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NOS Expression and NO Function in Glioma and Implications for Patient Therapies

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Abstract

Significance: Gliomas are central nervous system tumors that primarily occur in the brain and arise from glial cells. Gliomas include the most common malignant brain tumor in adults known as grade IV astrocytoma, or glioblastoma (GBM). GBM is a deadly disease for which the most significant advances in treatment offer an improvement in survival of only ~2 months.

Critical Issues: To develop novel treatments and improve patient outcomes, we and others have sought to determine the role of molecular signals in gliomas.

Recent Advances: One signaling molecule that mediates important biologies in glioma is the free radical nitric oxide (NO). In glioma cells and the tumor microenvironment, NO is produced by three isoforms of nitric oxide synthase (NOS), NOS1, NOS2, and NOS3. NO and NOS affect glioma growth, invasion, angiogenesis, immunosuppression, differentiation state, and therapeutic resistance.

Future Directions: These multifaceted effects of NO and NOS on gliomas both *in vitro* and *in vivo* suggest the potential of modulating the pathway for antiglioma patient therapies. *Antioxid. Redox Signal.* 26, 986–999.

Keywords: nitric oxide/NO, glioma, nitric oxide synthase/NOS, cancer stem cell, therapy

Introduction

GLIOMAS ARISE FROM glial cells in the brain or spine, the most common type being astrocytomas. Astrocytomas are currently graded according to the World Health Organization based on histologic features associated with malignancy from least (grade I) to most (grade IV; glioblastoma [GBM]) aggressive, although an updated classification system based on our novel molecular understanding of these tumors is likely imminent. Current treatment options for the most malignant gliomas are limited and offer no cure, indicating the importance of furthering our understanding of the disease. Identification of mechanisms leading to disease development, maintenance, and therapeutic resistance will facilitate development of new therapies and biomarkers for therapeutic response.

One mechanism that has been and continues to be elucidated as important for glioma growth involves the signaling molecule and free radical nitric oxide (NO). NO and the enzymes producing it, the nitric oxide synthases (NOSs), regulate glioma growth. Multiple mechanisms to modulate NO levels are therefore being evaluated as antiglioma treatments. In this study, we review the expression of NOS, production of NO, and their potential biological and signaling

roles in malignant glioma, as well as the implications of these findings for patient therapies.

Heterogeneity in GBM

The most commonly occurring form of primary malignant glioma in adults is grade IV astrocytoma, or GBM, a highly aggressive and invasive brain tumor (35, 127). Those diagnosed with GBM have a rapid onset of disease and progression, with patients surviving an average of only 14 months from the initial discovery of the tumor. Treatment options for GBM tumors are limited; the current standard of care is surgical resection to remove as much of the tumor mass as possible followed by concurrent radiation and chemotherapy with the drug temozolomide (47, 109, 110). Temozolomide is an alkylating agent that damages DNA in the tumor cells and has been a recent advancement in the treatment of GBM that has added on average just 2 months to the survival time of patients (54).

In addition to treatment for primary GBM, there is also a therapy that has recently obtained FDA approval for recurrent GBM known as bevacizumab (Avastin®) (16). Bevacizumab is a monoclonal antibody therapy designed to neutralize vascular endothelial growth factor (VEGF), a master regulator of

proangiogenic signaling (8, 11, 123). Although this drug was originally believed to be effective against the highly vascularized GBM tumors, recent clinical trials have brought the effectiveness of this treatment into question (14, 38). Thus, discoveries leading to novel clinical treatments of GBM are greatly needed, as current standard treatments are minimally effective.

Treating GBM is challenging due to its expansive inter-tumoral heterogeneity between patients created by different transcriptional signature, methylation patterns, and mutational genotypes, leading to the identification of tumor subtypes (9, 83, 120, 122). Molecular subtypes identified by unique transcriptional signatures have been described and stratify GBM into neural, proneural, classical, and mesenchymal subtypes. These subtypes vary in mutational status of key oncogenes, as well as patient survival outcome (122). In addition to inter-tumoral heterogeneity, understanding the impact of intratumoral heterogeneity is critical. Intratumoral heterogeneity reflects differences in both the tumor microenvironment (extracellular conditions such as hypoxia or the presence of secreted factors from endothelial and microglial cells) and the neoplastic compartment, presenting major challenges to GBM treatment, because of the inability to adequately eliminate all populations of tumor cells (41, 74).

