Astrocyte-specific Expression of Activated p21-ras Results in Malignant Astrocytoma Formation in a Transgenic Mouse Model of Human Gliomas

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INTRODUCTION

Astrocytomas are the most common primary tumor affecting the adult human CNS. Currently, these glial tumors are classified on the basis of their histological appearance into increasing grades of malignancy, with the grade IV, or GBM being the most common and, unfortunately, the most lethal adult human astrocytoma, with a median survival of only 9–12 months (1). Many adjuvant therapies demonstrating promise in various in vitro and in vivo models of astrocytomas have been applied in the clinical setting to patients with little success. The development of rational and targeted therapies for human astrocytomas is heavily dependent on an improved knowledge of the molecular pathogenesis of astrocytomas combined with the generation of appropriate preclinical mouse models that closely reproduce the clinical, histological, and molecular characteristics of human astrocytomas.

The histological grading of human astrocytomas reasonably correlates with the molecular pathogenesis of these tumors. At least two main genetic changes are presumed to underlie the pathogenesis of GBMs: (1) those alterations which result in the increased activation of growth factor signaling pathways (2) and those alterations which alter cell cycle regulatory pathways (2, 3). Augmented signaling through growth factor receptor mitogenic pathways is an important determinant of astrocytoma formation and progression. Amplification, overexpression, and mutations of the EGFR on chromosome 7, are found in ~40–50% of all GBMs (4) and not in lower grade astrocytomas. The mutant EGFR (EGFRvIII) harboring a large in-frame deletion of the extracellular domain that results in a novel glycine residue is the most common mutation associated with amplification of EGFR in GBMs (5, 6). EGFRvIII has attracted a lot of attention because it is constitutively activated, provides a mitogenic stimulus in experimental paradigms (6–8), and perhaps represents a negative clinical prognostic marker (9–11). Its specific expression in GBMs is being evaluated as a potential target for antibody-mediated biological therapies (12, 13).

The intracellular signal transduction pathways in GBMs that transmit the mitogenic signals initiated by EGFR and its mutants, as well as other overexpressed cell surface receptors such as PDGFR (14, 15), are important in considering potential targets for glioma therapy. Among the signaling pathways activated by these receptor tyrosine kinases, p21-ras is of importance as reflected by the presence of oncogenic activating p21-ras mutations in 30% of all human cancers (16). Astrocytomas lack primary oncogenic p21-ras mutations (16), but demonstrate elevated levels of activated p21-ras that approach v-ras transformed cells (17). The increased levels of p21-ras-GTP in sporadic human GBMs is not secondary to decreased p21-ras-GAPs, such as neurofibromin or p120-GAP (18), except in NF1-associated astrocytomas (19). The lack of either primary p21-ras mutations or the loss of p21-ras-GAPs in GBMs therefore suggests that the elevated p21-ras-GTP levels are secondary to activation of this signaling pathway from overexpressed and activated receptor tyrosine kinases such as EGFR. Activation of the p21-ras pathway is functionally important in stimulating astrocytoma proliferation and angiogenesis (17, 20) and may be a potential therapeutic target with agents that inhibit p21-ras isoprenylation, such as FTIs (21, 22).

To date, there are few accurate preclinical models for human astrocytomas. Xenograft models from established human GBM cell lines or tumors explanted into the rodent brain do not recapitulate the invasion, heterogeneity, angiogenesis, and other histopathological fea-
ures that characterize these tumors in humans and render them resistant to conventional therapy. In addition to providing a better preclinical model for the testing of novel and conventional therapeutic agents, a spontaneously occurring small animal model would also facilitate our understanding of the molecular pathogenesis of astrocytomas. Toward this goal, we have used an ES cells-based transgenic approach with in vitro selection to overexpress oncogenic V12Ha-ras specifically in astrocytes using the GFAP promoter. Using this ES cell transgenic approach, two mouse lines were derived. All of the mice with the highest levels of V12Ha-ras and p21-ras-GTP activity developed multifocal GBMs leading to their death 2 weeks postnatally. Ninety-five percent of the mice with moderate V12 Ha-ras expression specifically in astrocytes using the GFAP promoter and the molecular characteristics of these mouse astrocytomas, which are mice developing their astrocytomas by 3 months. The histological and molecular characteristics of these mouse astrocytomas, which are transplantable in another host, are similar in many aspects to those found in human astrocytomas and, therefore, have the potential to serve as extremely useful models in neurooncology.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pGfa 2lac-1, containing a 2.2-kb fragment of the human GFAP promoter directing expression of the β-galactosidase gene (LacZ) was obtained from Dr. Brenner (National Institutes of Neurological Disorders and Stroke). The LacZ gene and the 530-bp 3′ untranslated region of the mouse protamine gene was removed by digestion with BamHI and replaced with an insert containing the V12Ha-ras cDNA with a HA tag at the NH2 terminus. The 2.7-kb GFAP-V12Ha-ras fragment was inserted into the Smal site of PloxP-neo vector in which the neomycin selection marker is flanked by two identically oriented loxP sites. An IRESLacZ cassette, in which the LacZ gene was fused to a nuclear localization signal and an IRES sequence, was introduced in the above vector to form GFAP-V12Ha-ras-IRESLacZpolyA-loxP-neo-loxP (Fig. 1A).

ES Cells Transfection and In Vitro Differentiation. The R1 ES cells were grown as described previously (23). For transfection, approximately 5 × 105 cells were mixed with 20 μg of linearized DNA and electroporated at 250 V, 500 μF in a Bio-Rad Gene Pulser. After selection with G418 (200 μg/ml; Life Technologies, Inc.) for 7–8 days, colonies were picked and grown in 96-well plates. In vitro differentiation of ES cells to astrocytes was performed as described previously (24).

