[CANCER RESEARCH 63, 2300–2305, May 1, 2003]

PTEN Decreases *in Vivo* Vascularization of Experimental Gliomas in Spite of Proangiogenic Stimuli[1]

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ABSTRACT

Approximately 30–40% of malignant glial tumors exhibit mutations in the tumor suppressor gene, *PTEN/MMAC*. Additionally, these tumors are associated with (*a*) mutations in epidermal growth factor receptor (EGFR), leading to a pro-oncogenic constitutive activation, as well as amplification of its gene, and/or (*b*) mutations in p53, disrupting normal cellular homeostatic processes. Whereas PTEN/MMAC has been shown to possess antiangiogenic action, constitutively active EGFR or p53 gene defects have been associated with proangiogenic action. In this article, we asked if *PTEN/MMAC* gene transfer into human glioma cells that possess inactivating mutations of the *PTEN/MMAC* gene but also express either constitutively active EGFR (U87ΔEGFR cells) or possess an inactivating mutation of p53 (U251 cells) still display inhibited angiogenesis in orthotopic and ectopic models of gliomas. Human glioma xenografts treated with *PTEN/MMAC* gene transfer exhibited significantly decreased vascularity both in an orthotopic and in an ectopic model. Taken in combination, these results provide strong evidence of PTEN/MMAC's role in regulating glioma angiogenesis even in the presence of strong proangiogenic signals provided by constitutive EGFR activation or p53 inactivation.

INTRODUCTION

PTEN/MMAC mutations have been reported in ~20–40% of GBM[3] and 60–80% of glioma cell lines. Both somatic and germ-line mutations cluster within the conserved regions of PTEN′s phosphatase domain (1–4). PTEN primarily possesses phosphatase activity for lipid molecules. In particular, PTEN can dephosphorylate lipid substrates of the PI3K proto-oncogene such as PIP2 (1, 2) and PIP3 (1–3). Because PI3K action may promote multiple aspects of tumorigenesis (4), PTEN should antagonize this. In fact, expression of PTEN cDNA suppresses tumor cell growth both *in vitro* and *in vivo* (5). These results thus strongly suggest that PTEN acts as a tumor suppressor gene for tumors such as GBM.

One downstream effector of PI3K is the proto-oncogene Akt, a serine/threonine kinase (6). Activated Akt has been shown to lead to the development of glioblastoma tumors in mice brains (7, 8). Akt activity appears to be essential for antiapoptotic effects mediated by several growth factors and integrins (7–15). In some cell types, Akt has also been reported to modulate G_1 progression via inactivation of glycogen synthase kinase-3 (16). Additionally, Akt has been reported to regulate VEGF induction under hypoxic conditions (17). Recently, PTEN has also been shown to directly inhibit angiogenesis in U87 glioma cells in an orthotopic brain tumor model (18). Thus, Akt has multiple roles in tumorigenesis through the deregulation of cell cycle, enhancement of apoptotic resistance, and alteration of angiogenic potential.

All tumors require angiogenesis for tumor expansion (19, 20). GBMs can be extremely vascularized tumors and express elevated concentrations of many proangiogenic genes such as VEGF and fibroblast growth factor (19). In addition, another frequent alteration in GBMs and glioma cell lines is amplification of the EGFR and/or expression of constitutive forms of EGFR (21). EGFR has also been recently shown to transcriptionally up-regulate VEGF expression in human U87 glioma cells via a pathway involving ras/PI3K and Akt that was distinct from the pathway induced by hypoxia (22). In fact, humoral (23, 24) or pharmacological (25) inhibition of EGFR function has been shown to inhibit angiogenesis, and EGF has been shown to modulate the expression of VEGF in tumor cells (26).

In a second type of malignant gliomas, p53 rather than *EGFR* gene mutations are often observed, and several reports have shown that malignant gliomas will either have *EGFR* or p53 mutations but almost never both (27). Although p53 gene transfer into cells with p53 gene mutations has been shown to lead to decreased angiogenesis (28), the mechanisms responsible for this effect remain the focus of investigation. For instance, in fibroblasts (29) wild-type p53 expression has been associated with decreased angiogenesis through its stimulation of thrombospondin-1 expression. However, other studies have not found a link between p53 and thrombospondin-1 expression in glioma cell lines or in glioma tumors (30) but have found that p53 expression regulates expression of GD-AIF (31), of BAI1 (32), and of HIF-1 (33). In addition, p53 expression has been linked to inhibition of angiogenesis by reducing VEGF expression in nonglial tumors (34).

Because both *PTEN* and *EGFR* or *PTEN* and *p53* gene mutations can occur concomitantly in a large proportion of glioma, we wondered if the inhibition of glioma angiogenesis by PTEN would still occur in the presence of mutant *EGFR* or mutant *p53*. In fact, one could speculate that increased activation of PI3K function by mutant *EGFR* or the loss of antiangiogenesis signaling by wild-type p53 could provide an avenue for tumor cells to evade PTEN′s inhibition of angiogenesis. Because the original study by Wen *et al.* (18) used human U87 glioma cells that possess both a wild-type EGFR and wild-type p53 to answer the above question, we used human U87 glioma cells that stably express a constitutively active form of the *EGFR* (Δ*EGFR*), as well as human U251 cells that possess an inactivating mutation in the p53 gene. Herein, we show that PTEN expression decreases vascularization of ectopic and orthotopic glioma tumors. Therefore, *PTEN* gene transfer or modulation of PTEN activity may provide a therapeutic avenue toward limiting GBM angiogenesis, regardless of constitutive EGFR activity or loss of p53 function.

