Essential Role for Ras Signaling in Glioblastoma Maintenance

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Abstract

Malignant gliomas can be induced in mice through the combined expression of activated forms of both KRas and Akt in glial progenitor cells. To determine the reliance of these tumors on continued KRas signaling in vivo, we generated a viral vector that allows the expression of KRas to be controlled post-delivery. Tumor-free survival rates were compared between those animals with continued KRas expression and animals in which KRas expression was suppressed. KRas signaling was found to be required for the maintenance of these tumors in vivo; inhibition of KRas expression resulted in apoptotic tumor regression and increased survival. Subsequent reexpression of KRas reinitiated tumor growth, indicating that a percentage of the progenitor cells survived and retained tumorigenic properties. (Cancer Res 2005; 65(18): 8250-5)

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor. It is also the most fatal; mean survival is <1 year from the time of diagnosis, with <10% survival after 2 years. Despite major improvements in imaging, radiation, and surgery, the prognosis for patients with this disease has not changed in the last 20 years. Treatment options for patients with GBM include surgery, radiation, and chemotherapy (1). However, surgery and radiation are extremely difficult due to the location and invasive nature of the tumor, and chemotherapy remains controversial, as many studies have failed to show prolonged median survival in treated patients (2). In the past several years, genes that are altered in tumor tissue relative to normal brain tissue have been found (3). However, the effects of these genetic alterations have not been fully characterized in vivo; those that can be productively targeted for therapeutic intervention in patients remain to be identified.

Glioblastomas are classified as either primary or secondary GBM (4). Primary GBM develops de novo, whereas secondary GBM progresses from a low-grade glioma to a high-grade glioma through the acquisition of additional genetic alterations (5). Primary glioblastomas comprise 80% of all GBM (6). Although these two types cannot be distinguished pathologically, they seem to have distinct genetic alterations (reviewed in ref. 6). Epidermal growth factor receptor amplification and overexpression, PTEN, and INK4a deletion are hallmarks of primary GBM. In contrast, platelet-derived growth factor receptor overexpression, p53 mutation, cyclin-dependent kinase 4 overexpression, or RB loss, and PTEN loss characterize secondary GBM (6). In both cases, activated receptor tyrosine kinases (i.e., epidermal growth factor receptor and platelet-derived growth factor receptor) activate common downstream signaling pathways including the Ras pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (1, 7). Loss of PTEN expression is also common in both primary and secondary GBM (6). PTEN is a phosphatase that functions to inhibit the PI3K/Akt pathway (7). In the absence of PTEN, Akt activity is elevated leading to increased proliferation and inhibition of apoptosis (8). Akt activation has also been documented in GBM as a result of increased PI3K activity due to mutation within the regulatory subunit of PI3K (9).

Recently, a mouse model of human GBM based on the avian RCAS/TVA system was developed (10). In this model, the retroviral receptor, TVA, is expressed under the control of the nestin promoter, which is active in neural and glial progenitors. This promoter was chosen because GBM is thought to arise from the supporting glial cells of the brain (11). Replicating mammalian cells that express TVA are susceptible to infection by the subgroup A avian leukosis virus–derived RCAS vectors (Fig. 1A and B; reviewed in ref. 10). Although replication-competent in avian cells, these viruses are replication-defective in mammalian cells; therefore, the viral vectors cannot spread in the target animals. In addition, because little viral envelope protein is produced, there is no interference to superinfection. Thus, there is theoretically no limit to the number of experimental genes that can be introduced into a TVA-expressing mammalian cell. The ability of these cells to be infected by multiple viruses allows efficient modeling of GBM, because multiple oncogenic alterations can be easily introduced into the same cell or animal.

In this study, we examined the role of Ras signaling in glioblastoma maintenance in the kras- and akt-induced glioblastomas in the RCAS/TVA model. Inhibition of KRas expression resulted in apoptotic tumor regression and increased survival of tumor-bearing mice, strongly suggesting that KRas signaling is required for tumor maintenance in vivo. Subsequent reexpression of KRas reinitiated tumor growth, indicating that some progenitor cells survived and retained tumorigenic properties.

