation assays. By comparison, there was no significant inhibitory effect on proliferation when $Eln^{-/-}$ cells were treated with type I collagen (100 μg/ml) (data not shown). These data demonstrate that elastin regulates vascular smooth muscle cell proliferation.

Elastin induces a mature contractile phenotype in vascular smooth muscle cells

The primary function of mature differentiated vascular smooth muscle cells is contraction. This ability requires highly organized actin myofilaments, often referred to as actin stress fibers (Burridge et al., 1996; Small and Gimona, 1998). To determine if elastin modulates the phenotype of vascular smooth muscle cells, we evaluated actin organization in $Eln^{+/+}$ and $Eln^{-/-}$ cells using direct immunofluorescent staining with FITC-conjugated SM α-actin antisera. Well-defined actin myofilaments were apparent in more than 95% of $Eln^{+/+}$ cells scored by observers blinded to genotype (Fig. 2A,E). By contrast, only 23% of $Eln^{-/-}$ cells had actin stress fibers (Fig. 2B,E). Furthermore, $Eln^{-/-}$ cells with actin stress fibers were qualitatively different than $Eln^{+/+}$ cells, with a distinctive, rounder morphology (data not shown). These data indicate that vascular smooth muscle cells lacking elastin fail to organize their contractile apparatus.

To confirm that the failure to form an organized contractile apparatus in $Eln^{-/-}$ cells resulted from the inability to synthesize and secrete elastin, we treated these cells with recombinant tropoelastin protein. Within 3 hours of exposure to tropoelastin, the percentage of $Eln^{-/-}$ cells with organized actin stress fibers increased threefold to 74% (Fig. 2D,E). This response was dose dependent and was also seen by phalloidin staining for F-actin (data not shown). By comparison, tropoelastin treatment had no effect on actin stress fiber organization in $Eln^{+/+}$ cells (Fig. 2C,E). Similar results were

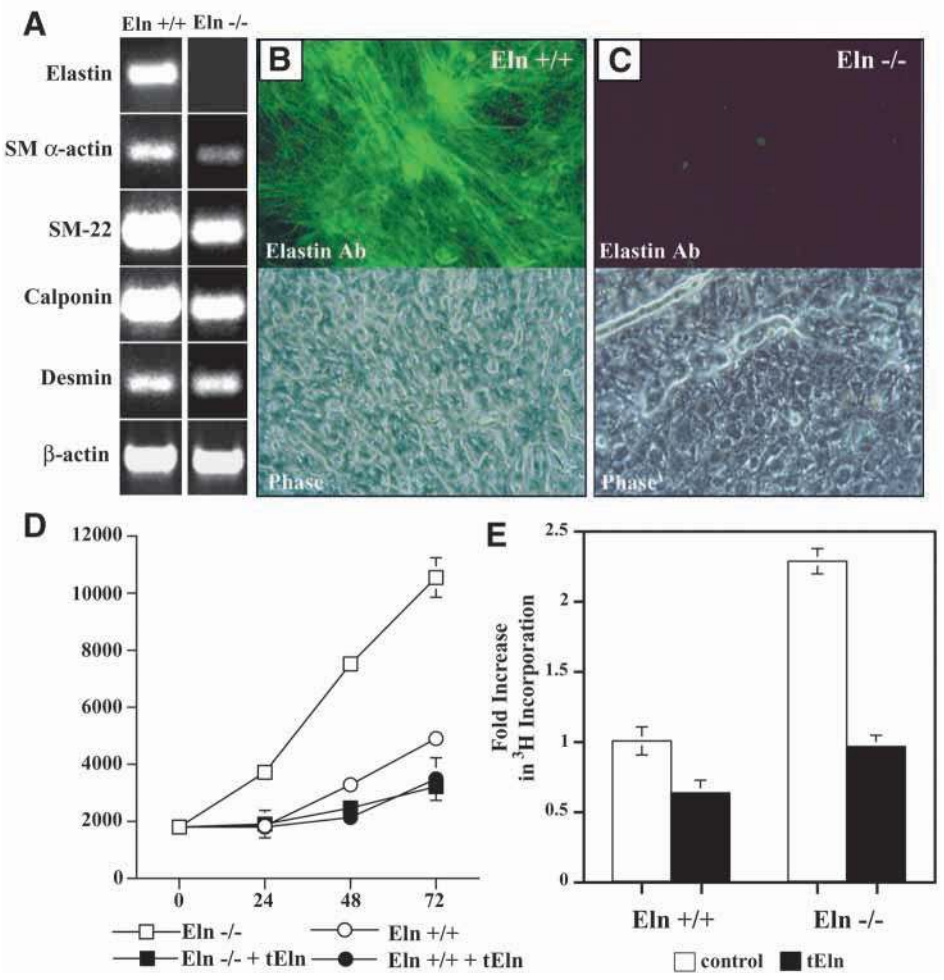


Fig. 1. Elastin inhibits proliferation of vascular smooth muscle cells. (A) RT-PCR experiments confirm that murine vascular smooth muscle cells lines express cell specific markers. The elastin gene product is detected in $Eln^{+/+}$ and not $Eln^{-/-}$ cells. Immunofluorescence analysis with elastin antibodies and accompanying phase photomicrographs demonstrate that $Eln^{+/+}$ vascular smooth muscle cells (B) synthesize and secrete elastin matrix, whereas $Eln^{-/-}$ vascular smooth muscle cells (C) do not produce elastin. (D) Assay measuring cell numbers demonstrates that $Eln^{-/-}$ vascular smooth muscle cells proliferate at a much higher rate than do $Eln^{+/+}$ vascular smooth muscle cells (P<0.0001, ANOVA). This difference is eliminated by the addition of recombinant elastin gene product or tropoelastin (tEln). (E) Assay measuring [^{3}H]thymidine incorporation assay demonstrates that $Eln^{-/-}$ vascular smooth muscle cells proliferate at a rate over twofold greater than that for $Eln^{+/+}$ cells. This difference is eliminated by the inhibition of $Eln^{-/-}$ cells proliferation by tropoelastin (P<0.0001, ANOVA).

