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Glioma Stem Cell Proliferation and Tumor Growth Are Promoted by Nitric Oxide Synthase-2

Christine E. Eyler,[1,2,3] Qiulian Wu,[1] Kenneth Yan,[1] Jennifer M. MacSwords,[1] Devin Chandler-Militello,[5] Katherine L. Misuraca,[2] Justin D. Lathia,[1] Michael T. Forrester,[6] Jeongwu Lee,[1] Jonathan S. Stamler,[7] Steven A. Goldman,[5] Markus Bredel,[10,11] Roger E. McLendon,[4] Andrew E. Sloan,[8] Anita B. Hjelmeland,[1,*] and Jeremy N. Rich[1,9,*]

[1]Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA
[2]Department of Pharmacology and Cancer Biology
[3]Medical Scientist Training Program
[4]Department of Pathology
Duke University Medical Center, Durham, NC 27710, USA
[5]Department of Neurology, University of Rochester Medical Center, Rochester, NY 14642, USA
[6]Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
[7]Institute for Transformative Molecular Medicine and Department of Medicine
[8]Departments of Neurological Surgery, Pathology, and Translational Neuroscience
[9]Department of Molecular Medicine at the Cleveland Clinic Lerner College of Medicine
Case Western Reserve University School of Medicine and University Hospitals, Cleveland, OH 44106, USA
[10]Department of Radiation Oncology, University of Alabama Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AB 35249, USA
[11]Department of Neurosurgery, Neurocenter and Comprehensive Cancer Center, University of Freiburg, D-79106 Freiburg, Germany
*Correspondence: hjelmea@ccf.org (A.B.H.), richj@ccf.org (J.N.R.)
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SUMMARY

Malignant gliomas are aggressive brain tumors with limited therapeutic options, and improvements in treatment require a deeper molecular understanding of this disease. As in other cancers, recent studies have identified highly tumorigenic subpopulations within malignant gliomas, known generally as cancer stem cells. Here, we demonstrate that glioma stem cells (GSCs) produce nitric oxide via elevated nitric oxide synthase-2 (NOS2) expression. GSCs depend on NOS2 activity for growth and tumorigenicity, distinguishing them from non-GSCs and normal neural progenitors. Gene expression profiling identified many *NOS2*-regulated genes, including the cell-cycle inhibitor cell division autoantigen-1 (*CDA1*). Further, high *NOS2* expression correlates with decreased survival in human glioma patients, and NOS2 inhibition slows glioma growth in a murine intracranial model. These data provide insight into how GSCs are mechanistically distinct from their less tumorigenic counterparts and suggest that NOS2 inhibition may be an efficacious approach to treating this devastating disease.

INTRODUCTION

Malignant gliomas are highly lethal brain tumors that portend a dismal prognosis for patients. Despite modern surgical and medical treatments, the median survival for glioblastoma patients (WHO grade IV astrocytoma) remains only 14.6 months (Stupp et al., 2005), emphasizing a need for improved therapies. The identification of highly tumorigenic subpopulations within gliomas has fueled enthusiasm for development of novel anti-glioma therapeutics. Due to their high tumorigenic potential and stem cell-like behavior, these cells have earned a variety of names, including tumor-propagating cells or cancer stem cells (CSCs). Unlike the bulk tumor mass, CSCs exhibit sustained self-renewal and produce secondary tumors that recapitulate the parent tumor's features and cellular diversity (Bonnet and Dick, 1997; Galli et al., 2004; Lapidot et al., 1994; Singh et al., 2003; Yuan et al., 2004). The concept of CSCs provides a rational hierarchical explanation for cellular heterogeneity observed within tumors (Reya et al., 2001), which is complementary to stochastic mutations with clonal outgrowths (Shackleton et al., 2009). Regardless of the etiology for tumor heterogeneity, the potent tumor-propagation capacity of CSCs suggests a utility for glioma stem cell (GSC)-directed therapies.

As their name suggests, CSCs share features with nonneoplastic stem cells. Gene expression profiles of GSCs resemble those of embryonic stem cells (Ben-Porath et al., 2008) and nonmalignant neural stem cells (Taylor et al., 2005). Disruption of several stem cell-specific pathways (Bar et al., 2007; Clement et al., 2007; Fan et al., 2006) abrogates CSC proliferation and tumorigenesis, though canonical stem cell signals (e.g., Hedgehog, Notch, and Wnt) are clearly critical to normal stem cell physiology as well (Androutsellis-Theotokis et al., 2006; Reya et al., 2003; Wechsler-Reya and Scott, 1999). Development of strategies that target CSCs while sparing normal stem

cell function is therefore necessary to attain a CSC-selective therapeutic index, a notion that has been supported by studies of leukemic versus hematopoietic stem cells (Yilmaz et al., 2006). In contrast, this concept is relatively unexplored in GSCs versus neural stem cells.

