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Fibronectin is an important regulator of flow-induced vascular remodeling

Hou-Yu Chiang, **Vyacheslav A. Korshunov**, **Andrew Serour**, **Feng Shi**, and **Jane Sottile**[1]

Aab Cardiovascular Research Institute University of Rochester School of Medicine and Dentistry 601 Elmwood Ave, Box CVRI Rochester, NY 14642 Phone: 585-276-9780 Fax: 585-276-9829

Abstract

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 periadventitially in pluronic gel immediately after surgery. Animals were sacrificed 7 or 14 days post 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 (60-70%), and reduced fibronectin and collagen I accumulation in the vessel wall. In addition, the fibronectin inhibitor prevented SMC phenotypic modulation, as evidence 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 pathologic vascular remodeling.

Keywords

extracellular matrix; fibronectin; collagen; vascular remodeling; smooth muscle cell

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Extracellular matrix (ECM) molecules, including fibronectin, have direct effects on the growth and migration of endothelial cells, smooth muscle cell (SMCs), and myofibroblasts[1-5]. 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, 7]. ECM molecules also play a critical role in stabilizing blood vessels. Mice lacking fibronectin die during embryogenesis due to cardiovascular defects[8,9]. 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)

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[1]Corresponding author: jane_sottile@urmc.rochester.edu .

DISCLOSURES None

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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 manuscript, 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[12]. 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 prior to the development of atherosclerotic lesions[14,15]. Changes in cell growth, migration, and matrix synthesis contribute to vascular remodeling following 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 periadventially 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. These studies are the first to demonstrate that fibronectin is an important regulator of vascular remodeling in vivo.

METHODS

Peptides

The pUR4 and control III-11C peptides were produced and purified as described[23, 24]. Details are provided in the data supplement (see http://atvb.ahajournal.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[18]. Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) as described[18]. The left common carotid artery was dissected free of the surrounding connective tissue and 50 µl of F-127 pluronic gel (BASF, Florham Park, NJ) containing 20 µM pUR4 or III-11C was applied around the carotid artery. Animals were separated into five groups containing 5-10 animals each: (1) sham-operated, in which the branches of the left 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, Ithaca, NY) 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 5µm were cut from the bifurcation every 200 µm through the first mm length of the carotid artery as described[18].

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Sections were stained with Verhoeff-van Gieson elastic stain then subjected to morphometric analysis.

An expanded Methods section is available in the data supplement (see http://atvb.ahajournal.org).

RESULTS

The fibronectin inhibitor, pUR4, blocks fibronectin and collagen deposition in SMC

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[5,23]. As shown in Fig. 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[6]. When SMCs were cultured in the presence of pUR4, deposition of endogenous collagen I into matrix fibrils was also inhibited (Fig. 1E). The control peptide had no effect on fibronectin or collagen I deposition (Fig. 1G,H). pUR4 binds to fibronectin[25], and inhibits fibronectin deposition by interfering with the binding of fibronectin to matrix assembly sites on the cell surface[23]. pUR4 does not inhibit cell spreading or adhesion to collagen (unpublished data, 2008) or fibronectin[23] and does not bind to other ECM proteins, including collagen I, fibrinogen, and laminin (Supplemental Fig. I, see http://atvb.ahajournal.org). In addition, pUR4 does not inhibit fibronectin or collagen mRNA synthesis in SMCs (Supplemental Table 1, see http://atvb.ahajournal.org).

The pUR4 fibronectin inhibitor blocks vascular remodeling

Fibronectin is known to affect SMC growth, migration and differentiation in vitro[2,26-28]. 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[18,29]. 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). Following ligation of the carotid artery, pUR4 and the control peptide were embedded in pluronic gel, and applied periadventitially. Supplemental Fig. II demonstrates that the pUR4 peptide can be readily detected in the vessel wall in both the media and adventitia 1-3 days following 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[29,30], FVB mice exhibited significant IMT 14 days after ligation (compare sham vs. ligated, Fig. 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 (Fig. 2A). Morphometry of the carotid compartment was performed 7 and 14 days post ligation (Fig. 2B-E). Lumen volume was significantly increased after ligation in control peptide treated mice in comparison to shams (Fig. 2B), consistent with our published data in FVB mice[29]. This increase was prevented by pUR4 treatment. There was a dramatic effect of pUR4 on vascular wall remodeling (Fig. 2C-E). Ligation of the carotid artery resulted in a 3 fold increase in intima-media volume at 14 days (Fig. 2C). The pUR4 peptide reduced the extent of IMT by 40% in comparison with control peptide treated animals at 14 days (Fig. 2C). When vessel compartments were analyzed separately (supplemental Fig. III, see http://atvb.ahajournal.org), pUR4 was found to reduce intima thickening by 63%, and media