One promising treatment strategy for GBM is targeting the cancer stem cell or brain tumor initiating cell (BTIC) fraction of these malignant tumors. TICs were first described in the blood cancer leukemia, where researchers found that only a subset of these cells was capable of establishing disease when injected in mice (61). These cells shared similarities to normal stem cells; they contained the same stem cell surface markers and were self-renewing, producing daughter cells that could either be stem cell like or differentiated cancer cells. This finding launched an effort to identify these cells in other cancers, ultimately leading to discoveries in solid tumors, including brain cancer (105).

The glioma stem cell fraction in GBM is the focus of current efforts for targeted therapies, as this subset of cells is known to be resistant to radiation and conventional chemotherapy (4, 66, 84). These cells express a combination of stem cell markers, CD133, CD44, SOX2, and NANOG, and can be functionally identified by their ability to form neurospheres in culture conditions, capability to differentiate into multiple lineages, and tumor formation *in vivo* when orthotopically injected into immunocompromised mice with a minimal amount of cells (5, 45, 63). The current understanding of the origin of glioma stem cells is unclear; experts have theorized that glioma stem cells originate from mature glial cells that have “dedifferentiated” into plastic stem-like cells *via* mutations or epigenetic changes, neural progenitors that have acquired mutations that allow for stem-like characteristics, or a population from the neural stem cell pool that has acquired mutations that lead to their tumorigenicity (19–21).

NOS Expression in Malignant Glioma Cell Lines and Rodent Models

One signaling molecule important for glioma and glioma stem cell biology is NO, which is produced from arginine and oxygen by NOSs. NOSs include NOS1 (neuronal or nNOS), NOS2 (inducible or iNOS), and NOS3 (endothelial or eNOS). Early studies using enzymatic assays detected NOS activity induced by lipopolysaccharide (LPS) or cytokine treatment

of rat C6 glioma cells in culture, consistent with the known inducible expression of NOS2. Treatment of C6 glioma cells with LPS, interferon-gamma (IFN- γ), interleukin (IL)-1 beta, and tumor necrosis factor-alpha either alone or in combination induced NOS activity (30, 42, 91, 102–104, 139). Immunohistochemistry of sections of rat gliomas confirmed expression (53), and a novel *in vivo* imaging probe using NOS2 antibody was also able to detect NOS2 in rodent models (118). Removal of serum, a prodifferentiating agent absent from BTIC cultures, from C6 glioma cell media increased responsiveness to LPS (138). Cytokine and LPS induction of NO and *Nos2* was also increased by some anesthetics (31) as well as thrombin (75) in C6 glioma cells. The induction of NOS2 activity post-LPS or cytokine treatment reached peak between 4 and 8 h and could be maintained for longer periods with additional stimulation (87). *NOS2* expression was similarly shown to be induced by IFN- γ , IL-1 beta, and tumor necrosis factor-alpha in human A-172 and T98G GBM cells (34, 46, 91), and *NOS2* cloned from these cells was shown to produce NO (46). The induction of *NOS2* was demonstrated to involve transcription regulation as *NOS2* mRNA was elevated and a *NOS2* promoter activated in the A-172 cells (28, 34). Studies in human T67 astrocytoma cells also showed the presence of *NOS3* mRNA (17). Together, these data demonstrate that mouse and human glioma cell lines express NOS, with a large number of publications demonstrating cytokines can stimulate NOS2 expression in GBM cells.

NOS Expression in Malignant Glioma Patient Specimens

Additional studies went beyond established cell lines to address the expression of NOS in human glioma specimens. When human tumor sections were analyzed *via* immunohistochemistry for NOS levels, NOSs were elevated in comparison to the normal adult brain in many gliomas (10, 15, 49). For example, higher expression of NOS1, NOS2, and/or NOS3 was often observed in grade III astrocytomas and GBM specimens in comparison to normal brain or grade II astrocytomas (10, 15, 49, 85, 115). *NOS2* mRNA was also expressed in human GBM and meningioma specimens (25), but immunohistochemistry results in one study determined NOS2 expression in grades I, II, and III astrocytomas with lower levels in GBMs (37).

NOS1, NOS2, and NOS3 proteins were observed in lysates derived from human GBM patient specimens and xenografts, with a consistent preferential increase in NOS2 rather than other NOSs in the BTIC fraction (29). *NOS2* mRNA also correlated with worse glioma patient prognosis, including decreased GBM patient survival (29). NOS1 strongly correlated with astrocytoma grade and proliferation (115), and NOS3 was strongly correlated with astrocytoma grade and VEGF expression (26). NOS3 expression also correlated with elevated tumor grade for ependymomas (134). Elevated expression of NOS2 was also observed in the majority of oligodendrogliomas (10). In contrast, NOS1, but not NOS2, was repeatedly elevated in juvenile pilocytic astrocytoma samples (15). Immunoreactivity of NOSs was heterogeneous across tumor cells, with additional expression of NOS2 and NOS3 in the tumor endothelial cells, and NOS2 in macrophages and microglia (3, 10, 15, 49, 68). Together the data demonstrate that gliomas express NOSs in the tumor cells and

the adjacent microenvironment, indicating that NO could be generated from multiple sources within a tumor.