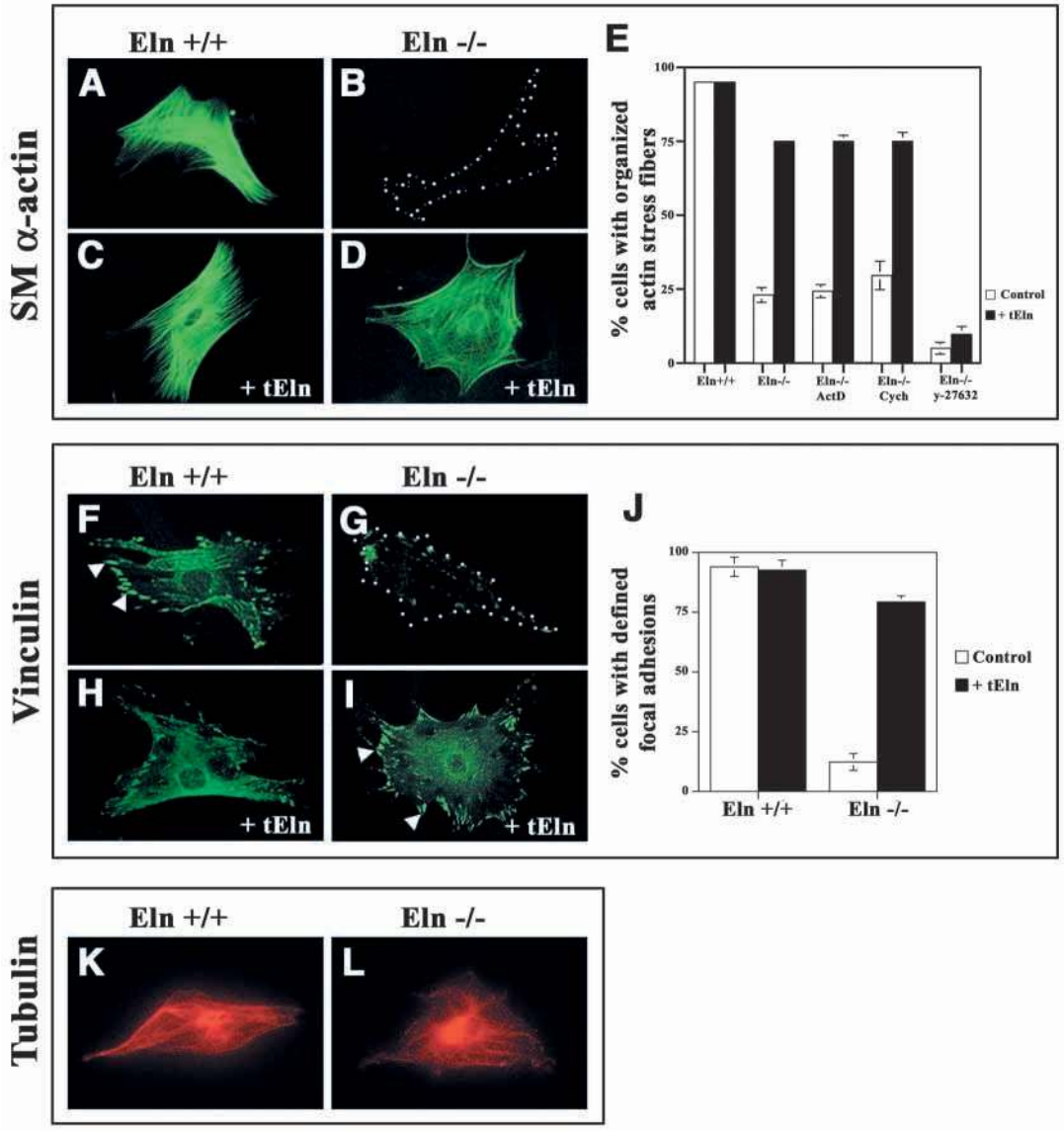


Fig. 2. Elastin induces a mature contractile phenotype in vascular smooth muscle cells. (A-E) Immunofluorescence analysis for SM α-actin reveals that $Eln^{+/+}$ vascular smooth muscle cells (A) have a highly organized network of actin stress fibers, a hallmark of mature contractile vascular smooth muscle cells. By contrast, there is a paucity of actin stress fibers in $Eln^{-/-}$ vascular smooth muscle cells (B; outlined by white dots). The elastin gene product, recombinant tropoelastin, induces the formation of organized actin stress fibers in $Eln^{-/-}$ vascular smooth muscle cells (D), but does not affect $Eln^{+/+}$ vascular smooth muscle cells (C). Scoring analysis demonstrates a significant increase in the percentage of $Eln^{-/-}$ vascular smooth muscle cells with organized actin myofilaments after elastin treatment (P<0.0001) (E). Tropoelastin mediated actin polymerization is unaffected when $Eln^{-/-}$ cells are treated with either a gene transcription inhibitor, actinomycin D, or a protein translation inhibitor, cycloheximide. The Rho kinase inhibitor, Y27632, blocks actin polymerization of tropoelastin treated $Eln^{-/-}$ cells. (F-J) Immunofluorescence analysis for vinculin reveals that $Eln^{+/+}$ vascular smooth muscle cells (F) have well-defined focal adhesions (arrowheads). By contrast, $Eln^{-/-}$ vascular smooth muscle cells (G; outlined by white dots) have poorly defined focal adhesions. Tropoelastin induces well-defined focal adhesions (arrows) throughout $Eln^{-/-}$ vascular smooth muscle cells (I), but does not affect $Eln^{+/+}$ vascular smooth muscle cells (H). Scoring analysis revealed a significant increase (P<0.0001) in the percentage of $Eln^{-/-}$ vascular smooth muscle cells with defined focal adhesions after tropoelastin treatment (J). (K,L) Immunofluorescence staining with antisera against tubulin reveals no difference between $Eln^{+/+}$ vascular smooth muscle cells (K) and $Eln^{-/-}$ vascular smooth muscle cells (L). Exposure times for all images are the same. Results represent the mean±s.d. from three individual experiments.

observed when $Eln^{-/-}$ cells were treated with crosslinked α elastin (data not shown). However, no change in actin stress fiber organization was observed when $Eln^{-/-}$ cells were treated with an equivalent concentration of type I collagen. In addition, the induction of actin polymerization by tropoelastin was unaffected by the presence or absence of serum in culture (data not shown). Thus, extracellular elastin is an important mediator of actin polymerization and contractile apparatus organization in vascular smooth muscle cells.

