Inhibition of Patched-1 Prevents Injury-Induced Neointimal Hyperplasia

Eileen M. Redmond, Katie Hamm, John P. Cullen, Ekaterina Hatch, Paul A. Cahill, David Morrow

Objective—To determine the role of patched receptor (Ptc)-1 in mediating pulsatile flow-induced changes in vascular smooth muscle cell growth and vascular remodeling.

Approach and Results—In vitro, human coronary arterial smooth muscle cells were exposed to normal or pathological low pulsatile flow conditions for 24 hours using a perfused transcapillary flow system. Low pulsatile flow increased vascular smooth muscle cell proliferation when compared with normal flow conditions. Inhibition of Ptc-1 by cycloamine attenuated low flow-induced increases in Notch expression while concomitantly decreasing human coronary arterial smooth muscle cell growth to that similar under normal flow conditions. In vivo, ligation injury–induced low flow increased vascular smooth muscle cell growth and vascular remodeling, while increasing Ptc-1/Notch expression. Perivascular delivery of Ptc-1 small interfering RNA by pluronic gel inhibited the pathological low flow–induced increases in Ptc-1/Notch expression and markedly reduced the subsequent vascular remodeling.


Key Words: atherosclerosis ■ Notch ■ patched ■ restenosis ■ SMC growth ■ vascular remodeling

Vascular remodeling occurs as a result of alterations in vascular smooth muscle cell (VSMC) proliferation and apoptosis, and is a key event in the pathogenesis of vascular disease. Atherosclerosis, arteriosclerosis, vascular rejection, venous graft restenosis, and coronary intervention are each characterized by increased VSMC growth and remodeling1-4 that occurs at vessel branch points and bifurcations where blood flow is perturbed. This pathological low flow or proatherogenic flow type occurs at the curves and bifurcations of large artery and arteriole branch points, which are the regions that remodel over time. Understanding how VSMC transduce pulsatile flow stimuli into intracellular biomechanical signals that promote VSMC growth and subsequent remodeling is unclear at present. We have previously shown that the biomechanical stimulation (shear stress and cyclic strain) of adult VSMC that contribute to the growth response of these cells involves Hedgehog and Notch signaling components.5-7 Moreover, we have recently shown that vascular injury–induced remodeling involves a marked upregulation of both Notch and Hedgehog signaling.5 However, how these signaling pathways coordinate in an altered hemodynamic environment to promote vascular remodeling in the pathogenesis of vascular disease remains unexplored.

Hedgehogs (Hh) are a class of 19-kDa morphogens that interact with heparin on the cell surface through an N-terminal basic domain and are tethered to the surface through cholesterol and fatty acyl modification.8 Sonic Hh is the most widely expressed hedgehog during development where lack of Sonic Hh is embryonically lethal.8 Signaling occurs through interaction with the Patched receptors (Ptc-1 and 2) that then activate the transcription factors Gli1, Gli2, and Gli3. The downstream targets of the Gli gene products include both Ptc and Gli themselves; thus, Ptc and Gli are both components and targets of the Hh signaling pathway. Hedgehog signaling, which has been well characterized as having an involvement in the development of embryonic lineages, has also been shown to promote VSMC growth and survival in adult tissue.9-11 In addition, Notch signaling has also been implicated as a critical determinant of VSMC survival and vascular structure through modulation of signaling pathways that regulate growth.12-15 We and others have recently demonstrated that Hh can regulate the expression of Notch target genes in a variety of cell types, supporting an interaction between these 2 pathways.5,7,11,16,17 Given these reports in the literature and our previous studies supporting biomechanical regulation of both Hh and Notch signaling components in VSMC in vitro and in the injury-induced remodeled vessel in vivo, we examined whether Ptc-1 mediates pathological low flow–induced neointimal hyperplasia via regulation of the Notch signaling pathway.
Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Ptc-1 Mediates Pulsatile Flow–Induced Changes in HCASMC Growth In Vitro via a Notch-Dependent Pathway
The effect of Ptc-1 inhibition on low pulsatile flow–induced Notch and human coronary arterial smooth muscle cell (HCASMC) growth was determined using cells grown in a perfused transcapillary culture system under different flow conditions. Low flow enhanced proliferating cell nuclear antigen (PCNA) expression and increased HCASMC cell number over time when compared with normal flow (Figure 1A). Ptc-1 inhibition with cyclopamine (40 μmol/L) treatment for 24 hours resulted in a complete attenuation of low flow–induced increases in HCASMC growth as determined by PCNA expression (24 hours) and cell counts during 5 days (Figure 1A). In parallel studies, inhibition of Ptc-1 signaling for 24 hours resulted in a decrease in low flow–induced Ptc-1 and Gli2 mRNA expression by 80.0%±6 and 72.0%±4, respectively (Figure 1B). Similarly, cylopamine attenuated the low flow–induced increases in Notch receptor 1 and Notch target genes Hairy-related transcription factors, Hrt-1 and 2 (Figure 1B), to near control levels, suggesting that low flow–induced stimulation of Notch signaling is a Ptc-1–dependent event. The inhibitory effects of Cyclopamine on PCNA expression were comparable with the effects of Ptc-1 knockdown with specific small interfering RNA (siRNA) duplexes (Figure 1 in the online-only Data Supplement).