Molecular Inhibition of NOS2 Expression in Glioma Cells

Among the NOSs expressed in gliomas, the molecular regulation of that expression has been most well defined for NOS2. However, regulators of *NOS2* expression have mostly been evaluated in rodent gliomas, and the known differences in the rodent and human *NOS2* promoters (18) suggest using caution when applying findings to human gliomas. However, conservation of NF- κ B and GAS binding sites within 300 base pairs of the transcriptional start site does hint that similar signaling pathways can be involved.

The importance of NF- κ B signaling in C6 glioma cells for *Nos2* expression has been demonstrated through expression of a nondegradable form of I κ B α , which prevents NF- κ B activation and LPS or cytokine-stimulated *Nos2* expression (81, 82). Degradation of I κ B α in C6 glioma cells induced by treatment with the hormone leptin resulted in increased *Nos2* expression and NO production *via* NF- κ B activation (72). Additional studies with the *Nos2* promoter in C6 glioma cells show RhoA downregulation by lovastatin increases NF- κ B activation to increase *Nos2* and NO levels (91). It is also through inhibition of NF- κ B binding that the sesquiterpene lactone Nepalolide A is suggested to inhibit *Nos2* expression in C6 glioma cells (124).

Beyond NF- κ B, kinases are also thought to be involved in additional signaling important for *NOS2* expression. In rat C6 glioma cells, LPS- and cytokine-induced NOS activity and *Nos2* expression could be inhibited by the isoflavone genistein, suggesting the importance of tyrosine kinase activity or oxidative stress in NOS2 levels (30). Additional studies have suggested roles for p38 MAPK but not p42/p44 MAPK in the regulation of LPS- and cytokine-induced *Nos2* expression in C6 glioma cells (132). Pretreatment of cells with the p38 MAPK inhibitor SB202190 prevented NO induction and *Nos2* elevation post-LPS, TNF- α , and INF- γ exposure (132). Another p38 MAPK inhibitor, SB203580, was also sufficient to inhibit the induction of *NOS2* expression and NO production by advanced glycosylation end products (64).

Ethanol and other short chain alkanols inhibit *Nos2* expression, resulting in decreased NO production in C6 glioma cells (76, 112–114). Through the generation of multiple deletion mutants of the *rat Nos2* promoter as well as other techniques, a minimal LPS and cytokine responsive region was determined to contain C/EBP β , gamma-IRE, GAS, and Oct binding domains (96). Further experiments concluded that the octamer DNA binding motif was required for ethanol-mediated inhibition of *Nos2* promoter activity (96).

NOS2 expression in glioma cells can also be controlled by additional signals important for normal brain and nervous system function, although the direct mechanism for these effects is not completely understood. Activation of the serotonin receptor 5-HT $_{2A}$ receptor with 2,5-dimethoxy-4-iodamphetamine inhibits cytokine-mediated *NOS2* transcription (77). Dopamine may also inhibit NO production and *NOS2* expression through mechanisms involving dopamine receptor and the beta-adrenergic receptors (73). Similarly, higher levels of the dopamine precursor L-DOPA (500 μ M or greater) blocked NO production stimulated by LPS and IFN- γ in association with reduced NOS2 protein in C6 glioma cells

(106). Agonism of the cannabinoid receptor with WIN 55,212–2 is also sufficient to inhibit LPS- and IFN- γ -induced NO production and *NOS2* expression (27). The role of alpha 2 adrenergic and imidazoline receptors has been mixed, however, as both the antagonist Idazoxan and the agonist Clonidine inhibited LPS-induced *Nos2* expression and NO production in C6 glioma cells (32, 121). These data demonstrate that there are multiple signals important for normal brain and cancer that are expected to regulate NO production in glioma *via* regulation of NOS2 levels. We anticipate that, as our understanding of NOS regulation in human cells grows, we will discover more mechanisms to modulate its levels.

NO, NOS, and Effects on Glioma Cell Biology

With the findings that NO was produced and NOSs expressed in human gliomas, multiple studies have sought to determine the biological consequences of modulation of the pathway through NO donation, NO scavenging, NOS induction, and/or NOS inhibition (see Table 1 for a list of drugs and their biological effects in gliomas). These studies have elucidated a multitude of roles for NO and NOS in glioma. Those functions include the regulation of tumor initiation, angiogenesis, invasion, immune system function, therapeutic resistance, and BTIC maintenance. We have sought to elucidate each of these major biological effects of NO and NOS in gliomas in the context of the method of pathway targeting or activation in further detail below.

