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Perivascular delivery of Resolvin D1 inhibits neointimal hyperplasia in a rat model of arterial injury

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Abstract

Objective—Lipid mediators derived from omega-3 polyunsaturated fatty acids such as Resolvin D1 (RvD1) accelerate the resolution of inflammation, and have potential as vascular therapeutics. The objective of this study was to evaluate local perivascular delivery of RvD1 as a means to attenuate neointimal hyperplasia in a rat model of arterial injury.

Methods—Smooth muscle cells were harvested from rat aortas to study the effects of RvD1 on rat arterial vascular smooth muscle cell (RASMC) responses *in vitro*, with focus on inflammation, proliferation, migration, cytoskeletal changes and cytotoxicity. The safety and efficacy of perivascular delivery of RvD1 via thin biodegradable 3-layered PLGA wraps or 25% Pluronic F127 gels were studied in a rat model of carotid angioplasty. A total of 200 ng RvD1 was loaded into each construct for perivascular delivery after injury. Morphometric and histologic analyses were performed 3 and 14 days after injury.

Results—RvD1 attenuated RASMC inflammatory pathways, proliferation, migration and mitogen-induced cytoskeletal changes *in vitro*, without evidence of cytotoxicity. RvD1-loaded wraps reduced neointimal formation after carotid angioplasty by 59% versus no-wrap controls (P = .001) and by 45% versus vehicle-wrap controls (P = .002). RvD1-loaded pluronic gels similarly reduced neointimal formation by 49% versus no-gel controls (P = .02) and by 52% versus vehicle-gel controls (P = .02). No group was associated with infection, thrombosis or negative vessel remodeling. Wraps were found to be easier to apply than gel constructs. Ki67 proliferation index was significantly lower in RvD1-loaded wrap treated arteries compared to both no-wrap and vehicle-wrap controls at both 3 and 14 days post-injury (65% versus no-wrap group and 70% versus vehicle-wrap group at day 3, 49% versus both control groups at day 14, P < .05). Similarly, oxidative stress (30% and 29%, P < .05). and NF-kB activation (42% and 45%, p<.05) were significantly lower in the RvD1-loaded wrap group compared to both no-wrap and vehicle-wrap controls at three days post-injury

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Conclusions—Local perivascular delivery of RvD1 attenuates formation of neointimal hyperplasia without associated toxicity in a rat model of carotid angioplasty. This effect is likely due to attenuation of inflammatory pathways as well as decreased arterial smooth muscle cell proliferation and migration.

Introduction

Peripheral arterial disease affects 9–12 million Americans, causing considerable associated disability, suffering and burden on the healthcare system.[1,2] Each year, more than 500,000 open and endovascular peripheral interventions are performed in the United States, and the total national cost associated with peripheral vascular disease is approximately $21 billion/year.[3] Failure rates approach 50% or greater within two years depending on the specific anatomy treated and the modality employed, with a significantly higher rate of failure for endovascular interventions such as angioplasty and stenting than for open surgical interventions such as endarterectomy and bypass.[4,5] A final common pathway to failure is persistent inflammation leading to neointimal hyperplasia, which results in recurrent occlusive disease and need for additional procedures, incurring additional morbidity, mortality and costs.[6]

The termination of inflammation and restoration of homeostasis is of considerable interest in many disease states, including atherosclerosis and restenosis after vascular intervention. While resolution of inflammation was previously thought to be a passive decrescendo process, we now understand that resolution involves active counter-regulation by specific pro-resolving lipid mediators (SPMs) generated from omega-6 and omega-3 polyunsaturated fatty acids.[7] Several biochemical families of SPM have been described, including the E- and D-series resolvins derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) respectively. Recent work has demonstrated that SPMs have biologic activity relevant to vascular disease, with specific actions that attenuate inflammatory pathways and vascular smooth muscle cell phenotypic changes critical to the pathobiology of neointimal hyperplasia.[8–10] Previous studies have shown that intra-arterial or intra-peritoneal administration of SPMs attenuates neointimal hyperplasia in animal models of vascular injury;[11–13] however, these delivery methods are impractical for most clinical situations.

Local perivascular administration of bioactive compounds may allow for greater pharmacokinetic efficiency and relevance, particularly for surgical applications. Biomaterials such as ethylene vinyl acetate (EVA), polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) have been well described as drug delivery devices, with variable degradation and drug elution kinetics associated with each construct.[14–17] Amongst the available biomaterials, PLGA is particularly appealing as it is biodegradable and FDA approved for use as a drug-delivery device.[18] Another option for local perivascular delivery in animal models is Pluronic F127 gel, which has established utility in early proof-of-concept studies.[19] Unfortunately, previous attempts at local delivery of anti-inflammatory agents to vasculature beds have been limited by significant toxicity associated with the drugs themselves, which delay rather than accelerate healing.[20] Furthermore, the delivery of potent anti-proliferative agents to sites of vascular graft implantation has led to infection-related complications when advanced to clinical trials in humans.[21]

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SPMs have been demonstrated to orchestrate the process of resolution without associated toxicity while actually strengthening the immune system,[22–26] giving them distinct advantages over current anti-inflammatory agents. Here we report the safety and efficacy of local perivascular delivery of a well-described SPM, Resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid; RvD1), in a rat model of arterial injury. Our results provide further evidence for the vasculo-protective effects of these bioactive lipid mediators, and suggest they may be useful to modulate healing after vascular injury.

Methods

Rat Arterial Vascular Smooth Muscle Cells (RASMC)

RASMCs were isolated from uninjured control male Sprague-Dawley rats, as previously described.[27] Briefly, thoracic and abdominal aortas were harvested immediately after animals were euthanatized and perfused with heparinized saline. The vessels were incubated in collagenase for 15 minutes followed by mechanical denudation of the endothelium and stripping of the adventitia. The remaining tunica media was then minced and seeded into 12-well plates coated with 1% gelatin. Cells were initially grown in DMEM (low glucose; HyClone Laboratories, Logan UT) with 20% FBS (Invitrogen Life Technologies, Grand Island, NY) until passage 1, when this was exchanged for DMEM with 10% FBS. Cultures were confirmed to be >95% ASMC via staining for α-SM actin (1:200; Sigma, Aldrich, St. Louis, MO) with DAPI nuclear counterstaining (Southern Biotech, Birmingham, AL). Cells were used for experiments between passage 3 and 5.