Pathological Low Flow Promotes Neointimal Hyperplasia via a Ptc-1/Notch–Dependent Pathway
Partial ligation of the carotid artery in a wild-type C57BL/6J mouse led to a 90% reduction in pulsatile flow when compared with sham-operated controls as previously shown. This induced a reproducible remodeling response, assessed 2 weeks after ligation that included an increase in SMC growth as compared with sham-operated vessels (Figure 2A). Vascular remodeling was inhibited after perivascular delivery of Ptc-1 siRNA in mice when compared with perivascular delivery of control-scrambled siRNA (Figure 2B). The pathological low flow–induced increase in SMC medial and neointimal volume was reduced to sham-operated levels after localized Ptc-1 gene knockdown (Figure 2B), whereas the low flow–induced decreases in lumen volume were abrogated to sham-operated control levels after Ptc-1 knockdown (Figure 2B). Low-flow–induced intimal medial thickness volume was significantly reduced after perivascular delivery of Ptc-1 siRNA (Figure 2C).

Notch component and Ptc-1 mRNA levels were determined in the carotid arteries of ligated animals and compared with sham. There was a significant increase in Notch1 and Hrt-2 mRNA levels in ligated vessels (Figure II in the online-only Data Supplement). In addition, Ptc-1, Gli2, Notch 1, and Hrt-2 mRNA levels were determined in the carotid arteries of sham, control-scrambled siRNA ligated and Ptc-1 siRNA ligated vessels. The injury-induced low flow increases in Ptc-1, Gli2, Notch 1, and Hrt-2 mRNA levels were reversed after selective knockdown of Ptc-1 by siRNA (Figure 3A). Similarly, injury-induced increases in Ptc-1, Notch 1IC, and PCNA protein were significantly attenuated after Ptc-1 gene knockdown (Figure 3B). Parallel studies confirmed Ptc1-mediated inhibition of Notch1 stimulated HCASMC PCNA expression in vivo (Figure III in the online-only Data Supplement).

Immunohistochemical examination of fixed tissue sections of carotids from sham-operated control vessels, scrambled siRNA ligated vessels, and Ptc-1 siRNA ligated vessels revealed Ptc-1 expression in both the adventitia and SMC-rich media by costaining with SMC α-actin (Figure 3C). Decreased SMC Ptc-1 concomitant with a reduction in intima medial thickening and a decrease in SMC growth after Ptc-1 gene knockdown was evident by dual immunofluorescence staining with α-actin (Figure 3C).

Discussion
During the past decade, we and others have investigated a role for the Notch and Hh signaling pathways in adult vascular cells and, in particular, how these pathways arbitrate cell fate decisions in normal and disease states. A fundamental
role for these pathways in vascular development is well established, but their recapitulation in adult cells and subsequent pathological role warrants further exploration. To date we have determined that both Notch and Hh signaling pathways can mediate adult SMC growth, and that these pathways are sensitive to biomechanical regulation in this cell type and in endothelial cells.\(^7\),\(^21\),\(^22\) Moreover, the biomechanically mediated changes in SMC growth are at least, in part, a Hh and Notch-dependent event. The novelty of our current study is that for the first time we show that the pathological flow–induced changes in SMC growth seen both in culture and in the injury-induced vessel occur via Ptc-1 regulation of downstream Notch signaling.

