Somatic Induction of Pten Loss in a Preclinical Astrocytoma Model Reveals Major Roles in Disease Progression and Avenues for Target Discovery and Validation

Andrew Xiao, Chaoying Yin, Chunyu Yang, Antonio Di Cristofano, Pier Paolo Pandolfi, and Terry Van Dyke

Departments of Biochemistry and Biophysics, and of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; Human Genetics Program, Division Population Science, Fox Chase Cancer Center, Cottman Avenue, Philadelphia, Pennsylvania; and Cancer Biology and Genetics Program, Memorial-Sloan Kettering Cancer Center, New York, New York

Abstract

High-grade astrocytomas are invariably deadly and minimally responsive to therapy. Pten is frequently mutated in aggressive astrocytoma but not in low-grade astrocytoma. However, the Pten astrocytoma suppression mechanisms are unknown. Here we introduced conditional null alleles of Pten (Pten<sup>loxp/loxp</sup>) into a genetically engineered mouse astrocytoma model [TgG(ΔZ)I<sub>121</sub>] in which the pRb family proteins are inactivated specifically in astrocytes. Pten inactivation was induced by localized somatic retroviral (MSCV)-Cre delivery. Depletion of Pten function in adult astrocytoma cells alleviated the apoptosis evoked by pRb family protein inactivation and also induced tumor cell invasion. In primary astrocytes derived from TgG(ΔZ)I<sub>121</sub>; Pten<sup>loxp/loxp</sup> mice, Pten deficiency resulted in a marked increase in cell invasiveness that was suppressed by inhibitors of protein kinase C (PKC) or of PKC-ζ, specifically. Finally, focal induction of Pten deficiency in vivo promoted angiogenesis in affected brains. Thus, we show that Pten deficiency in pRb-deficient astrocytoma cells contributes to tumor progression via multiple mechanisms, including suppression of apoptosis, increased cell invasion, and angiogenesis, all of which are hallmarks of high-grade astrocytoma. These studies not only provide mechanistic insight into the role of Pten in astrocytoma suppression but also describe a valuable animal model for preclinical testing that is coupled with a primary cell-based system for target discovery and drug screening. (Cancer Res 2005; 65(12): 5172-80)

Introduction

Astrocytic glioma is the most common primary brain tumor in humans. Highly malignant forms (Grades III and IV) are fast growing, infiltrative, and angiogenic (1–4). Despite years of effort in clinical and basic research, tumor therapies currently available are rarely effective against malignant gliomas. Thus, the median survival of patients with Grade IV disease (glioblastoma multiforme, GBM) is 8 to 12 months beyond diagnosis (1–4).

Most human malignant gliomas (80–90%) harbor alterations that disrupt the pRb pathway (including pRb or p16/INK4a loss of function or CDK4 or Cyclin D activation), which controls the G1-S cell cycle transition. The mutation patterns are nonoverlapping, indicative of a linear epistatic relationship in this pathway for tumor suppression (1–4). We recently showed in a transgenic mouse model [TgG(ΔZ)I<sub>121</sub>] that astrocyte-specific inactivation of pRb and related proteins p107 and p130 predisposes mice to high-grade malignant astrocytomas that are accelerated by Pten heterozygosity (5).

Pten mutations are associated with 30% to 50% of gliomas (1–4). Most mutations result in loss of Pten expression, which can also be alternatively caused by epigenetic silencing (6). Through its lipid phosphatase activity, Pten antagonizes phosphatidylinositol-3 kinase (PI3K) signaling (7–9), which in various experimental systems, has been shown to affect cell division, apoptosis, cell size, mobility/migration, differentiation, and angiogenesis. The best-studied downstream mediator of PI3K is the Akt kinase, which has been shown to regulate cell division, apoptosis, cell size, and potentially angiogenesis (10, 11). Akt is constitutively activated in some gliomas and other human cancers (12, 13). Yet, Akt activation is not equivalent to Pten inactivation in tumorigenesis (14), indicating that Pten mediates other pathways important for tumor suppression in addition to, or instead of, the Akt pathways. Whereas much progress has been made in understanding the downstream pathways of PI3K/Pten that affect cell division and apoptosis, those regulating cell invasion and tumor angiogenesis, critical features of astrocytoma, are not well understood.