Received 4/19/02; accepted 8/2/02.

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[1] This work was supported by NIH/National Cancer Institute P01 (to E. A. C.) and the Berkowitz-Knott Fund for Brain Tumor Research. Support was also derived from donations made to the Brain Tumor Research Fund in memory of Anna Guglietta, Linda Todisco, Karen Backlund, and Dr. Thomas Shea.

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[3] The abbreviations used are: GBM, glioblastoma multiforme; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; GD-AIF, glioma-derived angiogenesis inhibitory factor; BAI1, brain-specific angiogenesis inhibitor-1; HIF-1, hypoxia-inducible factor-1; MOI, multiplicity of infection; PN, particle number.

MATERIALS AND METHODS

Cell Lines and Chemicals. Human U251 glioma cells were obtained from American Tissue Culture Collection. Human U87ΔEGFR and U87 glioma cells were a generous gift of Dr. H. J. Su Huang (University of California at San Diego). This cell line was established by retroviral transfer of mutant EGFR into parental glioma cell lines, enhancing their tumorigenic capacity in the brain of nude rats (35). U87 and U251 cells were propagated at 37°C in an atmosphere containing 5% carbon dioxide in DMEM supplemented with 10% FCS containing 100 units/ml penicillin and 100 μg/ml streptomycin. U87ΔEGFR cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml G418 (Sigma). LY294002 was obtained from Sigma Chemical Co., and a stock was prepared by dissolution in DMSO.

Viral Vectors. The MMAC1/PTEN vector, MMCB, was engineered by recombining the cDNA encoding full-length PTEN/MMAC1 (1) into the E1 region of the adenoviral vector under control of a human cytomegalovirus promoter. In addition to the E1 deletion, the genome of this vector also has deletions of the E3 region and protein IX gene. The control vector, BGCG, possesses a lacZ cDNA in the same E1 region under control of the cytomegalovirus promoter within the E1-, E3-, protein IX-deleted vector background. All constructs were produced at Canji, Inc. (San Diego, CA), as described previously (36). Virus particle concentrations were determined by Resource Q high-performance liquid chromatography. The ratio of plaque-forming unit to pn was 1:20. The primary structure of the transgenes was verified by automated sequencing of viral DNA.

Animal Studies. All animal studies were reviewed and approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care. Adult female nude rats (rnu/rnu), purchased from the NIH, were anesthetized with an i.p. injection of 0.5 ml of 0.9% NaCl containing 12.5 mg of ketamine and 2.5 mg of xylazine. After immobilizing the rats in a stereotactic apparatus and placing a linear skin incision over the bregma, burr holes (1 mm in diameter) were drilled in the skull ~1 mm anterior to and 2.5 mm lateral to the bregma on the right side. Two hundred thousand U87ΔEGFR cells (in a volume of 2 μl) were then injected to a depth of 4.5 mm from the dura, using a 5-μl Hamilton syringe. Five days later, animals were reanesthetized for direct injection. Adenovirus (7.3×10^9 pn; in 10 μl) was inoculated stereotactically using the same coordinates.

For vascularity and tumor volume assays, animals were sacrificed by anesthetic euthanasia 5 days after virus inoculation followed by intracardiac infusion of a solution containing 4% neutral paraformaldehyde in 0.9% sodium chloride and 10 mM sodium phosphate, pH = 7 (PBS). After harvesting, brains were kept in 4% paraformaldehyde for 1 d, transferred to 30% sucrose in PBS for 5 days, frozen over liquid nitrogen and stored at −80°C.

Histological and Quantitative Analysis of Tumor Vascularity and Tumor Volumes. For vascularity assays, brains were sectioned on a cryostat to a thickness of 10 μm and then air-dried at room temperature. Sections were then stained with H&E for anatomical detail or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for LacZ cDNA expression. Microvessel staining was performed using direct incubation with 3,3′-diaminobenzidine (Dako lab kit; Dako, Inc., CA) to detect expression of alkaline phosphatase in endothelium (37). For quantitative determination of microvessel density, five or more randomly selected sections of brain tumors were analyzed using an Olympus BX60 microscope. Each section was scanned by Sony 3-chip Color Video Camera at ×200 magnification and vessel numbers enumerated using Image-Pro PLUS software (Media Cybernetics, Silver Spring, MD). This was performed by selection of discrete and distinct areas that represented stained blood vessels. The number of vessels were then normalized to the tumor volume and calculated using the formula $4/3\pi r1r2r3$ (where r1 and r2 represent the perpendicular radii of the center cross-section of the tumor and r3 the longitudinal radius of the tumor).

Tumor volumes were assayed by sectioning the region of brain that contained the tumor on a cryostat. Section thickness was 20 μm. Every fifth section was collected starting from the most rostral portion of the tumor to the most caudal (range of sections collected was 10–24). For measurements of tumor volumes, the four most central sections within the tumor were analyzed and their radii calculated using computerized measurements with Image-Pro Plus.