Diploid ES Cell Embryo Aggregation. Positive ES clones after screening by in vitro ES cell differentiation were used for aggregation with embryo following described protocol (23). Some E16.5 chimeric embryos were dissected, fixed, and stained with X-Gal. Chimeras were tested for germ-line transmission by mating with ICR females.

Genotyping. To establish the genotype and transgene copy number in the GFAP-V12 Ha-ras transgenic lines, genomic DNA was prepared from ear punch or tail biopsy samples and used in PCR and Southern blot analysis. PCR amplification was performed with 100 ng of genomic DNA and a sense primer (5′-ACTCCTTCATAAAGCCCTCG-3′) located in the GFAP promoter and an antisense primer (5′-CTCGAATTTCTCAGGAGACCA-3′) located in the 3′ region of the Ha-ras cDNA in a 25 μl mix containing 1× PCR reaction buffer (Promega) supplemented with 0.1 m M deoxynucleotide triphosphate, 1.5 m M MgCl2, 3 pmol of each primer, and 1 unit of Taq DNA polymerase. PCR was performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. For Southern blot analysis, 10 μg of tail DNA were digested with AflIII, electro-
phoresed, and transferred to Hybond nylon membrane (Amersham Life Science). Prehybridization, hybridization with LacZ cDNA labeled with α-32P-dCTP, and washings were done according to standard protocols.

**Northern Blot Analysis.** Total mRNA from flash-frozen mouse tissues was extracted using TRZol (Life Technologies, Inc.). Ten µg of total RNA were fractionated on a 1% agarose-formaldehyde gel and transferred to Hybond nylon membrane (Amersham Life Science). The cDNA probes used in these experiments were 540 bp of Ha-ras fragment (coordinates 3–570 bp of the coding region). A mouse β-actin cDNA served as a control for RNA loading. Hybridization was carried out in ExpressHyb (Clontech) containing 2 × 10^6 cpm/ml probe according to the manufacturer’s protocol.

**Histology, Immunohistochemistry, immunofluorescence, and Electron Microscopy.** Tissue for histological examination was immersion-fixed in neutral buffered formalin, paraffin-embedded, sectioned, and stained with H&E according to standard protocols. Immunohistochemical analysis was performed according to standard procedures incorporating the avidin-biotin-peroxidase complex, using 3,3'-diaminobenzidine tetrachloride or AEC as a chromophore. Paraffin-embedded tissue sections were pretreated with pressure cooking for 6 min at high pressure and then blocked with either 20% goat serum (Life Technologies, Inc.) or 10% rabbit serum (Zymed) in PBS. Then tissue sections were incubated with primary antibodies overnight at 4°C. Primary antibodies used were rabbit anti-GFAP (dilution, 1:300; Dako), mouse monoclonal anti-β-actin (dilution, 1:80; Boehringer), mouse monoclonal anti-LacZ (dilution, 1:10000; BioLab), mouse anti-VEGF (dilution, 1:200; Upstate Biotechnology), rabbit anti-PTEN (dilution, 1: 500; Zymed), rabbit anti-VEGF (dilution, 1:1000; BioLab), mouse anti-phospho-p44/42 MAPK antibody (dilution, 1:1000; BioLab), mouse anti-p44/42 MAPK antibody (dilution, 1:10000; BioLab), mouse anti-VEGF (dilution, 1:200; Upstate Biotechnology), rabbit anti-PTEN (dilution, 1: 500; Zymed), anti-Akt/PKB or AEC was used for detection. Immunofluorescence on tissue sections was performed according to standard protocol. Rabbit anti-LacZ antibody (dilution, 1:10; Clontech) was applied for 2h. Fluorescence-conjugated secondary antibodies (dilution, 1:200) were from Jackson Immunoresearch. For ultrastructural analysis, tumors were fixed in 3% glutaraldehyde in PBS. Samples were post-fixed in osmium tetroxide, dehydrated, and epoxy-embedded, and 30-nm sections were mounted on copper grids, stained with lead citrate and uranyl acetate and examined on a Philips 400 electron microscope at 60 keV.

**Establishment of Astrocytes from Transgenic Mice and Growth in Nod-Scid Mice.** Astrocytes from postnatal transgenic mice or from newborn ICR strain mice were generated as described (25). The single cells were grown in astrocyte basal medium with supplements (Clenetics) and 10% horse serum (Life Technologies, Inc.) according to the manufacturer’s protocol. After 2–3 passages, astrocytes derived from the transgenic mice were maintained in DMEM supplemented with 10% FCS (Life Technologies, Inc.). In each astrocytoma or ES cell line designated as RasD7 had the highest transgene expression, followed by RasB8 with a moderate expression level. Southern hybridization analysis of the RasD7 chimeras or RasB8 transgenic mice, or from primary RasD7 astrocytomas that were inoculated into Nod-Scid mice and second generation (G2) astrocytoma lines derived, 20 metaphase spreads were studied. To determine the earliest passages of tumor cells in which trisomy of chromosomes 10 and 10, respectively, was present, interphase FISH was performed on 200 nuclei of RasD7 or RasB8 P0 cells using probes MMU 8, 8, 74 cM and MMU 10, and 58 cM (Applied Genetics Laboratories, respectively). These P0 cells were obtained 2 days after removal of the D7 or B8 mice brain, grown to about 70% confluence, and analyzed.

