Inhibition of Vascular Smooth Muscle Cell Proliferation, Migration, and Survival by the Tumor Suppressor Protein PTEN

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Abstract—Phosphatidylinositol (PI) 3-kinase signaling regulates numerous cellular processes, including proliferation, migration, and survival, which are required for neointimal hyperplasia and restenosis. The effectors of PI 3-kinase are activated by the phospholipid products of PI 3-kinase. In this report, we investigated the hypothesis that overexpression of the tumor suppressor protein PTEN, an inositol phosphatase specific for the products of PI 3-kinase, would inhibit the vascular smooth muscle cell (VSMC) responses necessary for neointimal hyperplasia and restenosis. Effects of PTEN were assessed in primary rabbit VSMCs after overexpression with a recombinant adenovirus and compared with uninfected or control virus-infected cells. PTEN was expressed endogenously in VSMCs, and PTEN overexpression inhibited PDGF-induced phosphorylation of p70s6k, Akt, and glycogen synthase kinase-3β but not ERK1 or -2. Overexpression of PTEN significantly inhibited both basal and PDGF-mediated VSMC proliferation and migration, the latter possibly due in part to downregulation of focal adhesion kinase. Moreover, PTEN overexpression induced cleavage of caspase-3 and significantly increased apoptosis compared with control cells. Taken together, these results demonstrate that PTEN overexpression potently inhibits the VSMC responses required for neointimal hyperplasia and restenosis. Adenovirus-expressed PTEN may therefore provide a useful tool for the local treatment of these and other vascular proliferative disorders. (Arterioscler Thromb Vasc Biol. 2002;22:745-751.)

Key Words: PTEN ■ vascular smooth muscle cell ■ phosphatidylinositol 3-kinase ■ p70S6K ■ neointimal hyperplasia

Restenosis after percutaneous coronary intervention is a significant clinical problem, occurring after 15% to 30% of angioplasty procedures or intracoronary stenting. Recent evidence has demonstrated that treatment with the immunosuppressive agent rapamycin (also known as sirolimus) dramatically reduces in-stent restenosis. Initial encouraging results in both canine and porcine models have been borne out in human studies in which rapamycin, delivered via drug-coated stents, led to restenosis rates near zero after 4 months and 1 year. Rapamycin acts specifically to inhibit the activity of mTOR (mammalian target of rapamycin), a serine/threonine kinase that acts as a central controller of cell growth. In response to growth factors and amino acids, mTOR has been shown to play a key role in regulating both transcription and translation. Phosphorylation of the translation inhibitor 4EBP1 by mTOR releases it from the translation initiation factor eIF4E to initiate cap-dependent protein translation. Similarly, phosphorylation by mTOR activates the p70 ribosomal protein S6 kinase (p70S6K), resulting in phosphorylation of the 40S ribosomal protein S6 and translation of key components of the cell’s translational apparatus. Although the exact signaling pathways upstream of mTOR are incompletely resolved, substantial evidence indicates that mTOR activation is dependent on phosphatidylinositol (PI) 3-kinase. PI 3-kinase is a lipid kinase that phosphorylates phosphatidylinositol at the D-3 position of the inositol ring. The predominant 3-phosphoinositide lipid products of PI 3-kinase, PI 3,4-bisphosphate and PI 3,4,5-trisphosphate (PIP3), are potent signaling molecules that regulate numerous cellular processes required for neointimal hyperplasia and restenosis, including cell proliferation, migration, and survival. These processes are regulated in turn by several intermediate signaling proteins, including the 3-phosphoinositide-dependent kinase PDK1 and Akt, both of which are recruited to and associate with the plasma membrane-bound 3-phosphoinositides. Notably, both PDK1 and Akt have been implicated in activation of mTOR. When PDK1 was originally identified, it was shown to activate p70S6K, although additional studies have indicated that both Akt and mTOR lie in a signaling pathway between PDK1 and p70S6K.
together, these studies point to the central role of PI 3-kinase and its lipid products in the regulation of mTOR and other key mediators of cell growth, proliferation, and survival. Moreover, they implicate PI 3-kinase as a potential upstream regulator of neointimal hyperplasia.

