Phosphatidylinositol 3-kinase is activated by vascular endothelial growth factor (VEGF), and many of the angiogenic cellular responses of VEGF are regulated by the lipid products of phosphatidylinositol 3-kinase. The tumor suppressor PTEN has been shown to down-regulate phosphatidylinositol 3-kinase signaling, yet the effects of PTEN on VEGF-mediated signaling and angiogenesis are unknown. Inhibition of endogenous PTEN in cultured endothelial cells by adenovirus-mediated overexpression of a dominant negative PTEN mutant (PTEN-C/S) enhanced VEGF-mediated Akt phosphorylation, and this effect correlated with decreases in caspase-3 cleavage, caspase-3 activity, and DNA degradation after induction of apoptosis with tumor necrosis factor-α. Overexpression of PTEN-C/S also enhanced VEGF-mediated endothelial cell proliferation and migration. In contrast, overexpression of wild-type PTEN inhibited the anti-apoptotic, proliferative, and chemotactic effects of VEGF. Moreover, PTEN-C/S increased the length of vascular sprouts in the rat aortic ring assay and modulated VEGF-mediated tube formation in an in vitro angiogenesis assay, whereas PTEN-wild type inhibited these effects. Taken together, these findings demonstrate that PTEN potently modulates VEGF-mediated signaling and function and that PTEN is a viable target in therapeutic approaches to promote or inhibit angiogenesis.

Vascular endothelial growth factor (VEGF) plays a key role in endothelial cell differentiation (vasculogenesis) and the sprouting of new blood vessels from preexisting ones (angiogenesis). Angiogenesis is critical for normal embryonic vascular development as well as a number of physiological and pathological conditions, including ischemic diseases, inflammation, and cancer. Binding of VEGF to VEGF receptor-2 (VEGFR-2) leads to receptor phosphorylation and subsequent activation of phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ1, Src family tyrosine kinases, and other signaling proteins (1–4). The key role of PI3K in VEGF-mediated signal transduction and angiogenic responses is well established (2, 5, 6). Experimental evidence has shown that activation of PI3K is critical for VEGF-mediated endothelial cell proliferation, survival, and migration. Moreover, downstream activation of Akt by PI3K is responsible for phosphorylation and activation of endothelial nitric oxide synthase (eNOS) by VEGF (6, 7).

PI3K catalyzes the phosphorylation of inositol phospholipids at the D3 position to generate phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate. These 3-phosphoinositides act as potent signaling molecules to regulate many cellular responses that are important for angiogenesis, including cell adhesion, proliferation, vesicular trafficking, protein synthesis, and cellular survival (8–11). Recent studies demonstrated that the 3-phosphoinositides are important substrates for PTEN (phosphatase and tensin homology deleted from chromosome 10) both in vitro (12) and in vivo (13, 14). PTEN was originally identified as a candidate tumor suppressor gene based on its high frequency of mutation in a variety of tumors (15). PTEN was subsequently found to exhibit both protein tyrosine phosphatase and inositol 3'-phosphatase activity. However, its lipid phosphatase activity appears to be primarily responsible for its tumor suppressor effects, as mutation or loss of PTEN results in increased 3-phosphoinositides and downstream activation of Akt (13, 16–18). Thus, PTEN functions in opposition to PI3K, and numerous studies have shown that overexpression of wild-type PTEN suppresses cell growth and proliferation through G1 cell cycle arrest and enhances apoptosis by down-regulating PI3K/Akt signaling (16, 18–23).

Most studies of PTEN have focused on its role in tumor cell biology; however, a recent report demonstrated that PTEN could modulate the response of cardiac myocytes to PI3K activation and thereby regulate myocyte survival and hypertrophy (24). Based on the roles of PI3K and Akt in signaling by VEGF, we hypothesized that PTEN could regulate VEGF-mediated endothelial cellular responses and angiogenesis. In this report, we demonstrate that inhibition of endogenous endothelial PTEN by adenovirus-mediated overexpression of a dominant negative PTEN mutant in cultured endothelial cells potently enhances a variety of VEGF-mediated cellular responses, including cell survival, mitogenesis, and migration. In contrast, these effects of VEGF are significantly inhibited by overexpression of wild-type PTEN. Moreover, overexpression of wild-type or dominant negative PTEN modulated endothelial tube formation in vitro and vascular sprouting in an ex vivo model of angiogenesis.
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EXPERIMENTAL PROCEDURES