 Invasion into surrounding brain tissue is a hallmark of human astrocytomas, making complete surgical removal of the tumor clinically impossible. Pten has been implicated in controlling cell migration and regulating other cell mobility-related activities both in vitro and in vivo. Pten plays a key role in the sensing of chemoattractant gradients to regulate directional cell migration (15, 16), and the migration rate in fibroblasts is increased in the absence of Pten (17, 18). Increased PI3K activity is a major event occurring at the leading edge of migrating cells (19). In the central nervous system (CNS), Pten-deficient human glioma cell lines are also highly invasive, and restoring Pten activity greatly reduces invasiveness (20, 21). Primary subventricular zone cells isolated from Pten<sup>−/−</sup> mice display increased migration in vitro (22). Furthermore, the architecture of the subventricular zone in Pten<sup>−/−</sup> mice is abnormal, indicative of aberrant cell migration (23). Finally, the highly organized architecture of the cerebellum is disrupted in the CNS-specific Pten conditional knockout mice, possibly resulting from abnormal cell migration (23, 24).

Angiogenesis, a pathologic hallmark of human GBM, is often associated with poor prognosis. At early stages, brain tumors acquire a blood supply by co-opting existing vessels (25). For higher grades, particularly GBM, angiogenesis plays a vital role in aggressive growth (26, 1–4). Some studies suggest a link between
inactivation of Pten and angiogenesis. In Pten-deficient glioma cell lines, transcription of vascular endothelial growth factor (VEGF), a potent proangiogenic growth factor, is induced under both normoxia and hypoxia conditions (27). Akt activation has been shown to activate VEGF and angiogenesis in glioma and other tumor cells, through Hif-1-dependent or Hif-1-independent pathways (28–30). Although Pten inactivation correlates with angiogenesis in a xenograft model (31), such a link has not been shown in spontaneous cancer models.

Here, we test the role(s) of Pten in glioma tumor suppression in vivo using the Tg(G(A2Z)T121) transgenic astrocytoma model in which pRB and related compensatory proteins p107 and p130 are inactivated in astrocytes by an NH2-terminal fragment of SV40 T antigen (5). We developed a somatic viral delivery approach to measure direct and long-term effects of depleting Pten locally in initiated cells already deficient in pRB pathway function, reminiscent of the clonal changes in human tumors. In addition, primary TgG(A2Z)T121 astrocyte cultures homozygous for floxed Pten alleles were also examined for cell growth and invasion properties. Using this primary cell-based system, we probed the contribution of pathways downstream of Pten using specific inhibitors. Results of these studies show that Pten inactivation, likely via multiple pathways, contributes numerous properties that facilitate progression to aggressive astrocytoma, including increased tumor cell survival and invasion along with angiogenesis.

Materials and Methods

Generation TgG(A2Z)T121 mice with conditional Pten alleles. Pten conditional mice were generated as described (32). Briefly, the Pten-loxp chimeras, which were bred to C57Bl6/J mice to generate sites (at the first 5’ XbaI site) downstream of exon 5 and a neo cassette flanked by two lox P sites (at the first 5’ BamHI site) upstream of exon 4 (Fig. 1A). The targeting construct was linearized and transfected into 129 SV/EV ES cell line via electroporation. Targeted ES cells were injected into blastocysts to generate neo/– Neo) and the LoxP-flanked alleles. The locations of inserted LoxP sites were determined by multiple experiments to target tumor cells. Coordinates: (A, L, D) = 1, 1, 1 and 1, 0.5, 0.5 from the Bregma line. These locations were determined by multiple experiments to target tumor cells. MSCV-Cre and MSCV-EGFP viruses were kindly provided by L. Su [University of North Carolina (UNC) at Chapel Hill] and propagated in 293T cells following standard protocols.

Immunohistochemistry and terminal deoxynucleotidyl transferase–mediated nick-end labeling. Brains were fixed, collected in 10% formalin for 16 to 19 hours, embedded in paraffin, and sectioned (5 μm). HE and immunohistochemistry staining were carried out as previously described (5), with some modifications. For most antibodies [except anti–proliferating cell nuclear antigen (PCNA), 1:200; Santa Cruz Biotechnology, Santa Cruz, CA], slides were boiled for 20 minutes in 10 mmol/L citrate buffer (pH 6) and cooled for 45 minutes before blocking. For mouse primary antibodies [anti–T antigen, NH2-terminal (1:25; Oncogene, Darmstadt, Germany); anti–PECAM/CD-31 (1:25-50; Pharmingen, San Diego, CA)], the M.O.M. kit (Vectorlabs, Burlingame, CA) was used according to manufacturer’s procedures. Alternatively, the bound antibodies were detected using the avidin-biotin complex method-peroxidase kit (Vectorlabs) with NovaRed as the substrate. Nuclei were counterstained with hematoxylin (Vectorlabs).