The potential role of PI 3-kinase as a mediator of restenosis has been addressed only recently. PI 3-kinase and p70S6K were found to upregulate the expression of several cell cycle proteins in coronary smooth muscle cells after both growth factor stimulation and balloon injury, consistent with a role in restenosis. In another study, balloon injury induced Akt phosphorylation and increased cyclin D1 expression, and this effect was blocked by the PI 3-kinase inhibitor wortmannin. In contrast to VSMCs, in tumor cells the effects of PI 3-kinase on cell proliferation and survival have been the subject of intense investigation. Recently, much of this attention has been directed toward the tumor suppressor protein, PTEN (phosphatase and tensin homolog deleted on chromosome 10), which functions as a phosphatidylinositol 3'-phosphatase to hydrolyze the lipid products of PI 3-kinase. Mutations that disrupt PTEN function occur in a high percentage of certain human cancers and result in an increase in 3-phosphoinositides, enhanced signaling through intermediates such as Akt and p70S6K, and enhanced cell growth, proliferation, and survival. In addition to its role in tumor cells, PTEN has recently been found to play a role in growth, proliferation, and survival. Moreover, they implicate PI 3-kinase as a potential upstream regulator of neointimal hyperplasia.

In VSMCs, PTEN regulates many of the angiogenic responses of endothelial cells. Because the phospholipid products of PI 3-kinase appear to regulate many of the cellular processes critical for the development of neointimal hyperplasia and restenosis, we hypothesized that PTEN would regulate these same processes in cultured vascular smooth muscle cells (VSMCs). In this report, we demonstrate that PTEN is expressed endogenously in VSMCs and that overexpression of PTEN with a recombinant adenovirus inhibits growth factor–induced activation of both Akt and p70S6K and induces downregulation of focal adhesion kinase (FAK). In addition, overexpression of PTEN inhibits growth factor–induced proliferation, migration, and survival of VSMCs. Taken together, these findings suggest that PTEN overexpression by using gene therapy approaches may be useful for the treatment of restenosis.

### Methods

#### Reagents

Anti-PTEN monoclonal antibody (clone A2B1) was from Santa Cruz Biotechnology. Anti-Akt, anti-phospho-Akt (Ser473), anti-phosphop44/42 ERK (Thr202/Tyr204), and anti-phospho-p70S6K were from Cell Signaling Technologies. Rat monoclonal anti-α-tubulin (clone YL1/2) was from Harlan Bioproducts. Mouse monoclonal anti-FAK was from BD Transduction Laboratories. Recombinant platelet-derived growth factor (PDGF-BB) was from R&D Systems. PD98059 and wortmannin were from Sigma Chemical Company.

#### Cell Culture

Rabbit VSMCs were obtained from Dr. Karsten Peppel (Duke University Medical Center) and used between passages 4 and 8. VSMCs were maintained at 37°C, 5% CO2 in 50% DMEM, 50% F-12 HAM (Life Technologies) containing 10% FBS (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Life Technologies).

#### Adenovirus Construction

A recombinant, replication-deficient adenovirus directing the expression of wild-type human PTEN (AdPTEN) has been described previously. A control, empty adenovirus containing no cDNA insert (AdEV) was obtained from Dr. Walter Koch (Duke University Medical Center). Adenoviruses were amplified in HEK-293 cells and purified by ultracentrifugation on a CsCl density gradient as described.

#### Adenovirus Infection

VSMCs were grown in wells of 6-well plates in DMEM/F-12 HAM containing 10% FBS. When the cells were nearly confluent, the medium was changed to DMEM/F-12 HAM containing 2% FBS, and viruses were added to the medium at a multiplicity of infection of 100. After 18 hours, the medium was changed to serum-free DMEM/F-12 HAM followed by different treatments or stimuli as indicated. As a control in all experiments, an identical group of cells was left uninfected but incubated 18 hours in DMEM/F-12 HAM containing 2% FBS.