Materials and Methods

Transgenic mice. Nestin-TVA mice have been previously described (12). The mice were maintained on standard food or doxycycline-containing food pellets (Harlan-Teklad, Madison, WI). All experiments were done in compliance with the guiding principles of the "Care and Use of Animals" (available at http://www.nap.edu/books/0399053773/html/) and were approved by the Van Andel Research Institute, Institutional Animal Care and Use Committee prior to experimentation.

Genotype analysis. DNA was prepared from tail biopsies using an AutoGenprep 960 automated DNA isolation system. PCR of the TVA allele was carried out in 25 μL total volume containing 2.5 μL 10× PCR buffer (Invitrogen, Carlsbad, CA), 1.0 μL DMSO, 1.25 μL 50 mmol/L MgCl2, 0.625 mmol/L of each nucleotide, 1 unit of Taq polymerase (Invitrogen), 6.25 μg/mL of primers TVA-386 sense and TVA-786 antisense. The following primer sequences were used: TVA-386 sense 5'-AGCTGGTGAGATGGGACT-GAAC-3'; TVA-786 antisense 5'-CGAAATCTAAAGCCTCGAG-3' (to detect a 400 bp fragment). Samples were amplified for 30 cycles (94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds).
Isolation of primary astrocytes. Primary astrocytes were isolated from the brains of Nestin-TVA-positive newborn mice. Single cells were obtained by mincing the tissue followed by digestion in 0.05% trypsin for 20 minutes at 37°C. The cells were resuspended in DMEM with 10% fetal bovine serum (FBS) and 1/100 penicillin/streptomycin. The cells were split 1:3 when confluent.

Vector constructs. The retroviral vectors used in this study were either replication-competent avian leukemia virus long terminal repeats, splice acceptor, and Bryan polymerase-containing vectors of envelope subgroup A (designated RCASBP(A); Fig. 1A and B), or no splice acceptor vectors (designated RCANBP(A); Fig. 1C and D). RCASBP(A)Akt has been previously described (8). The RCASBP(A) destination vector has also been described.

Figure 1. Schematic representation of the viral vectors and Western blot analysis of K-Ras expression. A, RCASBP(A); B, RCASBP(A)Tet-off; C, RCANBP(A); D, RCANBP(A)TRE-KRas. These vectors are all Gateway compatible to allow for the easy insertion of experimental sequences.

LTR, long terminal repeat; $\Psi$, packaging signal; SD, splice donor; SA, splice acceptor. E, nestin-TVA-positive astrocytes were infected in culture with RCANBP(A)TRE-KRas alone (lanes 1 and 2) or RCANBP(A)TRE-KRas with RCASBP(A)Tet-off (lanes 3 and 4). The samples in lanes 2 and 4 were treated with 2 $\mu$g/mL doxycycline for 48 hours. Lysates were separated on a 14% Tris-glycine gel, transferred to nitrocellulose, and probed with an antibody against the FLAG epitope tag on K-Ras (top). The membrane was reprobed with an antibody against $\alpha$-tubulin (bottom) to ensure equal loading.

Figure 2. Histologic examination of brain sections from a mouse injected with Akt, Tet-off, and TRE-KRas viruses at birth. All images are serial sections from the same mouse sacrificed at 24 days of age. A, section stained with H&E; B, TUNEL staining; C, immunoreactivity for the FLAG epitope tag on K-Ras (brown cells) and hematoxylin counterstain (blue nuclei); D, immunoreactivity for the HA epitope tag on Akt (brown cells) and hematoxylin counterstain (blue nuclei). Images were captured at 40× magnification. Bar, 0.05 mm.
To generate pENTR3C-Tet-off, pTRE (Clontech) was digested with
Competent Cells (Invitrogen). Transformations were plated on agar plates
and grown at 39°C. LR reactions were done for 2 hours at room

Cell culture. DF-1 cells were grown in DMEM-high glucose supplemented
with 10% FBS (Invitrogen), 1× penicillin/streptomycin, and maintained at
39°C (15, 16). The DF-1 cultures were passaged 1:3 when confluent.