NO, NOS, and Glioma Initiation

To define the role of NO and NOS function in tumor formation, utilization of animal models of glioma initiation or transformation of non-neoplastic cells is required. One mouse model of glioma initiation utilizes avian retroviruses in an RCAS vector to deliver oncogenes to specific cellular populations expressing the avian retroviral receptor (tv-a). When avian retrovirus expressing platelet-derived growth factor beta (PDGF β) is injected into the brains of mice expressing tv-a controlled by the promoter of the stem cell factor Nestin, oligodendrogliomas develop (101). Using this model system, tumors were initiated *via* PDGF β expression in Nestin tv-a transgenic mice with and without targeted deletion of *Nos3* (12). The survival of mice was extended with the loss of *Nos3*, indicating a protumorigenic role for NO and NOS3 during gliomagenesis.

While these data with models of glioma initiation suggest the benefit of targeting NO and NOS, it is important to note that their role may differ in other types of brain tumors. Medulloblastoma is a primitive neuroectodermal tumor and the most common malignant brain tumor in children. Mutations in protein patched homolog 1, PTCH1, a receptor for sonic hedgehog, are found in human medulloblastoma, and loss *Ptch1* can initiate the disease in mouse models. When mice with targeted deletion of *Nos2* were bred with those with heterozygous deletion of *Ptch1*, the incidence of medulloblastoma development increased approximately two-fold and mouse survival was significantly decreased (39). The data suggested an important role for NO signaling in development of the cerebellum and granule cell precursor migration and indicated an inhibitory role for NOS2 and NO in medulloblastoma initiation.

TABLE 1. LIST OF MOLECULES DONATING NITRIC OXIDE OR INHIBITING NITRIC OXIDE SYNTHASE AND A SUMMARY OF EFFECTS ON GLIOMA

<i>Molecules</i>	<i>Type</i>	<i>Effect</i>	<i>References</i>
JS-K (<i>O</i> ² -(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazene-1-ium-1,2-diolate)	GST-activated nitric oxide donor	Increase necrosis and apoptosis, decrease proliferation, open blood brain barrier	(126, 129)
L-arginine	NOS substrate, nitric oxide donor	Increase tumor permeability	(78, 137)
Hydroxyurea	Nitric oxide donor, antimetabolite	Increase tumor permeability	(6, 24, 92, 137)
CTX-NO	Tumor targeting nitric oxide donor	Induce chemosensitivity	(94, 95)
S-nitrosoglutathione (GSNO)	Nitric oxide donor	Reduce clonogenic potential when combine with alkylating agents	(136)
Diethylamine NONOate (DEA/NO)	Nitric oxide donor	Reduce clonogenic potential when combine with alkylating agents, growth inhibition and chemosensitization	(128, 136)
Sodium nitroprusside (SNP)	Nitric oxide donor	Reduce clonogenic potential when combine with alkylating agents, inhibit growth, radiosensitize, tumor cytotoxic	(7, 60, 136)
Disodium 1-[2-(carboxylate)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate methanolate (PROLI/NO)	Nitric oxide donor	Increase tumor permeability, growth inhibition and chemosensitization	(128, 130)
S-nitroso- <i>N</i> -acetylpenicillamine (SNAP)	Nitric oxide donor	Inhibit growth, radiosensitize	(7, 60)
O2-(58) 1-(<i>N,N</i> -dimethylamino) diazen-1-ium-1,2-diolate (PABA/NO)	Nitric oxide donor	Decrease cell proliferation and sensitize glioma cells to TMZ	(58)
Bradykinin TNP-470	eNOS inducer Indirect inhibitor of NOS	Increase tumor permeability Enhance effects of ionizing IR on tumor, prevent acute microvascular damage	(70, 78, 80) (69)
<i>N</i> -tert-butyl- α -phenylnitron	iNOS inhibitor	Decrease tumor cell proliferation, prevent vascular alteration	(22, 43)
OKN007	iNOS inhibitor	Increase apoptosis, decrease tumor hypoxia, angiogenesis and cell proliferation	(36, 43, 117)
Mercaptoethylguanidine	iNOS inhibitor	Enhance effects of IFN- γ -secreting glioma cells immunotherapy.	(2)
L-NIL	iNOS inhibitor	Enhance effects of IFN- γ -secreting glioma cells immunotherapy.	(1)
L-NAME	NOS inhibitor	Decrease tumor blood flow and vasodilation, decrease cell motility, enhance effects of IFN- γ -secreting glioma cells immunotherapy, regulate blood brain barrier permeability	(80, 89, 111, 131)

A large number of different NO donors and NOS inhibitors have been utilized to treat glioma cells with biological effects ranging from changes in cell growth and apoptosis to changes in tumor permeability or immunotherapy efficacy.