**RESULTS**

**ES Cells Mediated GFAP-V12-Ha-ras Transgenesis.** The GFAP-promoter-driven transgenesis construct, GFAP-V12-Ha-ras-IRESLacZ-polya-loxP-neo-loxP (Fig. 1A), expresses one mRNA encoding for both V12-Ha-ras and LacZ proteins. The IRES fragment allows independent translation of LacZ, thereby facilitating the location of V12-Ha-ras-expressing cells in vivo and identifying the origin of abnormalities by staining for LacZ. In addition, to detecting transgene-specific expression, the V12-Ha-ras protein was tagged with the HA epitope, leaving unaltered the subcellular localization and in vivo function of p21-ras (28). The transgene construct was stably transfected into R1 ES cells and clones selected by neomycin selection. Because the GFAP promotor is not active in ES cells, expression specificity of the transgene was screened further by in vitro differentiation of the neomycin-selected ES clones into astrocytic lineage by RA (24). Interestingly, only about 5% of the ES cell clones showed the expected RA-induced transgene expression regulated by the GFAP promotor, as detected by LacZ (Fig. 1B), or HA-tagged V12-Ha-ras expression (Fig. 1C). These doubly screened ES clones were selected for aggregation and predicted to be strong candidates for proper in vivo transgene expression. The in vivo expression specificity of these cells was tested with LacZ staining of E16.5 chimeric embryos produced by ES cell—embryo aggregation. The chimeric embryos from different candidate lines showed variable levels but highly CNS-specific expression in the brain and spinal cord (Fig. 1D). With this strategy, two ES cell chimeric lines demonstrating CNS-specific transgene expression were selected for additional studies. The line designated as RasD7 had the highest transgene expression, followed by RasB8 with a moderate expression level. Southern hybridization with internal LacZ cDNA probe on A/JIII-digested genomic DNA from these cell lines revealed that the RasD7 line had multiple-site and multiple-copy integration with frequent tail-to-tail junctions in contrast with the RasB8 line with a single-copy, single-site integration (Fig. 1E). The differences in the copy numbers of the transgene in the two lines was also reflected in the amount of HA-tagged V12-Ha-ras protein expressed in the astrocytes as detected by Western blot analysis with a HA antibody (Fig. 1F).

To analyze the tissue-specific expression of the V12-Ha-ras transgene, total RNA was extracted from various tissues of the RasB8 transgenic mouse. Northern blot analysis demonstrated a large 6-kb mRNA transcript corresponding to the V12-Ha-ras-IRESLacZ trans-
Development of Astrocytomas in GFAP-V\textsuperscript{12}Ha-ras Transgenic D7 and B8 Mice. All 30 RasD7 chimeric mice died between at 10–14 days of age and did not progress to germ-line transmission, Table 1. Upon histological analysis, the brains of all 30 RasD7 chimeras (Table 1) were diffusely hypercellular (Fig. 3A) and superimposed with multiple foci of frank tumor (Fig. 3B, arrowheads). These tumor foci were composed of cells with overtly atypical and pleomorphic nuclei and a spindled morphology (Fig. 3B) around zones of hemorrhagic necrosis containing reactive macrophages that stained for CD45 (Fig. 3C) The tumor cells strongly expressed GFAP and were increased around zones of necrosis (Fig. 3D). The V\textsuperscript{12}Ha-ras transgene was specifically located in GFAP-positive cells, as demonstrated by double indirect immunofluorescence staining for GFAP and nuclear localized LacZ (Fig. 3E). In addition to the pseudopalisading astrocytoma cells around zones of necrosis, other pathological similarities between the mouse astrocytomas and human GBMs were present. This included hypervascularity, characterized by an increased number of Factor VII positive microvessels (Fig. 3F), and increased perinecrotic staining for VEGF (Fig. 3G) in the GFAP- and LacZ-positive astrocytes. The mouse astrocytoma cells also contained numerous mitotic figures and had an elevated PCNA-labeling index (Fig. 3H). These histological features are compatible with the pathological diagnosis of a grade IV astrocytoma or GBM, according to the WHO classification scheme used for human astrocytomas (1). In addition, microglial activation often seen in human GBMs was also a constant feature, although these microglial cells did not express either the HA-tagged V\textsuperscript{12}Ha-ras transgene or the LacZ reporter gene.

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<th>Transgenic</th>
<th>Animal no.</th>
<th>Time to death</th>
<th>Incidence of tumor formation</th>
<th>Tumor no.</th>
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<td>RasD7 chimeras</td>
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<td>Multifocal</td>
<td>Grade 4 (100%; GBM)</td>
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<tr>
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<td>4 weeks (50%)</td>
<td>100%</td>
<td>Multifocal</td>
<td>Grade 3 (20%)</td>
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* WHO classification used for grading.
cytoma cells (F400). Incorporated astrocytoma cells, which also express the red/green (3400) astro-green/red fluorescence double-labeling for GFAP (3400), demonstrating proliferating astrocytoma cells with nuclear atypia.

E in the temporal cortex (D) of RasB8 transgenic mouse 3 months of age. C illustrates astrocytoma-like lesion, immunostaining with anti-GFAP antibody (image). Levels of p21-ras-GTP were approximately 10× and 2× higher in the RasD7 astrocytes compared with normal mouse astrocytes and RasB8 astrocytes, respectively (Fig. 6A). Both the RasD7- and RasB8-derived transgenic astrocytes had a much faster growth rate, a higher satura-

(data not shown). Other organs in the RasD7 mice were examined, and they were found to be normal and not expressing the transgene (data not shown).