We established in vitro that pathological low flow induces a significant increase in proproliferative Hh signaling via ptc-1 regulation of the Notch pathway. Ptc-1 inhibition with cyclopamine resulted in decreased Notch signaling concomitant with a decrease in the low flow–induced SMC growth. Moreover, the importance of these findings was confirmed in vivo using a model of arterial injury where Hh/Notch signaling and SMC growth were significantly increased in the ligated low flow artery in comparison with the normal flow controls. To confirm that these pathological flow–induced changes in Hh/Notch signaling were predominantly SMC specific, antibodies specific for Hh/Notch signaling components were used in tandem with α-actin staining antibodies. In addition, Laser Capture Microdissection was used to remove the SMC-rich medial layer, which was then analyzed for Hh/Notch RNA expression (data not shown). Both techniques in parallel with whole vessel analysis confirmed that low flow results in increased Hh/Notch signaling, which results in a proproliferative phenotype in these injured vessels promoting vascular remodeling.

We and others have previously established a functional relationship between Hh and Notch signaling and SMC growth in vitro and in vivo.\(^5\),\(^16\),\(^17\) Our previous studies had shown that normal vessels express Ptc-1 primarily in adventitial cells but after vascular injury, medial and intimal SMCs express Ptc-1 in abundance in vivo.\(^3\) In this context, injury-induced Hh stimulation leads to Notch 1IC activation, which in turn results in robust stimulation of downstream Notch target gene expression within these lesions. It is these hts, which feed into the proliferative (eg, c-myc) and antiapoptotic machinery of the cell by regulating both proapoptotic Bax and antiapoptotic Bcl-X\(_{L}\). However, to our knowledge, this is the first study that directly links Ptc-1 and Notch signaling together in controlling changes in SMC phenotype resulting from altered pulsatile flow.

In this study, we used a perivascular delivery system of gene-specific siRNAs by using a pluronic gel for localized delivery.\(^23\)–\(^25\) After pluronic gel delivery of Ptc-1 siRNA, which targets the desired siRNA to an area below the bifurcation of the ligated carotid artery, we achieved a significant inhibition of Ptc-1 mRNA and protein expression. Furthermore, this localized delivery of ptc-1 siRNA resulted in a marked inhibition of Notch signaling concomitant with decreased SMC medial growth and reduced low flow–induced remodeling. This study compliments recent work by Li et al\(^26\) that addressed the role of Notch 1
in neointimal formation after vascular injury. This study indicated a role for Notch 1, rather than Notch 3, in mediating SMC growth and neointimal formation after vascular injury through CHF1/Hey2 using heterozygous Notch1 mutants. In agreement, we demonstrate that Notch1, rather than Notch3, mRNA levels are elevated after injury, an effect that is abrogated after Ptc-1 knockdown. Although the level of neointimal hyperplasia was modest in our model, low flow was associated with significant vascular remodeling, albeit predominantly medial thickening. Vascular remodeling is a major manifestation of arteriosclerosis of the carotid artery, and it is an important predictor of cardiovascular events. The level of carotid remodeling has also a strong genetic component that underlies differences in neointimal hyperplasia among strains.

We have previously shown that the effect of vascular endothelial growth factor-A knockdown on sonic Hh–induced hrt-3 expression could be reversed after recovery with exogenous addition of recombinant vascular endothelial growth factor-A. Taken together with our previous work, it is clear that the Hh pathway, including Ptc-1, is an important regulatory molecule upstream of both vascular endothelial growth factor-A and Notch in adult SMCs that govern SMC growth and survival. However, how Ptc-1 is specifically modified by biomechanical forces, which elicit this signaling cascade, warrants further investigation. In addition, it is possible that localized treatment of stenosed vessels with ptc-1–specific siRNA could represent a novel therapy for either restenosis after vein grafting, after angioplasty or atherosclerosis.

In conclusion, we have shown for the first time that pulsatile flow mediates changes in SMC growth via regulation of Ptc-1 signaling, and that local inhibition of Ptc-1 by siRNA prevents low flow–induced vascular remodeling. From this data and our previous studies, it is tempting to speculate that a Hh/Notch axis may represent a novel therapeutic target for disease states where aberrant changes in SMC growth are key to disease pathogenesis. A greater understanding of this signaling cascade within vascular lesions warrants further investigation.

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Disclosures
None.