A terminal deoxynucleotidyl transferase–mediated nick-end labeling assay (Oncogene) was used to detect apoptosis in situ following protocols provided.

Primary astrocyte culture, adenovirus infection, and cell invasion assay. Primary astrocytes were isolated and cultured as previously described (5). Recombinant Ad-Cre and Ad-GFP viruses were provided by the UNC Viral Vector Core facilities. Primary astrocytes (passage 1) were infected with virus (multiplicity of infection = 10), in DMEM with 10% fetal bovine serum for 4 hours, washed with cold PBS, and cultured. Seven to 10 days after infection, cells were subjected to the invasion assay. Fifty microliters of Matrigel (Pharmingen) diluted 1:15 with DMEM was used to coat each 24-well insert. Coated chambers were incubated at 37°C for 30 minutes and at room temperature for 1 hour to dry. Cells were collected, a sample was counted using trypan blue, and diluted to 104 cells/mL with DMEM for plating. Inhibitors (Calbiochem, San Diego, CA) were diluted with DMSO and cells were preincubated with inhibitor-supplemented DMEM for 2 hours before plating. LX 294002 (50 μmol/L), GF109203 (20 μmol/L), and protein kinase C-δ (PKC-δ) pseudosubstrate (20 μmol/L) were used to inhibit the activities of PI3K, multiple PKCs, and PKC-δ only.

Figure 1. Generation of conditional Ptenneo/loxP mice and the somatic delivery strategy, A, schematic of the Pten floxed alleles. The locations of inserted LoxP sites and the LoxP-flanked neo cassette. PCR using primers flanking the LoxP sites (arrows, middle) were conducted to screen for correct targeted and floxed alleles (data not shown). B, somatic delivery strategy. The TgG(A2Z)T121 transgene was introduced by crossing Ptenneo/loxP mice with the TgG(A2Z)T121 mice. The genotypes of the mice generated and the number of mice of each genotype used for experiments are listed. Recombinant MSCV virus containing a Cre or GFP vector was delivered with stereotaxic instruments to brains of mice at five to eight weeks of age.
respectively. Cells (10⁵ in 100 μL) were loaded in triplicate into coated chambers. Each chamber was cultured with 600 μL medium at 37°C. After 48 hours, the inner side of each chamber was cleared with a cotton swab and extensively washed with PBS. Chambers were fixed in 4% paraformaldehyde, washed, and stained with crystal violet. Five random 20× fields were counted for each chamber. For cell growth assays, 10⁵ cells in triplicate were cultured with the same media and counted using trypan blue 48 hours after inhibitor treatment.

**Statistical analysis.** Data are presented as means ± SE for the absolute values or percent of controls as indicated in vertical axis legends. For one to one comparisons, the data were analyzed by individual t test; whereas for multiple factor experiments, the data were analyzed by ANOVA followed by Dunnett’s t test. P < 0.05 was considered statistically significant for the indicated n per group.

**Results**

**Generation of mice harboring conditional Pten alleles.** To understand the role of Pten in suppressing tumorigenesis, we generated mice in which Pten exons 4 and 5 are flanked by loxP sites (floxed, Pten<sup>loxp/loxp</sup>) using a standard knock-in approach (Fig. 1A; Materials and Methods). Pten exons 4 and 5 encode a phosphatase domain critical for regulating the PI3K pathways (35). Deletion of this region results in loss of stable transcript and protein expression (35). No difference has been observed between Pten<sup>loxp/loxp</sup> mice and their wild-type (wt) littermates for up to 15 months of age (data not shown). Consistent with previous results, Pten expression was undetectable in the brain following exposure to Cre (data not shown), indicating conditional inactivation. Pten conditional mice were crossed with TgG(AZ)T<sub>121</sub> mice to generate TgG(AZ)T<sub>121</sub>Pten<sup>loxp/loxp</sup> mice, which were interbred to generate TgG(ΔZ)T<sub>121</sub>Pten<sup>fl/fl</sup> and TgG(AZ)T<sub>121</sub>Pten<sup>fl/fl</sup>/C<sub>0</sub> cohorts. TgG(ΔZ)T<sub>121</sub> mice express T<sub>121</sub> under the GFAP transcriptional signals to inactivate pRb, p107, and p130 proteins in astrocytes, inducing aberrant proliferation and apoptosis, ultimately leading to genetically engineered mouse Grade III astrocytoma and death around 9 to 12 months of age (5). As expected, the presence of conditional Pten alleles does not affect survival time or phenotype (data not shown).

