Perivascular Delivery of Blebbistatin Reduces Neointimal Hyperplasia after Carotid Injury in the Mouse

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ABSTRACT

Proliferation and migration of smooth muscle cells (SMC) require myosin II activity; thus, we examined whether blebbistatin, a cell-permeable selective inhibitor of myosin II ATP activity, would impair neointimal hyperplasia after vascular injury. Delivery of blebbistatin via a perivascular polymer cuff reduced neointimal formation by 73% and luminal obstruction by 75% after carotid denudation injury in C57BL/6 mice. Blebbistatin treatment was also associated with a reduction in cell density within the neointima; total number of cells (76 ± 7 to 27 ± 3 cells/high-powered field) and actin-positive cells (64 ± 4 to 24 ± 2 cells/high-powered field) in the neointima were reduced in blebbistatin-treated mice compared with vehicle-treated mice. In a model of vascular injury with an intact endothelium, implantation of a blebbistatin-secreting cuff after carotid liga-

tion in FVB/N mice was associated with a 61% decrease in neointimal area and a significant decrease in luminal obstruction ($88 \pm 4\%$ in vehicle-treated mice versus $36 \pm 4\%$ in blebbistatin-treated mice; $p < 0.0001$). In cultured rat aortic SMC, blebbistatin disrupted cellular morphology and actin cytoskeleton structure, and these effects were rapid and completely reversible. Blebbistatin had a dose-dependent inhibitory effect on DNA replication and cell proliferative responses to platelet-derived growth factor-BB, angiotensin II, and α -thrombin, migratory responses to serum, and migratory responses after blunt injury. In summary, perivascular delivery of blebbistatin reduced neointimal hyperplasia after carotid injury in the mouse.

Introduction

Neointimal hyperplasia resulting from the proliferation and migration of vascular smooth muscle cells (SMC) contributes to restenosis after percutaneous coronary intervention, venous bypass graft disease, and atherosclerosis (Doran et al., 2008). Many studies have established that smooth muscle cells exhibit phenotypic changes during the process of vascular repair with alterations in cytoskeletal organization, composition, and distribution (Owens, 1995). These changes include distinct alterations in the expression of myosin heavy chain (MHC) isoforms, which is likely to directly influence smooth muscle contractility, migration, and proliferation (Gallagher et al., 2000). Three different types of MHC are expressed in SMC, including smooth muscle MHC and two forms of nonmuscle MHC (NMHC-A and NMHC-B) that are encoded by two distinct genes, *MYH9* and *MYH10*, respec-

tively. Two distinct isoforms of smooth muscle MHC are produced by alternative splicing at the 5' and 3' ends of the primary transcript.

Blebbistatin is a cell-permeable noncompetitive inhibitor of myosin heavy chain that binds in the large cleft in the motor domain and stabilizes the complex of myosin with ADP and inorganic phosphate that precedes the force-generating step when myosin rebinds to actin (Allingham et al., 2005). It is a specific inhibitor of ATPase and has no direct effect on myosin light chain (Straight et al., 2003). Blebbistatin disrupts directed cellular motility and cytokinesis in vertebrate cells and inhibits contraction of contractile ring assembly (Straight et al., 2003). Exposure to blebbistatin has profound effects on SMC *ex vivo*, including disruption of actin-myosin interactions (Wang et al., 2008), inhibition of contraction of cultured SMC (Katayama et al., 2006), inhibition of ATPase activity of smooth muscle myosin (Eddinger et al., 2007), inhibition of KCl-induced tonic contractions produced by rabbit femoral and renal arteries (Eddinger et al., 2007), and inhibition of chemotaxis of SMC toward sphingosylphosphorylcholine and platelet-derived growth factor-BB (PDGF-BB) (Wang et al., 2008).

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ABBREVIATIONS: SMC, smooth muscle cells; RASM, rat aortic SMCs; MePG, methoxypolyethylene glycol; MHC, myosin heavy chain; PCL, polycaprolactone; PDGF-BB, platelet-derived growth factor-BB; NM, nonmuscle; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; Ang II, angiotensin II; DMEM, Dulbecco's modified Eagle's medium.

