Restoring Soluble Guanylyl Cyclase Expression and Function Blocks the Aggressive Course of Glioma


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ABSTRACT

The NO and cGMP signaling pathways are of broad physiological and pathological significance. We compared the NO/soluble guanylyl cyclase (sGC)/cGMP pathway in human glioma tissues and cell lines with that of healthy control samples and demonstrated that sGC expression is significantly lower in glioma preparations. Our analysis of GEO databases (National Cancer Institute) further revealed a statistically significant reduction of sGC transcript levels in human glioma specimens. On the other hand, the expression levels of particulate membrane guanylyl cyclases (pGC) and cGMP-specific phosphodiesterase (PDE) were intact in the intact cells that we have tested. Pharmacologically manipulating endogenous cGMP generation in glioma cells through either stimulating pGC by ANP/BNP, or blocking PDE by 3-isobutyl-1-methylxanthine/zaprinast caused significant inhibition of proliferation and colony formation of glioma cells. Genetically restoring sGC expression also correlated inversely with glioma cell growth. Orthotopic implantation of glioma cells transfected with an active mutant form of sGC (sGCα1β1Cys105) in athymic mice increased the survival time by 4-fold over the control. Histological analysis of xenografts overexpressing α1β1Cys105 sGC revealed changes in cellular architecture that resemble the morphology of normal cells. In addition, a decrease in angiogenesis contributed to glioma inhibition by sGC/cGMP therapy. Our study proposes the new concept that suppressed expression of sGC, a key enzyme in the NO/cGMP pathway, may be associated with an aggressive course of glioma. The sGC/cGMP signaling-targeted therapy may be a favorable alternative to chemotherapy and radiotherapy for glioma and perhaps other tumors.

Introduction

Gliomas account for almost 75% of primary malignant brain tumors. Of 10,000 Americans diagnosed each year with malignant gliomas, only half will live beyond 1 year after diagnosis, and those will die within 2 years. Despite compelling advances in diagnostic imaging, surgery, radiation, and/or antineoplastic agents, the prognosis for people with glioma has remained largely unchanged (Jemal et al., 2008). Thus, new concepts in glioma etiology, therapy, and clinical management are needed.

The nitric oxide (NO) and cyclic 3′,5′-GMP (cGMP) pathway is one of the best characterized signaling cascades and plays a central role in several physiological processes, such as...
induction of vasodilation. Soluble guanylyl cyclase (sGC) is the only known receptor for NO. The α1β1 heterodimer is the predominant isoform of sGC that is obligatory for catalytic activity. Nitric oxide binds to the ferrous heme at histidine 105 of the β subunit and leads to an increase in sGC activity and cGMP production of at least 200-fold (Bian and Murad, 2003). On the other hand, the effects of NO can be attributed to the cGMP-independent pathway, which is mediated mainly by reactive oxygen/nitrogen species such as highly reactive peroxynitrite (ONOO−) (Bian et al., 2003, 2008). The role of NO and cGMP signaling in tumor biology has been extensively studied during the past 3 decades. Simple applications of NO or cGMP-regulating reagents to various cancer cell lines or animal models has generated controversial results, and whether the pathway is beneficial or detrimental in cancer is still open to question (Kimura and Murad, 1975; Criss and Murad, 1976; Rao, 2004; Mujoo et al., 2010). We suggest several reasons for this ambiguity: first, although the NO participates in normal signaling (e.g., vasodilation and neurotransmission), NO is also a cytotoxic or apoptotic molecule when produced at high concentrations by inducible nitric-oxide synthase (iNOS or NOS-2). In addition, the cGMP-dependent (NO/sGC/cGMP pathway) and cGMP-independent (NO/oxidative pathway) components may vary among different tissues and cell types. Furthermore, solid tumors contain two compartments: the parenchyma (neoplastic cells) and the stroma (nonmalignant supporting tissues including connective tissue, blood vessels, and, inflammatory cells) with different NO biology. Thus, the NO/sGC/cGMP signaling molecules in tumors as well as the surrounding tissue must be further characterized before targeting this signaling pathway for tumor therapy.

In this study, we demonstrate that perturbation of sGC/cGMP signal in glioma cells may play a significant role in tumor malignancy. Our experiments with genetic restoration of sGC gene expression or pharmacologic manipulation of endogenous cGMP generation in glioma cells resulted in inhibition of the growth of glioma cells and blockage of aggressive course of glioma. We suggest that manipulation of sGC/cGMP signaling may serve as a favorable alternative to current glioma treatment strategies. These concepts may also have applications to other tumors.