Virus propagation. Virus infection was initiated by calcium phosphate
transfection of plasmid DNA that contained the retroviral vector in proviral
form (17). In standard transfections, DF-1 cells were plated at 30% confluence, allowed to attach (2-3 hours), and 5 μg of purified plasmid DNA
was introduced by the calcium phosphate precipitation method previously
described (18), followed by a 5-minute glycerol shock at 39°C (15% glycerol
in the medium). Viral spread was monitored by assaying culture
supernatants for avian leukosis virus capsid protein by ELISA as previously
described (19). Virus stocks were generated from the cell supernatants. The
supernatants were cleared of cellular debris by centrifugation at 2,000 × g
for 10 minutes at 4°C, filtered through a 0.45-μm filter, and stored in
aliquots at −80°C. Virus was determined to be replication-competent by a
reading of 0.200 or greater on ELISA for the viral capsid protein (19).

Viral infections in vitro. Astrocytes were seeded in six-well plates at a
density of 5 × 10⁴ cells/well and were maintained in DMEM with 10% FBS,
1× penicillin/streptomycin, and stop

Western blotting. Infected astrocytes were washed with PBS and 150 μL
SDS-lysis buffer was added to each well of a six-well dish. The cell lysates
were boiled for 10 minutes and passed through a 26-gauge needle five times.
Following centrifugation, the proteins were separated on a 14% Tris-glycine
polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 hour
at room temperature in blocking solution (0.05% Tween 20 in TBS with 5%
nonfat dry milk). Blots were immunostained for FLAG-KRas using an anti-

Histology and histochemical staining. Mice were euthanized when they
displayed obvious signs of distress or as indicated. Brain tissues were
fixed in formalin overnight, cut into three sections, and then dehydrated
through a graded alcohol series in a Ventana Renaissance processor
(Ventana Medical Systems, Tucson, AZ). Tissues were paraffin-embedded
and 5 μm sections were adhered to glass slides. Sections were stained with
H&E or left unstained for immunohistochemistry.

Immunohistochemistry. The sections were deparaffinized and treated
with 3% hydrogen peroxide for 30 minutes to quench endogenous
peroxidase activity. Sections were blocked for 30 minutes at room

Figure 3. Kaplan-Meier percentage of tumor-free survival curve. All mice
were injected with Akt, Tet-off, and TRE-Kras viruses at birth. A, for
Nestin-TVA-negative mice n = 26 (---) and for Nestin-TVA-positive mice n = 50
(----), P < 0.00004. B, only tumor-bearing mice were included in this analysis.

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To generate pENTR3C-TRE-Kras, RCASBP(A)FLAG-Kras (a gift
from Eric Holland, Departments of Surgery (Neurosurgery), Neurology, and
Cell Biology, Memorial Sloan Kettering Cancer Center, New York, NY) was
digested with XhoI and XbaI and the FLAG-Kras fragment was cloned into
pENTR3C-TRE also digested with XhoI and XbaI. RCANBP(A)TRE-Kras
(Fig. 1D) was generated by mixing 300 ng of pENTR3C-TRE-FLAG-Kras
with 300 ng of the RCANBP(A) destination vector in the presence of the LR
Clonase Enzyme Mix as described above.

Cell culture. DF-1 cells were grown in DMEM-high glucose supplemented
with 10% FBS (Invitrogen), 1× penicillin/streptomycin, and maintained at
39°C (15, 16). The DF-1 cultures were passaged 1:3 when confluent.