#### Western Blotting

Following overnight infection with adenoviruses, VSMCs were serum-starved in DMEM/F-12 HAM for 5 hours then stimulated either 10 minutes or 1 hour with PDGF-BB (20 ng/mL). In some experiments, cells were treated for 15 minutes with PD98059 or wortmannin at the indicated concentrations or with an equivalent volume of dimethyl sulfoxide (DMSO). Cells were lysed in Triton X-100 and samples were separated by SDS 8% or 16% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Membranes were Western blotted with the indicated antibodies. Typically, blots were stripped and reprobed with additional antibodies as indicated.

#### Thymidine Incorporation and Cell Counts

To evaluate the effect of PTEN overexpression on PDGF-BB–mediated DNA synthesis in VSMCs, [3H]thymidine incorporation was assayed essentially as described previously. Briefly, VSMCs were either left uninfected or were infected with AdPTEN or AdEV as described above. The following day, cells were trypsinized and plated in triplicate wells of a 24-well plate at 25 000 cells per well. After 24 hours in DMEM/F-12 HAM containing 10% FBS, the cells were quiesced in serum-free DMEM/F-12 HAM and incubated for another 24 hours. The medium was replaced with fresh serum-free DMEM/F-12 HAM with or without PDGF (20 ng/mL), and the cells were incubated an additional 18 hours. The cells were pulse-labeled with [3H]thymidine (2 μCi/mL, Amersham-Pharmacia Biotech) for 3 hours, and thymidine incorporation was assessed by liquid scintillation counting.

For cell counts, VSMCs were plated in triplicate wells of a 24-well plate and either left uninfected or infected overnight with AdPTEN or AdEV. The medium was changed to serum-free DMEM/F-12 HAM, and the cells were treated 48 hours with or without PDGF (20 ng/mL) with one change of fresh medium and PDGF after 24 hours. The cells were then trypsinized and counted on a hemacytometer (Fisher) with an Olympus CK2 inverted microscope.

#### Cell Migration Analysis

The rate of migration of VSMCs was determined by using a scratch wound assay as described previously. Briefly, VSMCs were grown in 60-mm dishes until confluent then either left uninfected or infected with AdPTEN or AdEV for 18 hours. The cell monolayer was disrupted with a sterile rubber policeman to create a cell-free
PTEN Overexpression Inhibits Activation of p70\(^{60k}\) but Not ERK

We first evaluated the effects of PTEN overexpression on PI 3-kinase-mediated signaling pathways that have been shown to play roles in VSMC proliferation. Primary rabbit VSMCs were infected with a recombinant adenovirus expressing wild-type PTEN (AdPTEN) or an identical empty adenovirus lacking a cDNA insert as a control (AdEV). As an additional control, uninfected cells were evaluated in an identical manner. Cells were then treated with or without PDGF to induce cellular proliferation. PTEN was expressed endogenously in rabbit VSMCs, and AdPTEN induced appreciable overexpression (Figure 1). PDGF treatment of infected or AdEV-infected rabbit VSMCs increased phosphorylation of p70\(^{60k}\), ERK1, and ERK2. Phosphorylation of these proteins correlates with activation of their respective kinases. Overexpression of PTEN inhibited PDGF-mediated p70\(^{60k}\) phosphorylation but not ERK phosphorylation, suggesting that PTEN overexpression could have effects on VSMC growth and proliferation by inhibiting p70\(^{60k}\) activation.