Materials and Methods

Cell lines, Cell Culture, and Biochemical Characterization of sGC/cGMP Signaling Molecules. Glioma cell lines U87, U251, U373, A172, LN18, LN229, and D54 were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) plus 1% penicillin/streptomycin mixture. The confluent cells were harvested, lysed, and processed by Western blot analysis, regular reverse transcription-PCR analysis, and quantitative real-time reverse transcription-PCR analysis as described in Supplemental Materials and Methods.

Generation of U87 Stable Transfectant Lines. The protocol for generation of U87 stable transfected clones is described in Supplemental Materials and Methods.

Assay of cGMP/cAMP in Intact Cells. For the activity assay, accumulation of cGMP/cAMP in tumor cells was determined in Dulbecco’s phosphate-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (preincubation for 10 min with an sGC activator, 1 μM BAY 41-2272 or vehicle control). Then, an NO donor, spermine NONOate (0.1 mM) or forskolin (10 μM) was added and incubation continued for another 10 min. Medium was aspirated and cGMP/cAMP was extracted by rapid freezing of the plates at −80°C in the presence of 50 mM sodium acetate, pH 4.0, (0.3 ml per well), then measured by enzyme-linked immunosorbent assay as described previously (Kots et al., 2008). For the assay of the basal level, tumor cells were harvested without IBMX preincubation and further treatments.

Cell Viability and Colony Formation Assays. MTT (3-(4,5-diethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used to quantify surviving/proliferating cells as described in Supplemental Materials and Methods. The detailed soft agar assay protocol for colony formation is described in Supplemental Materials and Methods.

Establishment of Xenograft Tumors, Efficacy Evaluation, and Characterization of Intracranial Xenografts. Human glioma cell lines with or without stable transfection (at a concentration of 106 cells/5 μl) were resuspended in PBS and injected into the right frontal lobe of nude mice using a guide-screw system implanted within the skull as described previously (Mccutcheon et al., 2000). Detailed protocols are described in Supplemental Materials and Methods. When the animals became moribund due to tumor progression, they were euthanized, and the brains were removed for histological and molecular analysis. The vasculature (CD31 staining) and cellular proliferation (Ki67 staining) in the tumors were evaluated by immunohistochemistry as described in Supplemental Materials and Methods.

Statistical Analysis. Results are expressed as mean ± S.E.M. A one-way analysis of variance was performed for multiple comparisons, and if there was a significant variation between the treatment groups, the mean values for a treated group were compared with those of the control by Student’s t test; p values of less than 0.05 were considered statistically significant. The n values indicate the numbers of animals used in the experiment.

Results

Absence of sGC Signaling in Human Glioma Tissues and Cells. The key intracellular receptor for NO is sGC, which produces the second messenger cGMP. Pathologically high concentrations of NO are produced by iNOS, which is overexpressed in various inflammation-related conditions, including glioma (Cuny et al., 2002). To explore the functional significance of increased NO on sGC, we examined cGMP accumulation in primary cultured brain tumor cells, including glioma specimens. As demonstrated in Supplemental Fig. S1 (also refer to Fig. 4B), the basal levels of cGMP in malignant brain tumors were markedly low, and unresponsive to stimulation by either NO donor spermine NONOate or 3-(4-amino-5-cyclopentyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrrozolo[3,4-b]pyridine (Bay41-2272), an allosteric sGC activator. Thus, the sGC-catalyzed cGMP production seems to be impaired or abolished in glioma and other malignant brain tumors.

sGC is a heterodimer composed of α and β subunits (Kaminski et al., 1986). To date, four isoforms of αGC subunit have been identified (α1, α2, β1, and β2) in mammals (Zabel et al., 1998), and the majority of functional sGC heterodimer is α1β1, which is expressed ubiquitously. To further explore the status of sGC expression in gliomas, we collected glioma cell lines of U87, U251, U373, A172, LN18, LN229, and D54, and investigated protein expression levels of the sGC α1 and β1 (Fig. 1A). The sGCα1 subunit was undetectable in most glioma cell lines. The sGCα1 subunit was significantly decreased in all glioma cells. Furthermore, the protein levels of the sGCα1 subunits were either undetectable or very low in...
approach, we quantified the amount of mRNA transcripts of tide (BNP) (Murad et al., 1987). Using a real time-Q-PCR atrial natriuretic peptide (ANP) and brain natriuretic pep-
gC) are stimulated with natriuretic peptides including

Fig. 1. Reduction of sGC expression in human glioma tissues and cell lines. sGC expression in glioma cell lines (U87, U251, U373, A172, LN18, LN229, and D54) was examined by Western blot (A) and real time-Q-PCR (B) and compared with that in BE2 human neuroblastoma cell line, which normally expresses both sGC α1 and β1 subunits at levels similar to those in normal human cortex (D) (Bonkale et al., 1995; Corbáln et al., 2002; Sharina et al., 2008). The GEO database analysis of sGC gene expression in human glioma tissues of different grade (C) showed that the expression of sGC α1 and β1 is markedly decreased in astrocytoma (n = 50), oligodendrocytoma (n = 26), glioblastoma multiforme (n = 81) compared with normal brain tissues (n = 23). Data are mean ± S.E.M. n = 3 to 6 per group for glioma cell lines. ***, P < 0.01 (versus normal brain tissues).

