Fibronectin Is an Important Regulator of Flow-Induced Vascular Remodeling

Hou-Yu Chiang, Vyacheslav A. Korshunov, Andrew Serour, Feng Shi, Jane Sottile

Objective—Fibronectin is an important regulator of cell migration, differentiation, growth, and survival. Our data show that fibronectin also plays an important role in regulating extracellular matrix (ECM) remodeling. Fibronectin circulates in the plasma and is also deposited into the ECM by a cell dependent process. To determine whether fibronectin affects vascular remodeling in vivo, we asked whether the fibronectin polymerization inhibitor, pUR4, inhibits intima-media thickening, and prevents excess ECM deposition in arteries using a mouse model of vascular remodeling.

Methods and Results—To induce vascular remodeling, partial ligation of the left external and internal carotid arteries was performed in mice. pUR4 and the control peptide were applied periadventitialy in pluronic gel immediately after surgery. Animals were euthanized 7 or 14 days after surgery. Morphometric analysis demonstrated that the pUR4 fibronectin inhibitor reduced carotid intima (63%), media (27%), and adventitial thickening (40%) compared to the control peptide (III-11C). Treatment with pUR4 also resulted in a dramatic decrease in leukocyte infiltration into the vessel wall (80%), decreased ICAM-1 and VCAM-1 levels, inhibited cell proliferation (60% to 70%), and reduced fibronectin and collagen I accumulation in the vessel wall. In addition, the fibronectin inhibitor prevented SMC phenotypic modulation, as evidenced by the maintenance of smooth muscle (SM) α-actin and SM myosin heavy chain levels in medial cells.

Conclusions—These data are the first to demonstrate that fibronectin plays an important role in regulating the vascular remodeling response. Collectively, these data suggest a therapeutic benefit of periadventitial pUR4 in reducing pathological vascular remodeling. (*Arterioscler Thromb Vasc Biol. 2009;29:00-00.*)

Key Words: extracellular matrix ■ fibronectin ■ collagen ■ vascular remodeling ■ smooth muscle cell

Extracellular matrix (ECM) molecules, including fibronectin, have direct effects on the growth and migration of endothelial cells, smooth muscle cell (SMCs), and myofibroblasts.\(^1\) In addition, our data show that the deposition of fibronectin into the extracellular matrix (ECM) controls the deposition, organization, and stability of other matrix molecules, including collagen I, collagen III, and thrombospondin-1.\(^6\) ECM molecules also play a critical role in stabilizing blood vessels. Mice lacking fibronectin die during embryogenesis because of cardiovascular defects.\(^9\) SMC migration and proliferation, as well as excess deposition of ECM molecules, are major factors contributing to vessel narrowing in certain types of vascular remodeling, including intima-media thickening (IMT) of the carotid artery.\(^10\) Hence, fibronectin and fibronectin deposition could play an important role in regulating vascular remodeling.

Vascular remodeling is a response of the vessel to hemodynamic changes or injury.\(^11,12\) In this article, vascular remodeling is defined as any change in the geometry of the vessel or vessel wall. Remodeling events can result in compensatory changes in the vessel wall that normalize wall stress.\(^13\) However, these compensatory changes are frequently inadequate, and further remodeling can result in narrowing of the vessel lumen.\(^12,13\) Carotid IMT in humans is a predictive indicator of cardiovascular disease and often occurs before the development of atherosclerotic lesions.\(^14,15\) Changes in cell growth, migration, and matrix synthesis contribute to vascular remodeling after injury or in response to changes in flow.\(^16-19\) Although much attention has been focused on the contribution of medial SMC during vascular remodeling, changes in the adventitia and in adventitial fibroblasts also occur and contribute to intimal thickening.\(^11,20\)

Fibronectin is produced and secreted by numerous cell types including SMCs, fibroblasts, and myofibroblasts and is widely distributed in ECM in vivo.\(^21\) Soluble fibronectin is deposited into tissue ECM by a cell-dependent process.\(^22\) In this study, we used a recombinant peptide derived from the F1 adhesin, pUR4, to inhibit fibronectin polymerization, and assessed its effects on an in vivo flow-induced vascular remodeling model in mice.\(^18\) We introduced pUR4 periadventitialy using pluronic gel and assessed its effects on carotid IMT, ECM deposition, and cell proliferation and differentiation. Our studies show that local delivery of a fibronectin polymerization inhibitor reduces early leukocyte infiltration and cell proliferation and attenuates the excess deposition of fibronectin and collagen that occurs during remodeling.