NF-κB Activation in-vitro (p65 translocation)

RASMCs were seeded onto 8-well chamber slides at 15,000 cells/well in 10% DMEM. After adhering, cells underwent pretreatment with RvD1 (10 nM; Cayman, Ann Arbor, MI) or vehicle for 30 minutes, followed by addition of IL-1β (50 ng/ml; Sigma, Aldrich, St. Louis, MO) for 2.5 hours. At the end of the experiment, slides were washed in ice-cold PBS then fixed in 4% formaldehyde and treated with 0.5% Triton X-100 (Sigma, Aldrich, St. Louis, MO). Blocking with 2% BSA (Sigma, Aldrich, St. Louis, MO) in 0.3% Triton X-100 was performed prior to overnight incubation at 4°C with an anti-p65 antibody (1:50; Santa Cruz Biotechnology, Dallas, TX). An Alexa Fluor® 488 tagged secondary antibody (1:200; Life Technologies, Carlsbad, CA) was then used, followed by DAPI nuclear counter staining (Southern Biotech, Birmingham, AL). Images were taken with a fluorescence microscope and analyzed via ImageJ (NIH). NF-κB activation was quantified by the ratio of fluorescent intensity in the nucleus versus cytoplasm. At least 24 randomly selected cells were analyzed per well.

Cell Proliferation (Direct Cytometry)

RASMC were seeded onto 6-well plates at 33,000 cells per well. After a 72 hour period of serum starvation in 0.5% DMEM, cells were exposed to either new 0.5% DMEM (negative control) or 10% DMEM with either vehicle (positive control) or RvD1 (10nM). After a 48 hour exposure period, cells were counted manually using a Neubauer hemocytometer.

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Cytotoxicity (MTT Assay)

RASMCs were grown to confluence on 24-well plates in 10% media and exposed to various concentrations of RvD1 (0.01–100nM). Cytotoxicity was quantified at 24 hours using a standard MTT assay (Sigma, St Louis, Mo).

Cytoskeletal Changes

RASMCs were seeded onto 8-well chamber slides at 15,000 cell/well, then serum starved in 0.5% DMEM for 24 hours. Cells were then pretreated with RvD1 or vehicle for 2 hours, prior to treatment with PDGF-BB (50 ng/ml) for 1 hour. Cells were subsequently washed twice in PBS, permeabilized with 0.1% Triton X-100, fixed in 3.7% formaldehyde, labeled with Alexa Fluor® 568 phalloidin (Invitrogen, Grand Island, NY), and mounted with DAPI containing mounting medium (Southern Biotech, Birmingham, AL). Length:width ratios were determined using ImageJ analysis software (NIH). For each well, length:width ratios were measured for all cells within 5 randomly selected fields (≥67 cells per well).

Cell Migration

RASMCs were grown to confluence on 24-well plates. After an overnight period of serum starvation, a mechanical “scratch” wound was made across each well with a sterile 200 μL pipette tip. Detached cells were washed away with PBS and remaining cells were exposed to fresh 0.5% DMEM with either no agonist (negative control), PDGF-BB (10ng/mL; Sigma Aldrich, St. Louis, MO), ATII (1μM; Sigma Aldrich, St. Louis, MO) or Thrombin (1U/m; Enzyme Research Laboratories, South Bend, IN). RvD1 (10nM) or vehicle was added to appropriate wells just prior to treatment with agonist. Wounds were observed at baseline and at 24 hours and wound closure quantified via ImageJ (NIH).

Perivascular Delivery Constructs

Polymer thin films were spun-cast from solutions of PLGA (PolySciTech, West Lafayette, IN) in 2,2,2-trifluoroethanol (Sigma-Aldrich, St. Louis, MO). PLGA compositions of varying ratios of lactic acid:glycolic acid were used for each layer, and are reported as the ratio of lactic:glycolic acid (e.g. 75:15 PLGA). A total of 200 ng of RvD1 was loaded between layers of PLGA to create a tri-layered construct composed of 85:15 PLGA / 100 ng RvD1 / 75:15 PLGA / 100 ng RvD1 / 50:50 PLGA. The asymmetry of the PLGA layers facilitated unidirectional release towards the layer with a relatively lower concentration of lactic versus glycolic acid (50:50 PLGA). Vehicle-wraps were loaded with ethanol. Total thickness of each device was kept under 50 μm in order to maintain construct compliance. Both RvD1 and vehicle-wraps were sized at 8 × 12 mm to accommodate the rat carotid artery size after angioplasty. A second delivery approach utilized Pluronic F127 gel (Sigma-Aldrich, St. Louis, MO). A total of 200 ng RvD1 was dissolved into 100 μL of 25% Pluronic F127 gel for delivery to arteries after angioplasty, allowing for complete circumferential coverage of the injured artery. Vehicle-gels were loaded with ethanol. Initial elution studies were performed *in vitro* using a standard EIA (Cayman, Ann Arbor, MI). Wrap and gel constructs were placed into transwell inserts (Corning Inc, Corning, NY) over 24-well plates and underlying media analyzed for cumulative elution between 3 hours and 14 days.

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Rat Carotid Artery Angioplasty

Male Sprague-Dawley rats (400–500g) were used in compliance with an IACUC-approved protocol (UCSF #AN108115-01A). Arterial injury was produced by balloon angioplasty to the left common carotid artery, as previously described.[27,28] Specifically, we cannulated the left common carotid artery with a 2Fr balloon (Edwards Lifesciences, Irvine, CA) inserted through the external carotid artery and inflated the balloon to 5 atm for 1 minute using a calibrated balloon inflation device (Boston Scientific, Marlborough, MA). After angioplasty, the external carotid artery was ligated, and either no-wrap, a vehicle-wrap or an RvD1-wrap was applied around the common carotid at the site of angioplasty. Both vehicle-wraps and RvD1-loaded wraps were oriented with the 50:50 PLGA side facing “inwards” towards the target vessel. The contralateral carotids were used for negative (uninjured) controls. Additional groups underwent perivascular application of 100 μL of vehicle-gel or RvD1-gel after angioplasty. Gels were kept on ice until perivascular application after angioplasty and subsequently allowed to solidify at body temperature, as previously described.[19] Rats were euthanized at either 3 or 14 days after angioplasty and specimens harvested and immediately frozen after perfusion with heparinized saline (for 3 day analyses) or fixed after perfusion with heparinized saline followed by 4% formaldehyde (for 14 day analyses).

Morphometric analysis

After fixation in 4% formaldehyde and processing in 70% ethanol, specimens were paraffin-embedded and 6 μm sections were taken throughout the zone of injury. Sections were stained with an Elastin Kit (Thermo Fisher Scientific, Waltham, MA) and analysis performed using ImageJ (NIH). Three sections within the zone of injury were analyzed for each specimen. Standard morphometric measurements were recorded including luminal area, neointimal area (area inside the internal elastic lamina minus luminal area) and medial area (area inside the external elastic lamina minus area inside the internal elastic lamina). Vessels were harvested within the surgical plane and periadventitial area was also calculated as all tissue outside of the external elastic lamina.