The 40 RasB8 transgenic mice were indistinguishable from their littermates at birth and up to 1 month of age, despite the expression of the transgene as detected by LacZ staining (Fig. 4A and B). However, they started to die, at about 2 months of age, with progressive ataxia, growth retardation, and hydrocephalus, with 50% dying by 3 months of age and 95% of the mice ultimately succumbing to astrocytomas by 4–6 months (Table 1; Fig. 5B). Histological evaluation of the RasB8 transgenics revealed mild, widespread astrocytic hyperplasia without the severe hypercellularity and multiple tumor foci described above for the RasD7 chimeras (Fig. 3A). A majority (80%) of the RasB8 mice had a solitary focus of astrocytoma-like lesions, detected in the septal area, the basal forebrain, and the temporal cortex (Fig. 4C), which consisted of GFAP-positive astrocytoma-like lesions expressing astrocyte markers with distinct nuclear atypia (Table 1; Fig. 4, D and E), equivalent to grade II or low-grade human astrocytomas according to the WHO classification scheme (1). Similar to human astrocytomas, the astrocytoma cells were infiltrative into the surrounding brain, as demonstrated by the double-labeled LacZ- and BrdUrd-positive astrocytoma cells, (Fig. 4F).

In ~20% of the 40 RasB8 mice analyzed, multifocal astrocytoma-like lesions were found with similar pathological characteristics to those described above or with more aggressive features comparable with grade-III human astrocytomas according to the WHO classification scheme (Table 1). These astrocytomas consisted of even more cellularity, nuclear atypia, occasional mitotic figures, and increased vascularity as detected by factor VIII and VEGF staining (data not shown), with the tumor foci usually accompanied by a microleral reaction.

Astrocytoma Formation Is Dependent on V12Ha-ras Gene Dosage. To further augment our findings between the RasD7 and RasB8 mice with regards to the influence of the V12Ha-ras transgene dosage on astrocytoma development, RasB8 transgenic mice were crossed to produce homozygosity for the V12Ha-ras transgene. HA-tagged V12Ha-ras transgene expression in the brains of homozygotes was ~2-fold higher than that of hemizygotes (Fig. 5A). This led to a dramatic reduction in survival, with 50% of the 20 homozygous mice analyzed were deceased or had to be killed because of their histologically verified astrocytomas at 3 weeks as compared with 12 weeks for the hemizygote mice (Fig. 5B). In addition, all of the 20 homozygous RasB8 (+/+) mice developed multifocal astrocytomas, some of which demonstrated increased pathologically aggressive features, comparable with grade III human astrocytomas (Table 1). At 3 weeks of age, there was no difference in the degree of hypercellularity in nontumorous regions or other parts of the CNS in the B8 homozygous mice when compared with B8 heterozygous mice of similar age. This can be compared with the slightly decreased incidence and longer latency of the heterozygous RasB8 (+/−) mouse astrocytomas (Table 1).

Tumorgenicity of Astrocytes Derived from GFAP-V12Ha-ras Mice. To address whether the astrocytes in astrocytoma-like lesions of GFAP-V12Ha-ras transgenic mice represent transformed cells, derivative astrocyte cultures (three independent) from RasD7 or RasB8 brains were established. The relative expression levels of the HA-tagged V12Ha-ras transgenes in these two cell lines were verified by Western blot analysis with HA antibody (Fig. 1E). Levels of p21-ras-GTP were approximately 10× and 2× higher in the RasD7 astrocytes compared with normal mouse astrocytes and RasB8 astrocytes, respectively (Fig. 6A). Both the RasD7- and RasB8-derived transgenic astrocytes had a much faster growth rate, a higher satura-

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tion density, and fewer serum requirements compared with normal mouse astrocytes in anchorage-dependent and -independent assays, which did not change even after 50 passages (data not shown). The D7 cells had increased both in vitro and in vivo growth in Nod-Scid mice, compared with the B8-derivative cells (data not shown).

To determine the role of the p21-ras-MAPKinase pathway in the derived mouse transgenic astrocytoma cells, we undertook cell viability assays using both a FTI as well as a specific MEK inhibitor. Similar to the previous results in human malignant astrocytoma cells (21), FTI (L-744,832) treatment strongly inhibited RasD7 or RasB8 cell viability in a dose-dependent (0–50 μM) manner (Fig. 6B). FTI treatment also reduced p21-ras-GTP and phospho-MAPKinase activity in these cells (Fig. 6B), indicating that the p21-ras-MAPK pathway plays an important role in proliferation of these cells. Because the anti-proliferative effects of FTIs may involve not only inhibition of p21-ras activation, but other p21-ras-dependent and -independent mechanisms (29), we also directly inhibited the major downstream mitogenic effector of p21-ras-GTP mediated by activation of MAPKinase by MEK, using a specific inhibitor of MEK (U0126; Ref. 30). Similar to FTI treatment, viability of the mouse astrocytoma cells and the human U87 malignant astrocytoma cell line were also decreased with increasing doses of U0126, with decreased levels of phospho-MAPKinase (Fig. 6C). However, in contrast to FTI treatment, application of U0126 did not cause any reduction of p21-ras-GTP levels upstream of MEK and MAPKinase (data not shown).