The Rho signal transduction pathway is known to be a central converging step in the formation of actin stress fibers through a post-translational mechanism (Mack et al., 2001;

Bishop and Hall, 2000). We investigated the role of this pathway in the regulation by tropoelastin of actin polymerization. The addition of actinomycin D (Greenburg et al., 1986), an inhibitor of gene transcription, or cycloheximide (Greenburg et al., 1986), an inhibitor of protein translation, did not block tropoelastin-induced actin polymerization in $Eln^{-/-}$ cells (Fig. 2E). Furthermore, a specific inhibitor of the Rho signaling pathway that targets Rho kinase, Y27632 (Mack et al., 2001; Ushata et al., 1997), blocked tropoelastin mediated actin polymerization of $Eln^{-/-}$ cells (Fig. 2E). Additionally, tropoelastin treatment of $Eln^{-/-}$ vsmc did not alter the amount of SM α actin transcript or protein levels as quantified by northern and western blot analysis but did shift the filamentous:globular (F:G) actin ratio from 1:1 to 3.1:1 (data not shown). These experiments, in combination with the failure of either transcription or translation inhibitors to inhibit tropoelastin-induced actin stress fiber formation in $Eln^{-/-}$ vsmc, suggest that elastin modulates vsmc phenotype by regulating actin treadmilling via a signal transduction pathway involving Rho GTPases and their effector proteins.

To evaluate further the effect of elastin on the contractile phenotype in vascular smooth muscle cells, we examined the organization of vinculin, tubulin and desmin in $Eln^{+/+}$ and $Eln^{-/-}$ cells (Burridge et al., 1996). Vinculin is concentrated in focal adhesion plaques that bind the actin cytoskeleton and connect with the cell membrane. Indirect immunofluorescent staining for vinculin revealed abundant focal adhesion plaques in 94% of $Eln^{+/+}$ cells when scored by observers blinded to genotype (Fig. 2F,J). By contrast, only 12% of $Eln^{-/-}$ cells had a normal distribution of defined focal adhesion plaques (Fig. 2G,J). Within 3 hours of exposure to recombinant tropoelastin protein, the percentage of $Eln^{-/-}$ cells with defined focal adhesions increased 6-fold to 79% (Fig. 2I,J). Treatment had no effect on focal adhesion organization in $Eln^{+/+}$ cells (Fig. 2H,J). Immunostaining for other cytoskeletal proteins, tubulin (Fig. 2K,L) and desmin (data not shown) showed no difference between $Eln^{+/+}$ and $Eln^{-/-}$ cells. Thus, the loss of elastin in vascular smooth muscle cells does not lead to a broad disruption of cytoskeletal architecture. These results indicate that elastin induces a mature, contractile phenotype in vascular smooth muscle cells by regulating the organization of specific cytoskeletal proteins.

Elastin controls the migration of vascular smooth muscle cells

Vascular proliferative disease involves the migration of smooth muscle cells from the arterial media to the subendothelial space, forming a neointima (Schwartz, 1997). The same phenomenon is observed during the development of $Eln^{-/-}$ arteries. We postulated that the elastin matrix surrounding each concentric layer of vascular smooth muscle cells provides a cue that localizes these cells to the arterial media, and prevents their migration to the neointima. To test this hypothesis, we used a modified Boyden chamber chemotaxis assay. We discovered that vascular smooth muscle cells migrate to tropoelastin in a dose-dependent manner (Fig. 3A). Cell migration depended on the concentration gradient of tropoelastin, not the total amount used in the assay. Chemotaxis was minimal for cells in control (0/0 ng/ml) and static fields of tropoelastin (200/200 ng/ml), but increased significantly as the concentration gradient of tropoelastin rose to 200 ng/ml. A similar but less dramatic

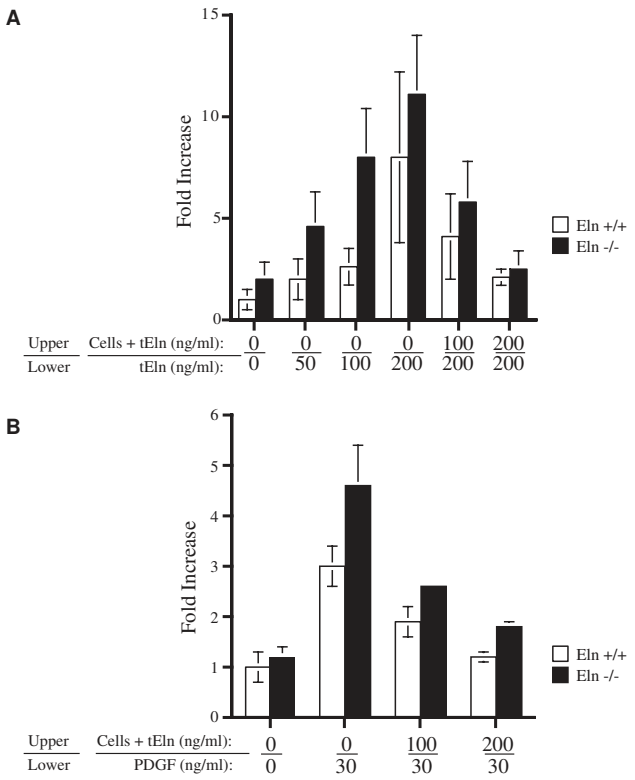


Fig. 3. Elastin controls migration of vascular smooth muscle cells. (A) $Eln^{+/+}$ and $Eln^{-/-}$ vascular smooth muscle cells migrate to tropoelastin in a concentration-dependent manner, indicating that tropoelastin is a specific and direct stimulus for vascular smooth muscle cell localization. In addition, $Eln^{-/-}$ cells consistently migrated at a higher rate than $Eln^{+/+}$ cells, suggesting that the autocrine production of elastin by $Eln^{+/+}$ cells reduces their chemotaxis to external stimuli. (B) Tropoelastin inhibits vascular smooth muscle cell migration to the chemotactic growth factor PDGF. A modified Boyden chamber assay was used for all experiments to determine the total number of migrated cells in 15 randomly selected high power microscope fields (HPF). Data shown are the mean±s.e.m. from three independent wells.

effect was seen with cells that produce elastin (Fig. 3A), suggesting that the synthesis and secretion of elastin by $Eln^{+/+}$ cells reduced migration to external stimuli. These data demonstrate that elastin is a potent chemoattractant for vascular smooth muscle cells.