Reagents—Anti-PTEN monoclonal antibody (clone A2B1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, anti-phasopho-Akt (Ser-473), anti-phasopho-p44/42 mitogen-activated protein (MAP) kinase (Thr-202/Tyr-204), and anti-phasopho-p44/42 MAP kinase polyclonal antibodies were purchased from New England Biolabs (Beverly, MA). Anti-cleaved caspase-3 (D175) was from Cell Signaling Technology (Beverly, MA). Recombinant human VEGF	extsubscript{165} and tumor necrosis factor-α (TNFα) were purchased from R&D Systems (Minneapolis, MN).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics Corp. (San Diego, CA) and were used between passages 4 and 8. HUVECs were grown on 0.1% gelatin-coated (Sigma) plates in endothelial growth medium (EGM, Clonetics Corp.) in a 37 °C, 5% CO\textsubscript{2} incubator. EA.hy926 (25) and Py-4-1 (26) endothelial cells were gifts from Dr. Cora-Jean Edgell and Dr. Victoria Bautch, respectively (University of North Carolina, Chapel Hill, NC). EC-RF24 cells were provided by Dr. Hans Pannekoek (University of Amsterdam, The Netherlands) (27). NIH 3T3 cells and human embryonic kidney 293 cells were from American Type Culture Collection. 3T3 cells expressing fms-Tie2 have been described (30).

Adenovirus Construction—cDNAs encoding wild-type (WT) and catallytically inactive PTEN, in which cysteine 124 has been mutated to serine (CS), were generously provided by Dr. Charles Sawyers (University of California, Los Angeles). To generate adenoviruses directing the expression of these proteins, cDNAs encoding PTEN were subcloned into pShuttle-CMV then recombined with pAdEasy-1 by electroporation into BJ5183 Echerichia coli (Stratagene, La Jolla, CA) (29). The recombinant phagemid vector DNA was transfected into human embryonic kidney cells. Budding and purification of the recombinant adenoviral vector DNA was transfected into human embryonic kidney cells and EUVECs were serially amplified in 293 cells, purified on a CsCl density gradient, and titered as described previously (30). A control adenovirus consisting of the identical adenovirus backbone without a DNA insert ("empty virus," AdEV) was provided by Dr. Walter J. Koch (Duke University Medical Center, Durham, NC) (31).

For most experiments, HUVECs were grown in EGM-MV (containing 5% FBS, Clonetics Corp.), and the cells were treated as indicated.

Western Blotting—Cells were lysed in Triton lysis buffer (137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0) containing protease inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μM pepstatin). An aliquot of each lysate was separated by PAGE and an aliquot of each cell lysate was examined daily on an Olympus IX-70 microscope (100×) (Olympus, Tokyo, Japan) then incubated at 37 °C until the Matrigel was dried. The greatest distance from the aortic ring body to the end of the vascular sprouts was measured at three distinct points per ring using Image J software (5 × 1011 viral particles of AdPTEN-WT, AdPTEN-CS, or AdEV in 1.0 ml of DMEM, 200 μl of Matrigel) then incubated at 37 °C until the Matrigel polymerized. The wells were then overlaid with 0.5 ml of EB medium containing 2% FBS and 5% CO\textsubscript{2} for 3 h. The rings were maintained at 37 °C for up to 10 days with medium changes every 2 days. Vascular sprouting from each ring was examined daily on an Olympus IX-70 microscope (100× magnification), and digital images were obtained. Quantitative analysis of endothelial sprouting was performed using images from day 5, and sprout length was quantified in NIH Image (v.1.62) using a calibrated micrometer. The greatest distance from the aortic ring body to the end of the vascular sprouts was measured at three distinct points per ring and in three different rings per treatment group.

Statistical Analysis—All results were expressed as the mean ± S.E. Statistical analysis was performed using the one-tailed Student’s t test (two sample, unequal variance), and p < 0.05 was considered statistically significant.