References
Significance

The onset of atherosclerosis as a result of flow-limiting stenosis is a leading cause of heart attack, which contributes to the 800,000 cardiovascular disease–related deaths annually. The arterial remodeling responsible for this onset is characterized by a pathology where medial/neointimal thickening is the predominant feature. Changes in vascular smooth muscle cell growth play an important role during this remodeling; however, the mechanisms remain unclear. Because similar changes are also apparent during modeling of the embryonic vasculature, we and others have postulated that the control of vascular smooth muscle cell growth and subsequent remodeling after vascular injury may share similar signaling patterns. This study has shown for the first time that pulsatile flow mediates changes in vascular smooth muscle cell growth via regulation of patched receptor 1 signaling allowing us to postulate that a patched receptor 1/Notch axis represents a novel therapeutic target for disease states where aberrant changes in smooth muscle cell growth are key to disease pathogenesis.
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Supplemental Figure I:

(A) Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis of Ptc-1 mRNA expression in HCASMC following Ptc-1 inhibition with Ptc-1 siRNA (2.0μM) and Cyclopamine (40μM) for 24 h. Data are normalized to GAPDH and represent the mean ± SEM, n=3. (B) Representative Western Blot together with cumulative data (C) of proliferating cell nuclear antigen (PCNA) protein expression in HCASMC following Ptc-1 inhibition with Ptc-1 siRNA (2.0μM) or Cyclopamine (40μM) for 24 h. (Mean ± SEM, n=3).
Supplemental Figure II:

Suppl. Figure II: The Effect of Injury-induced Low flow on Notch component mRNA Expression. QRTPCR analysis of Notch/Ptc-1 mRNA expression 2 weeks post carotid artery ligation. Data are normalized to GAPDH and represent the mean ± SEM, n=6
**Supplemental Figure III:**

Representative Western Blot together with cumulative data of proliferating cell nuclear antigen (PCNA) protein expression in HCASMC following Ptc-1 inhibition with Cyclopamine (40μM) for 24 h +/- Notch 1 IC overexpression. (Mean ± SEM, n=3).

**Suppl. Figure III: Ptc-1 inhibition decreases SMC Growth.**
Supplemental Figure IV: Ptc-1 inhibition attenuates Low pulsatile flow-induced increases in HCASMC Growth. Representative Western Blot together with cumulative data of proliferating cell nuclear antigen (PCNA) protein expression in HCASMC exposed to normal or low flow conditions in the absence or presence of Ptc-1 inhibition with Cyclopamine (40μM) over 5 days. (Mean ± SEM, n=3).
Suppl. Figure V. Perivascular Alex fluor 555-tagged siRNA transfection efficiency. (A) Photomicrographs of immunofluorescence of carotid artery wall 7 days post perivascular delivery of Alexa Fluor 555-tagged siRNA. Representative images of vessels wall with arrows indicating nuclei stained positive for DAPI, Alex fluor 555-tagged siRNA and merged DAPI/Alex Fluor 555-tagged siRNA. All images were visualized under 30/60 X magnification by confocal microscopy. (B) Cumulative data of transfected nuclei stained positive for Alexa fluor 555-tagged siRNA. (Mean ± SEM, n=6).
Materials and Methods

**Cell Culture.** Human Coronary Artery Smooth Muscle Cells (HCASMC) were obtained from Lonza (Walkersville, MD) and cultured in optimized Smooth Muscle Cell Medium (Clonetics<sup>R</sup> Lonza), supplemented with hEGF, Insulin, hFGF and 10% FCS. Cells were characterized by staining positive for smooth muscle cell α-actin, calponin, myosin and smoothelin.

**Western Blot Analysis.** Proteins from cell lysates (12-15 μg) were resolved on SDS-PAGE (12% resolving, 5% stacking) prior to transfer onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were stained with Ponceau S and probed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure equal protein loading and transfer and rinsed in wash buffer (PBS containing 0.05% Tween-20) before being probed as described previously.<sup>1</sup>

**Quantitative real-time RT-PCR.** Total RNA (0.5-1 μg), isolated from cells using Qiagen RNeasy kit (Valencia, CA) was reverse-transcribed using iscript<sup>TM</sup> cDNA Synthesis kit from BIO-RAD (Carlsbad, CA). The gene-specific oligonucleotide sequences were as previously described.<sup>1</sup> Real-Time RT-PCR was performed using the Stratagene MX3005 machine and the SYBER green jumpstart PCR kit (Sigma, St. Louis, MO) as described by the manufacturer.

**Cell Proliferation.** Changes in HCASMC cell number were determined by cell counting following exposure to pulsatile flow. Cells were seeded at $1 \times 10^4$ cells/well onto 6-well plates in 10% growth media. Cells were counted each day where the average of three wells was quantified by using a hemocytometer. In parallel studies, proliferating cell
nuclear antigen (PCNA) protein expression was determined by western blot following exposure to pulsatile flow.