Cytokines and growth factors, such as platelet-derived growth factor (PDGF), are thought to mediate the subendothelial migration of vascular smooth muscle cells in occlusive vascular lesions (Lusis, 2000; Bornfeldt et al., 1993). To determine if elastin matrix deposited in the arterial media can counteract the potent chemotactic activity of PDGF, we repeated the Boyden chamber experiments. At a PDGF concentration of 30 ng/ml, $Eln^{-/-}$ cells showed a fourfold increase in chemotactic activity (Fig. 3B). Increasing concentrations of tropoelastin prevented $Eln^{-/-}$ cell migration towards PDGF (Fig. 3B). Similar results were observed in $Eln^{+/+}$ cells. These data suggest that elastin provides a local cue that prevents vascular smooth muscle cells from migrating away from the arterial media to the subendothelial space. Together, these in vitro experiments demonstrate that elastin is

a potent and specific regulator of vascular smooth muscle cell maturation, migration and proliferation.

Elastin signals via a non-integrin, G-protein coupled signaling pathway

To determine whether elastin has a direct signaling effect, we examined the molecular mechanism of the regulation by elastin of vascular smooth muscle cells. Though various binding proteins, chaperones and other matrix elements that interact with elastin have been identified and cloned, the mechanism of elastin signaling remains to be elucidated (Mecham and Hinek, 1986; Hinek et al., 1988; Mecham et al., 1991; Grosso et al., 1991b; Hinek, 1995; Hinek, 1996; Privitera et al., 1998). Using in vitro assays of migration and actin polymerization, we examined the signaling cascade stimulated by elastin in *Eln*$^{-/-}$ vascular smooth muscle cells. Above, we demonstrate that elastin mediates actin polymerization through a Rho mediated signal transduction pathway (Fig. 2E). Cell-surface receptors that are known to regulate the Rho signaling pathway include the integrins and G-protein-coupled receptors (Seasholtz et al., 1999; Wei et al., 2001). Integrins are a well-characterized family of receptors that recognize matrix proteins such as collagen, vitronectin, fibulin and fibronectin. Although elastin is not a known ligand for integrins, recent work suggests that the integrins may be involved in elastin signaling through an intermediary, fibulin 5 (Nakamura et al., 2002; Yanagisawa et al., 2002). Fibulin 5 interacts directly with elastin, and serves as a ligand for cell-surface integrins. Integrins require extracellular divalent cations to bind their matrix ligands, and low doses of chelators such as EDTA block these cell-matrix interactions (Brockdorff et al., 1998). As expected, control experiments demonstrated that integrin mediated migration of vascular smooth muscle cells to collagen was EDTA sensitive (Fig. 4A). However, EDTA did not interfere with tropoelastin-mediated migration or actin polymerization of *Eln*$^{-/-}$ cells (Fig. 4A,B). In addition, blocking antibodies to integrins known to bind other matrix proteins such as collagen and fibulin 5 did not perturb elastin-mediated actin polymerization or chemotaxis (data not shown). Thus, these data strongly suggest that the integrin family of receptors is not involved in elastin signaling.