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temperature with 1% bovine serum albumin and 5% normal goat serum in PBS. KRas expression was detected using a monoclonal antibody to the FLAG epitope (Sigma) at a 1:250 dilution, and Akt expression was detected using a monoclonal antibody to the HA epitope (Covance, Berkeley, CA) at a 1:1,000 dilution. The sections were incubated with the primary antibodies for 1 hour at room temperature and were then washed in PBS-T. The signal was detected with the Vectastain ABC kit (Vector Labs, Burlingame, CA) and visualized with 3,3′-diaminobenzidine tetrahydrochloride (Vector Labs). Sections were counterstained with hematoxylin. Apoptosis was detected by terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) staining using the in situ cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer’s specifications.

**Results and Discussion**

Intracranial infection of Nestin-TVA mice with either RCASB-P(A)Akt or RCASBP(A)KRas alone is insufficient to form tumors, but the combination of activated forms of both Akt and KRas induces glioblastomas that are histologically similar to human GBM (8). To determine the reliance of these tumors on continued KRas signaling, we generated a viral vector in which expression of the inserted gene could be regulated post-delivery using the tetracycline (tet)-inducible system. We used the RCANBP(A) virus (14), which lacks the splice acceptor at the 3′ end of the envelope gene (Fig. 1C), so that sequences inserted into this region are transcribed from an internal promoter and not the viral long terminal repeat. A tet-responsive element (TRE) was inserted upstream of the KRas gene to generate RCANBP(A)TRE-KRas (Fig. 1D). The virus was propagated in DF-1 cells, an immortalized chicken embryo fibroblast cell line (15, 16). No KRas expression was observed in these cells, because expression from the TRE requires the presence of a tetracycline transcriptional activator such as Tet-off or a reverse tetracycline transcriptional activator such as Tet-on. In the Tet-on system, the Tet-responsive gene is only expressed in the presence of

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**Figure 4.** Histologic examination of brain sections from a tumor-bearing mouse treated with doxycycline for 3 days. This mouse was injected with Akt, Tet-off, and TRE-KRas viruses at birth. All images are serial sections from the same mouse sacrificed at 27 days of age. A, section stained with H&E; B, TUNEL staining; C, immunoreactivity for the FLAG epitope tag on KRas (brown cells) and hematoxylin counterstain (blue nuclei); D, immunoreactivity for the HA epitope tag on Akt (brown cells) and hematoxylin counterstain (blue nuclei). Images were captured at 40× magnification. Bar, 0.05 mm.

**Figure 5.** Histologic examination of brain sections from a tumor-bearing mouse treated with doxycycline for 14 days. This mouse was injected with Akt, Tet-off, and TRE-KRas viruses at birth. All images are serial sections from the same mouse sacrificed at 40 days of age. A, section stained with H&E; B, TUNEL staining; C, immunoreactivity for the FLAG epitope tag on KRas (brown cells) and hematoxylin counterstain (blue nuclei); D, immunoreactivity for the HA epitope tag on Akt (brown cells) and hematoxylin counterstain (blue nuclei). Images were captured at 40× magnification. Bar, 0.05 mm.
doxycycline; in the Tet-off system, the Tet-responsive gene is repressed in the presence of doxycycline (20).

**Tet-regulated expression of KRas in primary astrocytes in vitro.** To test the ability of KRas expression to be induced from the TRE-KRas virus, Nestin-TVA-positive astrocytes were infected in culture with the TRE-KRas virus alone or with both the TRE-KRas and Tet-off viruses. The infected cells were cultured in the presence or absence of doxycycline and KRas expression was visualized by Western blot (Fig. 1E). KRas expression was observed only in those cells infected with both the TRE-KRas and Tet-off viruses. This expression is tightly regulated, because no KRas expression was detected in cells infected with the TRE-KRas virus alone or in cells infected with both the TRE-KRas and Tet-off viruses in the presence of doxycycline (Fig. 1E).