**Somatic induction of Pten deficiency suppresses apoptosis and promotes progression.** The latency of astrocytoma development in TgG(AZ)T<sub>121</sub> mice is reduced in a Pten<sup>fl/fl</sup> background (5). To determine the mechanism(s) by which Pten mutation within tumor cells contributes to reduced latency, we developed a somatic delivery approach, allowing the induction of Pten loss regionally in adult brain. Most CNS cells in normal adult mice (>5 weeks), including neurons and glial cells are nondividing and therefore resistant to standard retroviral infection (36). However, because TgG(ΔZ)T<sub>121</sub> astrocytes are highly proliferative at all ages (5), we reasoned that these cells but not surrounding normal cells would be susceptible to retroviral gene delivery.

Brains of TgG(ΔZ)T<sub>121</sub> mice homozygous for wild-type or floxed Pten alleles were injected using stereotaxic methods with MSCV-Cre or MSCV-GFP at 5 to 8 weeks of age (Fig. 1B). By this method, 10⁶ infectious units were reproducibly delivered to the same region of multiple mice with high accuracy. In this study, frontal cortex was injected because of the high frequency of proliferating tumor cells in this region. Injection of control virus (MSCV-GFP) generated limited scarring localized to the injection site. After infection (36). However, because TgG(ΔZ)T<sub>121</sub> mice developed a somatic delivery approach, allowing the induction of Pten loss regionally in adult brain. Most CNS cells in normal adult mice (>5 weeks), including neurons and glial cells are nondividing and therefore resistant to standard retroviral infection (36). However, because TgG(ΔZ)T<sub>121</sub> astrocytes are highly proliferative at all ages (5), we reasoned that these cells but not surrounding normal cells would be susceptible to retroviral gene delivery.

**Figure 2.** Somatic delivery of Cre induces accumulation of neoplastic cells. PCNA immunohistochemistry staining of brains 5 weeks after retroviral delivery. MSCV-Cre virus delivery to TgG(AZ)T<sub>121</sub>Pten<sup>fl/fl</sup> brains induced dense neoplastic cell foci at the injection sites (C) and in the regions as far as 150 μm away (D). The neoplastic cell foci were not present at the comparable region of the un.injected contralateral hemisphere (B). Focal tumor development was not due to retrovirus infection (A, TgG(AZ)T<sub>121</sub>Pten<sup>flo/flxop</sup> + GFP) or Cre protein (TgG(AZ)T<sub>121</sub>Pten<sup>fl.fl</sup> + Cre; data not shown) alone, because no such nodules were found in these controls. The only morphologic difference observed between these brains and those not injected (data not shown) is the needle track (A, arrows).

5 A. Xiao and T. Van Dyke, unpublished observations.
sites (the injection track; Fig. 2A), which did not obviously affect tumor development or survival. The injection track was noticeable as early as 2 weeks after injection and diminished after 6 weeks.

Five weeks after injection, TgG(ΔZ)T121PtenloxP/loxP brains receiving the Cre virus (three of four) developed dense neoplastic cell clusters near the injection track, as highlighted by a large number of PCNA-positive cells (Fig. 2C). Within these clusters, >90% of cells expressed T121 as well as phospho-Akt (data not shown), the latter indicating increased PI3K signaling. Although these regions could be seen clearly after PCNA staining, there was little morphologic distinction by H&E staining and boundaries were not well defined. Control TgG(ΔZ)T121PtenloxP/loxP sites injected with MSCV-GFP (Fig. 2A) contained a density of PCNA-positive cells that was similar to adjacent and contralateral uninjected regions (Fig. 2B). A similar density was observed with TgG(ΔZ)T121Pten+/− + MSCV-Cre (data not shown).