Expression of NPR, PDE, and PKG in Glioma Cells. cGMP is also produced when natriuretic peptide receptors (NPRs = membrane-bound [particulate] guanylyl cyclase, pGC) are stimulated with natriuretic peptides including atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (Murad et al., 1987). Using a real time-Q-PCR approach, we quantified the amount of mRNA transcripts of the receptors, and detected the expression of NPR1 (GC-A), NPR2 (GC-B), and guanylyl cyclase type C (GC-C) in all tested samples (Fig. 2A). It has been known that NPR2 represents the main natriuretic peptide receptor in the brain (Wilcox et al., 1991), and it shares structural similarities with NPR1.

Inhibition of specific phosphodiesterases (PDEs) results in cellular responses by elevating cGMP signals derived from the activation of sGC and/or pGC (Bian et al., 2008). We examined the profile of PDE expression in U87 cells (Fig. 2B) and detected PDE2, PDE5, PDE8, PDE10, and PDE11 expression. PDE5 is a highly cGMP-specific isoform. PDE5 inhibitors have been the subject of many recent clinical and basic-science studies beside erectile dysfunction (Bhuiyan et al., 2006). We performed Q-PCR analysis with various glioma cell lines (Fig. 2C) and found that U87, A172, and LN18 have higher levels of PDE5 expression.

One of the major receptors for cGMP is the cGMP-dependent protein kinase (PKG), which phosphorylates serine and threonine residues in many cellular proteins. Two PKG families (PKG 1 and PKG 2) are derived from separate genes (prkg1 and prkg2). Our Q-PCR analysis (Fig. 2, D and E) with glioma cell lines revealed at least three patterns of expression: PKG1-dominant (U87), PKG2-dominant (A172 or Ln18), equal expression of PKG1 and PKG2 (D54), and PKG-negative (U373).

Inhibition of Glioma Cell Proliferation by Pharmacologically Restoring cGMP Signaling. To verify whether restoring sGC function through cGMP elevation may influence proliferation of glioma cells, we investigated the effect of 8-
bromo-cGMP, a membrane-permeable cGMP analog resistant to PDE hydrolysis. As demonstrated in Fig. 3A, 8-bromo-cGMP significantly reduced proliferation of glioma cells in a concentration-dependent manner.

On the basis of NPR expression profile in glioma cell lines (Fig. 2A), we tested the hypothesis that elevating endogenous cGMP through NPRs stimulation should have similar effects as 8-bromo-cGMP on tumor proliferation. As demonstrated in Fig. 3B, ANP did inhibit proliferation of U87 glioma cells in a concentration-dependent manner.

Inhibiting PDE activity is another approach to elevate intracellular cGMP. Considering the fact that multiple PDE isoforms are expressed in U87 cells (Fig. 2B), we used a nonselective PDE inhibitor IBMX and showed (Fig. 3C) that IBMX concentration-dependently suppressed glioma cell proliferation. As shown in Fig. 2C, U87 cells express higher levels of PDE5. We then treated the cells with a PDE5-specific inhibitor zaprinast and also demonstrated a significant attenuation of cell proliferation (Fig. 3D). To further confirm our observations, we treated U87 cells with an NPR agonist in combination with a PDE inhibitor. The combined treatment concentration-dependently inhibited tumor cell growth for up to 6 days (Fig. 3E). We did not observe marked morphological changes of the glioma cells treated with 8-bromo-cAMP, ANP, and IBMX.

To verify the effects of these pharmacological agents on the levels of intracellular cGMP, the assay for cGMP accumulation was performed. As demonstrated in Fig. 3F, 24-h treatment with ANP (1 μM) or IBMX (1 mM) markedly increased cGMP accumulation in U87 glioma cells. In summary, glioma cell proliferation was significantly inhibited upon pharmacological restoration of sGC function through increasing intracellular cGMP levels. To further rule out possible cAMP involvement, we measured cAMP accumulation (Fig. 3, G and H) in U87 glioma cells under stimulation of the cGMP-promoting agents. In contrast to the magnitude of forskolin-stimulated cAMP, IBMX, ANP, and BNP failed to significantly influence the levels of cAMP.