Tumor Angiogenesis Assay in Mice. A dorsal air sac was created in 5–7-week-old male mice according to published methods (38). Subconfluent monolayers (80%) of glioma cells were infected in P100 dishes with adenoviral vectors at a MOI of 10 in 500 μl of culture medium for 1 h, after which 10 ml of fresh medium were added for an additional 12-h incubation time. Cells were then harvested by trypsinization, counted using a Coulter counter, and then suspended in HBSS at a concentration of 1×10^7 cells/ml. As a control, a suspension consisting of HBSS alone was also prepared. Tumor or HBSS suspension (0.2 ml) was then injected into a chamber (Millipore Corp.) that was then implanted into a dorsal air sac produced in the mouse by injecting 10–15 ml of air. Five days later, five mice from each group were euthanized, and the dorsal air sac with surrounding skin carefully dissected. After removing the implanted chamber, a ring without filters was placed on the same site and photographed by digital video (JVC, Tokyo, Japan). The dense capillary network in the area of the dorsal air sac was quantified with Image-Pro PLUS software using this photograph, where the red capillaries were selected for counting by the software.

RESULTS

***In Vivo* Decrease in Angiogenesis in Orthotopic Brain Tumors Treated by Adenovirus-*PTEN* Gene Transfer.** An intracerebral orthotopic brain tumor model was established in athymic rat brains using U87ΔEGFR cells. These tumors establish rapidly, leading to the demise of the animal usually by 2 weeks after implantation because of ΔEGFR's stimulating effects on tumor proliferation and possibly angiogenesis (39). Tumor vascularity was assayed by histochemical staining for alkaline phosphatase in vessels (Fig. 1*A*). Tumors treated by PTEN-adenoviral vector gene transfer qualitatively exhibited less tumor angiogenesis than those treated with lacZ-expressing adenoviruses or control tumors. Semiquantitative analyses showed a decrease in microvessel density in PTEN-adenovirus treated tumors when compared with lacZ-adenovirus-treated or control tumors (Fig. 1*B*). Adenovirus-lacZ-treated tumors did not exhibit a qualitative increase in microvessel density when compared with control brain tumors (Fig. 1, *A* and *B*). When U87 tumors were grown in animal brains, both growth and vascularization were retarded compared with U87ΔEGFR (data not shown), in agreement with published studies (39). The volume of U87ΔEGFR tumors was decreased both 2 and 5 days after injection with the PTEN-adenovirus compared with the control, lacZ-adenovirus (Fig. 1*C*). These results thus showed that PTEN gene transfer could inhibit *in vivo* tumor vascularity and growth, despite constitutive ΔEGFR expression in tumors.

PTEN Inhibition of Angiogenesis in Ectopic U87ΔEGFR Tumors. To provide a more detailed quantitative analysis of effects of PTEN gene transfer on tumor angiogenesis, a dorsal air sac method of analysis was used (38). Implantation of U87ΔEGFR cells in the s.c. tissue of the mouse provoked exuberant neovascularization by day 5 (data not shown). When U87ΔEGFR cells, infected with the control lacZ-expressing adenovirus (BGCG), were injected, this neovascularization was still present (Fig. 2*A*). However, injection of cells infected with the PTEN-expressing adenovirus (MMCB) resulted in a visible decrease in neovascularity. Image-computer analysis of capillaries that had developed in the dorsal air sac ring showed that PTEN-expressing adenovirus significantly inhibited ($P < 0.01$) the development of capillary networks in U87ΔEGFR tumors (Fig. 2*B*) and, in fact, vascularization approached that of control dorsal air sac where medium without tumor cells were injected. These results thus additionally confirmed that *PTEN* gene transfer significantly inhibited neovascularization of glioma tumors even if they expressed ΔEGFR.

***PTEN* Gene Transfer also Decreases the Neovascularization of U251 Tumors.** To provide additional confirmatory evidence of a role for *PTEN* gene transfer in decreasing *in vivo* glioma angiogenesis despite constitutive presence of proangiogenic signals, an additional