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Fibronectin and Collagen Deposition in SMCs

The Fibronectin Inhibitor, pUR4, Blocks IMT

Effect of pUR4 on FN and collagen I deposition in SMCs. SMCs were grown in serum containing media in the absence (A through C) or presence of 500 nmol/L pUR4 (D through F) or control III-11C (G through I) peptides for 3 days. Cells were fixed, then incubated with a polyclonal antibody to fibronectin (A, D, and G) or collagen I (B, E, and H). Corresponding differential interference contrast images are shown in C, F, and I. Bar, 20 μm.

Methods

Peptides

The pUR4 and control III-11C peptides were produced and purified as described. Details are provided in the Data Supplement (available online http://atvb.ahajournals.org).

Carotid Ligation Surgery

Blood flow in the left carotid artery of 10-week-old FVB/NJ (FVB) mice was reduced by ligating the external and internal carotid arterial branches as described. Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) as described. The left common carotid artery was dissected free of the surrounding connective tissue and 50 μL of F-127 pluronic gel containing pUR4 or III-11C was applied around the carotid artery. Animals were separated into 5 groups containing 5 to 10 animals each: (1) sham-operated, in which the branches of the carotid artery were exposed and dissected out, but not ligated; (2) ligation; (3) ligation with pluronic gel; (4) ligation with pluronic gel containing III-11C; (5) ligation with pluronic gel containing pUR4.

Tissue Collection and Processing

Blood flow in the left and right common carotid artery was measured using an ultrasonic transit-time volume flowmeter (Transonic Systems) before sacrifice. Animals were perfusion-fixed with 10% formalin 7 or 14 days after surgery. The common carotid arteries were harvested and embedded in paraffin. Cross-sections of 5 μm were cut from the bifurcation every 200 μm through the first mm length of the carotid artery as described. Sections were stained with Verhoeff–van Gieson elastic stain then subjected to morphometric analysis.

An expanded Methods section is available in the Data Supplement.

Results

The Fibronectin Inhibitor, pUR4, Blocks Fibronectin and Collagen Deposition in SMCs

To determine the effect of fibronectin on vascular remodeling, we used the fibronectin polymerization inhibitor, pUR4. pUR4 has been shown to inhibit fibronectin polymerization in fibroblasts, osteosarcoma cells, and endothelial cells. As shown in Figure 1D, addition of pUR4 to cultured SMCs inhibited the deposition of endogenous fibronectin into the ECM. Our published data show that fibronectin regulates the deposition of other proteins into the ECM, including type I collagen. When SMCs were cultured in the presence of pUR4, deposition of endogenous collagen I into matrix fibrils was also inhibited (Figure 1E). The control peptide had no effect on fibronectin or collagen I deposition (Figure 1G and 1H). pUR4 binds to fibronectin, and inhibits fibronectin deposition by interfering with the binding of fibronectin to matrix assembly sites on the cell surface. pUR4 does not inhibit cell spreading or adhesion to collagen (unpublished data, 2008) or fibronectin and does not bind to other ECM proteins, including collagen I, fibrinogen, and laminin (supplemental Figure I). In addition, pUR4 does not inhibit fibronectin or collagen mRNA synthesis in SMCs (supplemental Table I).

The pUR4 Fibronectin Inhibitor Blocks Vascular Remodeling

Fibronectin is known to affect SMC growth, migration, and differentiation in vitro. Hence, fibronectin could promote vascular remodeling by multiple mechanisms. To determine whether the pUR4 fibronectin inhibitor blocks IMT, we used a flow-induced model of vascular remodeling in which the internal and external branches of the common carotid artery are ligated. Blood flow in the common carotid artery was significantly reduced after ligation (0.13±0.01 mL/min) compared to shams (0.59±0.07 mL/min). There were no differences among experimental groups treated with pluronic gel (unpublished data, 2008). After ligation of the carotid artery, pUR4 and the control peptide were embedded in pluronic gel, and applied periadventitiously. Supplemental Figure II demonstrates that the pUR4 peptide can be readily detected in the vessel wall in both the media and adventitia 1 to 3 days after periadventitial application. The levels of the peptide were significantly reduced by 7 days. The control peptide could also be detected in the vessel wall (unpublished data, 2008).

