Adenovirus-Mediated Intraarterial Delivery of PTEN Inhibits Neointimal Hyperplasia

Jianhua Huang, Xi-Lin Niu, Anne M. Pippen, Brian H. Annex, Christopher D. Kontos

Objective—Phosphoinositide (PI) 3-kinase promotes vascular smooth muscle cell (VSMC) responses necessary for neointimal hyperplasia. We recently demonstrated that the inositol 3-phosphatase PTEN is expressed in VSMCs and that its overexpression inhibits these cellular responses. The purpose of this study was to determine the effects of adenovirus-mediated overexpression of PTEN on neointimal hyperplasia in vivo in the rat carotid injury model.

Methods and Results—Rat carotid arteries were balloon-injured and treated with a recombinant control adenovirus (AdEV) (n = 6), an adenovirus encoding wild-type PTEN (AdPTEN) (n = 8), or phosphate-buffered saline (sham) (n = 5). Injured vessels demonstrated PTEN overexpression by Western blotting and immunohistochemistry after AdPTEN treatment. Neointimal hyperplasia was assessed 2 weeks after balloon injury and adenovirus administration. Compared with controls, AdPTEN treatment significantly decreased neointimal area and percent stenosis. To investigate the mechanisms of action of AdPTEN, vessels were harvested 3 days after balloon injury and virus infection. AdPTEN significantly increased medial cell apoptosis while decreasing proliferation of the remaining viable cells.

Conclusions—PTEN overexpression potently inhibits neointimal hyperplasia through induction of apoptosis and inhibition of medial cell proliferation. These findings suggest that modulation of PTEN expression or activity may be a viable approach to treat neointimal hyperplasia. (Arterioscler Thromb Vasc Biol. 2005;25:354-358.)

Key Words: apoptosis ■ PTEN ■ vascular biology ■ neointimal hyperplasia ■ phosphoinositide 3-kinase

N eointimal hyperplasia contributes to restenosis after percutaneous coronary intervention, venous bypass graft disease, and atherosclerosis.1 Vascular injury in each of these conditions results in the release of mitogenic growth factors and hormones that contribute to pathological vascular growth.2 Many of these molecules contribute to neointimal hyperplasia by activating phosphoinositide (PI) 3-kinase in vascular smooth muscle cells (VSMCs). Consistent with activation of this pathway after percutaneous coronary interventions, the drug Sirolimus (rapamycin) has recently shown promising results in the prevention of in-stent restenosis.3-5 Sirolimus inhibits a downstream effector of PI 3-kinase, mammalian target of rapamycin (mTOR), which regulates initiation of protein translation.6 However, PI 3-kinase activates a host of other effector molecules that promote cellular proliferation, migration, and survival, including PDK1, Akt, Rac, p70S6k, and FAK.7 Thus, we hypothesized that inhibition of PI 3-kinase signaling upstream of mTOR would have potent effects on neointimal hyperplasia.

Somewhat surprisingly, very few studies have investigated the effects of PI 3-kinase inhibitors on vascular injury in vivo. Shigematsu et al treated rats with wortmannin, a highly selective PI 3-kinase inhibitor, either locally or systemically before carotid arterial injury.8 Wortmannin blocked increases in arterial Akt activation and cyclin D1 expression, and these effects correlated with inhibition of medial VSMC proliferation. In a recent study, stent implantation in rat aortas was found to induce activation of PI 3-kinase and its downstream effector Akt.9 Treatment with wortmannin prevented PI 3-kinase/Akt activation, as well as neointima formation after stent-implantation. Together, these studies suggest that arterial PI 3-kinase inhibition may be an effective strategy to target pathological VSMC growth after vascular injury.

We recently investigated this possibility by evaluating the role of an endogenous inhibitor of PI 3-kinase in VSMCs. PTEN (phosphatase and tensin homology deleted on chromosome 10) is an inositol 3-phosphatase that was originally identified as a tumor suppressor protein.10 PTEN hydrolyzes the 3-phosphoinositide lipid products of PI 3-kinase, PI 3,4-bisphosphate and PI 3,4,5-trisphosphate, to prevent downstream activation of a number of PI 3-kinase effector molecules.11 Overexpression of PTEN in VSMCs with a recombinant adenovirus inhibited PDGF-induced cellular proliferation, migration, and survival,12 suggesting that overexpression of PTEN in vivo might disrupt neointimal hyperplasia. In this report, we tested this hypothesis and found that...