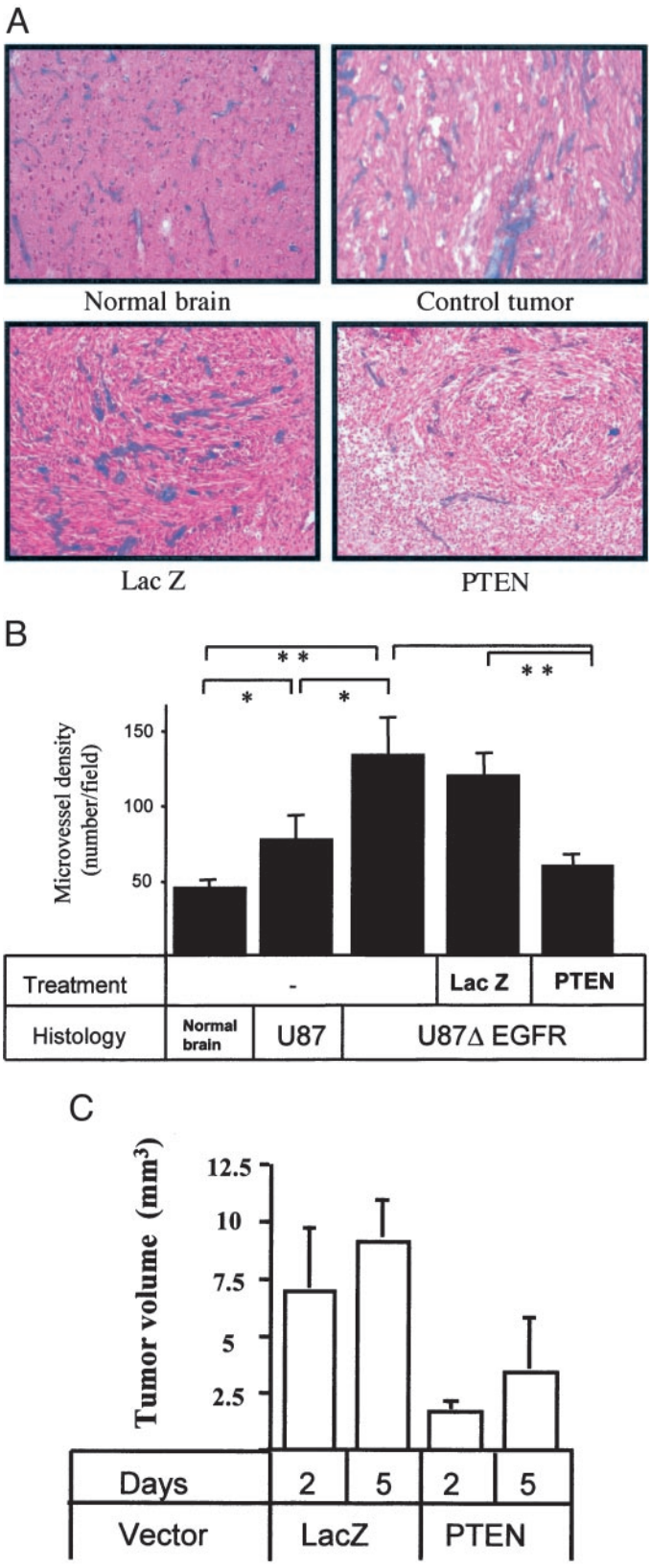


Fig. 1. PTEN-adenovirus infection of orthotopic brain tumors suppresses their *in vivo* angiogenesis. In *A*, histochemical analysis for alkaline phosphatase provides a qualitative estimate of microvessel density in control, BGCG, (lacZ-adenovirus), and MMCB (PTEN-adenovirus)-infected tumors. All analyses of tumor vascularity were performed 5 days after adenoviral infection. Approximately 80% of the tumor expressed β-galactosidase when infected with the lacZ-adenovirus. In *B*, microvessel density was calculated by computer-assisted image analysis. Five or more randomly selected sections were counted for each experimental group using a microscope. Values were then normalized according to tumor volumes. *Error bars* represent the SD. $*$, $P < 0.05$; $**$, $P < 0.01$ (Student's *t* test). In *C*, brain tumor volumes were measured in animals 2 or 5 days after injection of lacZ-adenovirus or PTEN-adenovirus. Volumes were measured after sacrificing animals ($n = 2$) and staining brains and tumors with H&E. Microscopic visualization of the tumors was carried out, and tumor volume measurements were carried out as detailed in "Materials and Methods."

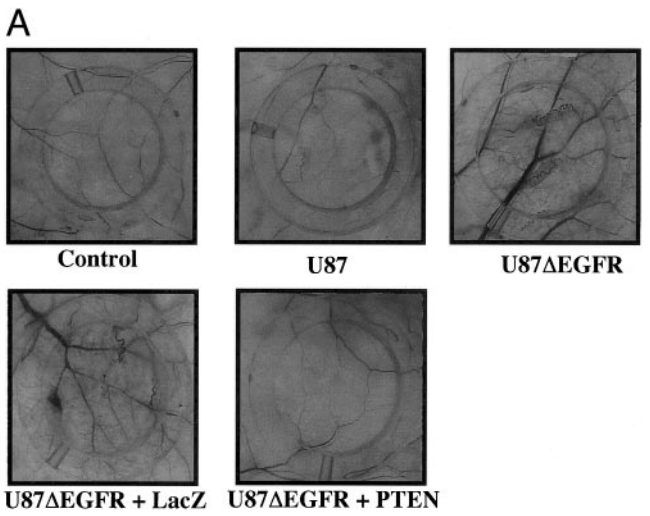
tumor model was tested. U251 cells possess a frame shift inactivating mutation at codon 241 of *PTEN* (40). Unlike U87 cells, U251 cells also possess an inactivating mutation in the p53 gene (40). Because it has been reported that p53 contributes to the maintenance of angiogenesis homeostasis through a variety of mechanisms (including VEGF regulation) in several tumors and tumor cell lines (28, 31, 33, 34, 41–48), we asked if *PTEN* gene transfer could overcome the proangiogenic stimulus provided by loss of p53 gene function. When studied in a dorsal air sac model, *PTEN* gene transfer into U251 tumors decreased capillary networks both by visual inspection (Fig. 3*A*) and by enumeration of microvessel density (Fig. 3*B*). Treatment with LY294002 (50 μM) also markedly inhibited the induction of angiogenesis. These results thus provided confirmatory evidence for an *in vivo* role for *PTEN* gene transfer in decreasing tumor vascularization despite proangiogenic, inactivating mutations in p53.

DISCUSSION

The primary objective of this study consisted of determining the effect of PTEN on angiogenesis in human glioma xenografts even when such tumor cells expressed proangiogenic phenotypes. We determined that: (*a*) *PTEN* gene transfer inhibited the vascularity of U87ΔEGFR xenografts in both orthotopic and ectopic models in U87 glioma cells that express constitutively active EGFR; and (*b*) this inhibition of vascularization also occurred in a second xenograft model, composed of U251 cells, circumventing proangiogenic stimuli that may have been provided by p53 gene defects. As an ectopic model, we selected a dorsal air sac method (38), which has been reported to reproduce vascular endothelial tubular morphogenesis. Findings in this model mirrored those in the orthotopic model in terms of reduced angiogenesis for tumors treated with PTEN.