PTEN Blocks Akt-Mediated Survival Signaling and Downregulates FAK Expression

Activation of Akt by PI 3-kinase initiates a potent survival signaling cascade, and PDGF is known to activate this pathway. Treatment of uninfected or AdEV-infected VSMCs with PDGF induced the phosphorylation of Akt, which correlates with Akt activation (Figure 2). Once activated, Akt in turn phosphorylates and inactivates several downstream targets that induce apoptosis, including glycogen synthase kinase (GSK)3-α and -β. PDGF treatment of control cells resulted in phosphorylation of GSK3-α and -β. Overexpression of PTEN in VSMCs blocked PDGF-induced phosphorylation of Akt and GSK3-α and -β. Moreover, PTEN overexpression induced cleavage of caspase-3, a marker of apoptosis, in both the basal state and after treatment with PDGF. These findings indicate that PTEN overexpression induces apoptosis in both resting and PDGF-treated VSMCs. Because the PI 3-kinase pathway also regulates cell migration,\(^{13,30,31}\) we investigated one protein involved in this pathway, FAK.\(^{32–34}\) Whereas PDGF induced an increase in FAK expression, overexpression of PTEN markedly downregulated expression of FAK in both the presence and absence of PDGF (Figure 2), suggesting that PTEN could also have effects on cell migration.

PTEN Does Not Affect ERK Activation in Rabbit VSMCs

To confirm that PTEN overexpression had no effect on ERK activation in our system, we performed a side-by-side comparison of PDGF activation of ERK and Akt after pretreatment with known pharmacological inhibitors of mitogen activated protein kinase kinase (MEK) and PI 3-kinase and overexpression of PTEN. PDGF again activated both ERK
and Akt, and PTEN inhibited phosphorylation of Akt but not ERK (Figure 3). At the same time, the MEK inhibitor PD98059 blocked activation of ERK but not Akt. In contrast, the PI 3-kinase inhibitor wortmannin blocked activation of Akt but not ERK. These findings confirm that PI 3-kinase is not required for ERK activation in the rabbit VSMCs used here, and that any effects of PTEN on VSMC proliferation should not be due to effects on ERK.

PTEN Inhibits VSMC Proliferation

We next investigated whether the effects of PTEN on PI 3-kinase-mediated signaling pathways correlated with effects on physiological responses relevant to restenosis and neointimal hyperplasia. Primary rabbit VSMCs were infected with AdPTEN, AdEV, or left uninfected, and PDGF-mediated DNA synthesis was measured by [3H]thymidine incorporation. PDGF induced significant increases in DNA synthesis in uninfected and AdEV-infected VSMCs (Figure 4A). PTEN overexpression significantly decreased basal thymidine incorporation. Moreover, PTEN completely blocked the PDGF-induced increase in DNA synthesis.

To confirm these findings, cell counts were performed on adenovirus-infected and uninfected VSMCs treated with or without PDGF. As in the thymidine incorporation experiments, PDGF induced a significant increase in the number of uninfected or AdEV-infected cells (Figure 4B). PTEN overexpression significantly decreased the number of untreated VSMCs compared with untreated control cells, suggesting that PTEN may enhance cell death. This finding is consistent with an increase in caspase-3 cleavage in PTEN-overexpressing cells (Figure 2). PTEN overexpression inhibited the PDGF-induced increase in cell number, and the number of AdPTEN-infected cells in the PDGF-treated group was still significantly less than in the control groups that were not treated with PDGF ($P<0.01$ for both). Together, these findings demonstrate that PTEN overexpression blocks PDGF-mediated increases in VSMC proliferation, possibly by enhancing apoptosis.

PTEN Inhibits VSMC Migration

VSMC migration is an important component of neointimal hyperplasia, and PDGF induces cell migration through PI 3-kinase-mediated pathways. To investigate whether PTEN alters VSMC migration, we measured the migration of adenovirus-infected or uninfected VSMCs after disruption of the cell monolayer. PDGF treatment significantly increased the migration of VSMCs into the wound (Figure 5A). In contrast, PTEN overexpression abrogated both basal and PDGF-induced VSMC migration (Figure 5).