Immunohistochemistry

Frozen sections (3 day analyses) or paraffin sections (14 day analyses) were processed and 6μm sections were taken throughout the zone of injury. Frozen sections were fixed in either 4% formaldehyde or acetone for 10 minutes prior to staining. Paraffin sections were first deparaffinized in xylene then rehydrated prior to antigen retrieval with a sodium citrate buffer (Sigma, Aldrich, St. Louis, MO) using a commercial microwave to heat to achieve >95°C for 20 minutes. Sections were then permeabilized with 0.2% Tween 20 (Sigma, Aldrich, St. Louis, MO) and blocked in 5% goat serum (Sigma, Aldrich, St. Louis, MO). Blocking of endogenous Avidin/Biotin was performed with a commercial kit (Vector, Burlingame, CA) prior to incubation with one of the following primary antibodies: rabbit-anti-Ki67 (1:50; Abcam, Cambridge, MA), rabbit-anti-phospho-p65 (1:100; Abcam, Cambridge, MA), rabbit-anti-CD45 (1:150; Abcam, Cambridge, MA) or rabbit-anti-α-SM actin (1:200; Sigma, Aldrich, St. Louis, MO). This was followed by incubation with a biotin-goat-anti-rabbit secondary (1:200; BioLegends, San Diego, CA) then a FITC-conjugated streptavidin (1:200; Vector, Burlingame, CA). Sections were then mounted in a

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DAPI mounting solution (Southern Biotech, Birmingham, AL). Additional double staining for Ki67 and α-SM actin was performed to further investigate cellular kinetics. Staining for apoptosis (TUNEL; Sigma, Aldrich, St. Louis, MO) and reactive oxygen species (DHE; Life Technologies, Carlsbad, CA) were performed using commercially available kits as per manufacturer protocol. Three sections within the zone of injury were analyzed for each specimen for each histologic target.

Statistical analysis

Initial comparisons between groups were performed using ANOVA on all data sets. Non-paired, two-tailed Student t-tests were used for individual group comparisons, with Bonferroni correction for multiple comparisons.

Results

RvD1 attenuates p65 nuclear translocation (NF-κB Activation) in RASMC

Nuclear translocation of the transcription factor p65 (Rel-A) is an important step in activation of the NF-κB inflammatory pathway. We induced p65 translocation in rat arterial smooth muscle cells with IL-1β (50 ng/mL) and quantified the nuclear to cytoplasmic ratio of p65 via immunohistochemistry. We observed that RvD1 (10 nM) inhibited p65 translocation in RASMC by 41% ($P < .01$) (Fig 1A–E).

RvD1 inhibits RASMC proliferation, without associated cytotoxicity

Rat arterial smooth muscle cell kinetics were studied in response to 10% serum with or without RvD1. Proliferation was quantified by direct cell counts and cytotoxicity through a standard MTT assay. RvD1 (10nM) inhibited RASMC proliferation at 48 hours by 30% ($P = .04$) after stimulation with 10% serum (Fig 2A). We encountered no evidence of cytotoxicity in RASMC across a range of RvD1 (0.01–100nM) using an MTT assay, with analysis performed between 24–96 hours (Fig 2B).

RvD1 inhibits PDGF-induced cytoskeletal changes in RASMC and impairs migration in response to various motogens

PDGF (50 ng/mL) induces motogenic changes in the RASMC cytoskeleton, including an increase in the length:width ratio. We found that these changes are attenuated by RvD1 (10 nM), whereas RvD1 alone does not appear to affect the cytoskeleton in the absence of an agonist (Fig 3A–E). A scratch injury model was used to evaluate RASMC migration, with three well-described motogens (PDGF-BB 10ng/mL, ATII 1nM and thrombin 1 U/mL) used to stimulate migration after injury. We found that RvD1 (10nM) inhibited migration response to PDGF-BB by 35% ($p<0.001$), to ATII by 57% ($P = .01$) and to thrombin by 48% ($P = .02$) (Fig 3F).

Local perivascular delivery of RvD1 in a rat model of carotid angioplasty

A rat model of carotid angioplasty was utilized to study the effect of local perivascular delivery of RvD1 after arterial injury. Drug delivery was achieved after carotid angioplasty through a thin biodegradable 3-layered PLGA wrap (Supp Fig 1A–B) or 100 μL of 25%

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Pluronic F127 gel, with total 200 ng of RvD1 loaded into each construct. *In vitro* elution studies demonstrated that the pluronic gel construct provided more rapid elution than the wrap construct; however, the total amount released from each system was similar during the intervals measured (13709 pg and 14957 pg, respectively) (Supp Fig 1C). A total of 76 rats were utilized for this study, divided into five different groups: angioplasty only, angioplasty with vehicle-wrap, angioplasty with RvD1-wrap, angioplasty with vehicle-gel and angioplasty with RvD1-gel. There was no correlation between wraps, gels or RvD1 with mortality (2.6% overall) or thrombosis (7.9% overall). No infections were observed.

Local perivascular delivery of RvD1 after arterial injury inhibits proliferation without inducing cytotoxicity

Proliferation was quantified by the Ki67 index (total number of Ki67 positive cells inside of the EEL divided by total number of nucleated cells inside of the EEL) at both 3 and 14 days after angioplasty. Perivascular application of RvD1-loaded wraps decreased the proliferation index by 65% compared to no-wrap controls and 70% compared to vehicle-wrap controls at day 3 after injury ($n=3$, $P < .05$ for both comparisons). This effect was still present at day 14 after injury, at which time the proliferation index was 49% lower for the RvD1-wrap group than both the no-wrap group and the vehicle-wrap group ($n=5$, $P < .05$ for both comparisons) (Fig 4). The majority (>95%) of Ki-67 positive nuclei were within smooth muscle actin-positive cells by double immunostaining (not shown). Apoptosis was evaluated at day 3 after injury via TUNEL staining using sections immediately adjacent to those analyzed for proliferation. While angioplasty induced an increase in the apoptosis index above uninjured controls, no difference was observed in apoptotic index across the treatment groups (ANOVA $P = .30$) (Supp Fig 2A–G).