These results provide confirmatory evidence for an *in* vivo role of PI3K activation in angiogenesis, as well as *PTEN* gene transfer in inhibiting this process. Furthermore, they show that *PTEN* gene transfer can circumvent the proangiogenic effect of p53 mutations. Modulation of PTEN function (either through gene transfer methods or pharmacological maneuvers) thus provides a powerful avenue for inhibition of angiogenesis even when proliferative and proangiogenic pathways (*i.e.*, constitutive EGFR activation and p53 tumor suppressor gene mutations) are operative.

Previously, Wen *et al.* (18) showed that reintroduction of PTEN in U87 glioma cells using a retroviral vector dramatically suppressed tumor angiogenesis in an orthotopic model. Through the use of a specific point mutant of PTEN, they were able to show that lipid phosphatase, but not protein phosphatase, activity was required for angiogenesis inhibition. Human U87 glioma cells express a wild-type EGFR. However, mutations leading to amplification and constitutive expression of EGFR are a common finding in malignant glioma specimens (27). This leads to activation of ras/PI3K, stimulating cellular proliferation (49), as well as tumor angiogenesis. Therefore, it was important to determine whether PTEN replenishment in the context of constitutive EGFR activation would also result in an inhibition of angiogenesis. U87ΔEGFR cells were generated by infection of U87 cells with a retroviral vector expressing a constitutively active mutant of EGFR (35). The proliferation of these cells is significantly enhanced both *in vitro* and *in vivo*, when compared with parental U87. Microvessel density in these cells is also increased



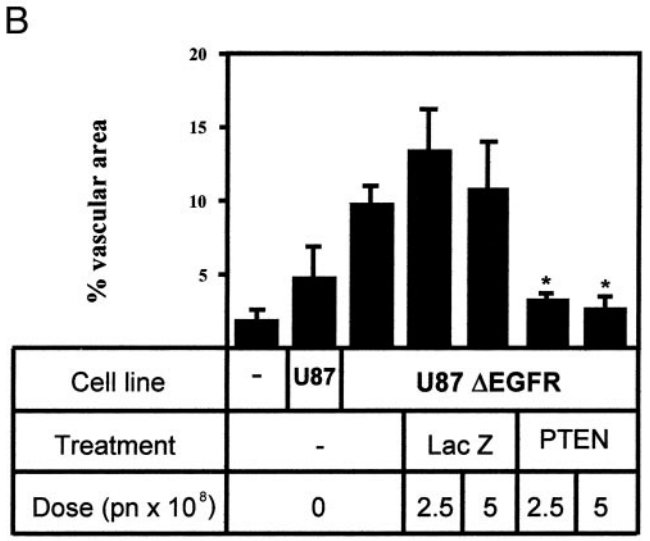
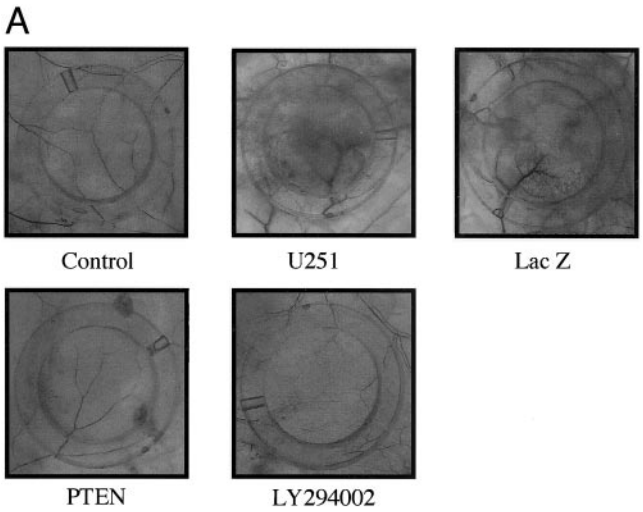


Fig. 2. PTEN-adenovirus infection of ectopic brain tumors (dorsal air sac model) suppresses their *in vivo* angiogenesis. U87ΔEGFR cells were infected at a dose of 5×10^8 adenovirus particles/ml with 500 μl of complete medium for 1 h (final MOI = 25) and then reincubated in 5 ml of complete medium for an additional 12 h. After washing, cells were resuspended in HBSS to achieve a final concentration of 1×10^7 cells/ml. A total of 0.2 ml of this suspension was then injected into a dorsal air sac chamber created s.c. on the flank of athymic mice, as described in "Materials and Methods." Five days later, the extent of neovascularity was assayed (*A*). The microphotograph shows representative dorsal air sac chambers with blood vessels feeding PTEN-adenovirus (MMCB)-infected tumors, lacZ-adenovirus (BGCG)-infected tumors, uninfected tumors, or controls (consisting of medium injection without tumor cells). This control provides a background estimate of the vascularization of normal epidermis. In *B*, semiquantitative analysis of microvessels nourishing tumors grown in the dorsal air sac chambers was performed. The capillary network visualized in each dorsal air sac chamber was quantified using a computer-assisted analysis method. Two doses of adenoviral vectors were tested (2.5 or 5 pn $\times 10^8$). *Error bars* represent the SD $*P < 0.01$ (Student's *t* test), upon comparison of PTEN-adenovirus-treated to lacZ-adenovirus-treated tumors.

when they are grown in athymic mice models (Abe and Chiocca, unpublished results). MMCB (PTEN-expressing adenovirus) infection of these cells led to a significant decrease in VEGF mRNA levels. This could also be accomplished with the PI3K inhibitor, LY294002. *In vivo*, this was associated with a decrease in tumor proliferation (data not shown), as well as a decrease in angiogenesis.