Inhibition of Glioma Cell Proliferation by Genetically Restoring sGC. To provide additional evidence supporting the inhibitory effects of cGMP on tumor growth, we established three stable clones of U87 cells overexpressing the sGCα1 subunit alone (Fig. 4A, Western blot, lanes 8–10) or in combination with the sGCβ1Cys105 mutant (Fig. 4A, lanes 11–13). The sGCβ1Cys105 mutant was created and characterized previously by our group (Martin et al., 2003). It has a constitutively elevated activity that allows observing the effect of sGC function with less inference from endogenous NO (Martin et al., 2003). It is worth noting that overexpression of the αβ1Cys105 sGC heterodimer resulted in higher basal level of cGMP (Fig. 4B).

To verify the effect of genetically restored sGC activity on glioma cell proliferation, the MTT assay was performed, and the proliferation curves were plotted in Fig. 4C. The prolif-
eration of U87 cells was markedly inhibited by overexpression of α1β1<sup>Cys105</sup> sGC. Delivery of only sGCc1 failed to suppress cellular proliferation, probably because the insufficient expression of sGCB1 in the cells could not generate threshold basal level of cGMP (Fig. 4B). To further clarify the action of sGC enzyme, we measured basal and NO-stimulated activity of the wild-type and mutant α<sub>1</sub>β<sub>1</sub><sup>Cys105</sup> sGC in generation of cGMP and cAMP (Fig. 4D). NO donor DEA-NO (1 μM) significantly increased cGMP generation of wild-type sGC, but had no influence on cGMP synthesis by the α1β1<sup>Cys105</sup> sGC mutant. Neither basal nor NO-stimulated wild-type and mutant α<sub>1</sub>β<sub>1</sub><sup>Cys105</sup> sGC catalyzed significant synthesis of cAMP.

Inhibition of Glioma Cell Colony Formation by Pharmacologically Increasing cGMP or Genetically Restoring sGC. The cellular microenvironment is important for modulation of cancer cell growth. Growing cells within a three-dimensional (3D) support system simulate a natural microenvironment affecting tumor cell proliferation, morphology, signaling, and responses to therapeutic agents (Fischbach et al., 2007). Thus, we used the colony formation assay to further evaluate the influence of sGC signaling on glioma cell growth. We treated the cells with 1 μM ANP and assessed the inhibitory effect of the natriuretic peptide on colony number and size. There were fewer colonies (the size ≥1 mm<sup>2</sup>) formed in the ANP-treated group than in the control group (Fig. 5, A and B). In addition, the average size of the colonies was reduced by 40% in the ANP-treated group (Fig. 5, A and C).

The effects of elevating intracellular cGMP by IBMX on U87 glioma colony formation are shown in Fig. 5, D to F. IBMX significantly decreased colony (with size ≥1 mm<sup>2</sup>) number and size by 33 and 67%, respectively. To further confirm the efficacy of PDE inhibition, we used the PDE5-specific blocker zaprinast and observed significant suppression of tumorigenesis (Fig. 5, G–I).

Genetically restoring sGC by overexpression of α1β1<sup>Cys105</sup> sGC in U87 glioma cells also significantly attenuated colony formation. The average size of the colonies was reduced from 3.5 mm<sup>2</sup> for the controls to 0.7 mm<sup>2</sup> for the α1β1<sup>Cys105</sup> sGC-overexpressing clones (Fig. 5, J and K). The number of larger colonies (size ≥1 mm<sup>2</sup>) was also markedly less in the α1β1<sup>Cys105</sup>sGC-transfected colonies (Fig. 5, J and L). Neither the U87 control nor U87 with an empty vector (pcDNA or pMG) showed any changes in the size or number of colonies. Similar results were obtained in the stable clones overexpressing the sGC α1 subunit.