As we previously showed, FVB mice exhibited significant IMT 14 days after ligation (compare sham versus ligated; Figure 2A). Application of pluronic gel in the absence or presence of the control peptide had no effect on vascular remodeling. Periadventitial administration of pUR4, but not the control peptide, dramatically reduced carotid remodeling (Figure 2A). Morphometry of the carotid compartment was performed 7 and 14 days after ligation (Figure 2B through 2E). Lumen volume was significantly increased after ligation in control peptide–treated mice in comparison to shams (Figure 2B), consistent with our published data in FVB mice. This increase was prevented by pUR4 treatment. There was a dramatic effect of pUR4 on vascular wall remodeling (Figure 2C through 2E). Ligation of the carotid artery resulted in a 3-fold increase in intima-media volume at 14 days (Figure 2C). The pUR4 peptide reduced the extent of IMT by 40% in comparison with control peptide–treated animals at 14 days (Figure 2C). When vessel compartments were analyzed separately (supplemental Figure III), pUR4 was found to reduce intima thickening by 63%, and media thickening by 27%. Interestingly, there was no statistical
The pUR4 Fibronectin Inhibitor Decreases ECM Accumulation

ECM accumulation is a hallmark of vascular remodeling in response to changes in blood flow or injury. Hence, we used immunohistochemistry (IHC) to determine whether pUR4 caused a reduction in fibronectin and collagen I deposition in the left carotid artery. IHC analysis indicates that there was a dramatic reduction in the accumulation of collagen I and fibronectin in the media and adventitia 7 and 14 days after surgery in pUR4-treated animals in comparison with control animals (Figure 3A). At 7 days after surgery, pUR4 totally prevented increased fibronectin deposition (Figure 3B and 3C). At 14 days after surgery, fibronectin and collagen levels were still decreased in pUR4-treated animals in comparison to control peptide–treated animals. The decrease in collagen deposition parallels the decrease in fibronectin deposition and is consistent with in vitro data showing that collagen deposition depends on fibronectin matrix polymerization.6,7,31

The pUR4 Fibronectin Inhibitor Decreases SMC Phenotypic Modulation

To begin to define the mechanism(s) by which fibronectin regulates vascular remodeling, carotid artery sections were analyzed by IHC to determine the effect of pUR4 on SMC differentiation, cell proliferation, and leukocyte infiltration. Arterial injury is known to decrease SMC differentiation markers at early times after injury or in response to decreased blood flow.16,19 This decline in SMC differentiation is thought to contribute to increased SMC migration and proliferation. Our quantitative data show that reduced flow in the left carotid artery results in decreased SMC α-actin and SM myosin heavy chain staining after treatment with III-11C peptide (Figure 4). However, the pUR4 fibronectin inhibitor prevented SMC dedifferentiation, as evidenced by the maintenance of SM α-actin and SM myosin heavy chain staining in the media (Figure 4).

The pUR4 Fibronectin Inhibitor Decreases Cell Proliferation

Our previous data with FVB mice showed that the greatest increase in cell proliferation and leukocyte infiltration was at 7 days.30 Our data show that ligation resulted in a significant increase in cell density in the media 7 days after surgery (6.7×10^3 cells/mm² in ligated animals versus 5.0×10^3 versus cells/mm² in sham operated animals). pUR4 treatment prevented this increase in cell density by 75% (5.3×10^3 cells/mm² in ligated animals versus 1.4×10^3 cells/mm² in pUR4-treated animals).
Further, ligation of the left carotid artery increased cell proliferation in the vessel wall as shown by PCNA staining (supplemental Figure IV); this increase in cell proliferation was drastically reduced in pUR4-treated animals. There was a significant reduction in cell proliferation in the intima-media (70%) and adventitia (61%) in animals treated with pUR4 (Figure 5A and 5B). These data indicate that fibronectin promotes vascular remodeling, in part, by enhancing cell proliferation.

The pUR4 Fibronectin Inhibitor Dramatically Reduces Accumulation of Inflammatory Cells in the Carotid Artery

Inflammation plays a prominent role in vascular remodeling after injury. Flow-induced vascular remodeling is also accompanied by an increase in inflammatory cells in the vessel wall.19,30 Our data show that reduced flow in the left carotid artery resulted in an increase in leukocyte numbers in the intima, media, and adventitia 7 days after surgery (Figure 5C and 5D, and supplemental Figure V). Inhibition of fibronectin polymerization significantly decreased (80%) leukocyte infiltration into the intima, media, and adventitia (Figure 5C and 5D). Further, pUR4 caused a dramatic decrease in adhesion molecule expression, as shown by the reduction in intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) levels in the vessel wall (Figure 6, supplemental Figure VI). These data are the first to demonstrate that fibronectin plays a critical role in regulating the inflammatory response during vascular remodeling.