INF- γ , interferon-gamma; IR, irradiation; NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, Ng-nitro-L-arginine methyl ester.

In addition to *in vivo* mouse models, tumor initiation can be evaluated *in vitro* using human and mouse cells. A study on gene and microRNA expression in GBM also found a significant downregulation of miRNAs targeting NO signaling pathways (23). With methods similar to the Weinberg method of cellular transformation (using hTERT, oncogenic Ras, and T antigen) (40), human astrocytes can be transformed to gliomas (93, 107, 108). However, the effect of NO and NOS on transformation of human astrocytes using similar systems has not yet been determined. In mouse astrocytes

where transformation was achieved *via* expression of the constitutively active epidermal growth factor receptor variant III (EGFRvIII), knockdown of NOS2 inhibited their growth *in vitro* and tumorigenic potential *in vivo* (23, 90). While the data suggest that NOS2 promotes astrocyte transformation, this hypothesis was not directly tested in terms of the effects of NO donors or NOS2 expression on soft agar formation or *in vivo* tumorigenic potential. Nor has the ability of NO or NOS2 to substitute for or synergize with key transforming pathways in astrocytes been determined. Thus, further

elucidation of the ability of NO- and NOS-mediated signals to transform cells to promote glioma initiation could provide additional benefit.

NO, NOS, and Glioma Growth

As a hallmark of cancer is uncontrolled proliferation, many studies have examined the impact of NO and NOS signaling on glioma cell growth. Indeed, when NOS2 expression was first evaluated, cytokines that increased *Nos2* were observed to increase the growth of rat C6 glioma cells. NOS2 inhibitors decreased the cytokine growth effect, indicating a proliferative role for NO (31,32). In the studies with EGFRvIII expressing transformed astrocytes, treatment with the NOS2 inhibitor 1400W or S-MIU significantly reduced their proliferation, whereas treatment with the NO donor SNAP increased cell growth (90). In contrast, NOS2 was not a critical mediator of nontransformed astrocyte cell growth, suggesting the potential for minimal toxicity against non-neoplastic cells in the brain (90). In BTICs, NOS2 inhibition with 1400W also decreased cell growth and NOS2 was found to suppress the cell-cycle inhibitor cell division autoantigen 1 (CDA1) to increase cell growth (29). Indeed, when NOS2 was targeted with shRNA or pharmacologic inhibitors in human or rat glioma cells, there was a significant delay in tumor growth *in vivo*, indicating glioma-derived NO is a critical factor for tumor growth (29, 86, 133).

NO, NOS, and the Regulation of Glioma Angiogenesis, Blood Flow, and Vascular Permeability

Malignant gliomas are known among human tumors for high vascular density. Furthermore, the moderate vascularization in low-grade comparing to prominent microvascular proliferation in high-grade gliomas suggests that activation of an "angiogenic switch" is critical for the progression of malignant gliomas (119). Angiogenesis is regulated by hypoxia through the stabilization of hypoxia inducible factor alpha (HIF α) and induction of VEGF, a critical proangiogenic factor in GBMs (88). NOSs are expressed in both tumor cells and the tumor vasculature, and NO regulates blood vessel relaxation and permeability, especially in the blood-brain barrier (50, 116). Thus, there are multiple mechanisms through which NO could regulate the tumor vasculature.

When the NOS inhibitor, L-NAME (Ng-nitro-L-arginine methyl ester), was used to treat rat C6 gliomas, tumor blood flow was reduced as much as 49% and tumor volumes were reduced (111). The decrease in tumor blood flow was associated with a significant increase in tumor necrosis, strongly suggesting that inhibition of NOS could be an effective method to reduce glioma growth (111). L-NAME was also used to more recently demonstrate a requirement for NOS (likely NOS1 and NOS3) in bradykinin-induced increases in glioma microvessel permeability (67). Additional experiments with *Nos2* targeting using antisense in rat C6 glioma cells demonstrated reduced tumor vascular development in association with reduced tumor perfusion (59). Similarly, use of an inducible *Nos2* antisense RNA in C6 glioma cells demonstrated decreased tumor vessel maturation when NOS2 expression and NO levels were reduced (86).