RESULTS

PTEN Is Expressed in Endothelial Cells—We first analyzed whether PTEN is expressed in endothelial cells. Lysates from several different immortalized endothelial cell lines as well as primary HUVECs and NIH 3T3 fibroblasts were analyzed by Western blotting with an antibody against PTEN. PTEN was expressed to varying degrees in both EA.hy926 cells (25) and HUVECs, but it was not detectable in EC-RF24 cells (27).
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1) PTEN was highly expressed in Py-4-1 cells, a murine microvascular endothelial cell line (26), as well as in parental NIH 3T3 cells and 3T3 cells stably expressing chimeric fms-Tie1 or fms-Tie2 receptors (28). The expression of PTEN in endothelial cells indicates that this protein could potentially modulate VEGF-mediated signaling.

Overexpression of PTEN Modulates VEGF-mediated Akt Phosphorylation—VEGF is known to activate the PI3K/Akt pathway (5); therefore, we investigated whether PTEN overexpression could alter VEGF-mediated Akt phosphorylation. Primary HUVECs were either left uninfected or were infected with recombinant adenoviruses encoding wild-type PTEN (AdPTEN-WT), a catalytically inactive mutant of PTEN in which cysteine 124 has been mutated to serine (AdPTEN-C/S), or an empty adenovirus as a control for viral infection (AdEV). After overnight virus infection, the cells were serum-starved for 6 h and then treated with or without VEGF. Endogenous PTEN was detectable at moderate levels in both uninfected and AdEV-infected HUVECs, whereas both wild-type and inactive PTEN were overexpressed after virus infection (Fig. 2). VEGF treatment increased Akt phosphorylation in uninfected and AdEV-infected cells. Importantly, overexpression of PTEN-WT abrogated this effect, whereas PTEN-C/S enhanced phosphorylation of Akt. Similar amounts of total Akt and tubulin were observed in each lane, demonstrating that the effects on Akt phosphorylation were a result of the PTEN proteins themselves. VEGF treatment also enhanced the phosphorylation of ERK1 and -2, but this was not altered by overexpression of either wild-type or inactive PTEN (Fig. 2), suggesting that VEGF-mediated ERK activation is not dependent on the production of 3-phosphoinositides.

PTEN Modulates VEGF-mediated Anti-apoptosis—Akt is known to play a critical role in cell survival mediated by VEGF (5). To determine whether the effects of PTEN on VEGF-mediated Akt phosphorylation in HUVECs correlated with the effects on apoptosis, we evaluated caspase-3 cleavage in HUVECs induced to undergo apoptosis. Caspase-3 is a key mediator of apoptosis, and cleavage of this enzyme to its active form correlates with the onset of apoptosis. HUVECs were infected with PTEN or control viruses, and the cells were serum-starved and treated with TNFα, which has been shown to induce endothelial cell apoptosis (38). In lysates from uninfected cells and those infected with AdPTEN-WT or AdEV, VEGF treatment blocked the TNFα-induced cleavage of caspase-3 (Fig. 3). Overexpression of PTEN-WT increased basal caspase-3 cleavage and appeared to reduce the protective effect of VEGF in these cells. In contrast, essentially no cleaved caspase-3 was detectable in cells expressing PTEN-C/S either with or without VEGF treatment. These findings indicate that overexpression of PTEN-WT enhances endothelial cell apoptosis, whereas inhibition of PTEN is protective.

To confirm the effects of PTEN overexpression on endothelial cell apoptosis, we assayed both caspase-3 activity and DNA fragmentation. HUVECs were infected with recombinant adenoviruses, and apoptosis was induced by serum starvation and TNFα treatment. Cell lysates were then used in a fluorimetric assay of caspase-3 activity. Consistent with the Western blotting results, VEGF treatment reduced caspase-3 activity in all cells, including those infected with AdPTEN-C/S (Fig. 4A). However, overexpression of PTEN-WT significantly increased, whereas PTEN-C/S significantly decreased caspase-3 activity compared with control cells, both with and without VEGF treatment. Similar results were obtained using a spectrophotometric assay of histone-associated DNA fragmentation, although the effects of PTEN-WT overexpression were not as pronounced (Fig. 4B). Taken together, these findings demonstrate that PTEN modulates the anti-apoptotic effects of VEGF.