Tumorgenecity in vivo was evaluated by injection of 1 × 10^5 – 1 × 10^6 primary transgenic astrocytes into Nod-Scid mice i.c. (Fig. 7A, C, and E) or s.c. (Fig. 7, B, D, and F), with both RasD7 and RasB8 astrocytes, forming GFAP-positive tumors within 10–20 days. The growth rate was faster in the i.c. or s.c. D7-injected Nod-Scids compared with B8 cells, with the s.c. D7-injected mice requiring killing by 1 month versus 2 months for the B8-injected Nod-Scids. These transplanted tumors maintained the morphological and immunohistochemical features of the parental astrocytomas and expressed the transgene as detected by LacZ staining (Fig. 7, E and F). Tumor implants from cultured cells derived from both RasD7 and RasB8 strains showed identical ultrastructural features, with organelle-poor cytoplasm containing glycogen and free ribosomes (Fig. 7, G and H). The cells gave rise to short processes containing small bundles of intermediate filaments, in keeping with the astrocytic origin of these cells, with neither microtubules, cell junctions, nor neurosecretory granules seen.

**Gene Expression Alterations in GFAP-V^12^Ha-ras Transgenic Models.** To characterize critical gene expression alterations implicated in human malignant astrocytomas, such as p53, Rb, p16^INK4a^, p19^ARF^, CDK4, EGFR, and PTEN (2, 3), in our GFAP-V^12^Ha-ras transgenic mouse model, three independent derivative astrocytoma lines from the RasD7 (2 weeks) and RasB8 (3 months) brains (passage numbers 5) were analyzed by Western blots. As shown in Fig. 8A, loss of p16^INK4a^ and p19^ARF^ expression was found in both transgenic mouse astrocytoma lines, whereas loss of PTEN expression was only observed in RasB8 cells. Sequencing the entire cDNA of PTEN in either D7- or B8-derivative lines did not yield any mutations (data not shown). p53 expression was low but similar in the transgenic lines as compared with control mouse astrocytes, although the expression of the p53-negative regulator MDM2 was higher, especially in the RasD7 cells. The amount of Rb expression was similar between the
transgenic and normal mouse astrocytes, although CDK4 levels were higher, especially in the RasD7 cells. EGFR was over-expressed in both of the transgenic mouse astrocytoma cell lines compared with normal mouse astrocytes, although, using an anti-EGFRvIII antibody, we could not detect any EGFRvIII expression in the cell lines or in the astrocytoma specimens (data not shown).

Analysis of the RasD7 and RasB8 astrocytomas in situ, using available immunohistochemical reagents directed at some of the genetic alterations noted in the derived cell lines above (Fig. 8A) was also undertaken. As demonstrated in Fig. 8B, immunohistochemical staining for PTEN in the RasB8 astrocytomas was positive in tumor-associated cells such as microglia cells (arrowhead) but not in the tumor cells (arrows). In contrast, PTEN was expressed in the RasD7 astrocytomas (data not shown) similarly to the Western blot analysis on the RasD7-derivative astrocytoma cell lines (Fig. 8A). Levels of activated/phosphorylated Akt/PKB, the downstream effector of the PTEN-regulated PI3Kinase pathway, was increased only in the PTEN-negative RasB8 tumor cells (Fig. 8C). A similar observation for PTEN and phospho-Akt/PKB expression was also noted in the s.c. implanted RasB8 tumors in Nod-Scid mice. PTEN expression was absent in the implanted RasB8 tumor (T), whereas it was detected in the surrounding s.c. normal (No) tissue (Fig. 8D). Adjacent sections demonstrated the PTEN-negative RasB8 tumor (T) to be positive for activated/phosphorylated Akt/PKB, unlike the PTEN-positive s.c. normal tissue (No; Fig. 8E). In both the RasB8 astrocytoma in situ (Fig. 8, B and C) and implanted tumors (Fig. 8, D and E), nonphosphorylated inactive levels of Akt/PKB were similar between the non-tumor and tumor regions (data not shown). We also were able to demonstrate the loss of p16 in both the RasB8 (Fig. 8F) and RasD7 (Fig. 8G) astrocytoma cells (arrows) in situ, consistent with the Western blot data on corresponding derivative cell lines (Fig. 8A).

Cytogenetic Analysis of GFAP-V12Ha-ras Astrocytoma Cells. SKY analysis on the transgenic ES cell lines, from which the RasD7 or RasB8 mice were derived, did not demonstrate any cytogenetic aberrations (Table 2). In the RasB8 transgenic astrocytoma cells, an abnormal clonal karyotype was found, consisting of trisomy of mouse chromosome 10 in 17 of 20 metaphase spreads examined. In the RasD7 chimera-derived astrocytoma cells, several chromosomal ab-

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Fig. 7. The RasD7 and RasB8 astrocytoma cells are transformed as they form tumors in a secondary host. RasD7 cells formed invasive and vascularized intracranial tumors (A, C, and E), whereas RasB8 cells formed s.c. tumors (B, D, and F). A and B, H&E staining (×200). C and D, immunostaining with anti-GFAP antibody (×200). E and F, immunostaining with anti-LacZ antibody (×200).