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thickening by 27%. Interestingly, there was no statistical effect of pUR4 on IMT at 7 days. The reduction of IMT by pUR4 at 14 days was due to prevention of both intimal and medial thickening compared to 7 day changes in control peptide treated animals (supplemental Fig. III). Similarly, pUR4 inhibited adventitial thickening by 38% compared to III-11C at 14 days (Fig. 2E). Finally, pUR4 treatment prevented outward remodeling over the time course, as there was no difference in EEL volume between pUR4 and shams 7 days post ligation (Fig. 2D). However, the remodeling index was not different between pUR4 and III-11C (unpublished data, 2008). These data are the first to demonstrate that fibronectin is an important regulator of vascular remodeling in vivo.

The pUR4 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 post surgery in pUR4 treated animals in comparison with control animals (Fig. 3A). At 7 days post surgery, pUR4 totally prevented increased fibronectin deposition (Fig 3B,C). At 14 days post 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 upon 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 following 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 (Fig. 4). However, the pUR4 fibronectin inhibitor prevented SMC de-differentiation, as evidenced by the maintenance of SM α-actin and SM myosin heavy chain staining in the media (Fig. 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 post surgery (6.7×10^{-3} cells/mm^2 in ligated animals vs. 5.0×10^{-3} vs. cells/mm^2 in sham operated animals). pUR4 treatment prevented this increase in cell density by 75% (5.3×10^{-3} cells/mm^2). Further, ligation of the left carotid artery increased cell proliferation in the vessel wall as shown by PCNA staining (supplemental Fig. IV, see http://atvb.ahajournal.org); 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 (Fig. 5A,B). These data indicate that fibronectin promotes vascular remodeling, in part, by enhancing cell proliferation.

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The pUR4 fibronectin inhibitor dramatically reduces accumulation of inflammatory cells in the carotid artery

Inflammation plays a prominent role in vascular remodeling following 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 post surgery (Fig. 5C,D, and supplemental Fig. V, see http://atvb.ahajournal.org). Inhibition of fibronectin polymerization significantly decreased (80%) leukocyte infiltration into the intima, media, and adventitia (Fig. 5C,D). 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 (Fig. 6, Supplemental Fig. VI, see http://atvb.ahajournal.org). These data are the first to demonstrate that fibronectin plays a critical role in regulating the inflammatory response during vascular remodeling.

DISCUSSION

In this manuscript we show that periadventitial delivery the fibronectin inhibitor, pUR4, reduces IMT in response to reduced blood flow (Fig. 2). pUR4 treatment also reduced fibronectin and collagen I accumulation in the vessel wall (Fig. 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 (Fig. 5), reduced ICAM-1 and VCAM-1 levels (Fig. 6), inhibited cell proliferation (Fig. 5), and prevented SMC phenotypic modulation (Fig. 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 polymerization is an important regulator of cell growth, migration, and ECM deposition and stability[4,6,7]. 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[23]. pUR4 can inhibit the polymerization of both endogenously produced (Fig. 1) and exogenously supplied fibronectin into the ECM[23]. pUR4 did not decrease fibronectin or collagen I mRNA production in cultured SMC (Supplemental Data, Table I, see http://atvb.ahajournal.org). However, there was a trend towards decreased fibronectin and collagen I mRNA in the carotid artery of animals treated with pUR4 in comparison with control peptide treated animals (Supplemental Data, Table II, see http://atvb.ahajournal.org). Since 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[2, 26, 27]. Further, fibronectin matrix deposition regulates the deposition and stability of other ECM proteins[6,7,31,35]. Our data show that pUR4 causes a significant reduction in cell proliferation. Previous studies have shown that

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fibronectin polymerization can positively affect myofibroblast, SMC and endothelial cell growth[2,4,5]. Fibronectin polymerization can also promote myofibroblast and epithelial cell migration[7,33]. 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[36]. Our data show that fibronectin plays an important role in regulating the recruitment of leukocytes into the vessel wall (Fig. 5). Fibronectin fragments are known to be chemotactic for neutrophils and monocytes[21,37,38]. However, most in vitro data suggest that intact fibronectin does not promote leukocyte chemotaxis[21,37,38]. 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 κβ (NF–κβ) in certain cell types[39-41], which in turn stimulates ICAM-1 and VCAM-1 production[42]. Hence, the downregulation of ICAM-1 and VCAM-1 levels in pUR4 treated animals could result from decreased NF–κβ activity.