**Tumor formation in Nestin-TVA mice infected with Akt, Tet-off, and TRE-KRas viruses.** Intracranial injection of Nestin-TVA mice with RCASBP(A)Akt and RCASBP(A)KRas induces GBM that are histologically similar to human GBM (8). To test the ability of the TRE-KRas virus to induce tumors in vivo, newborn Nestin-TVA mice were injected intracranially with Akt, Tet-off, and TRE-KRas viruses. As early as 3 weeks of age, several mice began showing signs of tumor formation (e.g., macrocephaly, lethargy, or cachexia). Histologic examination revealed tumors that were histologically similar to human GBM (8). To determine the reliance of these tumors on continued KRas signaling, tumors were induced by injection of Nestin-TVA mice with Akt, Tet-off, and TRE-KRas viruses at birth. Because ~50% of the injected Nestin-TVA-positive mice develop tumors (Fig. 3A), only tumor-bearing mice were selected for doxycycline treatment. Doxycycline was given continuously through the food to 14 tumor-bearing mice from the time of weaning (~21 days of age) until the experimental end point (4 months of age) to suppress KRas expression. Four of the treated mice expired shortly after the start of treatment as a result of tumor progression. The remaining 10 mice became asymptomatic. Tumor recurrence was not observed in any of the animals maintained on doxycycline. A separate cohort of 14 tumor-bearing mice was given standard feed (without doxycycline), and all eventually died of GBM. Tumor-free survival rates were compared between untreated mice and mice given doxycycline to determine whether the administration of doxycycline increased survival. The resulting Kaplan-Meier curve shows a significant increase in tumor-free survival for the mice treated with doxycycline ($P < 0.0006$; Fig. 3B).

**Inhibition of KRas expression results in apoptotic tumor regression.** To determine if the increased survival in the doxycycline-treated group was a result of reduced KRas expression, brain tissue from mice treated with doxycycline for 3 or 14 days was analyzed for the level of expression of both KRas and Akt by immunohistochemistry for the FLAG and HA epitopes on KRas and Akt, respectively. After 3 days of treatment, the level of KRas expression was significantly decreased relative to that in untreated mice, whereas the level of Akt expression remained unchanged (Fig. 4). After 14 days of doxycycline treatment, no tumor was visible and no KRas or Akt expression was detected (Fig. 5). A significant number of vacuolated cells were observed in sections from treated mice relative to untreated mice. Because the tumors from doxycycline-treated mice showed signs of regression, TUNEL staining was done to determine if apoptotic cells could be detected within the tumor. After 3 days of doxycycline treatment, a significant amount of apoptosis was detected within the tumor (Fig. 4B, compared with untreated mice Fig. 2B). After 14 days of doxycycline treatment, no tumor cells or apoptotic cells were visible (Fig. 5).

**Reexpression of KRas following doxycycline treatment reinitiates tumor growth.** To determine if reexpression of KRas could reinitiate tumor growth, doxycycline was withdrawn from animals treated for either 25 or 45 days. The mice were observed for signs of tumor growth, and tumor-free survival rates were compared between the two groups (Fig. 6). Interestingly, all seven mice in each group had recurrent disease. Upon reactivation of KRas, tumorigenesis quickly resumed in the mice that were treated for only 25 days. The majority of those animals (57%) displayed recurrent disease within 3 weeks of doxycycline withdrawal. In contrast, in mice that had been treated for 45 days, tumor formation was not observed until 3 months after doxycycline withdrawal. The tumors that formed after doxycycline withdrawal reexpressed Kras, as detected by immunohistochemistry for the FLAG epitope (data not shown). These data suggest that a percentage of the progenitor cells capable of reestablishing the tumor survived apoptotic cell death following the loss of KRas expression upon doxycycline treatment, and that this percentage decreases the longer KRas expression is suppressed. Future work will focus on determining the length of time required to eliminate all progenitor cells capable of reestablishing disease.
This model shows the critical importance of the Ras pathway in glioblastoma maintenance and indicates that continuous suppression of Ras signaling is necessary and sufficient to suppress the tumorigenic potential of the glial progenitor cells. In addition, this regulated expression system will allow the evaluation of the role of other genes and pathways in this context. This has important clinical implications for pharmacologic agents targeting these pathways in GBM patients.

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References


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