Because pRb function loss in astrocytes induces apoptosis as well as proliferation (5), we examined these activities in tumor cells surrounding injection sites within TgG(ΔZ)T121PtenloxP/loxP brains injected with Cre virus. Although these areas contained more PCNA-expressing cells than controls, the percentage of PCNA-positive cells compared with total cells was not significantly altered (P > 0.05; Fig. 3A). Thus, the high density of PCNA-expressing cells reflects increased cellularity of T121−, S-100+ (data not shown) astrocytoma cells, without increased proliferation. In contrast, the apoptosis levels in these regions (0.69 ± 0.04%) were significantly reduced (P < 0.05) when compared with the same region in the contralateral hemisphere (3.31 ± 1.24%) and with surrounding regions (>300 μm from the injection site; 3.51 ± 0.2%), where such tumor cell clusters were not observed (Fig. 3B). Apoptosis was not substantially reduced in controls TgG(ΔZ)T121PtenloxP/loxP brains injected with MSCV-GFP (2.5 ± 0.53%) or TgG(ΔZ)T121Pten+/− brains injected with MSCV-Cre (2.86 ± 0.3%). In these controls, microglial cells (T121+, S100−) accumulated near the injection track, which may account for a reduction in the absolute fraction of apoptotic cells (Fig. 3B). These data show that Pten inactivation directly reduces astrocyte apoptosis evoked by pRb inactivation, accounting for development of tumor cell–dense foci.

Pten regulates astrocytoma cell invasion. Surprisingly, in the above experiments, dense cell clusters could be detected at some distance from the injection site (±150 μm; Fig. 2D) in Cre virus–injected TgG(ΔZ)T121PtenloxP/loxP brains. This result indicates that Pten inactivation may also have induced invasiveness in pRb/p107/p130-deficient tumor cells. Invasion of tumor cells to surrounding brain tissue is a pathologic hallmark of aggressive astrocytomas. Such infiltration poses extreme difficulty for complete surgical removal of tumor tissue. Thus, we used primary astrocyte cultures from TgG(ΔZ)T121PtenloxP/loxP brains to address the following questions: Do Pten depleted astrocytoma cells have higher invasive capacity than cells with intact Pten function? If so, is increased invasion a direct effect of Pten deletion or a secondary effect of decreased apoptosis? Which pathway(s) downstream of Pten regulate(s) invasion?

A modified invasion chamber assay was used to assess the invasiveness of primary pRb/p107/p130-deficient astrocytes isolated from TgG(ΔZ)T121PtenloxP/loxP mice and treated either with Ad-Cre or Ad-GFP. Astrocytes from TgG(ΔZ)T121Pten+/− mice served as a control for Ad-Cre effects. This in vitro assay measures the capability of the astrocytoma cells to invade and travel within a matrix gel (primarily composed of laminin and collagen). Attributable to reduced apoptosis, Pten-deficient cells accumulated to higher numbers than did cells with intact Pten function. Thus, cells from each population were simultaneously cultured under the conditions of the invasion assay and the number of invading cells was normalized with the total cell counts (Fig. 4). pRb/p107/p130-deficient astrocytes with induced Pten deficiency (infected with Ad-Cre) effectively invaded the gel, whereas isogenic cells treated with the control GFP virus did not (Fig. 4A). Because cells were assayed within 48 to 72 hours of Cre-mediated Pten deletion, additional mutations cannot account for this difference. As confirmation, treating Pten-deficient cells with the PI3K-specific inhibitor LY292004 reduced invasiveness to a level similar to that of control cells infected with Ad-GFP. This result also shows that increased invasiveness is mediated...
by the Pten lipid phosphatase activity. Increased invasiveness could explain the tumor cell–dense foci found distal from the injection site upon localized induction of Pten deficiency in vivo (Fig. 2D).

In addition to inhibiting invasiveness of Pten-deficient astrocytoma cells, the PI3K inhibitor also reduced the overall cell number (Fig. 4B), consistent with survival suppression and restoration of apoptosis. Thus, it is possible that apoptosis suppression and increased invasiveness are linked to the same pathway. Whereas Akt, a PI3K-regulated kinase, has been linked to cell survival (10) and correlates with reduced apoptosis of TgGl(V2)T122;Pten flox/flox mice (ref. 5; see above), it has not been linked to cell invasiveness. However, PKC-ζ, a distinct PI3K-regulated kinase, has been shown to regulate astrocyte cell migration after wounding in culture (37). Using inhibitors in the in vitro invasion assay described above, we tested whether PKCs, and in particular PKC-ζ, are necessary to mediate cell invasion induced by Pten inactivation. When treated with the pan-PKC inhibitor GF109203 or with a PKC-ζ-specific pseudosubstrate peptide inhibitor, the invasiveness of Pten-deficient astrocytoma cells was significantly inhibited (by 13- and 6.5-fold, respectively, Fig. 4A). Consistent with previous reports (37), PKC inhibition did not cause a significant change (P > 0.05) in the cell number (Fig. 4B) thus separating apoptotic inhibition from increased invasiveness, both of which we show to be regulated by Pten in astrocytoma cells. The greater suppression of invasiveness with a pan-PKC inhibitor relative to that with the PKC-ζ-specific inhibitor could indicate a role for additional PKC isoforms or a difference in inhibitor effectiveness. These data indicate that PKC isoforms, including PKC-ζ, are important downstream mediators of Pten/PI3K regulated astrocytoma cell invasion.