These results were additionally confirmed with U251 cells. These cells possess an inactivating mutation in *PTEN*, but unlike U87 cells, also possess an inactivating mutation in *p53* (40). Expression of p53 has been linked to decreased angiogenesis in several tumor models. Recently, p53 defects have also been associated with enhancement of VEGF expression and neoangiogenesis in lung (41, 43), prostate (50), and breast cancers (44, 45). Wild-type p53 has been shown to transcriptionally suppress VEGF in sarcoma cells (34). It should be noted, although, that effects of p53 on VEGF expression are not completely agreed upon because some reports have failed to observe correlations between these two markers in glioma (51) or prostate tumors (52). Instead, in gliomas, p53 expression has been correlated with angiogenesis inhibition through its action on GD-AIF (31), of BAI1 (32), and of HIF-1 (33). In this study, we were able to provide a separate and independent confirmation of PTEN′s ability to circumvent a second pathway (*i.e.,* loss of p53 expression) through which glioma cells may stimulate angiogenesis. Therefore, the combination of these findings provide even additional evidence for PTEN′s significant role in inhibiting tumor angiogenesis, despite the presence of either p53 or EGFR mutations known to stimulate angiogenesis.

Mutations in PTEN accompany the progression of astrocytoma from lower to higher grades (53). Angiogenesis, the formation of new



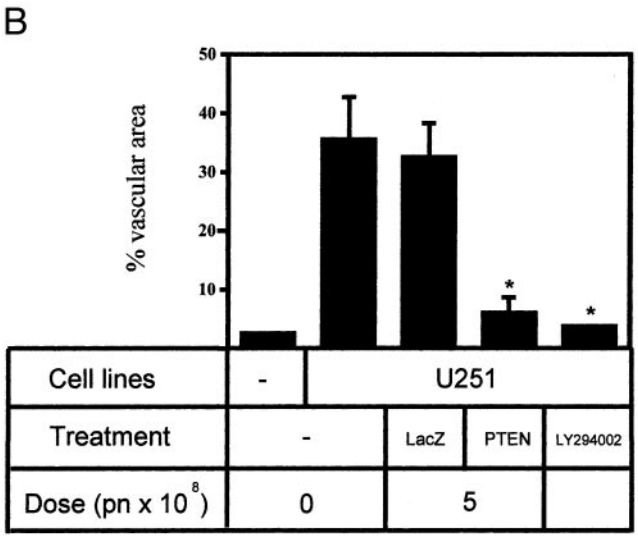


Fig. 3. PTEN-adenovirus infection of U251 tumors also suppresses angiogenesis. U251 cells were infected at a dose of 5×10^8 adenovirus particles/ml in 500 μl of complete medium for 1 h (final MOI = 25) and then incubated in 5ml of complete medium for 12 h. Treatment with 50 μM LY294002 was performed for 12 h. Shown are the dorsal air sac chambers (*A*), as well as the quantified capillary networks (*B*). In *A*, that the cloudy areas for parental U251 and LacZ-expressing U251 tumors represent a blush of capillaries sprouting from the larger microvessel seen within each dorsal air sac. *Error bars* = SD; *, $P < 0.01$ (Student's *t* test), upon comparison of PTEN-adenovirus-treated to lacZ-adenovirus-treated tumors.

blood vessels from existing vascular structures, is histologically associated with increases in microvessel density and increased invasion of tumor cells into brain parenchyma (54). During tumor angiogenesis, blood vessels are formed within the tumor parenchyma, and this process is thought to be controlled through a balance between the effective local concentration of stimulators and inhibitors of angiogenesis (20). EGFR-mediated transcriptional up-regulation of VEGF expression in human U87 glioma cells via a pathway involving ras/PI3K and Akt, distinct from the pathway induced by hypoxia, was recently characterized (55). In addition, several lines of evidence show that constitutive EGFR activation is associated with increased angiogenesis (56). Another common mutation in gliomas is in the p53 tumor suppressor gene, and this has been implicated to regulate angiogenesis by a variety of mechanisms, including modulation of GD-AIF (31), of BAI1 (32), and of HIF-1 (33). In light of these findings, it was important to determine whether the constitutive activation of EGFR function or the loss of p53 expression would shift the angiogenic balance and limit PTEN′s antiangiogenic action. Our findings negate this hypothesis and clearly provide *in vitro* and *in vivo* evidence of the potency of PTEN′s antiangiogenic action. It thus seems that therapies in which the purpose is to replenish PTEN function (gene therapy) and/or inhibit PI3K activation (small molecule inhibitors, such as LY294002) should be beneficial to the treatment of malignant gliomas through their modulation of VEGF expression.