Expression of VEGF is NO dependent, providing a direct mechanism through which NO could influence endothelial cell proliferation and/or migration. NO regulates the ex-

pression of VEGF gene in hypoxic A-172 GBM cells *via* the accumulation and translocation of HIF-1 α to the nucleus (57). HIF-1 α protein levels were significantly increased in NO-treated cells, through transcription-independent mechanisms (57). NO treatment using the donors S-nitroso-N-acetylpenicillamine (SNAP) and NOR3 also increased VEGF production in A-172 GBM cells. The half-life for VEGF mRNA was prolonged about 2.4-fold by SNAP at 0.5 mM, and SNAP-induced expression of VEGF was mediated through the cGMP pathway (13). However, effects of NO on VEGF expression are concentration/redox dependent since the addition of NO donors in a hypoxic environment can have an inhibitory effect on gene expression possibly due to toxicity (57).

Through mechanisms independent of VEGF, NO can also modulate endothelial progenitor cells (EPCs) to regulate angiogenesis. Produced by and associated with malignant glioma cells, osteopontin increased PI3-K/AKT activation in EPCs, which led to phosphorylation of NOS3 and elevated NO production (125). Elevated levels of circulating CD34+ CD105+ KDR+-activated EPCs were also observed in glioma patients in correlation with elevated plasma levels of NOS3 (140), suggesting that NOS-derived NO may contribute to EPC migration to promote glioma angiogenesis (59).

Angiogenesis was also reduced in rat C6 gliomas treated with *N*-tert-butyl- α -phenylnitron (PBN), a free radical spin trap that inhibits NOS2 and decreases NO production (22, 33). Magnetic resonance (MR) imaging and MR angiography of tumors pretreated with PBN showed reduced tumor growth and angiogenesis, leading to tumor regression. PBN treatment was able to induce complete regression and inhibit blood vessel formation in early stages. However, effects were less profound in established tumors, suggesting that the inhibitor can be used as a postsurgery prophylactic agent (22). OKN-007, a disulfonyl derivative of PBN, also decreased tumor angiogenesis, and this mechanism of action was not VEGF mediated (117). OKN-007 was more effective than PBN when administered to mice with established C6 gliomas (43), and OKN-007 showed potential for causing regression of U87MG human GBM cells. In addition to effects on angiogenesis, OKN-007 also decreased tumor cell proliferation and increased apoptosis (117).

NO donors have also been proposed for indirect tumor sensitization through increased drug delivery. In a rat syngeneic brain tumor model, oral administration of L-arginine (NOS substrate) or hydroxyurea (NO donor and antimetabolite) significantly increased uptake of a radiolabeled tracer selectively to the brain tumor. NO generated by the donor increased the tumor cGMP, leading to the activation of K_{Ca} channels in tumor microvessel through cGMP-dependent protein kinase, which in turn improved the vesicular transport of therapeutics (137). A phase II trial of hydroxyurea and imatinib mesylate showed benefit, while each agent alone did not have substantial effect (24, 92). In a more recent study, the short-acting NO donor PROLI/NO was shown to selectively increase intratumoral uptake of [¹⁴C]-aminoisobutyric acid and [¹⁴C]-sucrose through NO/cGMP pathway, independent on NOS activity. This permeabilization effect was achieved at PROLI/NO doses that did not cause significant unwanted changes in vascular tone and systemic circulation (130). The data therefore suggest the potential for drug

FIG. 1. Nitric oxide and nitric oxide synthases regulate glioma angiogenesis. NOS in the tumor vasculature and glioma cells produces NO to promote tumor angiogenesis, blood vessel permeability, and endothelial progenitor cell recruitment. The NOS/NO pathway in gliomas can lead to upregulation of VEGF to promote angiogenesis or mediate VEGF-independent effects downstream of osteopontin. NO, nitric oxide; NOS, nitric oxide synthase; VEGF, vascular endothelial growth factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

treatments that modulate NO to improve vascular permeability and drug efficacies (Fig. 1).

NO, NOS, and Glioma Migration and Invasion

GBMs most often recur within 2 cm of the original resection site due to the extensive ability of the tumor cells to migrate to distant locations in the normal brain. While the role of NO and NOS in glioma cell invasion has not yet been extensively evaluated, studies do suggest a potential role for the pathway in glioma invasion. For example, human U251 GBM cells overexpressing the metalloproteinase 9 (MMP9) or urokinase plasminogen activator receptor (uPAR) had less migratory and invasive potential after treatment with the NOS inhibitor L-NAME (141). Treatment of human U87MG GBM cells with the NO donor sodium nitroprusside also increased cell invasion *in vitro* through a mechanism thought to involve increased production of metalloproteinase 2 (65). Thus, there is potential for NOS inhibition to reduce glioma cell invasion, but additional studies are needed to clarify the extent of this effect *in vivo*.