**Astrocyte Pten deficiency promotes central nervous system angiogenesis.** Angiogenesis is a pathologic hallmark of the highest grade of human astrocytoma (GBM; see Introduction; ref. 2). To test whether Pten inactivation contributes to angiogenesis in astrocytoma, brains were examined 9 months after virus-mediated Pten deletion as described above. Vessels were highlighted by CD31 immunohistochemistry and counted. Each mouse homozygous for floxed Pten alleles and injected with Cre virus [TgGl(V2)T122;Pten flox/flox + Cre, six of six], showed increased vascular density in the injected cerebral cortex compared with control TgGl(V2)T122;Pten flox/flox mice also injected with MSCV-Cre (n = 3; Fig. 5A-B). The most striking difference associated with somatic Pten inactivation was the increased number of large (7-fold) and intermediate (2.1-fold) sized vessels compared with controls (Fig. 5B). In addition, evidence of branching and/or “merging” vessels, reminiscent of developing vasculature, was observed in mice with induced Pten deficiency (Fig. 5A, arrow), but not in control mice. These data suggest that induction of Pten deficiency, initially in a small population of pRb/p107/p130-deficient astrocytes, promotes an angiogenic process.

Importantly, increased vasculature was evident as far as 500 µm from the injection site in the cerebral cortex, as well as in specific regions of the contralateral hemispheres of mice with induced Pten deficiency (Fig. 5C). Although less advanced than in the injected hemisphere, the increased number of large vessels was significant when compared with controls (P < 0.05; Fig. 5D). In the injected hemisphere, tracks of tumor cell clusters seemed to gradually infiltrate into gray matter from the original injection sites. Six months after injection, these tumor cells were observed within the corpus colossum (two of two) and by 9 months, within the thalamus and caudal regions (six of six). These results are consistent with the in vitro demonstration that Pten depleted astrocytoma cells have a high invasive capacity (Fig. 4).

Whereas somatic induction of Pten deficiency promoted angiogenesis as measured by increased vessel density and sprouting, these changes were progressive. One month post injection, brains (three of three) showed no significant increase in vessel density or size compared with controls (data not shown), whereas increased vessel density and size were observed by 6 months (two of two; data not shown) and 9 months (six of six; above) after initial somatic delivery. Apparent latency of this effect indicates that secondary changes must accumulate to promote angiogenesis and/or that a threshold of tumor cell density, which takes time to accumulate, is required to achieve the response (Fig. 6).

![Figure 4. Pten regulates tumor cell invasion in vitro. Primary astrocytoma cells isolated from newborn TgGl(V2)T122;Pten flox/flox mice were first infected with Ad-Cre to delete the floxed Pten alleles (black column), or with Ad-GFP (gray column) as a negative control, and treated with inhibitors or the solvent DMSO (vehicle) only. Invasion data were normalized with the cell number data to control for the impact of Pten deficiency on cell numbers (Materials and Methods).](https://cancerrres.aacrjournals.org)
Discussion

Inactivation of pRb, p107, and p130 in mouse astrocytes leads to diffuse high-grade astrocytoma by 9 to 12 months of age (5). The primary effects of disrupting this pathway in astrocytes are persistent high-level proliferation and apoptosis. Heterozygosity for a Pten-null allele accelerates disease, and life-threatening high-grade disease develops by an average of <2 months (5). However, brain-specific conditional inactivation of Pten alone, including loss in glial cells does not elicit neoplastic changes (38, 39), indicating the requirement for preceding initiating activities. Here, we used a combination of somatic in vivo gene delivery and primary cell culture approaches to determine the mechanism(s) by which Pten inactivation suppresses astrocytoma progression. Via localized somatic induction of gene deletion, we assessed the effects of Pten inactivation within a limited population of astrocytes predisposed to tumorigenesis in vivo. Disruption of pRb function in astrocytes (by GFAP-driven T121 expression) induces widespread astrocyte proliferation predisposing to astrocytoma. The uniform proliferation of these cells also renders them susceptible to retroviral infection, facilitating selective retroviral gene transduction in vivo. By delivering Cre to these cells, we were able to determine the short- and long-term effects of Pten inactivation as it occurred within a limited population of cells in a defined region of the brain. We further explored the Pten-regulated pathways involved by conditional mutation of primary astrocytes. We show here that Pten suppresses the progression to aggressive astrocytoma by multiple mechanisms. Its loss directly reduces astrocytoma cell apoptosis, simultaneously increases invasion, and ultimately facilitates angiogenesis thus conferring multiple properties characteristic of human high-grade astrocytomas.