PTEN Enhances VSMC Apoptosis

Overexpression of PTEN was found by Western blotting to block signaling through PI 3-kinase/Akt and to enhance caspase-3 cleavage in VSMCs. To quantify this effect, we analyzed histone-associated DNA fragmentation in serum-starved VSMCs as a measure of apoptosis. Serum starvation of uninfected or AdEV-infected VSMCs induced very little cell death over 5 hours, but PDGF significantly reduced DNA fragmentation in AdEV-infected cells (Figure 6). After overexpression of PTEN, however, a significant increase in DNA fragmentation was observed, and this effect was not significantly reduced by treatment with PDGF. These findings indicate that PTEN induces cell death in VSMCs by inhibiting both basal and growth factor-induced cell survival pathways.

**Figure 3.** PI 3-kinase is not required for PDGF-mediated ERK activation in rabbit VSMCs. Uninfected or AdPTEN-infected VSMCs were pretreated for 15 minutes with DMSO or the indicated pharmacological inhibitors then stimulated with PDGF (20 ng/mL) for 10 minutes. Cell lysates were Western blotted with the indicated antibodies. Equal protein loading was confirmed by blotting with an antibody against α-tubulin.

**Figure 4.** PTEN inhibits VSMC proliferation. A, VSMCs infected were either uninfected (Un) or infected with AdPTEN or AdEV, quiesced in serum-free medium for 24 hours, treated with or without PDGF (20 ng/mL) for 18 hours, then pulse-labeled with [3H]thymidine. Thymidine incorporation was assessed by scintillation counting of precipitated DNA. *$P<0.005$ for −PDGF vs +PDGF; #$P<0.01$ vs Un or EV−PDGF; Δ$P<0.05$ vs Un or EV+PDGF. B, VSMCs were treated as in A except cells were grown 48 hours in the absence or presence of PDGF (10 ng/mL); then cells were trypsinized and counted. *$P<0.01$ for −PDGF vs +PDGF; #$P<0.005$ vs Un or EV−PDGF; Δ$P<0.0005$ vs Un or EV+PDGF.
Discussion

PI 3-kinase and its downstream effectors regulate an array of cellular processes, including proliferation, migration, and survival, which are essential for neointimal hyperplasia and restenosis. Inhibition of one of these effectors, mTOR, dramatically reduces in-stent restenosis. Activation of PI 3-kinase effectors is dependent on its 3-phosphoinositide products. As a result, hydrolysis of these phospholipids would be expected to inhibit the cellular processes required for neointimal hyperplasia and restenosis. In this report, we addressed this hypothesis in cultured VSMCs after adenovirus-mediated overexpression of PTEN, an inositol phosphatase specific for PI(3,4)P_2 and PIP_3. PTEN was expressed endogenously in VSMCs, and its overexpression led to inhibition of at least two effectors of PI 3-kinase, p70s6k and Akt. Moreover, PTEN overexpression significantly inhibited both basal and PDGF-induced VSMC proliferation, migration, and survival. Together, these findings suggest that PTEN overexpression has potential for the prevention of neointimal hyperplasia after percutaneous coronary intervention or other vascular interventions, such as coronary artery bypass grafting.

PI 3-kinase signaling has been linked to cellular proliferation in a variety of cell types, and this has been correlated with activation of both ERK and p70s6k. A large number of studies have investigated the interactions between PI 3-kinase and ERK. PI 3-kinase has been shown to be either upstream or downstream of ERK and to be activated as part of a parallel or independent pathway. In our study, overexpression of PTEN, which disrupts all 3-phosphoinositide-dependent signaling, inhibited activation of p70s6k and Akt but not ERK. This finding is consistent with our previous results in endothelial cells, in which PTEN overexpression did not alter ERK activation. To confirm that PTEN had no effect on ERK activation, we performed a side-by-side comparison of PTEN overexpression and pharmacological inhibitors of PI 3-kinase and ERK. Like PTEN, the PI 3-kinase inhibitor wortmannin inhibited Akt but not ERK, while the MEK inhibitor PD98059 blocked only ERK and not Akt. These findings demonstrate that PTEN, and thus PI 3-kinase, is not upstream of ERK in the rabbit VSMCs used here.