Local perivascular delivery of RvD1 after arterial injury attenuates oxidative stress and p65 nuclear translocation (NF-κB activation)

Dihydroethidium (DHE) staining was utilized to quantify oxidative stress within the vessel wall after arterial injury. DHE index was calculated by normalizing TRITC fluorescence inside the EEL to DAPI fluorescence inside the EEL for each specimen, with values subsequently normalized to uninjured vessels. Arteries treated with RvD1-wraps demonstrated a 30% decrease in DHE index versus the no-wrap group and a 29% decreased versus vehicle-wrap group ($n=3$, $P = .02$ for both comparisons) (Fig 5A–E). Staining for the activated form of p65 (phospho-S536) was also performed, as a marker for NF-κB activation. Arteries treated with RvD1-wraps demonstrated a 42% decrease in NF-κB activation versus the no-wrap group and a 45% decreased versus vehicle-wrap group ($n=3$, $P < .02$ for both comparisons) (Fig 5F–J). CD45 staining showed no difference in inflammatory cell recruitment within the vessel wall amongst the three injured groups at 3 days after angioplasty (Supp Fig 2H–L).

Local perivascular delivery of RvD1 after arterial injury attenuates neointimal hyperplasia

Morphometric analysis of arteries fixed 14 days after injury (Fig 6A–E) demonstrated decreased neointimal formation from perivascular delivery of RvD1 through both the wrap and gel constructs. Alpha smooth muscle cell actin and DAPI staining were utilized to further characterize the neointimal lesions (Fig 6F). The cellular composition of the

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neointima appeared similar for all groups. Perivascular applications of RvD1-wraps significantly decreased neointimal formation when compared to both the vehicle-wrap (0.073 mm^2 vs 0.132 mm^2, P = .002) and the no-wrap groups (0.073 mm^2 vs 0.176 mm^2, P = .001). These reductions in neointimal formation persisted after normalization for medial area (NI/M ratio; P = .0002 for RvD1-wrap vs vehicle-wrap group and P = .001 for RvD1-wrap vs no-wrap group). There was a trend towards less neointimal formation for the vehicle-wrap group versus the no-wrap group, however this did not reach statistical significance for either neointimal area or NI/M ratio (P = .13 and P = .12, respectively). Similar to the RvD1-wrap group, RvD1-gels significantly decreased neointimal formation when compared to both the vehicle-gel (0.090 mm^2 vs 0.189 mm^2, P = .02) and the no-gel groups (0.090 mm^2 vs 0.176 mm^2, P = .02). These reductions in neointimal formation persisted after normalization for medial area (NI/M ratio; P = .01 for RvD1-gel vs vehicle-gel group and P = .01 for RvD1-gel vs no-gel group). There was no significant difference between the RvD1-wrap group and the RvD1-gel group in terms of neointimal area or NI/M ratio (P = .43 and P = .57, respectively) (Fig 6G–I).

Further morphometric analysis demonstrated no evidence of negative remodeling associated with perivascular application of vehicle- or RvD1-wraps, or vehicle- or RvD1-gels (Supp Fig 3A). There did appear to be a more pronounced perivascular response with both the vehicle-wrap and the RvD1-wrap groups compared to the no-wrap group, however this only reached statistical significance for comparison between the vehicle-wrap and no-wrap groups (P = .002). A trend towards decreased perivascular response in the RvD1-wrap group compared to the vehicle-wrap group was observed, however this did not reach statistical significance (P = .07). Perivascular application of pluronic gels after angioplasty produced a similar perivascular response at 14 days post-injury as angioplasty alone (Supp Fig 3B).

Discussion

Neointimal hyperplasia results in clinical restenosis and is the predominant etiology of mid-term failure after vascular interventions. The lack of effective adjuvant therapies to address this pathology remains a critical limitation for all types of peripheral vascular reconstructions.[29,30] In particular, there are no clinically applicable therapies to prevent restenosis in surgical settings such as bypass grafting. Vascular biologists and surgeons have previously investigated a spectrum of pharmacologic and biologic approaches to address the pathobiology of neointimal hyperplasia.[5,6,14–17,19–21,27–31] These previous attempts have been limited by a redundancy of molecular targets, suboptimal delivery systems,[5,31] as well as inherent toxicities related to the drugs themselves.[20,21] The ideal therapeutic agent to prevent restenosis would accelerate vessel healing, maintaining luminal caliber without associated cytotoxicity. The ideal delivery system would provide adequate pharmacokinetics of the therapeutic agent in the target cells, with minimal adverse effects on the vessel and surrounding tissues.

In this study, we demonstrate that RvD1 has direct effects on RASMC, consistent with those of a candidate anti-restenosis agent. Inhibition of p65 nuclear translocation is of particular importance in that it is implicated not only in inflammatory signaling, but also in vascular smooth muscle cell proliferation, migration and oxidative signaling pathways.[10,33–35]

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Importantly, no cytotoxicity was observed across a wide range of RvD1 concentrations (0.01–100 nM) *in vitro*. Similarly, we did not encounter evidence of clinical toxicity or apoptosis associated with perivascular delivery of RvD1 in an *in vivo* model of arterial injury. Specialized proresolving mediators (SPMs) such as RvD1 may thus offer an advantage over previous agents targeting neointimal hyperplasia by promoting homeostasis without cytotoxicity.[7–13]

Local perivascular delivery of RvD1 was effective at reducing neointimal hyperplasia in a rat model of balloon angioplasty, employing either a thin film PLGA device or a simple pluronic gel for drug delivery. The RvD1-eluting PLGA wraps afforded a 45% reduction in neointimal area versus vehicle-wrap controls and a 59% reduction in neointimal area versus no-wrap controls. Similar results were found with our RvD1-gels, which afforded a 52% reduction in neointimal area versus vehicle-gel controls and a 49% reduction in neointimal area versus no-gel controls. Thus, we consistently observed that perivascular delivery of RvD1 attenuated neointimal hyperplasia by approximately 50% after rat carotid angioplasty. Importantly, perivascular delivery of RvD1 using either of these delivery systems (PLGA wrap or pluronic gel) was not associated with thrombosis, infection or death in this model.

While we observed a similar reduction in neointimal hyperplasia with our RvD1-wrap and RvD1-gel, we believe the thin-film perivascular wrap to be the more clinically relevant construct for several reasons. First, although the required pharmacokinetics for RvD1 in this context are unknown, the cellular kinetics of neointimal hyperplasia are more prolonged in larger animal species and humans. Thus, the rationale for the PLGA device was to provide a platform for sustained elution lasting beyond one week, in contrast to pluronic gel which completely dissolves within 1–3 days. Further studies are needed to determine the temporal requirements for RvD1 delivery in clinical settings of vascular injury. From a practical standpoint, pluronic gel is temperature sensitive and difficult to apply in a controlled, consistent fashion to achieve uniform product dosing to the vessel. Thus, while these gels may be useful for external applications,[32] they are less ideal for intracorporeal application. In contrast, the thin film PLGA construct is easy to handle surgically and facilitates sustained drug-release over at least 14 days in-vitro. In-vivo pharmacokinetic studies are technically challenging in these small animal models, but will be required in subsequent stages of development of this approach.