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Fig. 8. Aberrant expression of cell cycle regulatory genes in GFAP-V12Ha-ras transgenic tumor cells. A, Western blot analysis of RasD7 and RasB8 cell lysates for cooperating genetic aberrations leading to astrocytomas demonstrate several which are common to human astrocytomas. Data shown are representative of three independent experiments using early-passage (3–5) cell cultures. B–E, loss of PTEN and activation of Akt/PKB in RasB8 transgenic astrocytoma in situ (B and C) and s.c. implanted RasB8 tumor (D and E). B and D, immunostaining with anti-PTEN antibody. B (×1000), the RasB8 astrocytoma cells (arrows) do not express PTEN, whereas tumor-associated cells are positive (arrowhead). D (×1000), PTEN is expressed in the normal (No) s.c. tissue but is absent in the implanted RasB8 tumor (T). C and E, immunostaining with anti-phospho-Akt/PKB. C (×1500), the RasB8 tumor cells are positive for activated/phosphorylated Akt/PKB, similarly to the implanted RasB8 tumor (T), as detected in E. Nontumor cells or tissue are negative. F and G, immunostaining with anti-p16 antibody on RasB8 (F) and RasD7 (G) astrocytoma in situ. Arrows, tumor cells that are p16-negative.
normalities were identified, but the abnormal karyotype was not clonal in nature. However, an abnormal clonal karyotype was found in second generation RasD7 cells (G2), which were derived after in vivo propagation of the primary RasD7 astrocytoma cell lines in Nod-Scid mice. Similar to the RasB8 cells, the RasD7G2 astrocytoma cell lines exhibited clonal trisomy of chromosome 10 in 17 of 20 metaphase spreads examined (Fig. 9A). In addition, the RasD7G2 astrocytoma cells exhibited trisomy of chromosome 8 (in 9 of 20 metaphase spreads) and partial trisomy of chromosome 3 with additional genetic material derived from chromosome 3 on part of the derivative chromosome 18 in 17 of 20 metaphase spreads examined (Fig. 9A). Trisomy of chromosome 19 in 2 of 20 metaphase spreads (Table 2) was also noted. To confirm that the trisomies of chromosomes 8 and 10, respectively, were present in tumors at the time of removal from mice, P0 cultures were directly processed after culture for ~2 days when they were ~70% confluent by interphase FISH (Fig. 9, B and C). All of the RasD7G2 and RasB8 cells exhibiting >88% trisomy also had significant trisomies in the P0 cultures after 2 days of establishment (range, 10–70% of nuclei), when they would be comprised of both transformed astrocytoma cells and nontransformed astrocytes and other cellular elements.

**DISCUSSION**

To develop a transgenic mouse model of human astrocytomas, we chose ES-mediated transgenesis over standard pronuclear DNA injection because of several advantages. Conventional pronuclear DNA injection frequently results in multiple-copy integration of a transgene, which can result in variation of transgene expression (31), whereas ES cell-mediated transgenesis provides a higher frequency of low copy numbers (like our RasD7 line) or even single-copy (RasB8) of transgene integration (Fig. 1E). In addition, the antibiotic-selected transfected ES cell clones also underwent a secondary in vitro selection using RA-induced astrocyte differentiation assay (Fig. 1B). This in vitro screen increases the specificity of transgene expression driven by the GFAP promoter. In addition, the ES cell-mediated transgenic approach also allowed us to circumvent the potential hurdle in a number of cases where expression of the transgene is lethal, because it involves chimera production with transgenic ES cells contributing to different levels in the animals.

We have demonstrated previously that one of the most important mitogenic signaling pathways in human malignant astrocytomas involves activation of p21-ras (17, 21). In this study, we tested the hypothesis that p21-ras activation in astrocytes was sufficient for astrocytoma formation, using ES cell-mediated transgenesis, with overexpression of oncogenic V12-Ha-ras specifically in astrocytes using the GFAP promoter. Our results are the first demonstration that activated p21-ras by itself can lead to transformation of astrocytes in vivo, with the transgenic mouse astrocytomas mirroring both the histopathology and the molecular profile of human malignant astrocytomas. The mouse astrocytomas were composed of GFAP-positive, highly mitotic, pleomorphic, and infiltrative astrocytes, which are associated with increased vascularity and areas of necrosis, all typical histopathological features of human malignant astrocytomas (Figs. 3 and 4). These mouse astrocytes are truly transformed, as evidenced by their ability to form tumors in another host (Fig. 7). The transgenic mouse astrocytoma cells, in addition to elevated p21-ras-GTP levels as reported by us previously in human malignant astrocytoma cells and tumor specimens (17), also harbored some genetic and chromosomal aberrations, which are known to be relevant in the molecular pathogenesis of human astrocytomas. These properties—especially in the RasB8 line, which has gone to germ-line transmission with the availability of large numbers of mice that develop astrocytomas with high penetrance—makes this a good preclinical model of human malignant astrocytomas.

Despite the high prevalence of p21-ras mutations in a variety of human tumors, mutational activation of p21-ras is not an obligatory event for tumorigenesis and progression, as evidenced by the fact that

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**Table 2** Description of the karyotype of GFAP-V12 Ha-ras mouse astrocytoma cell lines

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<tbody>
<tr>
<td>RasB8 ES</td>
<td>G-banding</td>
<td>40,XY</td>
</tr>
<tr>
<td>RasB8</td>
<td>G-banding, SKY</td>
<td>40-41,XY,+10[17]/42,XY,+3,+10[3]</td>
</tr>
<tr>
<td>RasD7 ES</td>
<td>G-banding</td>
<td>40,XY</td>
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</tbody>
</table>