Because myosin expression is differentially regulated after vascular injury and has been implicated in the control of important reparative processes such as smooth muscle cell proliferation (Simons and Rosenberg, 1992) and migration (Wang et al., 2008), we sought to test the hypothesis that inhibition of myosin activity would limit neointimal formation after vascular injury and thereby potentially represent a novel therapeutic option for preventing restenosis after revascularization.

Materials and Methods

Reagents and Assays. Reagents were obtained from the following sources: recombinant PDGF-BB (R&D Systems, Minneapolis, MN), α -thrombin (Hematologic Technology, Essex Junction, VT), and angiotensin II (Sigma-Aldrich, St. Louis, MO). Blebbistatin (Enzo Life Sciences, Plymouth Meeting, PA) was dissolved in dimethyl sulfoxide (DMSO), which was used as vehicle control for the *in vitro* studies. Rat aortic SMC (RASMC) isolated from the aortas of Sprague-Dawley rats were cultured, and proliferation, migration, cell adhesion, and Western Blotting assays were performed as described previously (Stouffer and Owens, 1994; Huang and Kontos, 2002). Histone-associated DNA fragmentation was analyzed in cell lysates by using the Cell Death Detection ELISA-PLUS kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions (Huang and Kontos, 2002).

For the *in vitro* wound healing assay, RASMC were grown to confluence in medium containing 10% FBS and then growth-arrested in 60-mm dishes. The cell monolayer was disrupted with a sterile rubber policeman to create a cell-free zone. Cells were washed with phosphate-buffered saline (PBS) and refreshed with medium containing 10% FBS $\pm$ blebbistatin at different concentrations. For the chamber cell-migration assay, a total of 5 to 10×10^4 cells in serum-free medium (300 μ l) $\pm$ blebbistatin were added to the upper chamber of a Boyden apparatus [8.0- μ m (pore size) polyethylene terephthalate membrane with Falcon cell-culture insert (BD Biosciences, San Jose, CA)], and 500 μ l of appropriate medium with 10% FBS was added to the lower chamber. Transwells were incubated for 4 to 6 h at 37°C. Cells on the inside of the transwell inserts were removed with a cotton swab, and cells on the underside of the insert were fixed and stained.

Carotid Injury Mouse Model. Male 8-week-old mice (C57BL/6 strain) were subjected to endothelial denudation carotid artery injury by repeated withdrawal of an epoxy resin probe slightly larger than the diameter of the carotid as described previously (Lindner et al., 1993). As an additional model of vascular injury, male 8-week-old mice (FVB/N strain) had suture ligation of the common carotid artery (Kumar and Lindner, 1997). All animals recovered and showed no symptoms of a stroke.

To ensure that the extent of vascular injury was similar between blebbistatin- and vehicle-treated groups, all mice underwent surgery on the same day by the same individual. Mice were randomly assigned to receive a perivascular cuff loaded with blebbistatin or vehicle at the time of surgery. The cuff was placed loosely around the injured carotid artery and was nonconstrictive. Blebbistatin-loaded polymer cuffs were made as described (Signore et al., 2001) with some modifications. Blebbistatin was first blended with methoxy-polyethylene glycol (MePG; MW 350). Polycaprolactone [PCL; -O(CH₂)₅CO-*n*] (Absorbable Polymers International, Cupertino, CA) was heated in a thermo-block for 20 min at 60°C in a 1.5-ml Eppendorf tube followed by adding the premixed MePG with blebbistatin. The blend of blebbistatin, PCL, and MePG was mixed well and incubated at 70°C (molten polymer) for 30 min. The PCL/MePG ratio was 4:1 (w/w). The concentration of blebbistatin in drug-loaded cuffs was 1% (w/w). Polymer cuffs made with MePG and PCL without blebbistatin were used in the control group.

In vitro release of blebbistatin was determined by placing polymer

cuffs ($n = 6$) in 15-ml tubes containing 5 ml of PBS. The tubes were incubated at 37°C with gentle rotating at 8 rpm. Supernatant was withdrawn every 24 h, and levels of blebbistatin were measured at a wavelength of 265 nm as described previously (Kolega, 2004). A standard curve of blebbistatin dissolved in PBS was made in the 0- to 100- μ M range and used to quantitate the amount of blebbistatin.