PLGA is an attractive option as a drug delivery device as it is both biodegradable and biocompatible, and is FDA approved for use as a drug delivery device in vascular settings.[18,21] A previous report has suggested that PLGA is associated with an increased risk of arterial thrombosis in a rat model of carotid angioplasty,[17] however this result is to be interpreted cautiously given its small sample size (n=4). In addition, the high rate of thrombosis seen with the rapamycin-loaded PLGA-construct used might have been more strongly related to inherent cytotoxicity associated with the drug rather than to the biomaterial. As previously noted, neither the vehicle-loaded PLGA device nor the RvD1-loaded PLGA device was associated with thrombosis in this study. Enthusiasm for PLGA as a vascular drug-delivery device has also previously been tempered by increased rates of infection seen with a paclitaxel-eluting mesh in an earlier clinical study.[21] However, these complications might be more likely attributed to the immune-suppressing drug released

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rather than the PLGA delivery device itself. As previously noted, no incidences of infection were encountered in this study.

A few limitation of our study warrant discussion. First, the precise mechanism for the observed *in-vivo* effects of RvD1 in this model remains uncertain. We observed reduced proliferation, oxidative stress and NF-κB activation in treated vessels. However, in contrast to prior studies in mice and rabbits,[11,12] we were unable to demonstrate reduced leukocyte recruitment to injured arteries treated with perivascular RvD1 *in vivo*. We speculate this may in part reflect a modest peri-vascular response to the delivery devices, however more comprehensive kinetic studies of leukocyte subpopulations are beyond the scope of the present investigation. Similarly, quantification of cellular migration is difficult to ascertain *in vivo*, thus we are left to rely on *in vitro* studies to investigate the effects of RvD1 on RASMC migration responses.

We selected the rat model of carotid angioplasty for this proof-of-concept study because it is well described in the literature and relatively cost-effective. However, models of arterial injury might not be generalizable to restenosis seen in venous or synthetic grafts, such as those used for peripheral bypass and hemodialysis access. Furthermore, as with any animal model, applicability to human disease may be limited. For example, the absence of infectious complications in this study may be attributable to the robust immune system in rodents. Thus, this finding and others are taken with cautious optimism. Finally, although sustained drug release (via perivascular wraps) did not appear to confer additional therapeutic benefit over immediate drug release (via pluronic gels) in this model, we believe sustained release to be necessary in human applications. Future studies in larger animal models will be needed to test this hypothesis.

SPMs are endogenously derived bioactive lipids demonstrating pro-resolving activity in nanomolar concentrations. The organic synthesis of several SPM compounds has facilitated investigations on their pharmacologic properties.[7–13] These data, combined with prior reports, support further investigation of SPMs as candidate vascular therapeutics. While we chose RvD1 for this preliminary study, this choice was largely due to the availability of an RvD1-specific EIA to facilitate initial elution studies for the perivascular delivery constructs. Moving forward, other specific SPMs or combinations of SPMs might be explored. More studies are needed to optimize perivascular delivery of SPM, and various other models of vascular injury (i.e. venous and/or prosthetic bypass) will need to be explored prior to translation to clinical studies.

Conclusion

Perivascular delivery of RvD1 significantly attenuates neointimal hyperplasia in a rat model of carotid angioplasty. This effect was achieved without apparent toxicity. The short-term results observed in this study provide cautious optimism to evaluate perivascular SPM delivery in larger animal models of vascular injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Relevance

Failure of peripheral vascular interventions remains the single greatest challenge in management of vascular disease, with failure rates approaching 50% or greater within two years for many interventions. In contrast to the currently available cytotoxic drugs used on vascular devices that delay vessel healing, novel endogenous “pro-resolving” lipid mediators (such as RvD1) have potential to reduce neointimal hyperplasia by accelerating homeostasis. This study provides proof-of-concept for local perivascular delivery of “pro-resolving” agents to improve the healing response after acute vascular injury.

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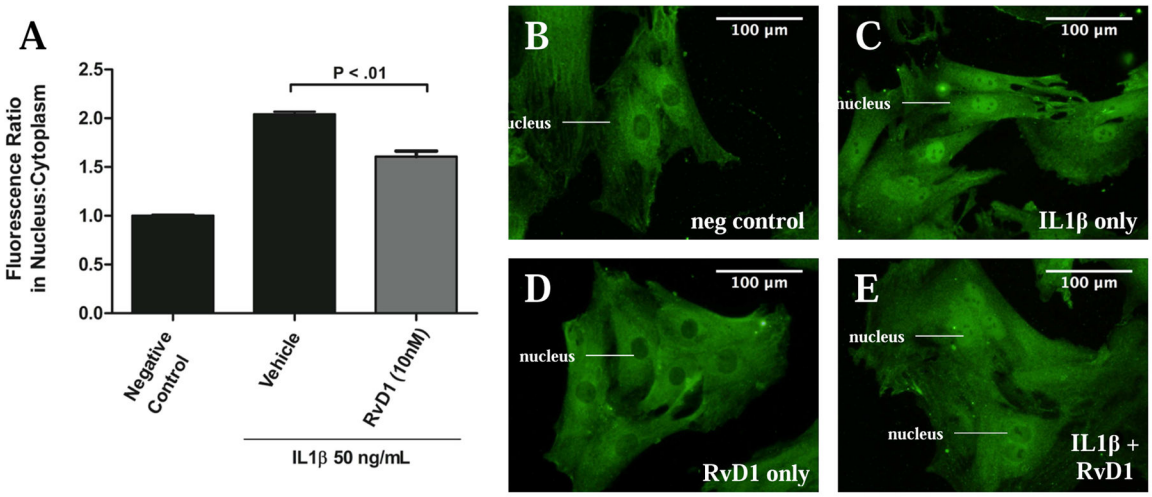

Fig. 1.