**Pten inactivation alleviates apoptosis induced by pRb/p107/p130 inactivation.** Here we show that Pten deficiency suppresses the apoptosis of astrocytes induced in vivo by inactivation of pRb pathway. This result shows a cell autonomous role for Pten in the apoptotic defense to unregulated proliferation occurring in astrocytes. There was no significant effect on the proliferation rate of tumor cells lacking Pten. Previous studies attributing increased proliferation of cultured cells to Pten inactivation indicated a role for Akt, with p27KIP1 (a major inhibitor for G1 cyclin-dependent kinases) as a target to regulate the G1-S cell cycle transition (40, 41). In T121-expressing astrocytes, activation of G1 cyclin-dependent kinases would be inconsequential due to the preexisting inactivation of pRb function. Indeed, most human astrocytomas (80-90%) harbor alterations in the pRb pathway. Because up to 50% of
astrocytomas also carry Pten mutations, it is unlikely that increased proliferation provides the selective pressure for Pten inactivation in these cancers. Rather, our results indicate that decreased apoptosis provides the selective advantage for Pten disruption. In the current report, we model this aspect of tumor evolution by limiting Pten inactivation to a small subset of initiated cells.

**Pten deficiency induces astrocytoma cell invasion.** Tumor cell invasion is a pathologic hallmark of human astrocytoma, making therapy exceedingly difficult (1). Previous work on rat and human glioma cell lines indicates that the Pten/PI3K-regulated pathways play an important role in regulating invasion (17, 20). The current studies provide *in vivo* and *in vitro* evidence to show that Pten inactivation promotes cell invasion. Using a primary cell culture system, we show that Pten-depleted tumor cells have increased invasive potential compared with isogenic cells with functional Pten alleles; that the Pten phosphatase activity is required for limiting invasiveness (based on the effects elicited by a PI3K inhibitor); and that PKC(s), especially PKC-ζ, is (are) required for invasiveness induced by Pten inactivation. Moreover, whereas PKC inhibitors suppressed invasion, they did not affect apoptosis as measured by cell survival *in vitro*. These data also imply that Akt, although critical, is not the only mediator for Pten tumor suppression.

Tumor cell invasion is a multifaceted process requiring changes in cell mobility/migration, cell-cell contact, cell adhesion, and extracellular matrix protein breakdown. This complex process requires the involvement of multiple cell types as well as other microenvironment interactions. Studies with Pten conditional null mice show that Pten inactivation in prostate epithelium leads to metastatic tumors (32, 42). Herein, we focused on the role of Pten in regulating intracellular signaling pathways within tumor astrocytes. Although we showed that PKCs, especially PKC-ζ, are involved in invasion, other downstream targets of Pten/PI3K may also mediate some aspects of astrocytoma invasion. Inhibiting the upstream PI3K had the strongest effect, whereas inhibiting all PKCs or only PKC-ζ had significant but reduced effects. This difference may indicate that additional PI3K regulated factors are involved or that the inhibitors varied in their effectiveness. The biochemical activities of some PKCs are regulated by PI3K-activated PDK1 (43, 44). Furthermore, PIP₃, the phospholipid product of PI3K function, can directly bind to PKC-ζ, possibly as a cofactor with PDK 1 for stable activation of this atypical PKC (45). The relative contributions of PI3K-regulated pathways along with the astrocyte-extrinsic determinants within the microenvironment that promote tumor cell invasion can be determined in the future using the model system and approaches described here.