Histopathological Assessment and Quantification of Neointimal Hyperplasia. Animals were euthanized on day 21, and 10% buffered formalin was infused at physiological pressure. The carotid arteries were removed en bloc with adjacent tissues, immersed in formalin for 24 h, and then transferred to 70% ethanol. Arteries were embedded in paraffin, and serial sections (5 μ m thick) were cut at 100- μ m intervals from distal to proximal, starting 500 μ m proximal to the bifurcation. Slides were stained with Verhoeff van Gieson stain and projected onto a calibrated digitizing pad for computer-assisted morphometric analysis by a blinded observer. Three equally spaced cross-sections of the vessel in all mice were analyzed. Paraffin-embedded sections were stained with fluorescein isothiocyanate-conjugated anti- α -smooth muscle actin (Sigma-Aldrich), and nuclei were counterstained with 4,6-diamidino-2-phenylindole in mounting medium. Immunofluorescence was visualized under a fluorescence microscope at 400 $\times$ magnification. Fluorescein isothiocyanate-conjugated anti- α -smooth muscle actin-positive cells were counted in 10 separate sections from different vessels of each group.

Statistical Analysis. Data are expressed as the mean $\pm$ S.E.M. Statistical analysis was performed across multiple groups by using analysis of variance and confirmed between individual groups by using a two-tailed Student's *t* test. IC₅₀ values were determined by curve-fitting the data in a nonlinear regression analysis using ED50plus software developed by M. H. Vargas (<http://www.softlookup.com/display.asp?id=2972>; accessed August 2010). $P < 0.05$ was considered statistically significant.

Results

Local Delivery of Blebbistatin Decreased Medial Cell Number and Neointimal Hyperplasia after Denudation Carotid Artery Injury. We used two different mouse carotid injury models to examine the effects of local delivery of blebbistatin on neointimal hyperplasia after injury (Lindner et al., 1993; Kumar and Lindner, 1997). Both models are characterized by medial SMC replication, followed by SMC migration from the media to the intima and then SMC replication within the intima (Lindner et al., 1993). The models differ in that probe withdrawal injury is characterized by endothelial denudation and maintenance of blood flow, whereas carotid artery ligation is characterized by the interruption of blood flow in the presence of an intact endothelium.

Twelve mice (C57BL/6 strain) had endothelial denudation of the left carotid artery followed by implantation of a non-constrictive perivascular cuff loaded with blebbistatin ($n = 6$) or vehicle ($n = 6$). PCL polymers are biocompatible and have been shown to result in a controlled release of drug (Signore et al., 2001; Pires et al., 2005); the cuffs used in this study released blebbistatin in a linear and sustained fashion for at least 21 days in *ex vivo* testing (data not shown). When analyzed 3 weeks after injury, neointima obstructed $40 \pm 6\%$ of the lumen in vehicle-treated mice, similar to prior reports using this model (Lindner et al., 1993). Perivascular delivery of blebbistatin resulted in a reduction in the amount of neointima that formed after injury ($p < 0.03$; Fig. 1) and in the amount of lumen obstruction by neointima ($p < 0.02$; Fig. 1). The area of the media was slightly smaller in the blebbistatin-treated mice, but this difference did not reach statistical

Fig. 1. Effect of perivascular release of blebbistatin on neointimal formation in a mouse model of endothelial denudation carotid injury. A, representative photomicrographs of arterial cross-sections from C57BL/6 mice 21 days after endothelial denudation of the left carotid artery followed by immediate implantation of a nonconstrictive perivascular cuff loaded with blebbistatin ($n = 6$) or vehicle ($n = 6$). B, the area of the lumen and the neointima were quantified on multiple sections from each vessel, and the degree of neointimal hyperplasia was expressed as neointimal area. *, $p < 0.05$ for control versus blebbistatin. C, luminal obstruction by neointima for individual mice is shown.

significance ($p = 0.12$; Fig. 1B). Blebbistatin had no effect on arterial remodeling because the area within the external elastic lamina did not differ between groups.