RvD1 attenuates p65 nuclear translocation (NF-κB Activation) in rat arterial vascular smooth muscle cells (RASMC). Nuclear translocation of the transcription factor p65 indicates activation of the NF-κB inflammatory pathway. IL1β (50 ng/mL) induces p65 translocation in RASMC, with maximum activation seen at 2.5 hours. RvD1 (10nM) attenuates this effect. Activation within each cell can be quantified after immunostaining for p65 by the ratio of mean fluorescence in the nucleus versus in the cytoplasm. Each group consisted of four wells (n=4) and quantification of the nuclear:cytoplasmic p65 fluorescence ratio was performed using 24 randomly selected cells for each well (**A**). Representative images are shown (**B–E**).

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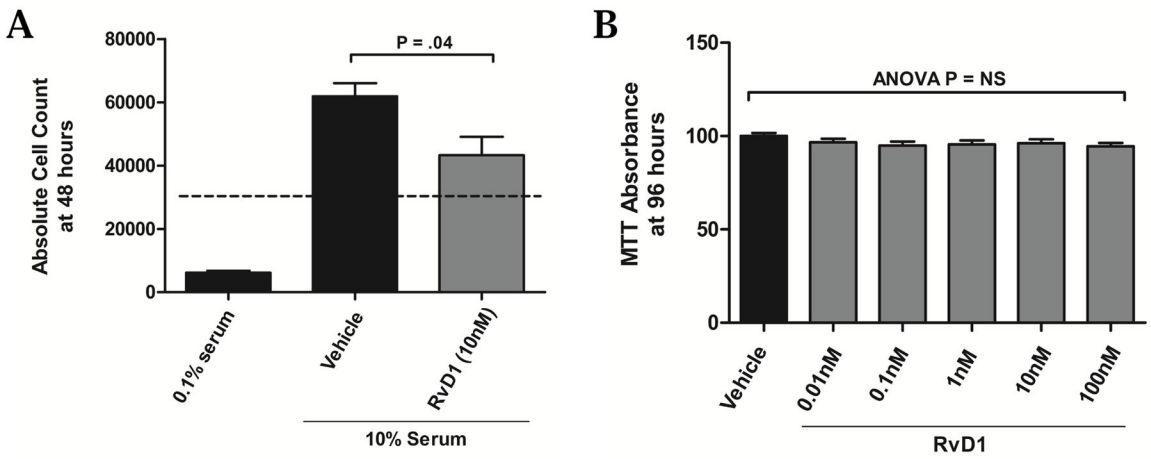

Fig. 2.

RvD1 inhibits RASMC proliferation, without associated cytotoxicity. Non-confluent cells were exposed to 10% serum with either vehicle (ethanol) or RvD1 (10nM) and proliferation quantified at 48 hours by direct cytometry (n=4). The dashed line represents the number of cells initially seeded per well (**A**). Confluent cells were exposed to 10% serum along with either vehicle or a range of RvD1 concentrations (0.01–100nM) and cytotoxicity quantified at both 24 and 96 hours using an MTT assay (n=4) (**B**).

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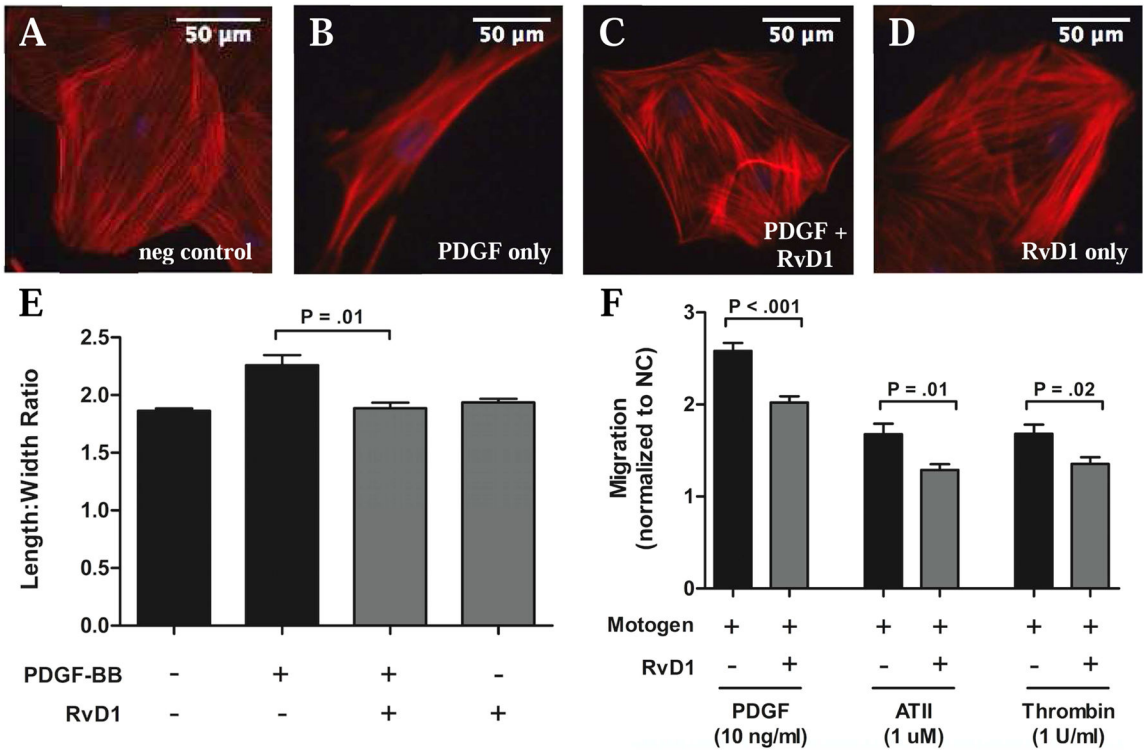

Fig. 3.

RvD1 inhibits PDGF-induced cytoskeletal changes in RASMC and impairs migration in response to various motogens. PDGF (50 ng/mL) induces motogenic changes in the RASMC cytoskeleton, including an increase in the length:width ratio (**A**, **B**). These changes are attenuated by RvD1 (10 nM) (**C**). RvD1 alone does not appear to affect the cytoskeleton in the absence of an agonist (**D**). Each group consisted of four wells (n=4) and quantification of the length:width ratio was performed within 5 randomly selected 20x fields for each well (**E**). RASMC migration was studied in response to PDGF (10 ng/mL), angiotensin II (1 uM) and thrombin (1 U/mL) using a scratch wound assay. Cells in each experiment were exposed to either no motogen (negative control), motogen only or motogen with RvD1 (10 nM). Wound closure was quantified at 24 hours and normalized to the negative control (NC) group for each experiment (n=7–8) (**F**).