REFERENCES

1. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., *15:* 356–362, 1997.
2. Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J., and Eng, C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat. Genet., *16:* 333–334, 1997.
3. Maehama, T., and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate. J. Biol. Chem., *273:* 13375–13378, 1998.
4. Fruman, D. A., Meyers, R. E., and Cantley, L. C. Phosphoinositide kinases. Annu. Rev. Biochem., *67:* 481–507, 1998.
5. Cheney, I. W., Johnson, D. E., Vaillancourt, M. T., Avanzini, J., Morimoto, A., Demers, G. W., Wills, K. N., Shabram, P. W., Bolen, J. B., Tavtigian, S. V., and Bookstein, R. Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated MMAC1/PTEN gene transfer. Cancer Res., *58:* 2331–2334, 1998.
6. Aoki, M., Batista, O., Bellacosa, A., Tsichlis, P., and Vogt, P. K. The akt kinase: molecular determinants of oncogenicity. Proc. Natl. Acad. Sci. USA, *95:* 14950–14955, 1998.
7. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, *91:* 231–241, 1997.
8. Holland, E. C., Celestino, J., Dai, C., Schaefer, L., Sawaya, R. E., and Fuller, G. N. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. Nat. Genet., *25:* 55–57, 2000.
9. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J., *16:* 2783–2793, 1997.
10. Pap, M., and Cooper, G. M. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J. Biol. Chem., *273:* 19929–19932, 1998.
11. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, *96:* 857–868, 1999.
12. Kops, G. J., and Burgering, B. M. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. J. Mol. Med., *77:* 656–665, 1999.
13. Davies, M. A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D., Yung, W. K., Mills, G. B., and Steck, P. A. Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. Cancer Res., *58:* 5285–5290, 1998.
14. Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G., and Stokoe, D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr. Biol., *8:* 1195–1198, 1998.
15. Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N., and Parsons, R. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res., *58:* 5667–5672, 1998.
16. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. Genes Dev., *12:* 3499–3511, 1998.
17. Mazure, N. M., Chen, E. Y., Laderoute, K. R., and Giaccia, A. J. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood, *90:* 3322–3331, 1997.
18. Wen, S., Stolarov, J., Myers, M. P., Su, J. D., Wigler, M. H., Tonks, N. K., and Durden, D. L. PTEN controls tumor-induced angiogenesis. Proc. Natl. Acad. Sci. USA, *98:* 4622–4627, 2001.
19. Wesseling, P., Ruiter, D. J., and Burger, P. C. Angiogenesis in brain tumors; pathobiological and clinical aspects. J. Neurooncol., *32:* 253–265, 1997.
20. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., *1:* 27–31, 1995.
21. Schober, R., Bilzer, T., Waha, A., Reifenberger, G., Wechsler, W., von Deimling, A., Wiestler, O. D., Westphal, M., Kemshead, J. T., Vega, F., *et al*. The epidermal growth factor receptor in glioblastoma: genomic amplification, protein expression, and patient survival data in a therapeutic trial. Clin. Neuropathol., *14:* 169–174, 1995.
22. Maity, A., Pore, N., Lee, J., Solomon, D., and O'Rourke, D. M. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3′-kinase and distinct from that induced by hypoxia. Cancer Res., *60:* 5879–5886, 2000.
23. Viloria-Petit, A., Crombet, T., Jothy, S., Hicklin, D., Bohlen, P., Schlaeppi, J. M., Rak, J., and Kerbel, R. S. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies *in vivo*: a role for altered tumor angiogenesis. Cancer Res., *61:* 5090–5101, 2001.
24. Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin. Cancer Res., *5:* 257–265, 1999.
25. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R., and Tortora, G. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin. Cancer Res., *7:* 1459–1465, 2001.
26. Ravindranath, N., Wion, D., Brachet, P., and Djakiew, D. Epidermal growth factor modulates the expression of vascular endothelial growth factor in the human prostate. J. Androl., *22:* 432–443, 2001.
27. Kleihues, P., and Ohgaki, H. Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro-oncol., *1:* 44–51, 1999.
28. Bouvet, M., Ellis, L. M., Nishizaki, M., Fujiwara, T., Liu, W., Bucana, C. D., Fang, B., Lee, J. J., and Roth, J. A. Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. Cancer Res., *58:* 2288–2292, 1998.
29. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science (Wash. DC), *265:* 1582–1584, 1994.
30. Tenan, M., Fulci, G., Albertoni, M., Diserens, A. C., Hamou, M. F., El Atifi-Borel, M., Feige, J. J., Pepper, M. S., and Van Meir, E. G. Thrombospondin-1 is down-regulated by anoxia and suppresses tumorigenicity of human glioblastoma cells. J. Exp. Med., *191:* 1789–1798, 2000.
31. Van Meir, E. G., Polverini, P. J., Chazin, V. R., Su Huang, H. J., de Tribolet, N., and Cavenee, W. K. Release of an inhibitor of angiogenesis upon induction of wild type p53 expression in glioblastoma cells. Nat. Genet., *8:* 171–176, 1994.
32. Nishimori, H., Shiratsuchi, T., Urano, T., Kimura, Y., Kiyono, K., Tatsumi, K., Yoshida, S., Ono, M., Kuwano, M., Nakamura, Y., and Tokino, T. A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. Oncogene, *15:* 2145–2150, 1997.
33. Blagosklonny, M. V., An, W. G., Romanova, L. Y., Trepel, J., Fojo, T., and Neckers, L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J. Biol. Chem., *273:* 11995–11998, 1998.
34. Zhang, L., Yu, D., Hu, M., Xiong, S., Lang, A., Ellis, L. M., and Pollock, R. E. Wild-type p53 suppresses angiogenesis in human leiomyosarcoma and synovial sarcoma by transcriptional suppression of vascular endothelial growth factor expression. Cancer Res., *60:* 3655–3661, 2000.
35. Nagane, M., Coufal, F., Lin, H., Bogler, O., Cavenee, W. K., and Huang, H. J. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. Cancer Res., *56:* 5079–5086, 1996.
36. Huyghe, B. G., Liu, X., Sutjipto, S., Sugarman, B. J., Horn, M. T., Shepard, H. M., Scandella, C. J., and Shabram, P. Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography. Hum. Gene Ther., *6:* 1403–1416, 1995.
37. Bell, M. A., and Scarrow, W. G. Staining for microvascular alkaline phosphatase in thick celloidin sections of nervous tissue: morphometric and pathological applications. Microvasc. Res., *27:* 189–203, 1984.
38. Abe, T., Okamura, K., Ono, M., Kohno, K., Mori, T., Hori, S., and Kuwano, M. Induction of vascular endothelial tubular morphogenesis by human glioma cells. A model system for tumor angiogenesis. J. Clin. Investig., *92:* 54–61, 1993.
39. Mishima, K., Johns, T. G., Luwor, R. B., Scott, A. M., Stockert, E., Jungbluth, A. A., Ji, X. D., Suvarna, P., Voland, J. R., Old, L. J., Huang, H. J., and Cavenee, W. K. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of monoclonal antibody