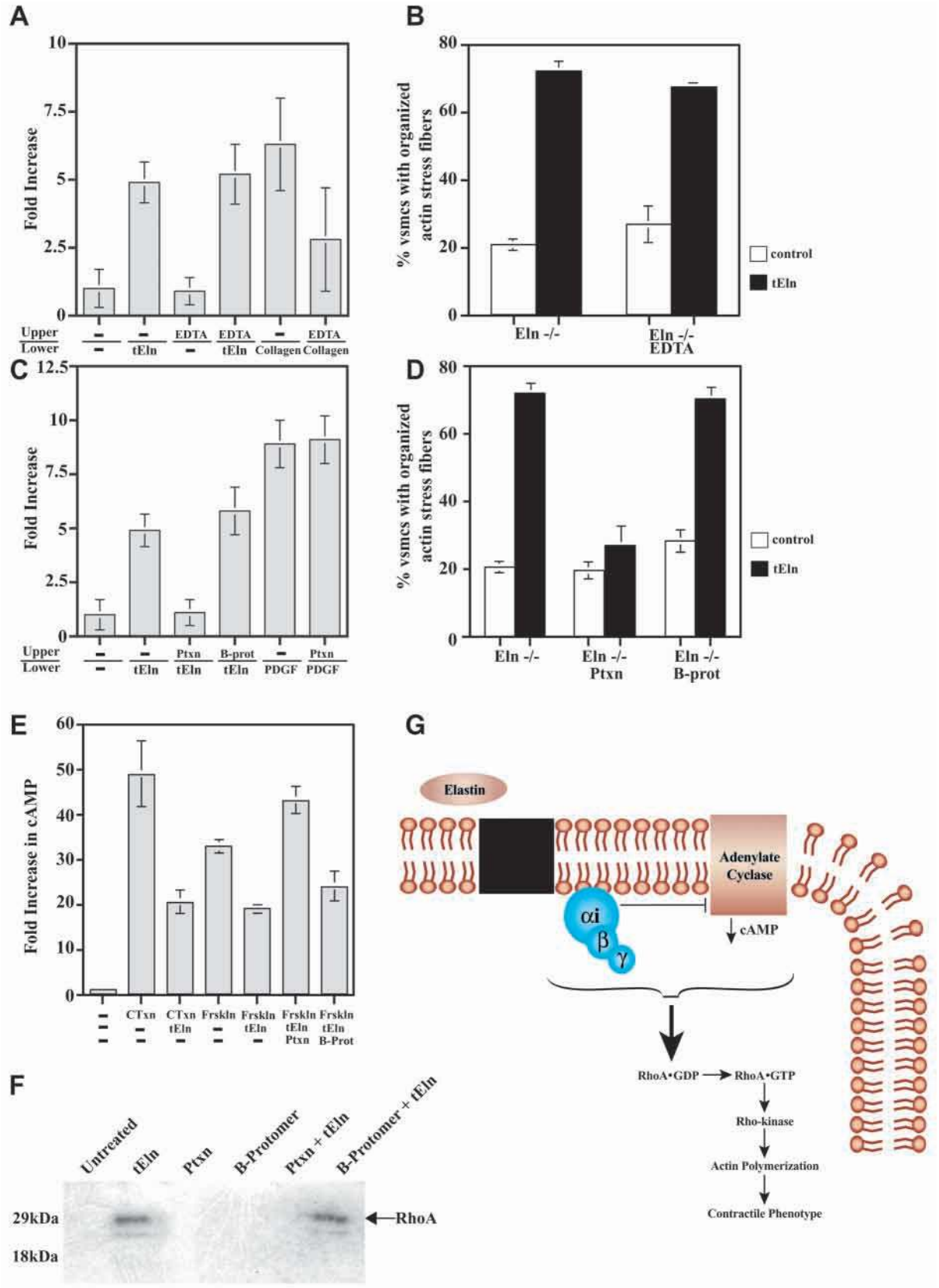
G-protein-coupled receptors (GPCR) are the largest family of transmembrane receptors and are known to activate Rho kinase (Seasholtz et al., 1999; Marinissen and Gutkind, 2001). GPCRs signal through heterotrimeric G proteins classified into four protein families: G_s, G_i, G_q and $G_{12/13}$ (Marinissen and Gutkind, 2001). Pertussis toxin, a specific inhibitor of G_i (Thomas et al., 2000), blocked tropoelastin-mediated migration and actin polymerization of vascular smooth muscle cells (Fig. 4C,D). The specificity of pertussis toxin inhibition was demonstrated in a series of control experiments. First, migration of vascular smooth muscle cells to platelet-derived growth factor is not dependent on a G-protein-coupled signaling pathway and was not disrupted by pertussis toxin (Fig. 4C). Second, pertussis toxin is composed of two subunits, A and B (Thomas et al., 2000). The A protomer inhibits G_i by ADP ribosylation, while the B protomer facilitates the entry of the toxin into the cell. In the presence of B protomer alone, there was no disruption of either migration or actin polymerization (Fig. 4C,D). Using a Ca^{2+}-sensitive dye, fura-2, and immunoprecipitations with specific antibodies, we detected no evidence that rapid Ca^{2+} influx, tyrosine kinase receptors or the Map kinase pathway was involved in elastin signaling (data not shown). Together, these experiments indicate that elastin signaling is mediated via a non-integrin and pertussis toxin-sensitive G-protein-coupled signaling pathway.

The pertussis toxin-sensitive G protein, G_i, inhibits adenylate cyclase, the enzyme responsible for generating cAMP (Thomas et al., 2000). To obtain direct biochemical evidence that tropoelastin signals through G_i, we measured the level of cAMP in tropoelastin-treated cells. To measure a reduction in cAMP level, it was necessary to increase the baseline cellular levels of cAMP using either forskolin or cholera toxin, agents that activate adenylate cyclase (Thomas et al., 2000; Coward et al., 1999). When tropoelastin was added to either forskolin or cholera toxin pretreated cells, the reduction in cAMP levels was between 40 and 60%, indicating that G_i was activated and inhibited adenylate cyclase activity (Fig. 4E). These reductions in cAMP levels are comparable with those observed with other ligands known to activate G_i via known G-protein-coupled receptors (Thomas et al., 2000; Coward et al., 1999). The reduction in cAMP was pertussis toxin sensitive, which confirmed the role of G_i. Thus, tropoelastin activates G_i and reduces cAMP levels. Finally, consistent with the activation of Gi, we found no evidence for rapid Ca^{2+} influx, a prominent feature of G_q activation, in our cellular system. Although GPCRs activate the G_i pathway and ligands for many of these receptors have not been identified, our data does not rule out the possibility that elastin might indirectly activate the GPCR pathway at the level of the heterotrimeric G proteins.

Others have shown that the GPCRs pathway can trigger the Rho signaling cascade (Seasholtz et al., 1999; Wei et al., 2001; Kabarowski et al., 2000). In this cascade, activated RhoA stimulates Rho kinase. We examined whether tropoelastin stimulated the RhoA signaling pathway via activation of a G-protein-coupled signaling pathway. Immunoprecipitation experiments demonstrate that tropoelastin activates RhoA through a pertussis toxin-sensitive mechanism (Fig. 4F). Together, our pharmacological and biochemical data lead us to propose a molecular mechanism for elastin signaling (Fig. 4G). In vascular smooth muscle cells, elastin activates a pertussis toxin-sensitive G-protein-coupled pathway that stimulates G_i, inhibits adenylate cyclase, reduces cAMP levels and stimulates Rho induced actin polymerization. In the absence of elastin synthesis, this mechanism is disrupted and vascular smooth muscle cells lose their contractile phenotype. Thus, there is a direct role for elastin in controlling vascular smooth muscle cells.

Elastin reduces the vascular proliferative response to arterial injury in vivo

The in vitro experiments described above demonstrated that elastin is an autocrine factor that induces a contractile state, inhibits proliferation and localizes vascular smooth muscle cells to the vessel wall. These data suggest that disruption of a crucial morphogenic signal in the vessel wall may release smooth muscle cells to dedifferentiate, proliferate and occlude mature arteries. To test this hypothesis in vivo, we used a porcine model to determine whether the application of



exogenous elastin to a site of vascular injury would reduce the neointimal accumulation of smooth muscle cells and arterial stenosis.