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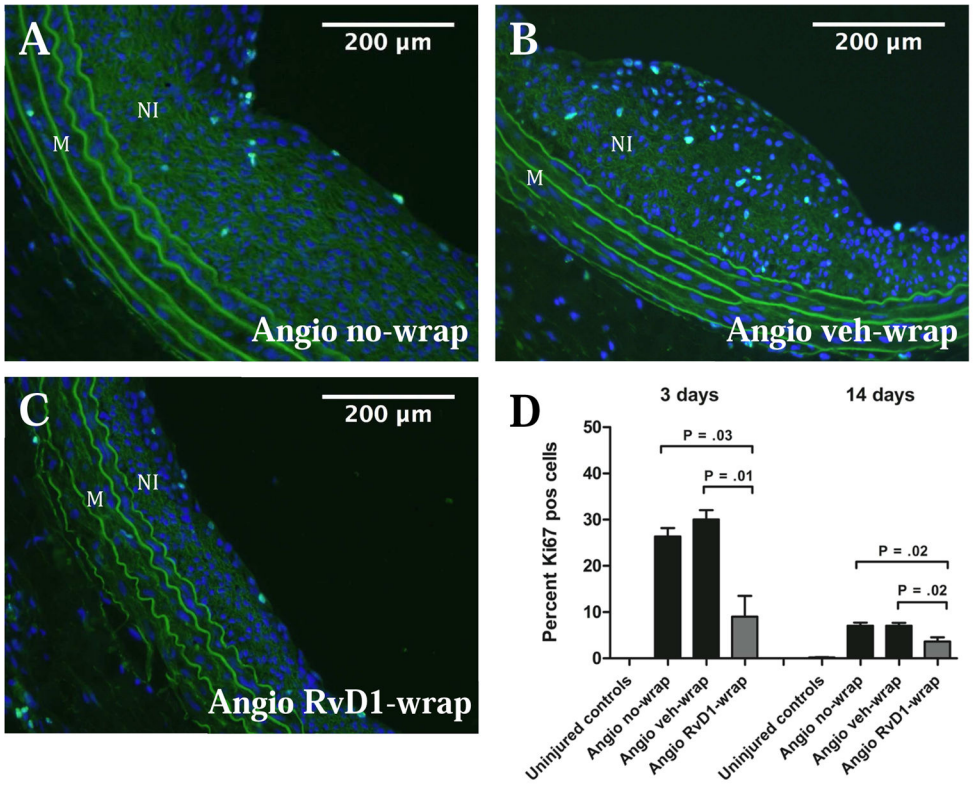

Fig. 4.

Local perivascular delivery of RvD1 after arterial injury inhibits proliferation. Unilateral balloon angioplasty was performed on rat carotid arteries, followed by perivascular application of no-wrap, vehicle-wrap or RvD1-wraps. The arteries were harvested at either 3 or 14 days post-injury and stained for Ki67 to detect proliferating cells. Nuclear staining for DAPI was also performed in order to calculate the percentage of proliferating cells per section. Three arteries per group (n=3) were used for the day 3 time point and 5 arteries per group (n=5) for the day 14 time point. Representative images from the day 14 staining are provided (NI = neointima, M = media) (**A–C**). Quantification of the percentage of Ki67 positive cells was performed using three sections along the zone of injury for each artery (**D**).

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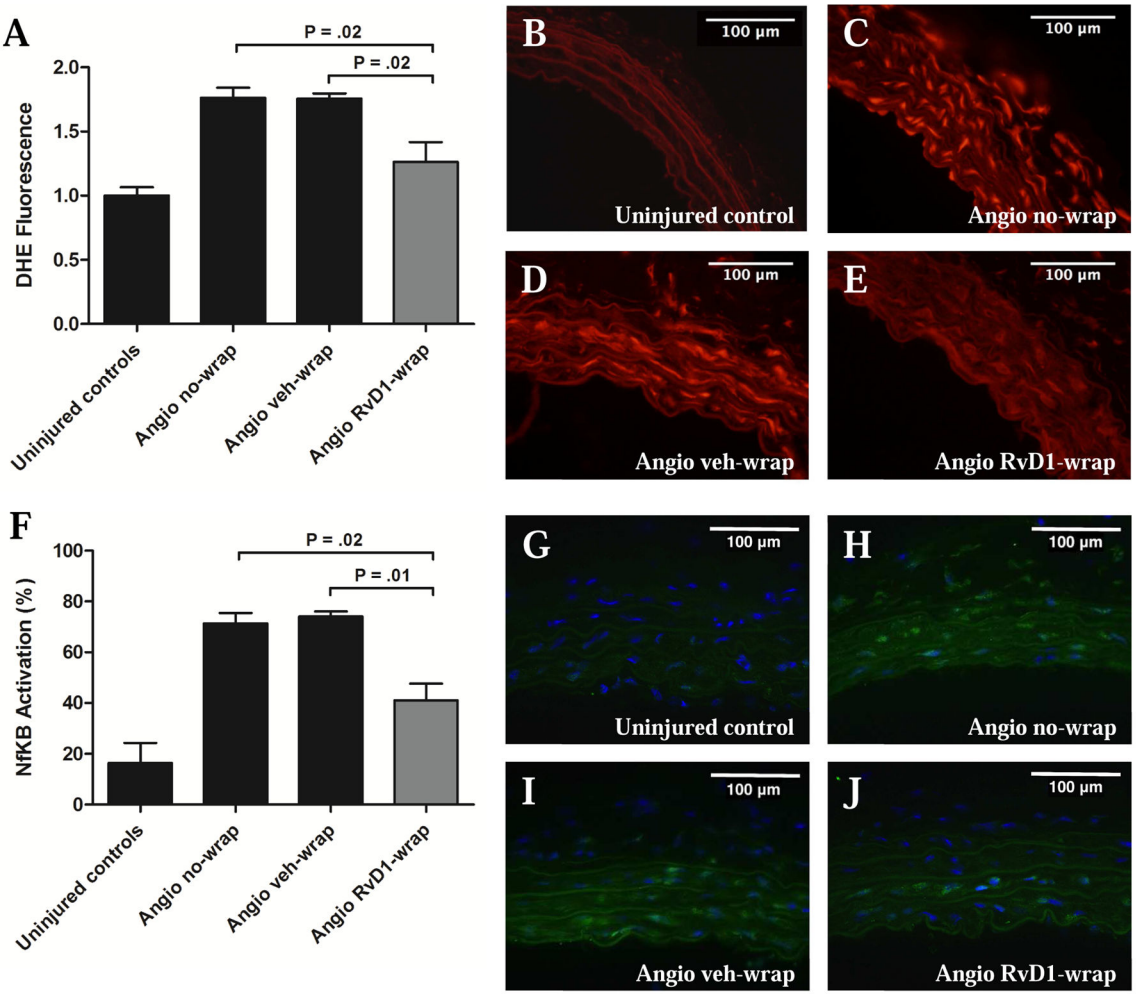

Fig. 5.