Dual fluorescence staining for double-stranded DNA and

α -actin showed that the predominant cell type within the neointima expressed α -actin, consistent with being smooth muscle cells (Fig. 2A). Local treatment with blebbistatin was associated with a 19% reduction in total cell number within

Fig. 2. Effect of perivascular release of blebbistatin on cell density within the media and neointima in a mouse model of endothelial denudation carotid injury. A, representative samples of fluorescence staining for double-stranded DNA (4'-6-diamidino-2-phenylindole; blue) and actin (green) and a merged image from C57BL/6 mice 21 days after endothelial denudation of the left carotid artery followed by immediate implantation of a nonconstrictive perivascular cuff loaded with blebbistatin ($n = 6$) or vehicle ($n = 6$). B and C, the number of cells per high-powered field in the media and intima were quantified (B) as were the number of cells within the neointima that stained with anti- α -smooth muscle actin (C). Data shown are mean $\pm$ S.E.M. *, $p < 0.05$ for vehicle versus blebbistatin.

the media ($p = 0.004$; Fig. 2B), a 64% reduction in total cell number within the neointima ($p < 0.001$; Fig. 2B), and a 67% reduction in α -actin-positive cells within the neointima ($p < 0.001$; Fig. 2C). These data demonstrate that local delivery of blebbistatin results in a reduction in smooth muscle cells within the neointima.

Local Delivery of Blebbistatin Decreased Neointimal Hyperplasia after Carotid Artery Ligation. In the mouse carotid artery ligation model, implantation of a blebbistatin-secreting cuff was associated with a 61% decrease in neointimal area compared with a vehicle-secreting cuff ($p = 0.01$; Fig. 3). This resulted in a significant decrease in the amount of the lumen that was obstructed by neointima ($p < 0.0001$; Fig. 3). The size of the artery as determined by area within the external elastic lamina was not different between the groups.

Blebbistatin Elicited Profound, Dose-Dependent, Reversible Effects on RASMC Morphology. Previous studies have shown that blebbistatin inhibits PDGF-BB-induced migration of SMC derived from the brain basilar artery of guinea pigs and an immortalized cell line of aortic smooth muscle cells obtained from Sprague-Dawley rat embryos (Wang et al., 2008). Effects of blebbistatin on SMC proliferation and migratory responses to injury are largely unknown; thus, additional studies exploring mechanisms of blebbistatin effects were performed with cultured RASMC.

Treatment of confluent RASMC with blebbistatin elicited a change in cell morphology characterized by dose-dependent cell retraction and cell rounding (Fig. 4A). Changes in cell shape occurred within 1 h of exposure to blebbistatin (data not shown) and were reversible on withdrawal of the drug, even after 18 h of exposure to blebbistatin (Fig. 4A). RASMC lost cell-cell contact with little effect on the organization of stress fibers at lower concentrations of blebbistatin (10 μ M), but there was complete disruption of the actin cytoskeleton, interruption of vinculin organization, and interference with the alignment of stress fibers at higher concentrations (Fig. 4B). Disruption in actin cytoskeleton and cell rounding did not cause detachment of RASMC to noncoated tissue culture plates because adhesion was not affected by blebbistatin, even at a concentration of 100 μ M (Fig. 4C).

Blebbistatin Inhibited Proliferation of Cultured RASMC. PDGF-BB, angiotensin II, and α -thrombin are SMC mitogens that have been implicated in mediating vascular responses after injury and thus were used to study the effects of blebbistatin on SMC proliferation. As expected, treatment with PDGF-BB elicited a significant increase in DNA synthesis and cell number in RASMC ($P < 0.05$ for comparison of treated versus nontreated controls; Fig. 5A). Blebbistatin had an inhibitory effect on PDGF-BB-induced DNA synthesis with an effect observed beginning at 10 μ M and complete inhibition of responses to PDGF-BB seen at 100 μ M. The IC_{50} for blebbistatin inhibition of [3 H]thymidine incorporation responses to PDGF-BB was 18 ± 3 μ M ($n = 4$). Angiotensin II and α -thrombin stimulated an increase in [3 H]thymidine incorporation, although the response was less robust than observed with PDGF-BB (118, 79, and 615% increases in [3 H]thymidine incorporation, respectively). At doses of 25 μ M and higher, blebbistatin had an inhibitory effect on DNA synthesis in response to each of these mitogens (Fig. 5A). IC_{50} values for blebbistatin inhibition of [3 H]thymidine incorporation responses to angiotensin and α -throm-