**Perfused transcapillary co-culture system.** The perfused transcapillary culture apparatus (Cellmax Quad artificial capillary culture system, Spectrum Laboratories) was used to expose cells *in vitro* to different flow rates corresponding to normal and pathological low flow as previously described. In the current study, pathologic low flow was modeled by a flow rate of 0.3 ml/min, corresponding to a pulse pressure of 24/18 mmHg, a frequency of 0.2 Hz, and amplitude of 6 mmHg in the extracapillary space. A normal flow rate of 25 ml/min was used corresponding to a pulse pressure of 64/14 mmHg, a frequency of 2 Hz, and an amplitude of 50 mmHg in the extracapillary space for 1-6 days as described. Cellular mRNA was harvested using TRIzol reagent (Invitrogen) and protein using lysis buffer from BIO-RAD (Carlsbad, CA) according to the manufacturer's specifications.

**Mouse Carotid Artery Partial Ligation.** The carotid artery ligation model of vascular injury and remodeling was performed essentially as described utilizing 6-8 week male C57BL/6 mice. All procedures were approved by the University of Rochester Animal Care Committee. After buprenorphine analgesia and induction of anesthesia using inhalational isoflurane, the mouse was positioned on a clean operating table, with a warming pad to maintain body temperature. The animal was clipped and the surgical site prepped using betadine solution and alcohol. A midline cervical incision was made. With the aid of a dissecting microscope, the left external and internal carotid arterial branches were isolated and ligated with 6-0 silk suture reducing left carotid blood flow to flow via the patent occipital artery. The neck incision (2 layers, muscle and skin) was closed with
a running suture using 4-0 coated Vicryl. Partial ligation of the left carotid artery in this manner resulted in a decrease in blood flow in the left carotid artery, concomitant with an increase in the right carotid artery, with an intact endothelial monolayer, when compared to sham-operated control.

siRNA delivery in vivo. Select in vivo ready siRNAs (Life Technologies, Grand Island, NY) were pre mixed with lipofectamine based transfection reagents and added to the pluronic gel. Pluronic gel solutions (Sigma, St. Louis, MO) at 1mg/ml were prepared and kept at 4°C. Following carotid ligation, 200μL of this solution was applied to the carotid artery. On contact with tissues at 39°C the pluronic gel solidifies instantaneously, generating a translucent layer that envelops the treated region. The wound was then closed immediately after the application of the gel. Treated vessels were removed at 14 days post ligation for analysis. siRNA transfection efficiency by pluronic gel was verified by Alexa Flour 555-tagged control siRNA (A kind gift from Nitin Puri, Life Technologies, Grand Island, NY) showing localized delivery throughout the vessel (Supplemental Figure 5).

Preparation of Carotid Artery RNA. Mice were perfused with heparin/saline solution. Carotid arteries were collected in Trizol (Invitrogen) and homogenized using an Ultra-Turrax tissue disperser and RNA was prepared according to the manufacturers specifications.

Immunohistochemistry and Histomorphometry. Mice were perfusion fixed with 10% paraformaldehyde in sodium phosphate buffer (pH 7.0), 14 days post ligation, and the carotids harvested and embedded in paraffin. Starting 500 μM below the carotid
bifurcation landmark, a series of cross-sections (10 x 5 μm) were made, every 200 μm through 2 mm length of carotid artery. Cross-sections were stained with Verhoeff-Van Gieson stain for elastic laminae and sections were imaged using a Nikon TE300 microscope equipped with a Spot RT digital camera (Diagnostic Instruments). Digitized images were analyzed using MCID image software. Assuming a circular structure in vivo, the circumference of the lumen was used to calculate the lumen area, the intimal area was defined by the luminal surface and internal elastic lamina (IEL), the medial area was defined by the IEL and external elastic lamina (EEL) and the adventitial area was the area between the EEL and the outer edge. All histomorphometric analysis was performed “blind analysis” by the same observer. Immunofluorescence for smooth muscle actin (Sigma; A2547), activated Notch1 (NICD; Abcam, ab8925), Patched-1 (Abcam ab39266), and proliferating cell nuclear antigen (PCNA) (Laboratory Vision; RB-9055) was performed by standard procedures.

Data Analysis. Results are expressed as mean ± SEM. Experimental points were performed in triplicate, with a minimum of 3 independent experiments (VSMC), or a minimum of 5 animals per group. An ANOVA test was performed on cell count data and a Wilcoxon Signed rank test was used for comparison of two groups when compared to normalized control. A value of p≤0.05 was considered significant.
Bibliography