Discussion

In this article we show that periadventitial delivery the fibronectin inhibitor, pUR4, reduces IMT in response to reduced blood flow (Figure 2). pUR4 treatment also reduced fibronectin and collagen I accumulation in the vessel wall (Figure 3). These data are consistent with in vitro data showing that inhibition of fibronectin polymerization decreases the deposition of fibronectin and collagen I in the ECM.6,7,31 Treatment with pUR4 also resulted in a dramatic decrease in leukocyte infiltration into the vessel wall (Figure 5), reduced ICAM-1 and VCAM-1 levels (Figure 6), inhibited cell proliferation (Figure 5), and prevented SMC phenotypic modulation (Figure 4). These data are the first to show that fibronectin is an important regulator of flow induced vascular remodeling.

Fibronectin is a secreted protein that is polymerized into ECM fibrils by a cell-dependent process.22 There is a large literature demonstrating the importance of ECM proteins, including fibronectin and collagen, in regulating cell migration, growth, and differentiation.1-4,32 Further, the ECM polymerized form of fibronectin has been shown to have distinct effects on cell behavior in comparison to protomeric fibronectin.2-4,7,33,34 Similarly, polymerized collagen I has distinct effects on SMC growth in comparison to nonpolymerized collagen.1 Our in vitro data indicate that fibronectin

Figure 4. The pUR4 fibronectin inhibitor maintains SMC differentiation. A, Immunohistochemistry for SMMHC and SM α-actin in the left carotid artery 7 days after ligation. Media=“M”. Quantitative results of immunostaining intensities (relative units per unit area) of SM α-actin (B) and SMMHC (C) in the media, Bar, 25 μm. *P<0.05, **P<0.01.

Figure 5. A and B, The pUR4 fibronectin inhibitor decreases cell proliferation in the left carotid artery. Sections of the left carotid artery 7 days after ligation were stained for PCNA and counterstained with hematotoxin. Quantitative IHC analysis of the proliferation index (PCNA (+/+) cells per total cell number) in the intima-media (A) and adventitia (B) 7 days after ligation. C and D, The pUR4 fibronectin inhibitor decreases leukocyte infiltration into the left carotid artery. Sections of the left carotid artery were stained with antibodies to CD45 7 days after ligation. Sections were counterstained with hematotoxin. Percentage of the area which is CD45 (+/+) was assessed in the intima-media (C) and adventitia (D) of the vessels as described in the Methods. *P<0.05, **P<0.01.

Figure 6. The pUR4 fibronectin inhibitor decreases ICAM-1 and VCAM-1 levels. Sections of the left carotid artery 7 days after ligation were stained with ICAM-1 or VCAM-1. Percentage of the intima-media area that was ICAM-1 (+) (A) or VCAM-1 (+) (B) was evaluated in the intima-media of the vessels. *P<0.05, **P<0.01.
polymerization is an important regulator of cell growth, migration, and ECM deposition and stability. These data suggest the possibility that fibronectin polymerization may be a key regulator of vascular remodeling in vivo. However, to date, in vivo evidence that fibronectin or fibronectin polymerization regulate SMC function or ECM remodeling has been lacking. In this study, we used the pUR4 peptide to inhibit fibronectin polymerization to determine the role of fibronectin matrix deposition in vascular remodeling. pUR4 binds to fibronectin, and inhibits its ability to be polymerized into ECM fibrils. pUR4 can inhibit the polymerization of both endogenously produced (Figure 1) and exogenously supplied fibronectin into the ECM. pUR4 did not decrease fibronectin or collagen I mRNA production in cultured SMC (supplemental Table I). However, there was a trend toward decreased fibronectin and collagen I mRNA in the carotid artery of animals treated with pUR4 in comparison with control peptide–treated animals (supplemental Table II). Because pUR4 does not decrease fibronectin or collagen I mRNA in vitro, it is likely that the effect of pUR4 on mRNA levels in vivo is indirect, perhaps resulting from altered cytokine production.