Fibronectin is also known to promote SMC de-differentiation[28,43]. 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 (Fig. 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 following ligation.

Other ECM molecules have been shown to play important roles in vascular remodeling, including thrombospondin I[44], vitronectin[45] and osteopontin[46]. Interesting, certain ECM and cytoskeletal proteins have been shown to influence both IMT and vessel size[47-49], 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 1 and collagen 1 into ECM fibrils[6, 7]. In addition, we previously showed that the ability of fibronectin to promote myofibroblast migration is due in large part to its ability to regulate the deposition of type I collagen[7]. 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 neotimal formation in vivo[50,51].

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 peradventitially and that the levels of the peptide peak 1-3 days post 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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Supplementary Material

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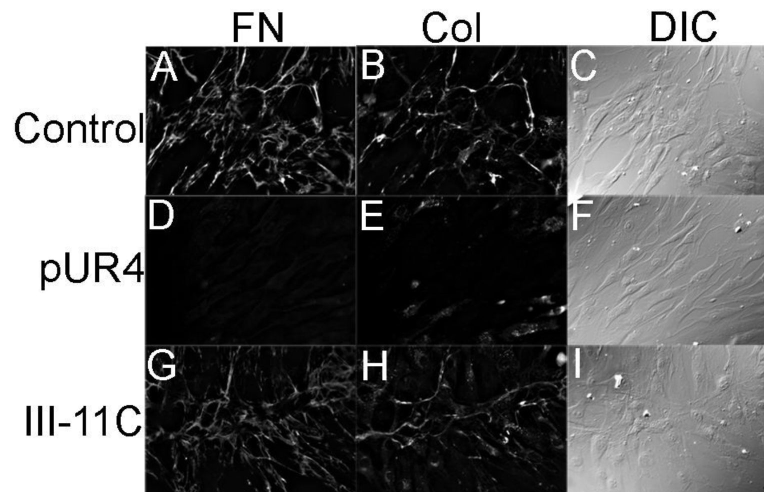

Figure 1.
Effect of pUR4 on FN and collagen I deposition in SMCs. SMCs were grown in serum containing media in the absence (A-C) or presence of 500nM pUR4 (D-F) or control III-11C (G-I) peptides for 3 days. Cell were fixed, then incubated with a polyclonal antibody to fibronectin (A,D,G) or collagen I (B,E,H). Corresponding differential interference contrast images are shown in C,F,I. Bar, 20μm.

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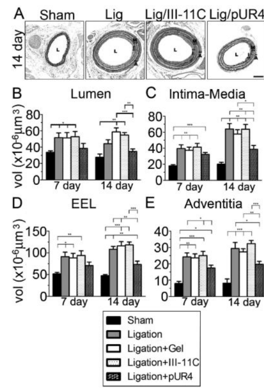

Figure 2.

The pUR4 fibronectin inhbitor decreases vascular remodeling in the carotid artery. (A) Representative photomicrographs of the left carotid artery 14 days after ligation. Lumen (L), intima (I), media (M) and adventitia (A) in ligated vessels are shown. Bar, 100μm. Morphometric analyses showing the volume (vol) of the lumen (B), intima-media (C), external elastic lamina compartment (EEL; D), and adventitia (E) 7 and 14 days after ligation. *indicates $p<0.05$, ** $p< 0.01$ and *** $p< 0.001$.

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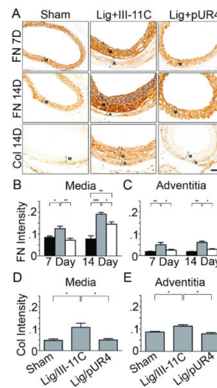

Figure 3.