bin were 36 ± 10 μ M ($n = 4$) and 48 ± 4 μ M ($n = 4$), respectively. Blebbistatin inhibited cellular proliferation as well as DNA synthesis. Blebbistatin at 25 μ M inhibited approximately 50% of the cell number increase in response to PDGF-BB or thrombin and approximately 40% of the response to angiotensin II (Fig. 5B). At a concentration of 100 μ M, blebbistatin completely inhibited proliferative responses to each of these mitogens.

To evaluate whether the inhibitory effects of blebbistatin on proliferative responses were caused by cell death, apoptosis of RASMC in the presence of blebbistatin was measured. Histone-associated DNA fragmentation, a sensitive measurement of apoptosis, was not increased by blebbistatin. Incubation of RASMC with blebbistatin for 36 h at various concentrations (10–100 μ M) alone or in the presence of PDGF-BB, angiotensin II, or thrombin did not increase the rate of RASMC apoptosis (Fig. 5C). The reversible effect in cell shape change after removal of blebbistatin (Fig. 4) further confirmed the viability of the RASMC exposed to blebbistatin.

Blebbistatin Has a Dose-Dependent Inhibitory Effect on RASMC Migration. As expected, blebbistatin inhibited migratory responses to PDGF-BB of a primary SMC line established from aorta of adult rats, similar to prior studies using SMC derived from the brain basilar artery of guinea pigs and an immortalized cell line of aortic smooth muscle cells obtained from Sprague-Dawley rat embryos (Wang et al., 2008). Blebbistatin also inhibited the migratory responses of RASMC to serum. In a modified Boyden chamber assay, the IC_{50} values of blebbistatin-induced inhibition of PDGF-BB and FBS-induced migration were 29 ± 5 μ M ($n = 4$) and 40 ± 1 μ M ($n = 4$), respectively (Fig. 6A).

To study directional cell migration in vitro, we used the well established wound healing assay. Migration occurs in the setting of more established cell-cell and cell-matrix interactions than the Boyden chamber assay, and the stimulus to migration is injury rather than a chemical gradient. We found that after blunt injury to a monolayer of RASMC blebbistatin caused a dose-dependent inhibition of migration (Fig. 6, B and C). At a dose of 50 μ M, blebbistatin inhibited 70% of the distance migrated by RASMC.

Discussion

Blebbistatin, a small molecule with inhibitory effects on both nonmuscle myosin and smooth muscle myosin, had profound effects on the amount and cellular content of neointima formed in response to carotid injury in the mouse. Perivascular delivery of blebbistatin reduced neointimal formation after probe withdrawal injury and ligation of carotid arteries in mice. The effects of blebbistatin were observed 3 weeks after injury, a time period in which SMC proliferation and migration are the most important contributors to neointima formation in both of these models (Lindner et al., 1993; Kumar and Lindner, 1997).

SMC exhibit phenotypic changes during vascular repair, and myosin expression is modulated by injury with levels of nonmuscle myosin increasing and levels of smooth muscle myosin decreasing (Kuro-o et al., 1991; Sartore et al., 1994, 1997; Aikawa et al., 1997; De Leon et al., 1997; Gallagher et al., 2000; Sajid et al., 2000; Christen et al., 2001; Herdeg et al., 2003). Our studies were not designed to determine the contribution of various isoforms of MHC to the observed

Fig. 3. Effect of perivascular release of blebbistatin in a mouse model of carotid ligation. A, representative photomicrographs of arterial cross-sections from FVB/N mice 21 days after ligation of the left common carotid artery followed by immediate implantation of a nonconstrictive perivascular cuff loaded with blebbistatin ($n = 6$) or vehicle ($n = 6$). B, the area of the lumen and the neointima were quantified on multiple sections from each vessel, and the degree of neointimal hyperplasia was expressed as neointimal area. Data shown are the mean $\pm$ S.E.M. *, $p < 0.05$ for vehicle versus blebbistatin.