Interestingly, arterial balloon injury has been shown to activate p70s6k and Akt as well as ERK, but in one study, pharmacological inhibition of ERK did not affect late injury-induced intimal SMC replication. In contrast, rapamycin appears to have effects on both early and late VSMC replication. When considered together with our results, these findings suggest that ERK and p70s6k differentially regulate VSMC proliferation and that inhibition of cell proliferation by PTEN in our study was likely due to inhibition of p70s6k and not ERK.
It is likely that inhibition of VSMC proliferation by PTEN was mediated in part by an increase in apoptosis, because the number of PTEN-expressing cells was significantly less than the number of control cells. Importantly, enhanced VSMC apoptosis was mediated specifically by PTEN and was not due to virus effects, because the response of control virus-infected cells was nearly identical to that of uninfected cells, which displayed little cell death. Enhanced apoptosis could also partly account for PTEN’s inhibition of cell migration. However, most of the PTEN-overexpressing cells were still viable in our migration assays (Figure 5B), so increased cell death cannot entirely explain this effect. One potential mechanism for this finding is the downregulation of FAK, which is known to play a role in PI 3-kinase–mediated cell migration.31–34 Because PI 3-kinase-mediated cell migration has also been linked to Rac,45 further studies will be required to clarify the roles of PTEN and PI 3-kinase in VSMC migration that occurs during neointimal hyperplasia.

In our studies, we evaluated apoptosis after serum starvation of VSMCs. Although this stimulus induced very little cell death in control cells, PTEN-expressing cells displayed dramatically higher levels of apoptosis, even when pretreated with PDGF. This finding indicates that PDGF-induced survival signaling in VSMCs is mediated primarily by PI 3-kinase, and likely by Akt. Interestingly, PTEN overexpression had significant effects on basal levels of proliferation and migration as well as survival. Some degree of basal signaling occurs in most cell types through autocrine pathways. As might be expected, an important component of this basal signaling appears to be mediated through PI 3-kinase because it was inhibited by PTEN.

The effects of PTEN on responses of VSMCs were evaluated in the absence and presence of PDGF. Along with other smooth muscle growth factors, PDGF is released after arterial injury and contributes to neointimal hyperplasia.46–49 PDGF is a potent VSMC mitogen, chemoattractant, and survival factor.50,51 In addition to PDGF, a number of other growth factors have been shown to regulate neointimal hyperplasia, including basic fibroblast growth factor, transforming growth factors, PDGF is released after arterial injury and contributes to neointimal hyperplasia.

Not only is PI 3-kinase activated by numerous cell receptors, but it is also expressed in nearly all cell types. As a result, systemic inhibition of PI 3-kinase signaling would likely result in significant toxicity. The signaling pathways inhibited by rapamycin are also broadly expressed, yet the recent studies with rapamycin-coated stents support the idea that local delivery to the vasculature is not only effective, but it also averts the toxicity associated with systemic delivery of this agent. By targeting the phospholipid products of PI 3-kinase, PTEN is proximal to PDK1, mTOR, Akt, and p70S6K in these PI 3-kinase–signaling cascades.22 As such, PTEN overexpression should inhibit the entire spectrum of downstream PI 3-kinase effectors, which may have more potent inhibitory effects on neointimal hyperplasia and restenosis than rapamycin does. In summary, our findings suggest that local delivery of AdPTEN holds great potential for the treatment of vascular proliferative disorders, and studies are currently underway in our laboratory to test this possibility.

Acknowledgments

This work was supported in part by a grant-in-aid from the Mid-Atlantic Affiliate of the American Heart Association (0051276U), a career development award from the National Institutes of Health (HL 03557), and a grant from the Proctor & Gamble Health Care Research Center.

References


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