The pUR4 fibronectin inhbitor decreases FN and collagen I deposition in the left carotid artery after ligation. (A) Immunohistochemistry of FN and collagen I 7 or 14 days post surgery. Intima (I), media (M), and adventitia (A) are shown. Quantitation of immunostaining intensity (relative unit per unit area) of FN (B,C) and collagen (D,E) in the media and adventitia 7 (fibronectin) and 14 days (fibronectin and collagen) after ligation. In panels B,C: sham (black box), ligation+III-11C (grey box), ligation+ pUR4 (open box). Bar, 25μm. *indicates $p<0.05$, ** $p< 0.01$ and *** $p< 0.001$.

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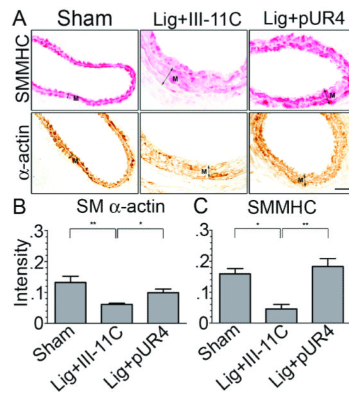

Figure 4.

The pUR4 fibronectin inhbitor 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, 25μm. *indicates $p<0.05$, ** $p<0.01$.

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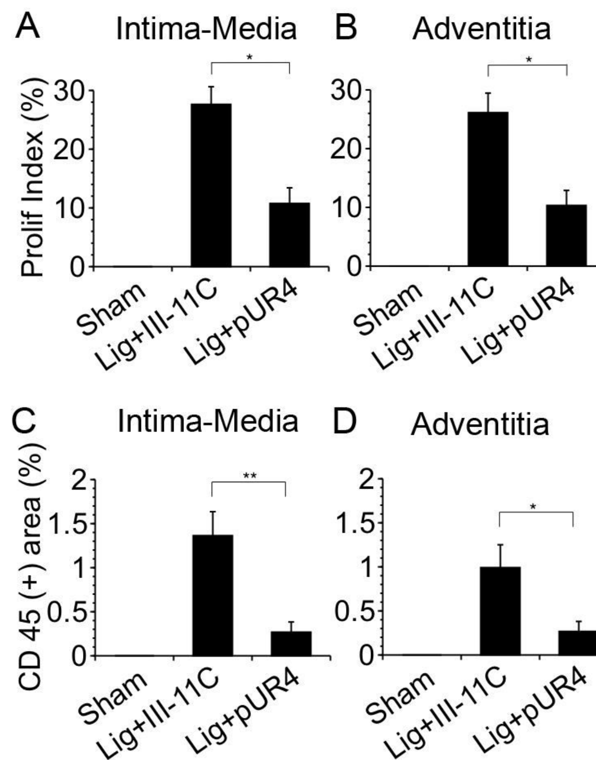

Figure 5.

A,B) The pUR4 fibronectin inhibitor decreases cell proliferation in the left carotid artery. Sections of the left carotid artery 7 days post ligation were stained for PCNA and counterstained with hematoxylin. Quantitative IHC analysis of the proliferation index (PCNA (+) cells per total cell number) in the intima-media (A) and adventitia (B) 7 days post ligation. C,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 hematoxylin. 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. *indicates $p<0.05$, **indicates $p< 0.01$.

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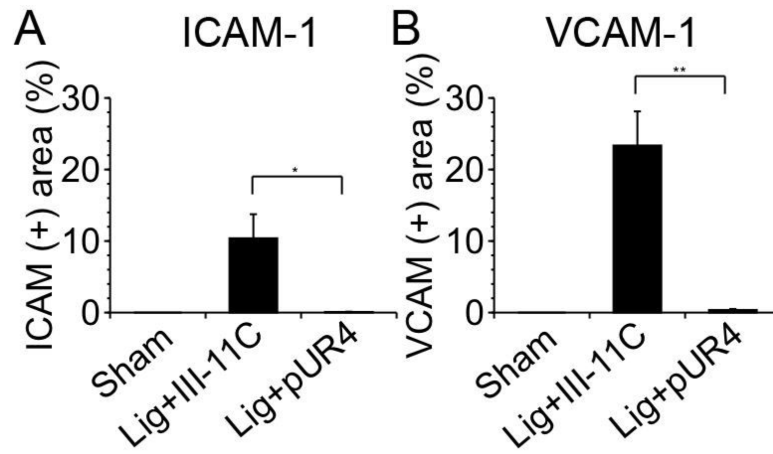

Figure 6.

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. *indicates $p<0.05$, **indicates $p< 0.01$.