A recent study reveals that in addition to its NH₂-terminal phosphatase activity, Pten can regulate cell migration through its COOH-terminal C2 domain (46). This may explain the partial inhibition of migration of Pten-deficient astrocytoma cells by the PKC-ζ inhibitor. In addition, for tumor cells to disperse in the brain, they must navigate through the brain matrix. Indeed, the invasiveness of astrocytomas is associated with their ability to secret proteases that disrupt the extracellular matrix. Matrix metalloproteinases (MMP) seem important in glioma invasion (47, 48). Pten has been shown to inhibit, whereas PKC can

![Figure 6](https://cancerres.aacrjournals.org/content/cancerres/65/12/5178/F6.large.jpg)

**Figure 6.** Model for astrocytoma initiation and progression. Inactivation of pRb family proteins (pRb) in normal astrocytes (dark cells with elaborate processes; neurons, clear cells with long axons) induces aberrant proliferation (cells with blue nuclei) and apoptosis (cells with brown nuclei). Localized Pten deficiency in initiated astrocytes alleviates apoptosis, which leads to focal hypercellularity, and promotes tumor cell invasion. Pten deficiency also predisposes to angiogenesis, which is apparent over time. Increased cellularity due to Pten deficiency and/or the requirement for other genetic/epigenetic events may explain this delay. Furthermore, due to cell invasion and/or other mechanisms (e.g., secretion of angiogenic inducers) angiogenesis occurs at large distances from the initial Pten deficiency.

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activate the expression of MMP-9 (49, 50). Hence, inhibition of PKC activity could provide an approach to reduce invasive potential of astrocytoma cells conferred by Pten inactivation.

**Pten deficiency in astrocytosis promotes angiogenesis.** Despite recent advances in understanding the mechanisms and pathways involved in angiogenesis, how tumor cells, and in particular the frequent genetic alterations they harbor, regulate angiogenesis is largely unclear. The results presented here provide the first in vivo evidence that Pten deficiency promotes angiogenesis spontaneously in cancer development. In human GBM, necrosis and angiogenesis are often observed together in fast growing tumor masses, raising the possibility that necrosis is a critical inducer of angiogenesis, presumably by creating hypoxic conditions (3). In the TgG(AlZ)121 model, astrocytoma is diffuse and solid necrotic tumor masses do not develop. This may be because the initiating event is induced in astrocytes throughout the brain. Alternatively, or in addition, further events may be required before or along with Pten inactivation to produce dense tumor masses. For example, the epidermal growth factor receptor is aberrantly activated with high frequency in GBM, but that event is not modeled here. Because Pten deficiency dramatically increases the invasiveness of pHb-deficient astrocytes, tumor masses may not accumulate without additional changes. Regardless of the reason for diffuse disease, it is notable that despite the absence of necrosis, vascularization increases, showing that necrosis is not a requirement. Further experimentation in this model and its associated primary cell cultures using modulated environments will facilitate the systematic determination of additional variables.

Extensive angiogenesis was not detected until many months post-injection. This apparent lag could be the result of a requirement for a threshold of Pten-deficient tumor cells. Alternatively, additional genetic, epigenetic, and/or environmental factors are required in tumor cells and/or the microenvironment. Even in this case, our findings indicate that Pten inactivation is required, if not sufficient, for CNS angiogenesis, because such effects are not observed in controls where Pten is intact (Fig. 6).

Further experimentation with sensitive assays of vascular development will distinguish these possibilities.

**One mutation, multiple advantages.** Recent studies in human breast tumors showed that carcinoma *in situ* and metastasized tumors share similar gene expression profiles (51). These and other data led to the hypothesis that alterations of the highly penetrant oncogenes and tumor suppressor genes contribute to tumor development by means in addition to regulating cell growth and death, including effects observed late in tumor progression (52, 53). Our data support this hypothesis for Pten inactivation in astrocytoma. Pten deficiency not only suppresses the apoptosis evoked by aberrant proliferation but also promotes cell invasion. Furthermore, Pten deficiency also promotes angiogenesis, suggesting that Pten deficiency provides advantages in late stages of tumorigenesis in addition to the immediate advantages of survival and invasiveness. The multiple roles for Pten in tumorigenesis shown here could account for its high mutation frequency in human tumors (astrocytomas in this case) along with limited alterations in other genes within PI3K-regulated pathways. The studies presented here predict that the most effective therapy for Pten-inactive astrocytomas would target PI3K. The ability to deliver drugs directly to the tumor may circumvent broad toxic effects of targeting such a central pathway. Alternatively, our studies also indicate that tumor invasiveness could be substantially inhibited by a more specific inhibitor of PKC-ζ. Such preclinical testing using the mouse model described here should be forthcoming.

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References


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Andrew Xiao, Chaoying Yin, Chunyu Yang, et al.


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