Intracerebral Glioma Xenograft Assay for sGC Transfectants. In vivo antitumor activity of sGC / cGMP was evaluated in athymic nude mice with intracerebral xenotransplantation of U87 cells with or without transfection. The data of Fig. 6A indicate that animals inoculated with α1β1<sup>Cys105</sup> sGC-transfected cells had significantly longer survival time. The 50% survival times of the mice inoculated with the α1β1<sup>Cys105</sup> sGC-expressing cells (n = 18) were extended to 73 days (332% of control group; Fig. 6, A and B). The longest average survival time of animals inoculated with the α1β1<sup>Cys105</sup> sGC-transfected cells was more than 124 days (4.6-fold over control). The average survival times and 50% survival times of different groups of tumorbearing mice are shown in Fig. 6, B and C. Unlike cell proliferation and colony formation assays, intracranial xenograft with the sGCc1 transfectant prolonged average survival time in a statistically significant manner (Fig. 6B).
Characteristic Glioma Xenograft Changes in sGC Transfectants. Histological analysis showed that glioma xenografts was clearly distinct from the surrounding host brain tissue and had pathological features of glioblastoma (Fig. 7A). The tumor tissue obtained from mice inoculated with the αβ1Cys105 sGC-transfected cells had significantly fewer microvessels as assessed by CD31 staining (Fig. 7, B and C) and quantified using a method previously developed by our group (Zhu et al., 2008). Immunostaining for Ki-67, a marker associated with cell proliferation, was used to evaluate the fraction of growing cells in malignant neoplasia. A marked reduction in Ki-67 positive cells was observed in double-sGC transfectant-derived tumors (Fig. 7, D and E). It is notable that xenografts in mice inoculated with the αβ1Cys105 sGC-transfected cells exhibited a heterogeneous population of tumor cells (Fig. 7A) with different nuclear density. We have observed decreased angiogenesis in both tumor cell populations. However, increased Ki-67 labeling occurred predominantly in the areas with higher nuclear density. It will be interesting to have more information about the heterogeneous population of tumor cells in the αβ1Cys105 sGC xenograft model.

**Discussion**

The role of cyclic nucleotides in the regulation of cell proliferation and tumor growth was noted as early as in the 1960s (Ryan and Heidrick, 1968). cAMP and cGMP were thought to act as biological antagonists in the regulation of cell growth at the beginning (Goldberg et al., 1975), and elevated cGMP levels have been observed in certain human tumor tissues (Hadden et al., 1972; DeRubertis et al., 1976; Takemoto et al., 1982). Increased urinary cGMP in rats with tumor tissues (Hadden et al., 1972; DeRubertis et al., 1976; Takemoto et al., 1982) and in rat brain tissue and had pathological features of glioblastoma (A, 100X magnification). The xenografts with αβ1Cys105 sGC-transfected cells had significantly fewer microvessels according to CD31 staining (B and C; 200X magnification). Significant reduction in Ki-67 staining, a marker associated with cell proliferation, was observed in sGC transfectant-derived tumors (D and E; 200X magnification). Data are mean ± S.E.M. n = 3 to 4 for each group. **p < 0.01 (versus control groups).
cell survival, proliferation, and mitochondrial function. cGMP inhibits proliferation (decreased MTT) but increases mitochondrial function (increased MTT) (Ruiz-Stewart et al., 2004). Thus, smaller effect of sGC/cGMP in the MTT assay versus the colony assay may imply an inhibition of cell-cell communication. Significant reduction of angiogenesis in xenografts of mice inoculated with α1β1<sup>Cys105</sup> sGC-transfected cells further supports this hypothesis (Fig. 7, B and C).

As illustrated in Fig. 7A, glioma xenografts of U-87 control or U-87 with the empty vector (pcDNA or pMG) exhibited morphology of cancer cells with hypertrophic nucleolus, decreased cytoplasmic/nuclear ratio, and shrunken cytoplasm. It is notable that xenografts expressing α1β1<sup>Cys105</sup> sGC had heterogeneous populations of tumor cells (Fig. 7, 400×), a large population of cells resembling the morphology of the normal cells. We propose that sGC/cGMP signaling normalizes glioma cellular architecture through a prodifferentiation mechanism. It is generally accepted that tumor malignancy correlates with undifferentiated (anaplastic) status. Our group has previously reported low levels of sGCα1 and β1 expression in undifferentiated embryonic stem cells from both human and mouse, and the embryonic stem cells regain sGC expression while entering differentiation (Krumenacker et al., 2006; Muijo et al., 2006). Tumorigenesis and organogenesis are similar in many respects, and many types of cancer (including brain tumor) contain cancer stem-like cells (Singh et al., 2004; Yuan et al., 2004). Thus, we isolated a population of CD133-positive cancer stem-like cells from human glioma and did not detect sGCα1 expression when the expression of sGCβ1 mRNA was very low (data not shown). Together with our findings that restoring sGC function inhibits glioma growth and normalizes cellular architecture, we suggest that involvement of a prodifferentiation mechanism in sGC-targeted therapy may be an alternative or complementary approach to toxic treatments such as chemotherapy and radiation.