Fibronectin matrix has the potential to influence multiple cell properties. Fibronectin can promote SMC growth and migration in vitro. Further, fibronectin matrix deposition regulates the deposition and stability of other ECM proteins. Our data show that pUR4 causes a significant reduction in cell proliferation. Previous studies have shown that fibronectin polymerization can positively affect myofibroblast, SMC, and endothelial cell growth. Fibronectin polymerization can also promote myofibroblast and epithelial cell migration. Hence, our in vivo data are consistent with much in vitro data that demonstrate a positive effect of fibronectin and fibronectin polymerization on cell growth and migration.

Inflammation has also been shown to play an important role in vascular remodeling in response to changes in flow. Our data show that fibronectin plays an important role in regulating the recruitment of leukocytes into the vessel wall (Figure 5). Fibronectin fragments are known to be chemotactic for neutrophils and monocytes. However, most in vitro data suggest that intact fibronectin does not promote leukocyte chemotaxis. Our data also show that the pUR4 fibronectin inhibitor causes a dramatic reduction in ICAM-1 and VCAM-1 levels in the vessel wall. It is likely that the effect of pUR4 on leukocyte infiltration is due to its ability to decrease VCAM-1 and ICAM-1 levels. Fibronectin is known to regulate the activity of nuclear factor κB (NF-κB) in certain cell types, which in turn stimulates ICAM-1 and VCAM-1 production. Hence, the downregulation of ICAM-1 and VCAM-1 levels in pUR4-treated animals could result from decreased NF-κB activity.

Fibronectin is also known to promote SMC dedifferentiation. However, the effect of matrix fibronectin on SMC differentiation has not been previously characterized. Our in vivo data show that inhibiting fibronectin polymerization results in maintenance of the SMC differentiated phenotype (Figure 4), indicating that fibronectin polymerization is an important regulator of SMC differentiation. Phenotypic modulation of SMC is thought to play a key role during vascular remodeling, contributing to increased SMC proliferation and migration. Hence, the ability of pUR4 to limit SMC dedifferentiation may be an important mechanism that contributes to reduced intima-media thickening after ligation.

Other ECM molecules have been shown to play important roles in vascular remodeling, including thrombospondin I, vitronectin, and osteopontin. Interestingly, certain ECM and cytoskeletal proteins have been shown to influence both IMT and vessel size, similar to our findings with fibronectin. These data suggest that the effects of ECM proteins on outward remodeling may be an important aspect of their ability to regulate vascular remodeling. Our in vitro data show that fibronectin polymerization controls the deposition of thrombospondin I and collagen I into ECM fibrils. In addition, we previously showed that the ability of fibronectin to promote myofibroblast migration is attributable in large part to its ability to regulate the deposition of type I collagen. Hence, one mechanism by which fibronectin may regulate vascular remodeling is by affecting the deposition of other matrix molecules, such as collagen I and thrombospondin 1. It is likely that ECM effects on vascular remodeling are regulated by integrins. Inhibition of integrin function using Arg-Gly-Asp (RGD) peptides or antibody blockade of αvβ3 integrin have been shown to inhibit neointimal formation in vivo.

Our data are the first to show that fibronectin is an important regulator of vascular remodeling in vivo. These results are particularly striking given that the pUR4 peptide was delivered peradventitiously and that the levels of the peptide peak 1 to 3 days after application. The ability of pUR4 to inhibit the vascular remodeling response long term (2 weeks), coupled with the reduction in leukocyte infiltration and cell proliferation, suggest that the fibronectin inhibitor acts by blocking an early step(s) in the remodeling response. This early step is likely to involve decreased leukocyte infiltration that occurs, at least in part, as a result of decreased VCAM-1 and ICAM-1 expression. Taken together, these data suggest the possibility that pUR4, or inhibitors that act by similar mechanisms, could have therapeutic applications in treating vascular occlusive diseases.

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Disclosures

None.

References


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SUPPLEMENT MATERIAL

Fibronectin is an important regulator of flow-induced vascular remodeling

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SUPPLEMENTAL MATERIALS AND METHODS

Proteins

Human fibronectin was purified as described\(^1\). Recombinant vitronectin was produced as described\(^2\). Laminin was purchased from BD Biosciences (San Jose, CA), collagen type I from UBI (Lake Placid, NY) and fibrinogen from Enzyme Research Laboratories (South Bend, IN). pUR4 cDNA was a generous gift from Dr. Hanski\(^3\), and was provided to us by Dr. Mosher (University of Wisconsin, Madison, WI). pUR4 (also known as FUD, functional upstream domain) is a 49-mer peptide derived from the bacterial F1 adhesin. We modified pUR4 cDNA to remove the coding region for a cys residue that was present in the N-terminal portion of the peptide. This cys is not part of the adhesin F1, but was added as a consequence of the original cloning strategy. The sequence of pUR4 that we used in this study is:

\[
\text{MRGSHHHHHHGSKDQSLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFS}
\]