Endogenous nitric oxide (NO) exhibits pleiotropic roles within cancer cells and tumors, and studies employing inhibition or genetic deletion of endogenous NO synthases (NOSs) support a tumor-promoting role for NO (Fukumura et al., 2006; Williams and Djamgoz, 2005). Downstream effects of endogenous NO in cancer include chemotherapeutic resistance (Fetz et al., 2009; Yang et al., 2002), evasion of apoptosis (Engels et al., 2008; Levesque et al., 2003), and enhanced proliferation (Lim et al., 2008). NOS isoforms exhibit heterogeneous expression patterns within glioma cell populations (Bakshi et al., 1998; Cobbs et al., 1995). This heterogeneity may reflect a NOS expression pattern that is restricted to specific glioma subpopulations. This raises the possibility that NOS activity could be unique to GSC subpopulations, as one determinant of glioma heterogeneity relates to the existence of GSCs. Along these lines, studies have suggested a protumorigenic role for NO in gliomas (Charles et al., 2010; Yamaguchi et al., 2002). Endothelial NOS3 localizes near neoplastic cells displaying stem cell markers, and exogenous NO donors support stem cell signaling pathways in murine glioma cells (Charles et al., 2010). However, the therapeutic possibilities of targeting NOS3 in glioma are limited, as previous human trials of nonselective NOS inhibitors (i.e., those with anti-NOS3 activity) resulted in adverse outcomes and increased mortality in sepsis (Alexander et al., 2007; Avontuur et al., 1998; López et al., 2004).

The possibility of GSC-specific endogenous NO synthesis remain unevaluated, and the contribution of other more targetable NOS isoforms to GSCs remains unexamined. Given the precedence that NO can support tumor growth and the aforementioned studies suggesting a pro-GSC effect for NO, we hypothesized that endogenous NO production might be augmented within GSCs relative to nonstem glioma cells (non-GSCs), thus promoting the established tumorigenic phenotype of GSCs.

RESULTS

Endogenous NO Contributes to Growth of GSCs, which Is Abrogated by Heterologous Expression of the Bacterial NO-Consuming Enzyme Flavohemoglobin

Employing techniques described in the Dirks group's original report first validating CD133 as a GSC cell surface marker (Singh et al., 2003), we characterized a variety of human tumor specimens and xenografts in which positive selection for CD133 segregates GSC-enriched populations from non-GSCs, as demonstrated by measures of self-renewal, stem cell marker expression, and tumor propagation potential (Bao et al., 2006a; Li et al., 2007). When CD133-based selection is utilized and stem cell-permissive culture conditions employed (Lee et al., 2006), CD133 marker expression is maintained (Figures S1A and S1B available online).

Using this CD133-based selection system, we compared the NO production capacity of CD133+ glioma cells (GSCs) with CD133– glioma cells (non-GSCs). We measured nitrite (NO_2^-), a stable byproduct of NO, in the culture medium using matched cultures from xenografted patient specimens. GSCs produced more NO_2^- than matched non-GSCs (Figure 1A), suggesting that elevated NO synthesis may be a distinctive feature of GSCs.

To examine the function of endogenous NO in GSCs, we designed and biochemically validated a strategy to deplete NO in mammalian cells (Forrester et al., 2011). While not conserved in mammals, bacteria and fungi employ flavohemoglobin (FlavoHb)—a potent NO-consuming enzyme that converts NO to nitrate (NO_3^-) (Figure 1B)—to protect from nitrosative stress (Gardner et al., 1998; Hausladen et al., 2001; Hausladen et al., 1998). Within GSCs and non-GSCs, we employed lentiviral-based expression of the *E. coli* FlavoHb. Efficient NO consumption by this approach was confirmed in HEK293 cells transfected with a CMV-driven NOS2, which results in supraphysiologic levels of NO (Figure 1C). Expression of FlavoHb impaired GSC growth (Figure 1D and Figure S1C) and neurosphere formation (Figure 1E and Figure S1D), though these effects were absent in CD133– non-GSCs and did not impact HEK293 cells that lack NO dependence (Figure S1E). Consumption of NO in GSCs via FlavoHb abrogated critical GSCs properties in vitro, suggesting a progrowth role for endogenous NO synthesis in GSCs.

Expression of NOS2 within GSCs Is Responsible for Their Distinctive NO Synthesis

While FlavoHb blocked NO availability and decreased GSC growth, the source of GSC-derived NO remained unclear. Glioma stem cells from primary patient specimens (Figure 2A) and human glioma xenografts (Figure 2B) displayed higher levels of NOS2 protein than matched non-GSCs, while no consistent expression pattern for NOS1 or NOS3 was observed. These data suggest that NOS2 expression in GSCs might contribute to their malignant properties, as (1) NOS2 is the most highly productive NOS, (2) *NOS2* is regulated largely at the level of transcription, and (3) GSCs demonstrated elevated endogenous NO production that contributed to GSC growth.

Although CD133 is useful for identifying GSCs (Bao et al., 2006a; Galli et al., 2004; Singh et al., 2003), it is not the only marker that may enrich for GSC phenotypes. The optimal method for defining GSC marker effectiveness likely depends on individual tumor characteristics and is a topic of active investigation. The marker stage-specific embryonic antigen-1 (SSEA1; CD15) has been reported to effectively isolate GSCs from some tumors with low CD133 expression (Son et al., 2009). Cell lysates from two of these previously reported tumors revealed elevated NOS2 expression in SSEA1+ GSCs relative to SSEA1– non-GSCs (Figure 2C). Positive selection for SSEA1 segregated for tumorigenic GSCs (as measured in transplantation assays) in these tumors from which we acquired SSEA1+ and SSEA1– protein lysates (Son et al., 2009). These data demonstrate that NOS2 cosegregates with GSC phenotypes in gliomas where CD133 or SSEA1 are useful for enriching GSCs.

To examine whether differential NOS2 expression is inadvertently driven by cell culture conditions, we used qRT-PCR to quantify *NOS2* messenger RNA (mRNA) derived from CD133+ and CD133– populations isolated by fluorescence-activated cell sorting (FACS) from fresh dissociated human gliomas