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adenovirus-mediated overexpression of PTEN potently inhibited the development of neointimal hyperplasia in a rat carotid arterial injury model by inducing medial cell apoptosis and by inhibiting proliferation of the remaining nonapoptotic cells.

**Methods**

**Adenovirus Construction**
Recombinant adenoviruses encoding wild-type human PTEN (AdPTEN) and a control virus containing no cDNA insert (empty virus, AdEV) were constructed using the AdEasy system (Stratagene). Briefly, briefly, rats weighing 450 to 500 grams were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg). The right external and common carotid arteries were surgically exposed and isolated, and the endothelium of the common carotid artery was demured with a 2-French Fogarty balloon catheter (Baxter Healthcare, Irvine, Calif). After balloon removal, the common carotid artery was flushed with phosphate-buffered saline (PBS), and a 1-cm segment was isolated with vascular clamps. A 100-μL solution of PBS alone (sham-infected) or PBS containing adenovirus (5 × 10^9 pfu) was injected and incubated in the common carotid artery for 30 minutes. After removal of this solution, the external carotid artery was ligated and blood flow to the common carotid artery was restored. PTEN expression was analyzed by Western blotting in a subset of 9 rats and blood flow to the common carotid artery was restored. PTEN vessel homogenates were separated by gel electrophoresis and Western

**Rat Carotid Injury Model and PTEN Expression**
Animal procedures were approved by the Duke University Institutional Animal Care and Use Committee. The rat carotid injury model and local adenovirus delivery were performed in a total of 54 male Sprague–Dawley rats essentially as described previously.15,16 Briefly, rats weighing 450 to 500 grams were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg). The right external and common carotid arteries were surgically exposed and isolated, and the endothelium of the common carotid artery was demurred with a 2-French Fogarty balloon catheter (Baxter Healthcare, Irvine, Calif). After balloon removal, the common carotid artery was flushed with phosphate-buffered saline (PBS), and a 1-cm segment was isolated with vascular clamps. A 100-μL solution of PBS alone (sham-infected) or PBS containing adenovirus (5 × 10^9 pfu) was injected and incubated in the common carotid artery for 30 minutes. After removal of this solution, the external carotid artery was ligated and blood flow to the common carotid artery was restored. PTEN expression was analyzed by Western blotting in a subset of 9 rats (n=3 per group) that were euthanized 3 days postoperatively. Vessel homogenates were separated by gel electrophoresis and Western blotted sequentially with antibodies against PTEN (clone A2B1; Santa Cruz Biotechnology; 1:1000) and α-tubulin (clone YL1/2; Harlan Bioproducts; 1:5000). PTEN expression was also analyzed by immunohistochemistry on vessel sections 3 days after vessel injury and virus infection. Vessels were frozen in optimal temperature cutting compound (Fisher) and 5-μm sections were cut on a cryostat. PTEN was stained with the same antibody as for Western blotting.

**Morphological Analysis**
In 19 rats (sham, n=5; AdEV, n=6; AdPTEN, n=8), vessels were harvested 14 days after injury and adenovirus infection; 5-μm fresh-frozen transverse vessel sections were stained with Accustain elastin stain (Sigma Diagnostics), and vessel measurements were made using SPOT Advanced software (v. 3.2.5; Diagnostic Instruments). For each measurement, 3 sections from different parts of the vessel were analyzed, and the area of the neointima, the media, and the lumen was quantified. Neointimal hyperplasia was expressed as the percentage of PCNA-positive nuclei. In a separate group of 17 rats (sham, n=5; AdEV, n=5; AdPTEN, n=7), apoptosis was analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining of perfusion-fixed, paraffin-embedded vessel sections (5 μm). Sections were stained with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s instructions. Fragmented DNA was labeled with TdT using digoxigenin-conjugated peroxidase staining. Negative controls were performed without active TdT. Sections were counterstained with methyl green and photographed through a blue filter at 400× magnification. TUNEL-positive and total nuclei were counted in 3 separate sections from different regions of each vessel. Apoptosis was expressed as the percentage of TUNEL-positive nuclei.

**Statistical Analysis**
Data are expressed as the mean±SEM. Statistical analysis was performed across multiple groups using analysis of variance (ANOVA) and confirmed between individual groups using a 2-tailed Student t test. P<0.05 was considered statistically significant.