TEDTKLN. Bold sequences were added as part of the cloning strategy, and to provide a his tag for purification purposes. The control peptide is a his tagged carboxyl terminal fragment (68-mer) of fibronectin’s III-11 module (III-11C) and was produced as described\(^4\). III-11C has been used in in vivo studies by others\(^5,6\), and has no known biological effects in vitro or in vivo\(^4-8\).

Endotoxin was removed from peptides using Detoxi-Gel (Pierce, Rockford, IL). Endotoxin levels were measured with a Limulus amebocyte lysate assay kit (QCL-1000, Lonza, Basel, Switzerland). Endotoxin levels were less than 0.1 endotoxin units (EU) per mg of peptide.

Enzyme linked immunosorbant assay (ELISA)

96-well tissue culture plates were coated with 10 µg/mL type I collagen, fibronectin, laminin, fibrinogen, or vitronectin overnight at 4°C. Denatured collagens were generated as described\(^9\). Plates were blocked with 1% bovine serum albumin (BSA) in Tris.
buffered saline (TBS) for 1 hour, then washed with TBS. pUR4 was serially diluted into coated wells, then incubated at room temperature for 90 min. Wells were washed with TBS containing 0.1% Tween 20. An anti his antibody (HisG, Invitrogen, Carlsbad, CA) that recognizes the his tag on pUR4 was added for 1.5 hour at room temperature. Wells were washed, then incubated with an horseradish peroxidase (HRPO) conjugated secondary antibody. After washing, peroxidase activity was quantified by using 2,2’-azino-bis-(3-ethylbenthiazoline-sulfonic acid). Measurements were done at 405 nm on a Wallac 1420 multilabel counter.

**Cell culture**

Rat aortic smooth muscle cells (RASM) were obtained from Cell Applications (San Diego, CA) and maintained in serum containing media (Cell Applications). SMC were used at passages 4-6.

**Animal Studies**

Both sexes were used for the carotid ligation experiments, since no significant differences in the remodeling response were reported between male and female mice \(^{10}\). All procedures were approved by the University of Rochester Animal Care Committee, and were performed in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals.

**Morphometry**

Digital images of Verhoeff-van Gieson elastic stained cross-sections were captured and morphometric analysis was performed using Image-Pro Plus software (Media Cybernetics, MD).
The circumferences of the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) in the sections were identified using the automatic trace mode in Image-Pro. The accuracy of the automated tracing was verified by visual inspection of the images. Cross-sectional areas of the lumen, neointima, media, adventitia, and the area encompassed by the EEL were measured. Vessel compartment volumes were calculated as described. Briefly, starting with the carotid bifurcation as the origin, a series of cross-sections of 5 µm were cut every 200 µm through the first mm length of the carotid artery. Morphometric analysis was performed at each point, and the average of each division was calculated, summed, and reported as the volume.

**Immunohistochemistry and quantitative analysis**

Sections selected from the first mm of the carotid artery in each group were used for immunohistochemistry (IHC). Endogenous peroxidase activity was blocked with 3% H2O2, followed by incubation with Dako serum-free blocking solution (Dako; Glostrup, Denmark). The primary antibodies used were: polyclonal anti-fibronectin (Chemicon/Millipore, Billerica, MA), polyclonal anti-collagen type I (LF-67; a gift from Dr. Fisher, NIH, Bethesda, MD), monoclonal anti-proliferating cell nuclear antigen (PCNA) (Sigma, St. Louis, MO), polyclonal anti-smooth muscle myosin heavy chain (SMMHC) (Biomedical Technologies Inc., Stoughton, MA), monoclonal smooth muscle α-actin (SM α-actin) (Dako), anti-leukocyte common antigen, CD45 (BD Pharmingen, San Jose, CA), monoclonal anti-intercellular adhesion molecule-1 (ICAM-1) (BD Pharmingen), and polyclonal anti-vascular cell adhesion molecule-1 (VCAM-1) (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen retrieval for anti-fibronectin and anti-collagen type I antibodies was performed by incubating sections with proteinase K in 0.05M Tris-HCL buffer. Antigen retrieval for CD 45 antibody was performed by incubating sections with 10 mM citrate buffer.
buffer (pH=6) at 120°C. Sections were then incubated with appropriate biotinylated secondary antibodies followed by avidin-biotin immunoperoxidase system (Vector Laboratories, Burlingame, CA). Liquid DAB Substrate Chromogen system (Dako) or Vector Red (Vector) was used for detection. PCNA, CD45, ICAM-1, and VCAM-1-immunostained sections were counterstained with hematoxylin.