Porous sheaths of elastin matrix were generated from porcine carotid arteries using established methods (Malone et al., 1984). The purity of the elastin was confirmed by scanning

Fig. 4. Tropoelastin regulates migration and actin polymerization via a non-integrin, G-protein-coupled signaling pathway. (A) *Eln*$^{-/-}$ vascular smooth muscle cells migrate to recombinant tropoelastin in an EDTA insensitive manner. By comparison, we show that vascular smooth muscle cell migration to collagen, which is mediated by integrins, is EDTA sensitive. (B) Tropoelastin mediated actin polymerization of *Eln*$^{-/-}$ cells is EDTA insensitive. (C) Migration of *Eln*$^{-/-}$ vascular smooth muscle cells towards tropoelastin is pertussis toxin sensitive. Control experiments with the B protomer of pertussis toxin alone demonstrate that the effect of pertussis toxin is specific to its ability to inhibit G-protein signaling. The migration of *Eln*$^{-/-}$ cells to PDGF (30 ng/ml) is insensitive to pertussis toxin. This control experiment demonstrates that *Eln*$^{-/-}$ cells treated with pertussis toxin can respond normally to stimuli other than tropoelastin. (D) Tropoelastin-mediated actin polymerization of *Eln*$^{-/-}$ vascular smooth muscle cells is pertussis toxin sensitive. Control experiments demonstrate that the B protomer of pertussis toxin does not inhibit actin polymerization. Together, these experiments indicate that tropoelastin regulates vascular smooth muscle cells through a pertussis toxin-sensitive G-protein signaling pathway. (E) Cholera toxin (ctxn) and forskolin (frskln) increase the baseline levels of cAMP in *Eln*$^{-/-}$ vascular smooth muscle cells by constitutive activation of the G_s pathway. In the presence of tropoelastin and forskolin, there is a marked decrease in cAMP. A similar decrease in cAMP levels is observed when tropoelastin is added to cholera toxin pretreated cells. The reduction in cAMP was blocked by pertussis toxin and not B-protomer indicating a G_i specific pathway. These experiments indicate that tropoelastin activates a receptor that signals through G_i to inhibit adenylate cyclase, and reduce cAMP levels. (F) Tropoelastin activates RhoA in a pertussis toxin-sensitive manner. Co-immunoprecipitation experiments demonstrate that tropoelastin treatment of *Eln*$^{-/-}$ cells results in a marked elevation of activated RhoA. This response is pertussis toxin sensitive and B-protomer insensitive indicating that tropoelastin activation of the Rho pathway requires G_i activity. (G) Proposed molecular mechanism for tropoelastin-mediated actin polymerization in vascular smooth muscle cells.

electron microscopy (Fig. 5A), immunohistochemistry, amino acid composition and desmosine content (Starcher, 1977) (data not shown). The amino acid composition and the concentration of the crosslinking amino acids desmosine, isodesmosine and lysinonorleucine revealed no microfibrillar proteins or other impurities. Elastin sheaths were secured to intracoronary stents (Fig. 5B) and successfully deployed in porcine coronary arteries using standard catheterization techniques. The biocompatibility of elastin sheaths was assessed 3 days and 14 days after placement in the porcine coronary artery. Elastin sheaths did not evoke an inflammatory or thrombotic response (data not shown). Moreover, elastin sheaths were biologically stable and did not degrade within the arterial wall during the course of animal studies. These experiments demonstrate that elastin sheaths can be used to restore elastin matrix to sites of vascular injury.

To determine if elastin sheaths would reduce vascular smooth muscle accumulation and neointimal formation, we used an established porcine coronary injury model of in-stent restenosis (Schwartz et al., 1992; Schwartz et al., 1994). Two major coronary arteries of domestic pigs received either a control stent or an elastin sheath-covered stent following vascular injury caused by balloon overexpansion. Four weeks after injury and stent placement, representative cross-sections taken from control arteries displayed a thick fibrocellular neointima (Fig. 5C). By contrast, neointimal formation was substantially reduced