**Results**

**Adenovirus-Mediated Overexpression of PTEN**
To investigate the effects of PTEN on neointimal hyperplasia, rat carotid arteries were balloon-injured, then infected with a recombinant adenovirus encoding PTEN, a control, empty adenovirus (AdEV), or sham-infected by treatment with PBS. Gene transfer was confirmed by Western blotting PTEN in homogenates of carotid arteries and by immunohistochemistry on frozen vessel sections. Three days after adenovirus infection, PTEN overexpression was observed in AdPTEN-infected vessels (Figure 1A and 1B), demonstrating the efficacy of transgene delivery. Immunohistochemical analysis demonstrated overexpression primarily in the intima and inner layer of the arterial media, as might be expected after local virus delivery (Figure 1B). PTEN expression was detectable at lower levels by both Western blotting and immunohistochemistry in normal carotid artery, and expression was not affected by balloon injury (sham infection) or control adenovirus infection.
PTEN Overexpression Inhibits Neointimal Hyperplasia

Fourteen days after balloon injury and adenovirus infection, the degree of neointimal hyperplasia was assessed morphologically and quantitatively (Figure 2). Compared with control-infected animals, AdPTEN treatment significantly reduced vessel stenosis ($P<0.001$ versus sham; $P=0.04$ versus AdEV) (Figure 2B) and neointimal area ($P=0.003$ versus sham; $P=0.04$ versus AdEV) (Figure 2C). The mean percent vessel stenosis in the AdPTEN-treated vessels was only $4\pm 2\%$ compared with $36\pm 4\%$ for sham-infected and $46\pm 14\%$ for AdEV-infected vessels. No significant difference in medial area was observed, although the mean area in the AdPTEN group was slightly less than that in the control groups (sham, $0.119\pm 0.032$ mm$^2$; AdEV, $0.130\pm 0.013$ mm$^2$; AdPTEN, $0.100\pm 0.010$ mm$^2$; $P=0.5$).

PTEN Overexpression Induces Apoptosis and Inhibits Proliferation of Medial Cells

To investigate the mechanisms by which PTEN overexpression reduced neointimal hyperplasia, additional groups of rats were evaluated 3 days after balloon injury and virus treatment. Vessel sections were analyzed for changes in cell number and nuclear morphology (Figure 3), apoptosis (Figure 4), and proliferation (Figure 5). Consistent with the slight medial thinning observed at 14 days, AdPTEN treatment induced a significant reduction in the number of cells in the media (by $60\%$) by 3 days after infection, as determined by nuclear staining with Hoechst 33342 (Figure 3A to 3C). Moreover, almost $50\%$ of these nuclei appeared fragmented or condensed, consistent with apoptosis (Figure 3D). To verify that PTEN overexpression increased medial cell apoptosis, TUNEL staining was performed on vessel sections 3 days after balloon injury and adenovirus infection (Figure 4A to 4C). Consistent with the results of Hoechst staining, the number of apoptotic cells was significantly increased after PTEN overexpression (Figure 4C). In addition, vessel sections stained for PCNA demonstrated that AdPTEN treatment...
PI 3-kinase plays a critical role in a wide variety of cellular processes, including proliferation, migration, vesicular trafficking, and survival. PI 3-kinase signals primarily through its 3-phosphoinositide lipid products, which regulate the plasma membrane recruitment and/or activation of a number of downstream effector molecules, including PDK1, Akt, p70S6k, and mTOR. Pharmacological inhibitors exist for some of these molecules, such as the drug Sirolimus (or rapamycin), which inhibits the activity of both mTOR and p70S6k. In addition, the lipid and protein kinase activities of PI 3-kinase itself can be blocked in a highly selective manner by the drugs wortmannin and Ly294002. In this report, we tested the effects on neointimal hyperplasia of disrupting PI 3-kinase–mediated 3-phosphoinositide signaling by overexpressing the inositol 3-phosphatase PTEN. This approach might be expected to have broader effects than inhibition of any one PI 3-kinase effector molecule alone. Adenovirus-mediated delivery of PTEN potently inhibited neointimal hyperplasia in the rat carotid injury model as a result of both pro-apoptotic and antiproliferative effects. Together, these findings indicate that PTEN may provide an important target for the modulation of PI 3-kinase signaling in the treatment of neointimal hyperplasia.