Quantitative IHC analysis was performed using Image-Pro Plus software. Data from 3-5 mice were averaged, and the average values ± s.e.m. determined. For fibronectin, collagen type I, SMMHC, and SM α-actin, color digital images were captured using a 40X objective and transformed into gray scale. 16 fields of view of equal size per section in the media or adventitia were randomly chosen, and the mean of the optical densities from the 16 fields of view was determined. 3 sections were analyzed per animal. For quantitative comparison of leukocyte infiltration and cell adhesion molecule expression, images were acquired using a 60X objective. The CD45, ICAM-1, and VCAM-1-positive areas were obtained using an automated programmed segmentation procedure in ImagePro. The intima plus media or adventitial regions were traced manually. The percent of the positively stained area to the total traced area was determined. For evaluation of proliferating cells, the percent of PCNA (+) cells to total cells was determined by counting cell numbers in PCNA-stained sections that had been counterstained with hematoxylin. The percent positive cells is reported as the proliferation index. To determine the SMC density, sections were stained with hematoxylin, and cells present in the media were counted. The data is expressed as number of cells/µm².
Detection of peptides in the vessel wall

pUR4 and III-11C were conjugated to Texas-Red, as per the manufacturer’s instructions (Molecular Probes/Invitrogen). 20 \( \mu \)M Texas-Red (TR) conjugated proteins were embedded in pluronic gel and applied to the vessel following ligation surgery. Mice were sacrificed 1, 3, or 7 days after surgery. Longitudinal frozen sections (20 \( \mu \)m thickness) were cut and immunostained with a polyclonal anti-TR antibody (Molecular Probes/Invitrogen). For quantitatively evaluating the efficacy of peptide delivery to the common carotid artery, 15-18 fields of view in the media from 2-3 mice at each time point were analyzed. The TR (+) areas were quantitated using an automated programmed segmentation procedure in ImagePro. The percentage of TR (+) area to the total medial area was determined.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). mRNA was isolated from RASMs 24 hours after incubation with 500 nM pUR4 or III-11C control peptides using RNAzol as per the manufacturer’s instructions (Invitrogen). cDNA was prepared using a Superscript First Strand cDNA kit as per the manufacturer’s instructions (Invitrogen). Mouse carotid arteries were harvested and frozen in liquid nitrogen. RNA was isolated using Trizol and purified using a QIAGen RNeasy Micro Kit. RNA integrity was examined with an Agilent 2100 Bioanalyser using RNA6000 NanoAssay (Agilent Technology). Whole Transcriptome Amplification was performed using Qiagen’s QuantiTect Whole Transcriptome Amplification Kit. Quantitative RT-PCR analyses were performed using ABI Prism 7900HT sequence detection system (Applied Biosystems). The qRT-PCR primers for 3 mouse genes were obtained from ABI: GADPH (4352932E), fibronectin (Mm01256744_m1), and collagen Ia1 (Mm00801666_g1). TaqMan probe chemistry was used according to the manufacturer’s
instructions. qRT-PCR reactions were run in triplicate for each sample. The data was normalized to the levels of GAPDH in each sample, and the results were averaged.

**Statistical analysis**

For animal studies, data are presented as the mean ± s.e.m. Comparisons were made with an unpaired, 2-tailed Student’s t test, or one-way ANOVA with GraphPad Prism software (San Diego, CA). A difference between the means was considered significant when \( p < 0.05 \).
SUPPLEMENTAL FIGURES

Figure I. Binding of pUR4 to ECM Proteins. 96-well plates were coated with 10 µg/mL type I collagen (COL), denatured type I collagen (dCOL), fibronectin (FN), laminin (LN), vitronectin (VN), or fibrinogen (FG) at 4°C overnight. pUR4 was added to the wells and serially diluted. Bound pUR4 was detected as described in the Supplemental Methods. Measurements were done at 405 nm on a Wallac 1420 multilabel counter. Data represents the average of duplicate determinations, and the error bars the range.