Our current study suggests two possible roles of NO/cGMP signaling in malignant tumors (also see Scheme 1). First, iNOS expression and NO overproduction may contribute to the formation of inflammatory cancer microenvironment. Second, sGC/cGMP signaling may influence proliferation and/or differentiation of the tumor cells. Our current observations suggest that although sGC-deficient glioma cells lack the cGMP-mediated response to NO, this highly permeant gas can act upon adjacent stroma and increase cGMP levels in nonmalignant supporting tissues, including connective tissue and blood vessels. Although the elevated cGMP in surrounding tissues cannot cross the cell membrane to enter the cancer cells, it can be released and detected in extracellular fluids of patients or animals with tumors as discussed above (Murad et al., 1975; Criss and Murad, 1976).

The cross-talk between the cGMP and cAMP pathways has been noticed for years. To clarify the involvement of cAMP in our experimental settings, we measured cAMP levels (Fig. 3, G and H) in glioma cells treated with cGMP-manipulating pharmacological agents. As shown in Fig. 3G, the 10 μM forskolin (in the presence of 1 mM IBMX) excited a 23-fold increase in cAMP compared with that stimulated by 1 mM IBMX alone. It is worth to note that 100 μM forskolin is commonly used for inhibition of cancer cell proliferation and growth both in vitro and in vivo (Yamanaka et al., 2010, 2011). In contrast, for human U138 glioma cells an enhanced proliferation by cAMP signaling was reported (Farias et al., 2008). To further verify the possible influence of sGC on the cAMP pathway, we examined the cAMP-forming capacity of both wild-type and Cys105-mutant sGC (Fig. 4D). We found only an insignificant formation of cAMP by NO-induced wild-type sGC and detected only traces of cAMP in samples with α1β1<sup>Cys105</sup> sGC enzyme (Fig. 4D). These data strongly suggest that the observed effect of sGC-dependent inhibition of glioma cell proliferation through a cAMP/protein kinase A-dependent pathway is unlikely.

cGMP-dependent protein kinase (cGK), also called protein kinase G (PKG), is a serine/threonine-specific protein kinase which exists in two isoforms, type-I and type II. PKG has been shown to have tumor suppressor properties in colon carcinoma (Deguchi et al., 2004). However, PKG1<sup>α1</sup> has been recently reported to promote DNA synthesis and cell proliferation in human ovarian cancer cells (Leung et al., 2010). PKG2 is widely expressed in normal brain tissue. We thus screened human glioma cell lines for expression of PKG isoforms (Fig. 2D) and found four different phenotypes. Although some cell lines express PKG1 (e.g., U87), PKG2 (e.g., A172 or Ln18), or both (e.g., D54), other cells are PKG-negative (e.g., U373). The U87 glioma cells are characterized as highly tumorigenic, low-invasive, and PKG2 deficient. Westernmark’s group (Swartling et al., 2009) reported that overexpression of PKG2 in U87 cells inhibited cell proliferation and xenograft growth. Together with our current findings, the experimental evidence supports our hypothesis that normal function of the sGC-cGMP signaling axis may be important for the prevention and/or treatment of glioma. Further studies are warranted to elucidate the detailed involvement of PKG, as well as protein kinase A, in sGC/cGMP signaling-targeted glioma therapy.

In summary, we found that sGC expression is lower or diminished in human glioma tissues and cell lines and pro-
pose that sGC is a novel tumor susceptibility gene in human glioma. Restoring sGC/cGMP signaling genetically or pharmacologically significantly inhibited glioma growth. Orthotopic xenograftment of glioma cells with the αβ1γδεθαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεثολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθοলαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθοলαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαεθολαеθολαεθολαεθολαεθολαεθολαеθολαеθολαеθολαεθολαеθολαеθολαеθολαеθολαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеθοлαеtheta epsilon omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda nu pi tau theta chi psi omega lambda numin onco. Enzyme 283:3121–3131.


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Restoring sGC expression and function blocks the aggressive course of glioma

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Lists of supplemental data

Supplemental Materials and Methods

Table S1. Primers used in Reverse Transcription PCR.

Table S2. Taqman Assays ID used in Quantitative real-time reverse transcription–PCR.

Figure S1. Absence of NO-dependent signaling in malignant brain tumors.

Figure S2. Altered expression of sGC in malignant brain tumor.

Figure S3. hTERT mRNA levels in benign vs malignant brain tumors.
Supplemental Materials and Methods

Reagents

Polyclonal anti-sGCα1 and monoclonal anti-α-tubulin antibodies were acquired from Sigma (St. Louis, MO), polyclonal anti-sGCβ1 antibodies were either generated by our lab or purchased from Calbiochem (Madison, WI); monoclonal anti-CD133 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and CD133 cell isolation kit was from Miltenyi Biotec (Bergisch Gladbach, Germany). Unless otherwise indicated, all routine chemicals and reagents were purchased from Sigma, and tissue culture media and reagents from Invitrogen (Carlsbad, CA).