PTEN Inhibits VEGF-mediated Proliferation—Although PTEN did not appear to alter VEGF-mediated MAP kinase activation, we evaluated the effects of wild-type and inactive PTEN on VEGF-mediated endothelial cell proliferation. HUVECs were either uninfected or were infected with AdPTEN-WT, AdPTEN-C/S, or AdEV, then DNA synthesis was assayed by [3H]thymidine incorporation. As expected, VEGF induced an increase in DNA synthesis in both uninfected and empty virus-infected HUVECs (Fig. 5A). Overexpression of PTEN-C/S resulted in a significant increase in VEGF-stimu-
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Fig. 4. Overexpression of wild-type or catalytically inactive PTEN modulates apoptosis. Apoptosis was induced in adenovirus-infected HUVECs by serum starvation and treatment with TNFα, and cells were treated with or without VEGF (50 ng/ml). Cell lysates were used in assays of caspase-3 activity (A) and histone-associated DNA fragmentation (B). WT PTEN significantly increased apoptosis in both assays, whereas catalytically inactive PTEN (C/S) significantly reduced apoptosis both in the absence and presence of VEGF. Data are expressed as the means ± S.E. Un, uninfected cells; EV, empty virus-infected cells; *, p < 0.05 versus AdEV-infected cells for –VEGF group; Δ, p < 0.05 versus AdEV-infected cells for +VEGF group.

Fig. 5. PTEN overexpression alters VEGF-mediated endothelial cell proliferation. A, adenovirus-infected HUVECs were plated in 24-well plates, quiesced in serum-free medium, then treated with or without VEGF. DNA synthesis was determined by incorporation of [3H]thymidine. Compared with uninfected (Un) or empty virus-infected (EV) cells, WT PTEN significantly reduced VEGF-mediated thymidine incorporation, whereas catalytically inactive PTEN (C/S) significantly enhanced it. Data are expressed as the means ± S.E. Δ, p < 0.05 versus AdEV-infected cells. B, adenovirus-infected or uninfected HUVECs were plated in 24-well plates and treated with or without VEGF for 48 h. Cells were trypsinized and counted. Cell numbers are expressed as the means ± S.E. of triplicate wells. *, p < 0.05 versus AdEV-infected cells for –VEGF group; Δ, p < 0.05 versus AdEV-infected cells for +VEGF group.

PTEN Modulates Tube Formation and Vascular Sprouting—Based on the effects of wild-type and catalytically inactive PTEN on HUVEC survival, proliferation, and migration, we next examined whether overexpression of PTEN would alter the endothelial tube-forming activity of HUVECs, often referred to as an in vitro angiogenesis assay (34–36). To do this, HUVECs were infected with PTEN-expressing adenoviruses and then plated on Matrigel and treated with or without VEGF, which is known to induce capillary morphogenesis (1). Within 24 h, uninfected and empty virus-infected cells formed an organized network of endothelial tubes, and this effect was enhanced by treatment with VEGF (Fig. 7, A and D). In contrast, PTEN-WT overexpression markedly inhibited tube formation (Fig. 7B) both in the presence and absence of VEGF.

PTEN Alters VEGF-mediated Endothelial Cell Migration—The lipid products of PI3K are known to regulate cell migration and chemotaxis (10, 11). To investigate whether PTEN overexpression modulates the effects of VEGF on endothelial cell migration, we performed a scratch wound assay (6, 32) on cultured HUVECs that were either uninfected or infected with the different recombinant adenoviruses used previously. Consistent with published results (1), VEGF enhanced the migration of HUVECs into the cell-free zone after wounding (Fig. 6). Overexpression of PTEN-WT significantly reduced HUVEC migration both at baseline and after VEGF treatment. In contrast, PTEN-C/S significantly enhanced VEGF-mediated cell migration, although it had no appreciable effect on basal migration. As seen in the thymidine incorporation experiment, numerous PTEN-WT-infected cells appeared apoptotic, suggesting that the adverse effects of PTEN-WT on migration could be due in part to enhanced cell death.

PTEN Alters VEGF-mediated Endothelial Cell Proliferation—To confirm the results of thymidine incorporation assays, we performed cell counts on uninfected HUVECs and on those infected with the PTEN or control adenoviruses. Cells were treated for 48 h with or without VEGF, then trypsinized and counted in triplicate. In all groups, VEGF induced an increase in cell number compared with untreated cells. As with changes in DNA synthesis, no differences were noted between uninfected and empty virus-infected cells (*, p < 0.05 versus AdEV-infected cells for –VEGF group; Δ, p < 0.05 versus AdEV-infected cells for +VEGF group) consistent with our earlier results demonstrating that PTEN-WT enhanced endothelial cell apoptosis.