Local perivascular delivery of RvD1 after arterial injury attenuates oxidative stress and decreases activation of the NF-κB inflammatory pathway. Unilateral balloon angioplasty was performed on rat carotid arteries, followed by perivascular application of no-wrap, vehicle-wrap or RvD1-wraps. The arteries were harvested at 3 days post-injury and stained for DHE as a marker for oxidative stress, followed by nuclear counterstaining with DAPI. DHE fluorescence was normalized to the DAPI signal within each arterial wall. Each group consisted of three arteries (n=3) and quantification of DHE fluorescence was performed using three sections along the zone of injury for each artery (**A**). Representative images are shown (**B–E**). Staining for the active form of p65 (phosphor-S536) was performed using sections immediately adjacent to those used for DHE staining, as an indicator of NF-κB activation within the artery wall. Nuclear staining for DAPI was also performed in order to calculate the percentage of NF-κB active cells per section (**F**). Representative images for each group are shown (**G–J**).

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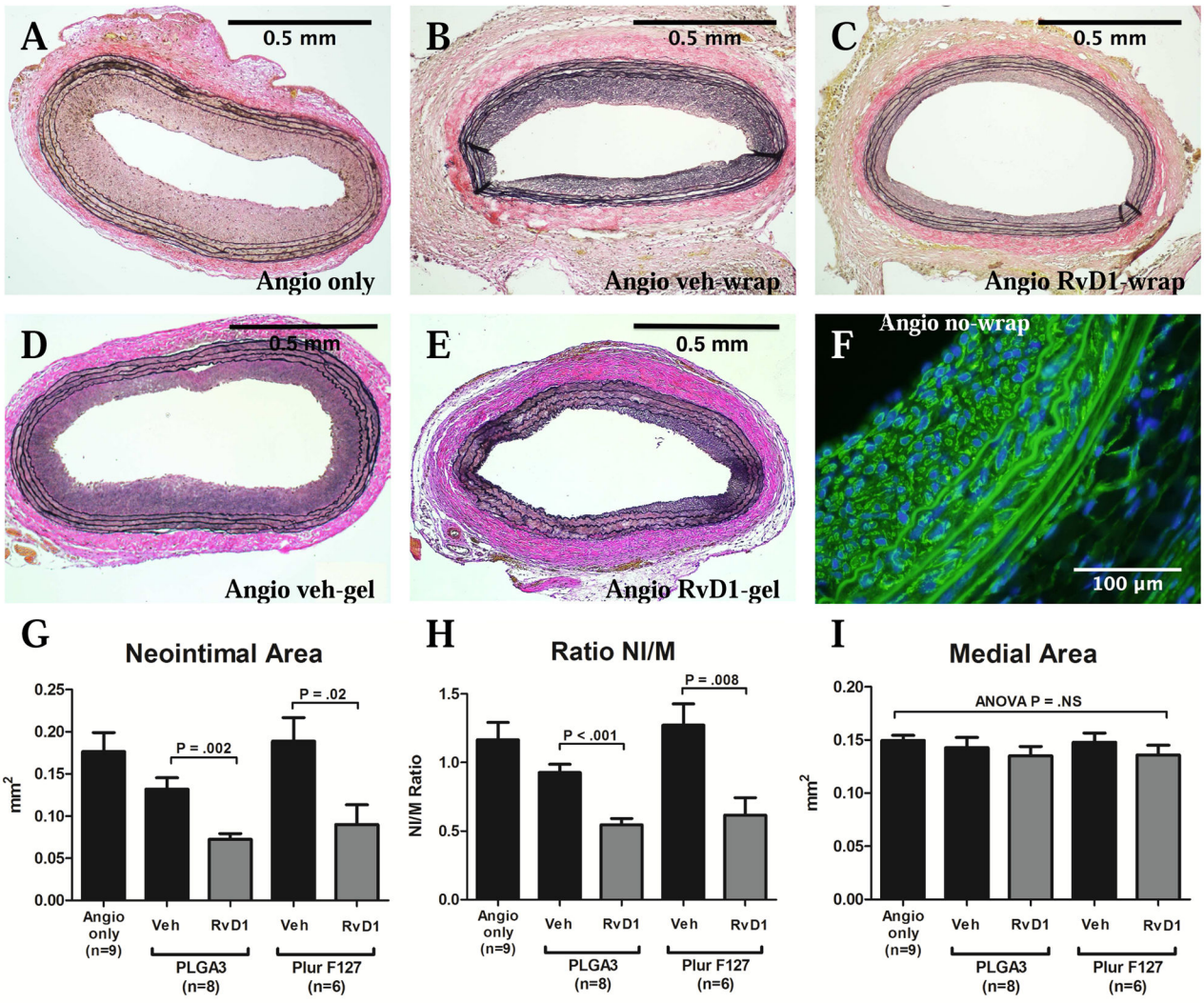

Fig. 6.

Local perivascular delivery of RvD1 after arterial injury attenuates neointimal hyperplasia. Unilateral balloon angioplasty was performed on rat carotid arteries, with or without subsequent perivascular application of a thin 3-layered PLGA wrap or 100 uL of 25% Pluronic F127 gel. Treatment groups consisted of no perivascular application (angio-only, n=9) or perivascular application of either vehicle-wraps (n=8), RvD1-wraps (n=8), vehicle-gels (n=6) or RvD1-gels (n=6). A total of 200 ng RvD1 was delivered for each drug-loaded group (wrap or gel). The arteries were harvested at 14 days post-injury and elastin staining performed to facilitate morphometric analysis (**A–E**). Further characterization of the neointima was performed by staining for α-smooth muscle actin (green), followed by nuclear counterstaining with DAPI (blue) (**F**). The cellular composition of the neointima appeared similar for all groups. Quantification of the neointimal and medial areas and the neointimal/medial ratio (NI/M ratio) was performed using three sections along the zone of injury for each artery (**G–I**). Both the RvD1-wrap treatment group and the RvD1-gel treatment groups demonstrated decreased neointimal formation versus their respective vehicle controls, as well as versus the angioplasty only control group (**G**). These findings

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persisted after normalizing for medial area (**H**). There was not a statically significant difference in neointimal response between the two treatment groups (RvD1-wrap and RvD1-gel) either in terms of neointimal area or NI/M ratio. The medial areas were similar for all groups (**I**).