(mAb) 806, a novel monoclonal antibody directed to the receptor. Cancer Res., *61:* 5349–5354, 2001.
40. Ishii, N., Maier, D., Merlo, A., Tada, M., Sawamura, Y., Diserens, A. C., and Van Meir, E. G. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol., *9:* 469–479, 1999.
41. Yuan, A., Yu, C. J., Luh, K. T., Kuo, S. H., Lee, Y. C., and Yang, P. C. Aberrant p53 Expression correlates with expression of vascular endothelial growth factor mRNA and interleukin-8 mRNA and neoangiogenesis in non-small cell lung cancer. J. Clin. Oncol., *20:* 900–910, 2002.
42. Tai, Y. T., Podar, K., Gupta, D., Lin, B., Young, G., Akiyama, M., and Anderson, K. C. CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. Blood, *99:* 1419–1427, 2002.
43. Fontanini, G., Vignati, S., Lucchi, M., Mussi, A., Calcinai, A., Boldrini, L., Chine, S., Silvestri, V., Angeletti, C. A., Basolo, F., and Bevilacqua, G. Neoangiogenesis and p53 protein in lung cancer: their prognostic role and their relation with vascular endothelial growth factor (VEGF) expression. Br. J. Cancer, *75:* 1295–1301, 1997.
44. Pal, S., Datta, K., and Mukhopadhyay, D. Central role of p53 on regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression in mammary carcinoma. Cancer Res., *61:* 6952–6957, 2001.
45. Linderholm, B. K., Lindahl, T., Holmberg, L., Klaar, S., Lennerstrand, J., Henriksson, R., and Bergh, J. The expression of vascular endothelial growth factor correlates with mutant p53 and poor prognosis in human breast cancer. Cancer Res., *61:* 2256–2260, 2001.
46. Galy, B., Creancier, L., Zanibellato, C., Prats, A. C., and Prats, H. Tumour suppressor p53 inhibits human fibroblast growth factor 2 expression by a post-transcriptional mechanism. Oncogene, *20:* 1669–1677, 2001.
47. Ravi, R., Mookerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L., and Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes Dev., *14:* 34–44, 2000.
48. Kieser, A., Weich, H. A., Brandner, G., Marme, D., and Kolch, W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene, *9:* 963–969, 1994.
49. Ram, T. G., and Ethier, S. P. Phosphatidylinositol 3-kinase recruitment by p185erbB-2 and erbB-3 is potently induced by neu differentiation factor/heregulin during mitogenesis and is constitutively elevated in growth factor-independent breast carcinoma cells with c-erbB-2 gene amplification. Cell Growth Differ., *7:* 551–561, 1996.
50. Kwak, C., Jin, R. J., Lee, C., Park, M. S., and Lee, S. E. Thrombospondin-1, vascular endothelial growth factor expression and their relationship with p53 status in prostate cancer and benign prostatic hyperplasia. BJU Int., *89:* 303–309, 2002.
51. Plate, K. H., Breier, G., Weich, H. A., Mennel, H. D., and Risau, W. Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. Int. J. Cancer, *59:* 520–529, 1994.
52. Strohmeyer, D., Rossing, C., Bauerfeind, A., Kaufmann, O., Schlechte, H., Bartsch, G., and Loening, S. Vascular endothelial growth factor and its correlation with angiogenesis and p53 expression in prostate cancer. Prostate, *45:* 216–224, 2000.
53. Nozaki, M., Tada, M., Kobayashi, H., Zhang, C. L., Sawamura, Y., Abe, H., Ishii, N., and Van Meir, E. G. Roles of the functional loss of p53 and other genes in astrocytoma tumorigenesis and progression. Neuro-oncol., *1:* 124–137, 1999.
54. Thorsen, F., and Tysnes, B. B. Brain tumor cell invasion, anatomical and biological considerations. Anticancer Res., *17:* 4121–4126, 1997.
55. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A. R., Ryan, H. E., Johnson, R. S., Jefferson, A. B., Stokoe, D., and Giaccia, A. J. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev., *14:* 391–396, 2000.
56. Yen, L., You, X. L., Al Moustafa, A. E., Batist, G., Hynes, N. E., Mader, S., Meloche, S., and Alaoui-Jamali, M. A. Heregulin selectively up-regulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. Oncogene, *19:* 3460–3469, 2000.

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Cancer Res 2003;63:2300-2305.