To confirm the results of thymidine incorporation assays, we performed cell counts on uninfected HUVECs and on those infected with the PTEN or control adenoviruses. Cells were treated for 48 h with or without VEGF, then trypsinized and counted in triplicate. In all groups, VEGF induced an increase in cell number compared with untreated cells. As with changes in DNA synthesis, no differences were noted between uninfected and empty virus-infected cells (*, p < 0.05 versus AdEV-infected cells for –VEGF group; Δ, p < 0.05 versus AdEV-infected cells for +VEGF group) consistent with our earlier results demonstrating that PTEN-WT enhanced endothelial cell apoptosis.
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Fig. 6. PTEN modulates VEGF-mediated endothelial cell migration. Adenovirus-infected HUVECs were plated in 6-well plates, and cell migration was quantified by the scratch method, as described under “Experimental Procedures.” Overexpression of PTEN-WT significantly reduced HUVEC migration in the absence or presence of VEGF. In contrast, inactive PTEN (C/S) enhanced VEGF-mediated endothelial cell migration. Data are expressed as the means ± S.E. Un, uninfected cells; EV, empty virus-infected cells; C/S, empty virus-infected cells plus 5 μg/ml of AdPTEN-C/S for the −VEGF group, δ, p < 0.05 versus AdEV-infected cells for the +VEGF group.

Nuclear staining of PTEN-WT-infected cells with Hoechst 33342 demonstrated nuclear condensation of many cells, consistent with enhanced apoptosis (data not shown). HUVECs infected with AdPTEN-C/S formed tubes earlier than uninfected or AdEV-infected cells, as capillary networks were observed as early as 6 h after plating on Matrigel (data not shown). Interestingly, by the following day PTEN-C/S expression had induced a dramatic change in the morphology of the endothelial networks (Fig. 7C). Although the number of tubes was essentially unchanged, the total number of endothelial cells in each field was markedly increased. Capillary networks were surrounded by clusters of viable endothelial cells that failed to properly assemble into tubes.

We next evaluated the effects of the PTEN adenoviruses in the rat aortic ring assay, an ex vivo assay of vascular sprouting that depends on the function of a variety of angiogenic growth factors and their receptors (37). Sections of rat aorta were infected with the different adenoviruses and then cultured in growth factor-reduced Matrigel, and the length of vascular sprouts was measured on day 5. Compared with uninfected or empty virus-infected rings, PTEN-WT induced a slight but statistically insignificant reduction in the length of vascular sprouts (Fig. 8). In contrast, sprouts from PTEN-C/S-infected rings were 2-3-fold longer than those in control rings. Taken together with the tube formation assays, these results indicate that PTEN has a potent inhibitory effect on angiogenesis.

**DISCUSSION**

Angiogenesis is a complex process mediated by several endothelial receptor tyrosine kinases and their ligands. These receptor-ligand systems regulate diverse endothelial cellular functions, including endothelial cell proliferation, migration, capillary morphogenesis, and survival, which are necessary for proper vascular development. VEGF/VEGF receptor signaling is known to regulate each of these processes (1), and many of them have been linked to PI3K signaling (2, 39, 40). Thus, the phosphoinositol second messengers generated by PI3K provide a common mechanism for multiple steps during angiogenesis. In this report, we have demonstrated that the inositol 3'-phosphatase PTEN, which hydrolyzes the PI3K lipid products phosphatidylinositol 3,4,5-trisphosphate, modulates VEGF-mediated signaling and cellular responses in cultured endothelial cells. Furthermore, PTEN altered capillary morphogenesis in an *in vitro* angiogenesis model as well as vascular sprouting in an *ex vivo* tissue model of angiogenesis.

PTEN was identified originally as a tumor suppressor protein (15) and, as a result, its effects have been studied primarily in tumor cells. Numerous studies have demonstrated that PTEN suppresses proliferation and survival of tumor cells by inhibiting PI3K/Akt signaling (13, 14, 17, 18, 20, 21, 23, 41). However, PI3K signaling is critical for a variety of cellular functions in diverse cell types (10). It seems likely, therefore, that PTEN should regulate PI3K signaling in other cell types in which it is expressed. In fact, a recent report demonstrated that overexpression of wild-type or catalytically inactive PTEN in cardiac myocytes had profound effects on myocyte hypertrophy and survival (24). Here, by overexpressing a dominant negative PTEN mutant (PTEN-C/S), we were able to demonstrate that inhibition of endogenous PTEN in cultured endothelial cells enhanced VEGF signaling. This finding correlated with the enhancement of a variety of VEGF-mediated cellular responses.