Figure II. Visualization of pUR4 in the vessel wall. Texas-Red (TR) conjugated pUR4 (20 µm) was embedded in pluronic gel and peri-adventitially delivered to the left carotid artery following ligation surgery as described in the Methods. The right carotid artery from the same animal was used as a control. Mice were sacrificed 1, 3, and 7 days after surgery, and the carotid arteries were harvested and flash frozen. Longitudinal sections (20 µm) were immunostained with an anti-TR antibody. Representative images from the right common carotid artery (A), and left common carotid artery of 1 day (B), 3 day (C) and 7 day (D) animals are shown. (E) Percentage of the area which is TR (+) was assessed in the media of the vessels. Media=M; adventitia=Ad; lumen=L. Bar, 20 µm.
**Figure III.** The pUR4 fibronectin inhibitor decreases intima and media thickening. Morphometric analysis of the intima (A) and media (B) 7 and 14 days after ligation. * indicates $p<0.05$, and ** indicates $p<0.01$.

**Figure IV.** The pUR4 fibronectin inhibitor decreases cell proliferation in the carotid artery. Sections of the left carotid artery were stained with antibodies to PCNA seven days post ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of PCNA-stained sections from sham-operated animals (A), ligated animals with III-11C treatment (B) and ligated animals with pUR4 treatment (C). Arrows indicate PCNA (+) cells in the media, and arrowheads indicate PCNA (+) cells in the adventitia. Bar, 50 µm.
Figure V. The pUR4 fibronectin inhibitor decreases leukocyte infiltration into the vessel. Sections of the left carotid artery were stained with antibodies to CD45 seven days after ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of CD45 (+) staining in the media (A,B,C) and adventitia (D,E) of the carotid artery from sham-operated animals (A), ligated animals with III-11C treatment (B and D) and ligated animals with pUR4 treatment (C,E). Arrows indicate CD45 (+) cells in the media and adventitia. Arrowheads indicate the location of the external elastic lamina. Bar, 25 µm in (A-C) and 12.5 µm in (D,E).

Figure VI. The pUR4 fibronectin inhibitor decreases ICAM-1 and VCAM-1 levels. Sections of the left carotid artery were stained with antibodies to ICAM-1 (A-C) or VCAM-1 (D-F) seven days after ligation. Sections were counterstained with hematoxylin. Representative photomicrographs of sections from sham-operated animals (A and D), ligated animals with III-11C treatment (B and E) and ligated animals with pUR4 treatment (C and F). Bar, 20 µm.
Table I. *qRT-PCR of fibronectin and collagen I in SMC*. Confluent cultures of rat aortic SMCs were incubated in the presence of 500 nM pUR4, control III-11C (C,D) peptide or an equivalent volume of PBS for 24 h. qRT-PCR was performed in triplicate for each sample using TaqMan chemistry as described in Methods. Data represent the average of duplicate samples, and the error bars the range. The relative levels of fibronectin and collagen are shown; the PBS control was set equal to 1.

<table>
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<th>Sample</th>
<th>Treatment</th>
<th>FN</th>
<th>Col Ia1</th>
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<tbody>
<tr>
<td>SMC</td>
<td>PBS</td>
<td>1.00 ± 0.28</td>
<td>1.00 ± 0.08</td>
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<tr>
<td></td>
<td>111-11C</td>
<td>1.16 ± 0.12</td>
<td>1.27 ± 0.13</td>
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<td>pUR4</td>
<td>1.43 ± 0.02</td>
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Table II. *qRT-PCR of fibronectin and collagen I in carotid arteries*. Mouse carotid arteries were harvested from pUR4 and III-11C treated animals 4 or 7 days post surgery. qRT-PCR was performed as described in Methods. qRT-PCR reactions were run in triplicate for each arterial sample. Three arteries were analyzed for each condition. The data represent the average of these 3 samples, and the error bars the s.e.m. The relative levels of fibronectin and collagen are shown; III-11C was set equal to 1. A two tailed homoscedastic (two sample equal variance) t-test was used to analyze the data. No statistically significant differences (p <0.05) were found between the groups.

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Day 7</th>
<th>Col Ia1</th>
<th>Day 4</th>
<th>Day 7</th>
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<td>Carotid Arteries</td>
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<td>1.00 ± 0.32</td>
<td>1.00 ± 0.16</td>
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<td>pUR4</td>
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<td>0.58 ± 0.36</td>
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**SUPPLEMENTAL REFERENCES**