NO, NOS, and Immune System Function in Glioma

Among the NOSs, NOS2 is responsive to immune stimuli and expressed in microglia, the macrophage which provides immunoprotection for the brain (79). The pro- and anti-glioma effects of microglia are still being determined, likely because macrophages have the capacity to be either pro- or anti-inflammatory, depending on their phenotype. These phenotypes include M1 macrophages that generate NO *via* NOS2

and secrete proinflammatory cytokines like IL-6. M2 macrophages in turn block NOS2 activity and secrete anti-inflammatory cytokines like transforming growth factor- β (TGF- β). Depending on the cell context, both pro- and anti-inflammatory cytokines like IL-6 and TGF- β can promote glioma growth or invasion. Thus, it is not surprising that the function of tumor-associated microglia and the potential role of NO and NOS in immune system regulation in gliomas are complex.

Interactions between glioma cells and microglia are likely to modulate NO release, as NO levels are greater when microglia are cultured on matrix derived from rat C6 glioma cells in comparison to astrocyte-derived matrix (56). Microglial conditioned media can promote apoptosis in glioma cells, but NOS inhibition dramatically reduced this effect (38,46). Importantly, conditioned media from microglia did not induce apoptosis in primary rat astrocytes, suggesting that the effect is specific to tumor cells (48). These data suggest an important role for microglia-produced NO in the inhibition of glioma cell growth through the promotion of cell death. However, microglia activated by glioma conditioned media elevated NOS2 resulting in NO production that increased the migration of glioma cells (49). Inhibition of NO signals in this context would appear favorable to prevent disease spread into the normal brain.

Glioma cells may also suppress the immune system in an NO-dependent manner. When splenocytes were extracted from rodents bearing gliomas, splenic cells failed to proliferate and dampened the IFN- γ and IL-10 response of splenocytes from tumor-free mice *in vitro*. This suppression was

reversed by the NOS inhibitor *N*-nitro-L-arginine methyl ester (44), indicating that malignant gliomas produced a systemic immunosuppressive effect through inhibition of splenic cell proliferation in an NO-dependent mechanism. NOS2 was also identified as the major mechanism through which myeloid-derived suppressor cells mediate T-cell suppression in response to glioma antigen immunization (52). Immunotherapy with a NOS2 inhibitor, mercaptoethylguanidine, demonstrated increased survival in rats with intracranial tumors when administered postimmunization with IFN- γ -secreting rat glioma cells (2). Together, these data would suggest the benefit of NOS2 inhibitor to promote immune system function.

NO, NOS, and BTIC Maintenance

BTICs are a cellular fraction critical for tumor maintenance and therapeutic resistance, and therefore, of significant interest for drug targeting. Recent studies in both mouse and human glioma models have defined an important role for NO derived from either the tumor cells or the tumor endothelial cells in BTIC maintenance. Platelet-derived growth factor (PDGF)-driven mouse gliomas were demonstrated to have elevated NOS3 in the tumor endothelium (12). As BTICs often reside in a perivascular niche, the potential for NOS3-mediated NO to facilitate BTIC maintenance was determined. The NO donor S-nitrosoglutathione (GSNO) increased the expression of side population that can mark BTICs in mouse glioma cells and increased activity of the promoter of the BTIC marker Nestin in human U251 GBM cells (12). GSNO also increased tumorigenic potential and neurosphere formation capacity, an *in vitro* assay that is a surrogate for self-renewal, of mouse glioma cells (12). These functional assessments of BTICs indicate that NO donors are capable of BTIC enrichment. Furthermore, as previously mentioned, converse experiments with NOS3 deletion in animal models with PDGF-induced gliomas demonstrated increased survival (12). The mechanism of NOS3 and NO donor effects on BTIC maintenance was determined to involve elevation of Notch signaling, a well-known stem cell maintenance pathway. These data strongly suggested that NO produced by the tumor endothelium was an important BTIC maintenance factor in the perivascular niche.

In addition to endothelium-produced NO promoting BTIC maintenance, an additional study demonstrated BTICs could produce NO directly *via* NOS2 (29). Even in the absence of endothelial cells, expression of the NO consuming enzyme flavohemoglobin in BTICs reduced their neurosphere formation potential *in vitro* and tumorigenic potential *in vivo* (29). These data demonstrated an autocrine role for NO in BTIC maintenance and self-renewal. Targeting NOS2 in BTICs with small-molecule inhibitors or shRNA *in vitro* also decreased their growth and neurosphere formation potential, indicating the importance of the NOS2/NO pathway for BTIC maintenance (29). NOS2 inhibition also decreased the most critical functional phenotype of BTICs, *in vivo* tumorigenic potential (29). Similar effects were not observed in non-neoplastic neural stem cells, once again suggesting limited toxicity of NOS2-directed therapies in the brain (29).