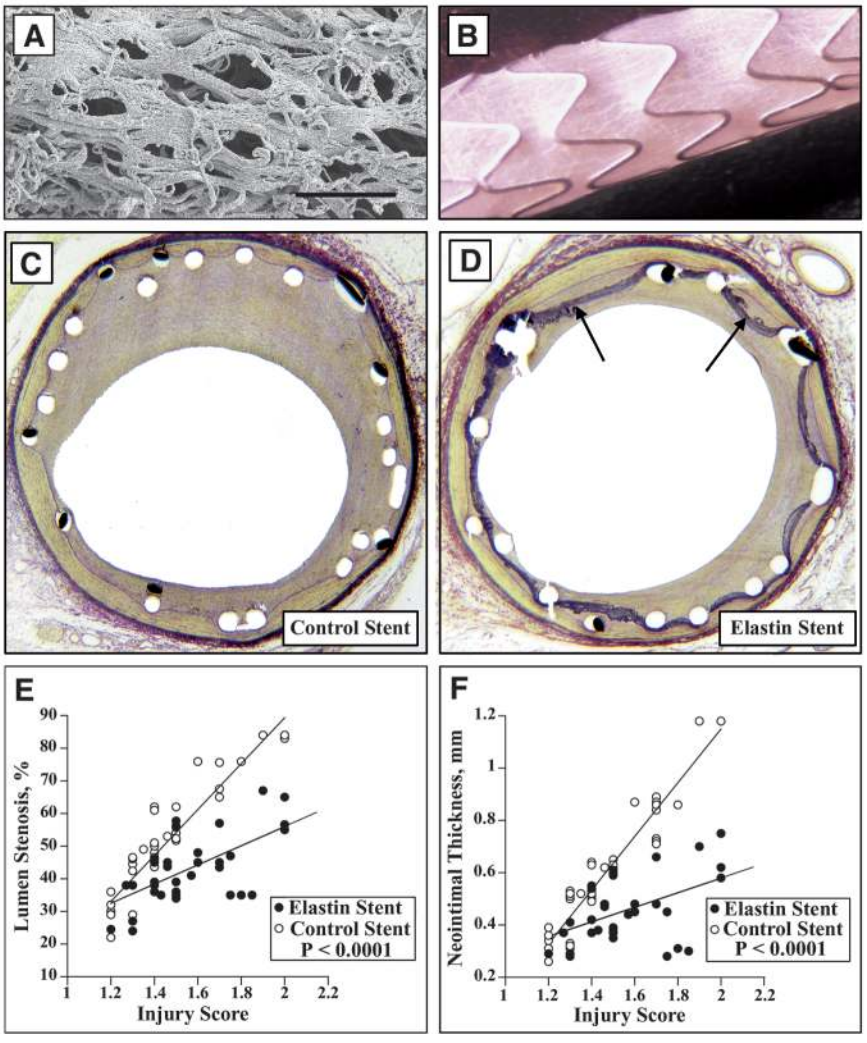


Fig. 5. Elastin therapy reduces vascular smooth muscle cell accumulation and arterial obstruction in vivo. (A) Scanning electron micrographs of elastin sheaths. Scale bar: 10 μm. (B) Elastin matrix sheath covering an expanded metal stent. (C,D) Representative cross-sections taken from control arteries (n=14) display the development of a thick fibrocellular neointima (C), whereas the neointima is substantially decreased in elastin sheath (arrows) treated arteries (n=13) with the same injury score (D). (E,F) Over a range of injury, the percent lumen stenosis (E) and mean neointimal thickness (F) were significantly reduced (both P<0.0001) in injured porcine coronary arteries treated with elastin sheath-stents compared with control stents.

in elastin sheath-treated arteries (Fig. 5D). Standard measurements of mean neointimal thickness (NIT) and percent stenosis were measured and correlated with the degree of injury induced by stent placement. The well-established correlation between the severity of injury and the amount of neointimal accumulation (Schwartz et al., 1994) is reproduced in our experiments with the control stents. Throughout the range of injury scores, there is a significant reduction in the NIT and percent stenosis in elastin sheath-treated arteries, with the greatest benefit at the highest injury scores (Fig. 5E,F). At a mean injury score of 2, when stent placement disrupts internal elastic lamella and lacerates the arterial media, elastin sheaths reduced the mean neointimal thickness by 52% as compared with controls. The specificity of the effect of elastin as a stent coating is supported by the work of our group and others with non-elastin sheaths. Sheaths made from collagen, fibrin or synthetic biopolymers failed to reduce neointimal thickness compared with bare stents and were frequently associated with a worse outcome (Goodwin et al., 2000; McKenna et al., 1998; van der Giessen et al., 1996; van Beusekom et al., 1998). Thus, consistent with the in vitro results, our in vivo experiments indicate that restoration of elastin matrix to a site of injury reduces vascular smooth muscle accumulation and limits neointimal formation.

DISCUSSION

Elastin is a critical regulatory molecule within the arterial wall

We conclude that elastin is a critical regulatory molecule that controls the phenotypic modulation, proliferation and migration of vascular smooth muscle cells. This conclusion is based on the following observations. First, cells that lack elastin proliferate at a rate twofold greater than wild-type cells. Second, cells that lack elastin fail to form a mature contractile phenotype, which is characteristic of wild-type cells. Third, cells that lack elastin migrate more aggressively to an exogenous chemoattractant than wild-type cells. Fourth, all of these aberrant phenotypes induced by the absence of elastin can be rescued in vitro by the addition of recombinant tropoelastin. Fifth, elastin exerts its regulatory function on vascular smooth muscle cells by activating a G-protein coupled signaling pathway. Finally, restoring elastin to a site of vascular injury in vivo reduces the neointimal accumulation of vascular smooth muscle cells. Thus, elastin matrix deposited by vascular smooth muscle cells in the arterial media provides a local cue that regulates these cells in vitro and in vivo.

Elastin-VSMC signaling is a critical morphogenic signal that is disrupted during the pathogenesis of vascular disease

Vascular smooth muscle cells are able to exist in a quiescent contractile state or a proliferative non-contractile state (Raines and Ross, 1993; Owens, 1998). This plasticity enables the vascular system to regenerate and grow. However, plasticity must be balanced by the need to maintain a mature and stable structure capable of circulating blood throughout a whole animal. Because vascular smooth muscle cells modulate their phenotype readily, external factors must instruct them to remain in a mature state if homeostasis is to be achieved. Our data indicate that the elastin matrix is a potent autocrine factor that regulates arterial morphogenesis by instructing vascular smooth muscle cells to localize around the elastic fibers in organized rings and remain in a quiescent, contractile state (Fig. 6A). This cell-matrix interaction is mediated via a heterotrimeric G-protein signaling pathway that activates downstream rho GTPases and appears to be crucial for stabilizing and maintaining the structure of the mature artery. When this morphogenic signal is absent during arterial development, the unregulated migration and proliferation of