Fig. 4. Reversible changes in morphology of RASM C after treatment with blebbistatin. A, representative photomicrographs showing morphologic changes in cell shape after treatment of RASM C with blebbistatin (various concentrations) or vehicle (DMSO) in the presence of 10% FBS for 18 h using an inverted microscope (representative photomicrographs were chosen from three independent experiments). To assess the reversibility of blebbistatin on cell shape, cells treated with blebbistatin for 18 h were then rinsed twice with PBS followed by incubation in 10% FBS/DMEM for 1 h. B, the effect of 1-h incubation with blebbistatin (10 or 100 μ M) or vehicle on actin and vinculin is shown (representative photomicrographs were chosen from three independent experiments). C, in the data shown, RASM C were grown to confluence, quiesced, and then treated with blebbistatin (various concentrations) or DMSO vehicle. One hour later, cells were trypsinized and suspended in 10% FBS medium containing blebbistatin or DMSO vehicle. Cells were plated on noncoated cultured dishes for 30 min at 37°C, and then the number of adherent cells was determined ($n = 3$; $p =$ not significant for comparison between groups).

Fig. 5. Blebbistatin inhibits RASM proliferation in a dose-dependent manner. RASM were grown until 80% confluence, growth-arrested, and then treated with various concentrations of blebbistatin or DMSO (as a control) in the presence of PDGF-BB (10 ng/ml), Ang II (1 μ M), α -thrombin (2 units/ml), or control (PBS) in DMEM. A and B, cell proliferation was measured by using [3 H]thymidine incorporation (A) and cell counting (B). C, histone-complexed DNA fragments were measured after treatment of RASM with various concentrations of blebbistatin or vehicle (DMSO) for 18 h in the presence of mitogens or vehicle (PBS) in DMEM. There were no statistically significant differences in apoptosis between the different groups. The data represent mean $\pm$ S.E.M. for a minimum of four independent experiments. *, $p < 0.05$; #, $p < 0.001$.

effects, but prior studies have implicated nonmuscle myosin in proliferative and migratory responses of SMC. Simons and Rosenberg (1992) showed that serum-induced proliferation of RASM was inhibited approximately 60% by treatment with an 18-mer antisense phosphorothiolate complementary to nucleotides 232 to 250 of the human NMHC-A. The inhibitory effect on growth was observed only if the NMHC-A antisense oligonucleotides were added to growth-arrested cells before serum stimulation; proliferation of actively growing

cells was not inhibited by antisense to NMHC-A. Proliferative responses returned to normal by 5 days, demonstrating that the inhibitory effect was reversible. In addition, both NM-A and NM-B have been implicated in cellular migration with important differences in localization and function. In their study of fibroblasts, Lo et al. (2004) found that NM-A was organized as sarcomere-like structures along stress fibers throughout the cell, whereas NM-B seemed to be more continuously distributed along stress fibers in the interior

A

B

C

Fig. 6. Blebbistatin inhibits RASMC migration in response to FBS or PDGF-BB and after blunt injury of RASMC monolayer. A, using a modified Boyden chamber cell-migration assay, RASMC $\pm$ blebbistatin were added to the upper chamber, and 500 μ l of appropriate medium with 10% FBS or PDGF-BB (10 ng/ml) was added to the lower chamber. Growth-arrested, confluent RASMC in a monolayer were disrupted to create a cell-free zone and then treated with 10% FBS $\pm$ blebbistatin at various concentrations. B and C, 24 h later, cells were fixed and visualized (B; 40 $\times$ magnification) with migration quantified by measuring the width of the cell-free zone at six distinct positions with a digitally calibrated micrometer (C). The data represent mean $\pm$ S.E.M. for three and six independent experiments in A and B, respectively. *, $p < 0.05$ versus FBS.

region. NM-B null cells migrated faster than control cells but there was a loss of direction stability.