Specimen collection, establishment of primary cultures

We obtained primary cell cultures from a set of pathologically confirmed human benign and malignant meningiomas and gliomas. Tissue was obtained from 10 patients who underwent therapeutic removal of primary intracranial tumors. Control tissue samples were obtained adjacent to each tumor from noneloquent white matter deemed normal based on radiographic and histological appearance. In addition, we measured human telomerase reverse transcriptase (hTERT) mRNA expression levels in both meningioma and glioma specimens using real-time PCR. hTERT levels were increased in malignant cells as expected (fig. S3). All samples were immediately used to establish primary cultures. To do so, tumor fragments were dispersed into individual cells by treatment with Dispase I for 15 to 30 min at 37°C. From each tumor, 10⁶ cells were then plated in a 100x20-mm tissue culture dish in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS)
plus 1% penicillin/streptomycin mixture. The cells were grown to confluence and then harvested, aliquoted, and stored in liquid nitrogen for future use.

**Primary tumor sphere culture**

Primary cultured human glioma cells were seeded in 24-well plates at $2 \times 10^4$ cells/well in DMEM/F12 medium containing 20 ng/mL EGF, 20 ng/mL bFGF, 10 ng/mL N2 supplement, plus 1% penicillin G/streptomycin mixture (Gibco, USA). After primary spheres were formed and reached the size of 100-200 cells, they were harvested, dissociated into single cells and the CD133 positive cells were separated by magnetic cell sorting technique (MACS; Bergisch Gladbach, Germany). The CD133-positive cells were diluted to yield 1 to 2 cells/10μl and plated into 96-well plates; fresh medium was added up to 100μl. The formed spheres were used for experiments.

**Quantitative real-time reverse transcription–PCR and reverse transcription PCR**

Total RNA was isolated using UltraSpec reagent (Biotecx, Houston, TX) according to the manufacturer’s instructions. Complementary DNA was synthesized by using a reverse transcription-PCR archive kit (Amersham Bioscience, Sweden) following the manufacturer’s protocol. All quantitative real-time PCR tests were performed using the standard real-time PCR-protocol of the ABI Prism 7700, with a total reaction volume of 25 μl as previously described (Bian et al., 2001). The temperature profile consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. The cycle threshold (Ct) for every sample is directly proportional to the amount of input template, so that with the use of a plasmid standard, the relative number of template molecules in the reaction was determined.
by using a plasmid standard containing the desired amplicon. Q-PCR data were normalized to the level of acidic ribosomal phosphoprotein P0 (36B4; housekeeping gene) or β-actin. Regular reverse transcription PCR was performed to identify the existence of PDE in U87 glioma cells, and identity of the bands was confirmed by sequencing. The primers used in reverse transcription PCR and the assay ID for real time PCR are listed in supplemental Table 1 and supplemental Table 2 respectively.

**Western blot analysis**

Cells were harvested and lysed by sonication in ice-cold PBS containing proteinase inhibitor cocktail. Equal amounts of protein (50 or 100 µg/well) were loaded and separated by 7.5% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane which was treated with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, 130 mM NaCl, pH 7.6 plus 0.1% Tween-20), then incubated at 4°C overnight with the specific antibodies. Secondary horseradish peroxidase-conjugated antibodies (Sigma) were used at 1:3000 dilution and protein bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). A monoclonal antibody to α-tubulin was used to monitor equal loading of samples.

**Generation of U87 stable transfectant lines**

Coding sequences of sGCα1 and sGCβ1<sup>Cys-105</sup> were subcloned into the pcDNA3.1 and pMG transfer vectors (Invitrogen, Carlsbad, CA), respectively, and orientation was confirmed by restriction enzyme analysis and DNA sequencing. For generation of the sGCα1 stable clone, pcDNA3.1-sGCα1 plasmid was transfected into human U87 cancer cells using
Lipofectamine 2000 according to manufacturer’s protocol (Invitrogen; Carlsbad, CA). Control cells were transfected with the native pcDNA3.1 vector (mock transfectants). After 24 h, the medium was replaced with selective medium containing 800 \( \mu g/mL \) G418. After two weeks, neomycin-resistant clones were expanded, and successful transfection was confirmed by Western blot analysis using an anti-sGC\( \alpha \)1 antibody. Four clones with the highest levels of sGC\( \alpha \)1 were selected for further study. To generate sGC\( \alpha \)1 / sGC\( \beta \)1\text{Cys-105} double transfection in U87 cells, the sGC\( \alpha \)1 stable clones were transfected with a pMG-sGC\( \beta \)1\text{Cys-105} plasmid. The clones were selected on 800 \( \mu g/mL \) of G418 and 400 \( \mu g/mL \) of hygromycin B. Clones with successful transfection were confirmed by Western blot.