* No two metaphase cells shared the same abnormal karyotype; only clonal changes are shown. Other non-clonal aberrations (present in one cell only) were observed within the six cells with a composite abnormal karyotype.
mutated p21-ras genes are rare in breast adenocarcinoma, neuroblastomas, ovary and esophageal carcinomas, and astrocytomas (16). However, several lines of evidence point to a “functional over-activation” of the p21-ras signaling pathway contributing to the growth of astrocytomas. First, direct measurement of p21-ras activity in sporadic astrocytoma cell lines and specimens indicates that activity of p21-ras is markedly elevated, and they are sensitive to growth inhibition by FTIs (17, 21). In these sporadic astrocytomas, there is abundant expression and activity of p21-ras-GAPs (i.e., p120ras-GAP and neurofibromin), the negative regulators of p21-ras-GTP (18). This would suggest that the increased p21-ras activity in these tumors most likely results from activation of overexpressed receptor tyrosine kinases such as PDGFRs and EGFRs. Second, in germ line-associated astrocytomas such as those in NF1 patients, levels of p21-ras activity is also increased (19). However, in contrast with sporadic astrocytomas, there is a loss of neurofibromin expression and decreased p21-ras-GAP activity, and not overexpression of receptor tyrosine kinases, in these astrocytomas (19). Third, reported mouse models of astrocytomas also indirectly implicates increased activity of p21-ras in the pathogenesis of astrocytomas: (a) expression of v-src in mouse astrocytes under control of the GFAP promoter leads to development of astrocytomas, although with a much lower prevalence compared with our direct expression of oncogenic p21-ras (32). Although not directly measured in this model, it is well known that v-src transformation leads to p21-ras activation (32); (b) expression of retrovirally transduced oncogenic p21-ras in combination with activated Akt/PKB into astrocytes expressing the retroviral receptor also induced astrocytoma formation (33); and (c) transgenic mice expressing mutations of the NF1 and p53 genes in cis, also develop astrocytomas with an accompanied loss of both the normal alleles in the astrocytomas (34). Although p21-ras activity was not directly measured in this mouse astrocytoma model, increased activity is implied because of a loss of neurofibromin expression similar to human NF1 astrocytoma specimens (19).

In our transgenic model, levels of p21-ras activity was directly measured, with the GFAP-V12Ha-ras transgenic RasD7 and RasB8 astrocytoma cell lines having markedly elevated levels of p21-ras-GTP compared with normal murine astrocytes (Fig. 6A) and similar to those previously reported in p21-ras-transformed RT8-murine fibroblasts or human astrocytoma cells (17). In addition, the onset of the development of malignant astrocytomas in these mice correlated with the level of p21-ras expression and activity. The stronger-expressor RasD7 line formed multifocal GBMs leading to death by 2 weeks of age (Table 1; Fig. 3), whereas the moderate-expressor RasB8 line, which had 2-fold-increased, HA-tagged V12Ha-ras expression and p21-ras-GTP activity, started to die from their astrocytomas at 2 months of age (Figs. 4 and 5). By interbreeding RasB8 transgenic mice, we generated homozygous with an increased dosage of the transgene, which led to a substantial shortening of the latency and increased penetrance of multifocal and pathologically more aggressive astrocytomas (Table 1; Fig. 5).

Oncogenic p21-ras alone is not transforming, though it is known to induce genomic instability and cooperate with other genetic alterations leading to transformation (35). In several types of primary cells, including neurons, oncogenic p21-ras alone causes a senescence response by inducing expression of cell cycle inhibitory molecules such as p53 and p16INK4a (36). In cells that are transformed by p21-ras, cooperating oncogenes or the loss of cell cycle regulatory genes are required to convert normal cells to tumorigenic ones (35, 37). Indeed, in the retroviral injection model, expression of both oncogenic p21-ras and activated Akt/PKB was required in the background of transgenic mice expressing the retroviral tv-a receptor under control of the nestin but not the GFAP promoter to induce GBMs pathologically similar to those in our model (33).

Our data demonstrates that overexpression of V12Ha-ras in astrocytes, although not a primary molecular aberration of human astrocytomas, can in fact initiate the sequence of additional genetic events that lead to the development of astrocytomas in mice. Astrocytes expressing elevated levels of Ras-GTP may be more genetically unstable, as has been demonstrated in other cell types (38, 39), making them more susceptible to additional genetic alterations that lead to transformation. This susceptibility is dose-dependent, as suggested by the shorter latency and multifocality of the GBMs in the D7 mice with higher-number of transgene integration sites and Ras-GTP levels.

Although, p53 or Rb expression levels were not directly altered in either mouse astrocytoma cell line, aberrations of the regulators of both these pathways prevalent in human astrocytomas were present. This is exemplified by the loss of p19ARF and over-expression of MDM2 (both regulators of the p53 pathway), and p16INK4a and over-expression of CDK4 (both regulators of the Rb pathway; Fig. 8A). The evidence that these cell cycle regulators cooperate in the pathogenesis of astrocytomas comes not only from their alterations in human astrocytomas, but also from the retroviral injection tv-a-expressing transgenic mouse model (40). Retroviral expression of mutated EGFRvIII, expressed in a large proportion of astrocytomas, by itself was not sufficient to lead to increased astrocytic foci. Additional genetic aberrations involving both the p53 or Rb pathways or its regulators, such as those found in the mice deficient for the INK4a/ARF locus harboring both p16INK4a and p19ARF, or simultaneous retroviral injection of CDK4, was required. Of note, overexpression of EGFR was present in both our mouse astrocytoma lines (Fig. 8A), although they do not harbor any amplifications or mutations (data not shown). The astrocytic foci in the retroviral injection experiments have pathological characteristics consistent with malignant astrocytomas; however, whether these are truly transformed astrocytes capable of developing tumors in a secondary host similar to our RasD7 or RasB8 cells (Fig. 7) remains to be demonstrated.