Blebbistatin is remarkably potent with an IC_{50} for inhibition of myosin ATPase activity of purified skeletal and non-muscle myosin between 0.5 and 5 μ M (Straight et al., 2003). In the same studies, however, blebbistatin concentrations approximately an order of magnitude higher were required to inhibit actin sliding over rabbit skeletal muscle heavy meromyosin, suggesting that myosin molecules may interact weakly with actin even in an inhibited state. In the present studies, IC_{50} values for blebbistatin inhibition of [3 H]thymidine incorporation of RASMCM in response to PDGF-BB, α -thrombin, and angiotensin were 18, 36, and 48 μ M, respectively. In a modified Boyden chamber assay, the IC_{50} values of blebbistatin-induced inhibition of PDGF-BB and FBS-induced migration were 29 and 40 μ M, respectively. Taken together with previous studies showing that blebbistatin inhibits the contraction of GbaSM-4 cells (derived from the basilar artery of guinea pigs) maintained in collagen-gel fiber at an IC_{50} of 23 μ M (Katayama et al., 2006) and migration of GbaSM-4 SMC to PDGF-BB and sphingosylphosphorylcholine at approximately 30 μ M (Wang et al., 2008), these results demonstrate that blebbistatin has wide-ranging inhibitory effects on SMC at an IC_{50} between 25 and 50 μ M.

Our study has several limitations. It was not designed to determine whether blebbistatin had a direct effect on SMC in the vascular wall, and other cells may have been affected. For example, myofibroblasts in the adventitia contribute to neointimal lesions in the murine carotid injury model (Li et al., 2000).

It is unlikely, however, that SMC were not affected to at least some degree because SMC proliferation and migration have been shown to play the predominant role in neointimal growth in both of these models at the time point studied (Lindner et al., 1993; Kumar and Lindner, 1997). Endothelial denudation is followed by formation of a platelet monolayer but not an occlusive thrombus. Medial SMC replication is apparent beginning at day 2, and SMC migration from the media to the intima occurs soon after that. Replicating SMC are detected within the intima at approximately 1 week (Lindner et al., 1993), and intimal SMC proliferation continues until endothelial regeneration. In contrast to endothelial denudation injury, carotid artery ligation results in cessation of flow because the common carotid artery in mice does not have side branches. The endothelium is not injured, and this model is characterized by rapid proliferation of SMC in the presence of functional endothelium (Kumar and Lindner, 1997). SMC migration from media to intima occurs within 1 week of ligation with cell replication in the media decreasing dramatically after 8 days (Choi et al., 2004). Intimal cell proliferation continues at a high rate until 2 weeks and is present for at least 3 weeks.

Another limitation of these studies is that we were unable to determine whether the inhibitory effect in the vascular wall was caused by inhibition of nonmuscle myosin, smooth muscle myosin, or a combination of both. This question will require the development of more specific inhibitors. Lastly, we did not examine the in vivo release of blebbistatin, other studies have demonstrated the efficacy of PCL polymer as a rational tool for local rapamycin and paclitaxel delivery in a mouse carotid injury model (Pires et al., 2005). Because the media and adventitia in murine carotid are relatively thin

and blebbistatin is a small and cell-permeable compound, it is plausible that blebbistatin released from a cuff would reach the inner layers of the treated vessels.

In summary, our results demonstrate that disruption of myosin II ATPase by blebbistatin leads to inhibition of proliferation and migration in RASMCM in vitro and attenuates neointimal hyperplasia after carotid injury in the mouse.

Authorship Contributions

Participated in research design: Huang, Pathak, and Stouffer.

Conducted experiments: Huang, Zhang, and Li.

Contributed new reagents or analytic tools: Huang.

Performed data analysis: Huang, Zhang, Pathak, and Stouffer.

Wrote or contributed to the writing of the manuscript: Huang, Pathak, and Stouffer.

Other: Stouffer acquired funding for the research.

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