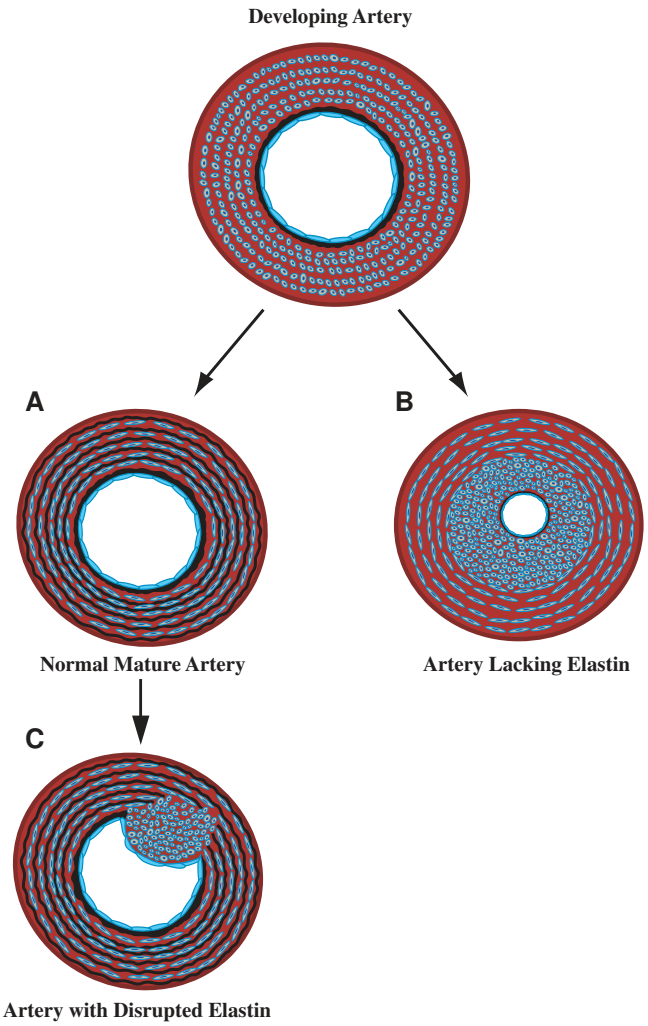


Fig. 6. Model of elastin-vascular smooth muscle cell interactions in development and disease. (A) During normal development, vascular smooth muscle cells synthesize and secrete elastin polymers that form concentric rings of elastic lamellae around the arterial lumen. Elastin provides mechanical support to the vessel wall, and signals vascular smooth muscle cells to localize around the elastic lamellae and remain in a quiescent, contractile state. (B) In the absence of elastin, this morphogenic signal is lost resulting in pervasive subendothelial migration and proliferation of vascular smooth muscle cells that occludes the vascular lumen. (C) This leads us to propose that the focal disruption and/or destruction of elastin in the mature artery by vascular injury releases smooth muscle cells to dedifferentiate, migrate and proliferate, and contributes to neointimal formation.

vascular smooth muscle cells results in occlusion of the arterial lumen (Li et al., 1998) (Fig. 6B). This leads us to propose that focal disruption or destruction of the elastin matrix in the mature artery by factors such as mechanical injury or inflammation play an important and direct role in the fibrocellular response characteristic of proliferative vascular diseases (Fig. 6C). Previously, emphasis was placed on the pivotal role of inflammatory cells in regulating vascular smooth muscle cells through the secretion of cytokines and growth factors (Lusis, 2000; Ross, 1995). In our model, the degradation of elastin by macrophages, T-cells and their proteases acts to release vascular smooth muscle cells from their mature contractile state to migrate, proliferate and form a neointima. Thus, the disruption of an essential morphogenic signal contributes to the pathogenesis of vascular disease.

Four lines of evidence support our model of elastin-vascular smooth muscle signaling in the pathogenesis of vascular proliferative diseases.

Pathology

Disruption of elastin matrix is consistently associated with vascular proliferative diseases in human pathological specimens (Sandberg et al., 1981; Sims, 2000; Sims et al., 1989). Moreover, the severity of occlusive vascular pathology increases in proportion to the magnitude of defects and discontinuities in the elastin matrix (Sims et al., 1989). It has also been noted that the programmed intimal hyperplasia and arterial occlusion required for the closure of the ductus arteriosis is associated with impaired elastic fiber formation (Hinek et al., 1991).

Genetic

We previously showed in human genetic studies that loss-of-function mutations in elastin is sufficient to cause a human vascular proliferative disease, supravalvular aortic stenosis and Williams-Beuren syndrome (Li et al., 1997; Curran et al., 1993; Ewart, 1993). In these diseases, there is an aggressive occlusive pathology that develops throughout the arterial tree of affected children in the absence of common risk factors for vascular disease.

Experimental

In murine gene-targeting experiments, we demonstrated that loss of elastin is sufficient to cause occlusive vascular pathology (Li et al., 1998). This pathology was caused by the unregulated proliferation, migration and accumulation of vascular smooth muscle cells in the subendothelial space (Fig. 6B). In this model, severe arterial obstruction occurs in the absence of an inflammatory response or hemodynamic stress.

Therapeutic

In this manuscript, we show that exogenous tropoelastin can control the proliferation, migration and maturation of vascular smooth muscle cells in vitro, and reduce the development of fibrocellular pathology in vivo. Other investigators have shown that matrix metalloproteinase inhibitors, which prevent the degradation of matrix components, can reduce neointimal formation (Zaidi et al., 2000). Together, these data indicate that the destruction of the elastin matrix is a critical step in the fibrocellular response characteristic of vascular proliferative disorders. Although strongly indicative, definitive proof of a role for elastin signaling in a vascular proliferative response requires further studies.

Elastin is unique among vascular extracellular matrix proteins

The extracellular matrix is known to play a crucial function in the regulation of vascular smooth muscle cell biology. The myriad associations and interaction between the many structural proteins, proteoglycans and growth factors of the vascular matrix makes it difficult to distinguish the effects of each element from one another. However, numerous in vitro studies have demonstrated the ability of matrix proteins such as collagen, fibronectin and laminin to affect vascular smooth muscle cell activity, including phenotypic modulation, migration and proliferation (Raines, 2000; Hedin et al., 1999). These data might suggest that there is overlap and redundancy with regard to the function of different vascular matrix proteins. Elastin, however, is unique among matrix elements in that the disruption of this gene leads to a vascular proliferative pathology in human and animal models. Disruption of other genes encoding vascular matrix proteins, including fibulin, fibrillin and collagen is associated with either arterial tortuosity, dissection or aneurysm formation in vivo, not proliferative or occlusive vascular pathology (Nakamura et al., 2002; Yanagisawa et al., 2002; Arteaga-Solis et al., 2000; Dietz and Mecham, 2000). Moreover, the specificity of the effect of elastin in vivo is supported by studies showing that collagen matrix-covered stents do not reduce neointimal formation in a porcine model of restenosis (Goodwin et al., 2000). Thus, elastin, when compared with other matrix proteins, is effective in both in vitro and in vivo experimental models. From these studies one would expect that targeted disruption of the elastin signaling mechanism would replicate phenotypes observed in $Eln^{-/-}$ cell and mice.

Restoration of elastin for the treatment of proliferative vascular diseases

Our work suggests that understanding the link between vascular development and disease may provide an alternative and potentially complementary strategy for the treatment of vascular proliferative diseases. Previously, emphasis has been placed on improving the outcome of vascular disease by inhibiting smooth muscle cell proliferation with coronary stents coated with either radioactivity or chemotherapeutic drugs such as rapamycin, actinomycin D and paclitaxil (Heldman et al., 2001; Leon et al., 2001; Sousa et al., 2001). These treatments use a common strategy of disrupting fundamental pathways such as microtubule assembly, DNA stability, and regulatory cell cycle proteins that are required in virtually all actively dividing cells. We present work that suggests restoring a natural morphogenic signal to the vessel wall may also be therapeutically beneficial. Clearly, additional work is needed to verify that results with murine and porcine models are recapitulated in humans.

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