PI3K was shown in one study to regulate VEGF-mediated endothelial cell proliferation, as the PI3K inhibitor wortmannin blocked both ERK phosphorylation and bromodeoxyuridine incorporation after VEGF treatment of HUVECs (2). In contrast to these results, Wu et al. (42) find that high dose wortmannin had no effect on VEGF-mediated ERK phosphorylation. Likewise, in our studies overexpression of PTEN-WT or PTEN-C/S had no effect on ERK phosphorylation, in contrast to the marked effects of both proteins on Akt phosphorylation. These findings indicate that PI3K and its lipid products, the 3-phosphoinositides, are not required for VEGF-mediated ERK activation. However, the effects of both wild-type and inactive PTEN on VEGF-mediated thymidine incorporation and cell counts suggest that a PI3K-dependent, ERK-independent signaling pathway is required for VEGF-mediated cell proliferation. Alternatively, the appreciable effects of PTEN-WT and -C/S on endothelial cell survival could have resulted in significant effects on cell proliferation. The effects of VEGF in both the thymidine incorporation assays and cell counts were performed after quiescence in serum-free medium, which by itself can initiate endothelial cell apoptosis. Thus, VEGF-mediated thymidine incorporation may have been blunted by PTEN-WT and enhanced by PTEN-C/S due to the respective effects of these proteins on cell survival, making it unclear whether the effects on proliferation were primary or secondary. Similarly, although PI3K is known to regulate cell migration and chemotaxis, it is possible that the effects of PTEN on VEGF-mediated cell migration were at least partly due to alterations in cell survival.

It is notable that both wild-type and dominant negative PTEN had significant effects on basal endothelial cell signaling and cellular responses in addition to their effects on VEGF-mediated processes. Based on its ability to hydrolyze the lipid products of PI3K, PTEN would be predicted to play a central role in the regulation of PI3K-mediated processes downstream of a variety of receptors and in virtually any cell type in which it is expressed. For example, PTEN has recently been shown to regulate IGF-1 signaling in cardiac myocytes (24). The fact that inhibition of endothelial PTEN with the dominant negative PTEN-C/S mutant resulted in enhanced basal endothelial signaling supports a key regulatory role for this enzyme in endothelial biology.

Based on the wide range of endothelial cellular effects of PTEN, we anticipated that PTEN overexpression might have significant effects on angiogenesis. Indeed, PTEN-C/S signifi-
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Fig. 7. PTEN modulates endothelial tube formation. HUVECs were either uninfected (A) or infected with PTEN-WT (B), PTEN-C/S (C), or empty virus (D), then plated on growth factor-reduced Matrigel in the wells of a 96-well plate and treated with VEGF (20 ng/ml) for 24 h. Overexpression of wild-type PTEN resulted in decreased tube formation, with numerous apoptotic-appearing cells (arrowheads), as determined by nuclear staining (not shown). In contrast, PTEN-C/S induced a marked increase in the number of endothelial cells in the tubes (arrows), which appeared viable by nuclear staining (not shown). Magnification, ×100.

Fig. 8. PTEN modulates angiogenesis in the rat aortic ring assay. Cross-sections of rat aorta were left uninfected (Un, A) or infected with recombinant adenoviruses encoding WT (B) or catalytically inactive PTEN (C/S, C) or with empty virus (EV, D) then embedded in growth factor-reduced Matrigel. Five days later, vascular sprouting was quantified by digital microscopy as the maximal length of sprouts from the body of the aortic ring at three different points per ring (e.g. arrows) and on three rings per group (E). Data are expressed as the means ± S.E. PTEN-WT significantly reduced sprout length, whereas PTEN-C/S increased it by ~3-fold. *, p < 0.05 versus AdEV-infected cells. Magnification in A–D, ×100.