Recombinant adenoviruses are effective gene delivery vectors, and they have been shown to effect high-level protein expression in the vascular wall. Moreover, adenoviruses can be delivered locally, thereby avoiding potentially adverse systemic side effects. Because PI 3-kinase regulates numerous and diverse cellular processes, systemic overexpression of PTEN might be expected to result in significant toxicity. Consistent with this possibility, we recently demonstrated that PTEN overexpression in cultured VSMCs or endothelial cells induces apoptosis. Accordingly, in the present study, a significant degree of apoptosis was observed in vivo in AdPTEN-infected arteries. With local delivery, the cytotoxic effects of PI 3-kinase inhibition were likely limited to the wall of the target vessel; in fact, they appear to have contributed to the beneficial effects on neointimal hyperplasia observed here.

The pro-apoptotic effect of PTEN overexpression appears to have been the primary mechanism for inhibition of neointimal hyperplasia, because the number of medial cells was markedly reduced early after virus infection, and a high percentage of the remaining cells demonstrated apoptotic morphological features and positive TUNEL staining. The proliferative capacity of these cells was also reduced by PTEN overexpression, as demonstrated by a decrease in nuclear PCNA staining. These findings suggest a possible dose-dependent effect of PTEN overexpression. Those cells expressing the highest levels of PTEN likely underwent apoptosis, whereas lower expression likely inhibited proliferation and possibly migration, as seen in vitro, without inducing cell death. This notion is supported by the results of a recent study by Moon et al in which PTEN overexpression in VSMCs induced cell cycle arrest and upregulation of the cyclin-dependent kinase inhibitors p21 and p27. These findings are consistent with earlier studies that demonstrated a role for PTEN in regulation of G1/S phase progression in tumor and embryonic stem cells. Moon et al also found that PTEN overexpression in VSMCs correlated with inhibition of matrix metalloproteinase-9 expression, which could partially explain any inhibitory effects on cell migration in our model of neointimal hyperplasia.

In this report, we tested whether PTEN overexpression would limit PI 3-kinase–induced 3-phosphoinositide production and subsequent VSMC signaling and growth. An important question that remains is whether neointimal hyperplasia that results from vascular injury is caused, at least in part, by altered expression or activity of endogenous PTEN within the vessel wall. Dysregulation of PTEN is responsible for a large percentage of human cancers, and many of the same cellular mechanisms that lead to tumorigenesis are also responsible for the development and progression of neointimal hyperplasia. Interestingly, deficiency of another tumor suppressor, p53, was recently shown to accelerate neointimal hyperplasia after arterial injury. Loss of an important regulator of cell growth like PTEN would be expected to have similar consequences. Notably, several studies have now described mechanisms regulating PTEN expression and activity. Although these regulatory mechanisms are not fully understood, the activity and subcellular localization of PTEN appear to be modulated in part by phosphorylation of serine and threonine residues in its carboxyl-terminus by the enzyme CK2. C-tail phosphorylation stabilizes PTEN protein, but this also appears to limit its enzymatic activity. Diphosphorylation of PTEN has been shown to lead to degradation of PTEN by the proteasome, as well as by caspase-3 and caspase-9. Thus, proteolytic degradation of PTEN may contribute to enhanced PI 3-kinase activity after vascular injury. Furthermore, PTEN’s active site cysteine residue can be oxidized by hydrogen peroxide, resulting in disulfide bonding with other cysteine residues and inhibition of PTENs enzymatic activity. Because hydrogen peroxide and other reactive oxygen species are released in the vessel wall after vascular injury, oxidation is another potential mechanism by which PTEN...
may be inhibited to contribute to pathological vascular growth.

As in tumors, loss of PTEN expression or activity would result in a shift toward cell survival and growth pathways, despite cellular injury that might otherwise induce apoptosis. A recent study found that exposure of airway epithelial cells to Zn²⁺ ions induced Akt activation and concomitant loss of PTEN expression. Loss of PTEN was proteasome-mediated and PI 3-kinase–dependent. Similarly, preliminary studies in our laboratory indicate that PTEN expression is reduced after vascular injury in a venous bypass graft model (J.H. and C.D.K., unpublished data). In both of these models, cellular injury may result in PTEN degradation and subsequent enhancement of PI 3-kinase activation, cell growth, and survival. When considered together with the results of our current study, these and other recent observations suggest that PTEN is a critical regulator of cell growth in the vasculature. Improved understanding of PTEN’s regulatory mechanisms may lead to the identification of novel targets to favorably modulate the activity of this protein for the treatment of neointimal hyperplasia. Based on recent experiences with rapamycin-coated stents, compounds targeting PTEN and its regulatory pathways may have wide usefulness.

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References

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