Loss of PTEN expression, found in majority of human GBMs (41, 42) was demonstrated in the RasB8 but not in the RasD7 astrocytoma cells (Fig. 8). Of interest, PTEN mutations were not found in the B8 lines, though no protein was detected. This is similar to human malignant astrocytomas where a large number lack PTEN expression with only a minority harboring mutations, suggesting alternate and yet-to-be-identified mechanisms of loss of PTEN expression in astrocytomas (41, 42). Loss of PTEN expression is associated with high-grade GBMs and not lower-grade astrocytomas in humans, so one would predict the more aggressive D7 tumors would be negative for PTEN expression. The reason for this discordance in PTEN expression and the associated astrocytoma grades between our two mouse astrocytoma lines is currently unclear. One may postulate that the extremely high levels of p21-ras-GTP in the RasD7 astrocytes (Fig. 6A), which results in postnatal lethality by 2 weeks and an extremely hypercellular astrocytic background (Fig. 3A), obviates the requirement for loss of PTEN that is associated with later tumor stages. In contrast, the RasB8 astrocytes, with lower levels of p21-ras-GTP and a normal cytoarchitecture of the brain, at least until 1 month postnatal (Fig. 4A), would be more dependent on additional genetic aberrations, including the loss of PTEN, to develop the grade II/III astrocytomas.

Our ongoing work examining the genetic changes during the various stages of astrocytoma development in the RasB8 mice, and crossing these mice with the PTEN heterozygotes, should be informative with respect to the role of PTEN in our model of human astrocytomas.

The cytogenetic analysis of the RasD7 and RasB8 astrocytomas are of ongoing interest (Table 2; Fig. 9). The lack of any karyotype abnormalities in the RasB8 and RasD7 ES cells supports the conclu-
sion that the noted genetic abnormalities in the corresponding tumors were related to the development of the astrocytomas. The lack of any clonal karyotype abnormalities in the primary astrocytes derived from the RasD7 chimeric mouse brain, in comparison with the clonal abnormalities seen in the secondary RasD7G2 astrocytoma cells (Table 2), we believe reflects the overall hypercellularity of the RasD7 brains (Fig. 3), with a large percentage of the astrocytes expressing high levels of the V12 Ha-ras transgene, (Fig. 1E). Not all of the cultured RasD7 astrocytes are truly transformed and have acquired the additional genetic alterations that are required for the formation of the multifocal malignant astrocytoma foci within the hypercellular background. These derived primary RasD7 astrocytoma cells would therefore not be expected to show any clonal chromosomal aberrations, which become evident when these cells are passaged through hosts (Fig. 7), thereby selecting for the truly transformed astrocytoma RasD7G2 cells. In primary RasB8 cells, derived from RasB8 transgenic astrocytomas with low levels of V12 Ha-ras transgene expression, the majority of astrocytoma cells that survive to grow in long-term culture for cytogenetic analysis are those that are transformed, with clonal chromosomal abnormalities.

Both established RasB8 and RasD7G2 astrocytoma cells had an extra copy of mouse chromosome 10 (in 17 of 20 metaphase spreads examined), using SKY analysis (Fig. 9A), with the chance of cell culture-induced artifacts minimized by observing the same results on early P0 cultures by interphase FISH analysis (Fig. 9, B and C). The P0 experiments were undertaken on 2-day-old cultures from D7 and B8 mice brain without passaging; hence the number of positive interphase FISH demonstrating trisomy varied and ranged between 10–70% of the nuclei seen, reflecting the heterogeneous population of cells at such an early time point. Mouse chromosome 10 harbors regions that are syntenic with a large portion of human chromosome 12q, the second most common amplified region after chromosome 7 in human astrocytomas (3). The chromosomal region 12q13-q14 contains the CDK4 and MDM2 genes, which are amplified and overexpressed in ~10% of human GBMs (43, 44). The extra copy of chromosome 10 was reflected in the overexpression of both CDK4 and MDM2 by both mouse astrocytoma cells compared with normal mouse astrocytes, and was especially prominent in the RasD7G2 cells (Fig. 8A). Trisomy of mouse chromosome 8 and an extra copy of chromosome 3 translocated to chromosome 18 were also detected at relatively high clonal frequency in the RasD7G2 cells, without any obvious syntenic relationship to any human astrocytoma-specific genetic alterations reported to date. These additional chromosomal changes detected by cytogenetic analysis, coupled with the aberrant expression of cell cycle regulatory genes, argue that tumor formation in GFAP-V12 Ha-ras mice, as in human astrocytomas, requires multiple genetic lesions, although overactivity of p21-ras and perhaps the resultant genetic instability in astrocytes was sufficient to initiate the process.

In summary, we have shown that overexpression of oncogenic p21-ras specifically in astrocytes with in vitro selected ES transgen- es leads to astrocytoma formation. In addition to the retroviral injection model discussed above, knockout mice such as those lacking p19ARF (45) or those expressing various oncogenes under control of the GFAP promoter also develop gliomas, though the incidence is lower, unpredictable, and with a longer latency (32). The main advantage of our model, especially the RasB8 line, is the ability to readily obtain large numbers of immunocompetent germ-line mice with reproducible astrocytomas that exhibit many of the key pathological and molecular features of human astrocytomas. Our transgenic mice develop astrocytomas at a high frequency and with a relatively short latency. These advantages make this model valuable to studies aimed at assessing the contributions of genetic modifiers to tumorigenesis as well as a preclinical model of brain tumors.

ACKNOWLEDGMENTS

We thank Dr. Fabrizio Mastronardi for assistance with making figures and developing the immunofluorescence assay.

REFERENCES

TRANSGENIC ASTROCYTOMA MOUSE MODEL


Astrocyte-specific Expression of Activated p21-ras Results in Malignant Astrocytoma Formation in a Transgenic Mouse Model of Human Gliomas

Hao Ding, Luba Roncari, Patrick Shannon, et al.


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