Significantly altered tube formation and vascular sprouting in two separate angiogenesis assays, whereas PTEN-WT dramatically limited tube formation, an effect that appeared to be largely because of reduced endothelial cell survival. However, the combined effects of PTEN-WT on endothelial cell proliferation, migration, and survival may have resulted in a synergistic effect greater than that of blocking any one cellular response alone. In contrast, overexpression of catalytically inactive PTEN resulted in a striking effect on tube formation that is difficult to explain based on its effects in cellular assays. Although the number of tubes and networks appeared the same as for control cells, the endothelial tubes were comprised of far more endothelial cells, as if cellular proliferation were out of proportion to migration and/or morphogenesis, and these endothelial cells appeared viable, unlike those overexpressing PTEN-WT. Notably this tube formation phenotype resembles that recently observed by Bussolati et al. (43) after the inhibition of VEGFR-1. In that report, VEGFR-1 signaling was found to inhibit VEGFR-2-mediated endothelial cell proliferation via nitric oxide (NO). Inhibition of either VEGFR-1 signaling or NO production by VEGFR-1 resulted in enhanced endothelial cell proliferation, but these cells appeared unable to contribute to normal capillary morphogenesis. It was suggested from these findings that VEGFR-2 primarily regulates endothelial cell proliferation, whereas VEGFR-1 is required for endothelial differentiation. These findings were confirmed in part by Zeng et al. (44), who demonstrated negative regulation of VEGFR-2 by VEGFR-1 that was mediated by PI3K signaling (44). A common thread between these two studies appears to be that PI3K/Akt signaling activates eNOS, resulting in increased nitric oxide release (7, 45). Interestingly, these findings would appear to conflict with our results, which suggest that enhanced Akt activation by PTEN-C/S should result in increased NO production and a resultant inhibition of VEGFR-2-mediated endothelial cell proliferation. Additional studies will be required to determine the effects of PTEN on NO production and the role of NO in the PTEN-C/S-induced phenotype in the tube formation assay.

Our current studies demonstrate that PTEN can significantly modulate angiogenic cellular responses through direct
effects on the endothelium. However, several recent reports have indicated that PTEN expressed in tumor cells can have indirect effects on angiogenesis as well through at least two distinct mechanisms. Expression of VEGF and other angiogenic growth factors is regulated by tissue hypoxia via the hypoxia-inducible factor-1 (HIF-1) transcription factor complex (1). Loss of PTEN in glioblastoma cells has been correlated with increased stabilization of HIF-1α and resultant increases in VEGF expression (46). In another study, reconstitution of wild-type PTEN expression in U87MG glioma cells had no effect on cell proliferation in vitro but reduced tumor growth and angiogenesis in vivo, and these findings were attributed to increased expression of the angiogenesis inhibitor thrombospondin in PTEN-expressing cells (47). In a study of prostate cancers, however, PTEN deletion was found to correlate with increased tumor microvesSEL density, but loss of PTEN did not correlate with thrombospondin-1 expression (48), suggesting that additional mechanisms exist through which PTEN regulates tumor angiogenesis.

To our knowledge, no other studies have investigated the possibility that PTEN could directly regulate angiogenesis by modulating endothelial cellular functions. Taken together with results from tumor cells, our findings suggest that overexpression of PTEN in whole cells could disrupt tumor angiogenesis through both tumor cell-mediated effects and effects on endothelial cells. This would result in induction of apoptosis in both cell types, which would be expected to be more potent than targeting either cell type alone. Another important implication of our results is that PTEN could be an important target for therapeutic pro-angiogenesis in ischemic heart and vascular diseases. Because PI3K signaling regulates many of the endothelial cellular responses required for angiogenesis, inhibition of PTEN could significantly enhance these responses. PI3K is activated downstream of other endothelial receptor tyrosine kinases that are required for vascular development, including Tie2 (28) and Tie1,2, which play important roles in vascular maturation. The observed effects on basal endothelial signaling and cellular processes in this study indicate that PTEN plays a central role in the regulation of a variety of angiogenic cellular responses. Inhibition of PTEN might allow potentiation of PI3K-mediated responses downstream of numerous receptors in an appropriate temporal and spatial context. This contrasts with the delivery of one specific growth factor, such as VEGF, which may be insufficient to induce appropriate vascular maturation of nascent blood vessels. Notably, a preliminary study has suggested that adenovirus-mediated delivery of constitutively active Akt to the endothelium enhances angiogenesis in a model of hindlimb ischemia (49). Targeting PI3K or PTEN may have advantages over this approach, since many of the effects of PI3K are independent of Akt. Studies are currently under way to test the effects of PTEN inhibition on angiogenesis in vivo.

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