**Immunohistochemistry**

Paraffin embedded tissue sections were first deparaffinized and rehydrated. Then, primary anti-CD31 and anti-Ki67 (Cell Signaling, Danvers, MA) antibodies were applied to the sections. The biotinylated secondary antibody (Abcam, Cambridge, MA) was used and streptavidin-biotin-horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) was further applied for signal detection. Diaminobenzindine (Vector Laboratories, Burlingame, CA) was used for staining and the sections were counterstained with hematoxylin. Endogenous peroxidase activity was blocked with 3% \( H_2O_2 \) (Fisher Scientific, Fair Lawn, NJ).

**Cell viability assay**

To quantify surviving/proliferating cells, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and incubated for 90 min. Medium and MTT were removed, dimethyl sulfoxide was added for 1 min to solubilize the dye, and
absorption was read at 570 nm in a spectrophotometer. In each experiment, cells were plated in quadruplicate and the average of the relative absorption (OD$_{570}$) was used as an estimate of the number of metabolically active cells. Percentage of surviving treated cells compared to control cells was calculated from the average OD$_{570}$ values obtained in each experiment.

**Colony formation assays**

We adopted the traditional soft agar assay protocol for colony formation with some modifications for current tumorigenesis study. U87 glioma cells ($3 \times 10^3$) were seeded in an agar-agarose semi-solid gel system (30mm cell culture dish) which was covered by culture medium containing FBS and the medium was changed every three days. After 21 days, the colonies were stained with 0.005% crystal violet solution for 1 h and washed with PBS three times and then photographed. The number and size of colonies were examined.

**Establishment of xenograft tumors, efficacy evaluation, and characterization of intracranial xenografts**

**Animals**—Protocol for animal use was approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center, and all experiments were done in accordance with National Institutes of Health guidelines.

**Procedure**—A total of 30 female mice, 8–10-weeks-old ($nu/nu$ athymic; Charles River Laboratories) were used in the experiments. Human glioma cell lines with or without stable transfection and meningioma-derived stem cell like cells (at a concentration of $1 \times 10^6$ cells/5 µL) were resuspended in PBS and injected into the right frontal lobe of nude mice using a guide-screw system implanted within the skull as described previously. Animals were
anesthetized with xylazine/ketamine during the procedure. When the animals became moribund due to tumor progression, they were euthanized and the brains were removed for histological and molecular analysis. The vasculature (CD31 staining) and cellular proliferation (Ki67 staining) in the tumors were evaluated with immunohistochemistry.
Table S1. Primers used in Reverse Transcription PCR.

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Table S2. Taqman Assays ID used in Quantitative real-time reverse transcription–PCR

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**Supplemental Figure S1.** Absence of NO-dependent signaling in malignant brain tumors. Measurement of cGMP levels in benign and malignant meningioma as well as glioma cells treated with Bay41-2272 (1 μM), spermine/NONOate (0.1 mM) or Bay41-2272 (1 μM) plus spermine/NONOate (0.1 mM) revealed that cGMP levels in glioma and malignant meningioma cells were unresponsive to the stimulations. Data are mean ± s.e.m. n = 6 per group, P < 0.01 (malignant vs benign in every experimental group).
**Supplemental Figure S2. Altered expression of sGC in malignant brain tumors.** Protein expression levels by Western blot (A) showed the 82 kDa sGCα1 subunit was undetectable in primary cultures of malignant meningioma cells (benign meningioma cells were used as control) and in malignant glioma tissue (normal autologous brain tissue was used as control).
The sGCβ1 subunit (78 kDa) was also absent in human malignant meningioma and its level was very low in malignant glioma tissue, while the protein was abundant in normal brain tissue or benign tumor samples. RT-Q-PCR (B) demonstrated sGCα1 mRNA was undetectable and sGCβ1 mRNA levels were very low in malignant tumors, while their levels were significantly higher in benign tumor samples.
Supplemental Figure S3. hTERT mRNA levels in benign vs malignant brain tumors. The quantitative real-time PCR analysis of hTERT mRNA levels in benign and malignant meningioma and glioma. The hTERT mRNA levels were quantified after normalizing to 36B4 (a housekeeping gene) mRNA. (M= meningioma; U87 and D54 are gliomas).