Furthermore, a study in GBM has shown that PDGF–NOS2–ID4–miR129 regulatory axis activated JAGGED1–NOTCH signaling in both BTICs and tumor endothelial cells,

promoting tumor progression, BTIC proliferation, and angiogenesis (51). Together, these data demonstrated the NO was a critical BTIC maintenance factor that could be produced in an autocrine or paracrine manner to promote glioma growth.

NO, NOS, and Glioma Therapeutic Resistance

BTICs are of significant interest for therapeutic targeting due to their ability to survive current therapeutic approaches. The current standard of care for GBM includes surgery, radiation, and chemotherapy with temozolomide, but tumors inevitably recur. Recurrence is associated with residual BTICs, which can be enriched post-therapy due to the preferential death of non-BTICs (4). A recent study showed that irradiation (IR) increased the BTIC subpopulation, thereby promoting a malignant phenotype, *via* elevated NO production (55). NOS2 levels and NO production were significantly increased in glioma cells treated with three IR doses of 2 Gy, correlating with expression of BTIC markers, including Nestin, Sox2, and Notch2 (55). These data and others suggest the importance of NOS and NO BTIC phenotypes and for radioadaptive response (Fig. 2).

Radioadaptive response occurs when cancer cells gradually change and become capable of surviving subsequent exposures to higher radiation doses. Chronic or repeated low-dose treatment with γ -IR induces NOS2-mediated radioadaptive response in A-172 human GBM cells (71). Acute doses of X-IR on chronically γ -irradiated cells are significantly less effective than the same doses on parental or acutely γ -irradiated cells. The adapted cells have increased activation of p53-negative regulator Hdm2, which in turn reduces p53 expression and allows the upregulation of NOS2 when the cells are exposed to high doses of X-IR (71).

Fractionated ionizing radiation was similarly found to increase NO and NOS2 expression in U87MG and U373MG GBM cells (55). Elevation of NOS2 and NO was associated with increased survival post-IR in colony formation assays (71) and resistance to cell death induced by IR as determined through propidium iodide staining (55). NOS2 induced by fractionated radiation was also critical for mediating resistance to cisplatin and taxol-induced cytotoxicity in human GBM cells (55), although NOS2 overexpression in rat C6 glioma cells did not affect the toxicity of cisplatin (135). NOS2 elevation in C6 glioma cells did prevent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) induced cell death (135). Additional experiments confirmed that a NO donor, isosorbide dinitrate (ISDN), promoted radioresistance of human glioma cells (71). These data would suggest the benefit of novel treatment paradigms that reduce endogenous NO and NOS levels to sensitize glioma cells to conventional therapies, thereby preventing therapeutic resistance and tumor recurrence.

Radiation-induced bystander effect is the radiobiological phenomenon in which irradiated cells exert certain effects on adjacent nonirradiated cells, including changes in protein expression, DNA damage and mutation, genomic instability, malignant transformation, and radioresistance. When only a small fraction of glioma cells were targeted using a unique approach in which single-cell microbeams were used to individually irradiate nuclei of T98G GBM cells with exact numbers of helium ions, the micronuclei yield on total cell population exceeded one predicted based on whole

FIG. 2. Nitric oxide and nitric oxide synthases can mediate therapeutic resistance in gliomas. NOS and NO promote a BTIC/glioma stem cell phenotype, which is associated with therapeutic resistance. NO is elevated by irradiation of glioma cells, and the NO can then have bystander effects on adjacent cancer and stromal cells. BTIC, brain tumor initiating cell. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

population IR, suggesting bystander effects (98). NO stood out as one of the mediators, as 40% of the cells in the population had increased NO level when only 1% was targeted. Treatment with c-PTIO inhibited bystander signals, effectively reducing micronuclei yield to expected levels (100). Similarly, T98G GBM cells also exert bystander effects on adjacent noncancerous fibroblast cells (97). Further investigation of the

mechanism showed that TGF-β1, together with NO, mediated the bystander response in irradiated T98G GBM cells. The presence of a fraction of irradiated cells elevates NO and TGF-β1 in the media, causing DNA damage in adjacent cells, as evidenced by increased yield of micronuclei. Addition of either exogenous NO or TGF-β1 exacerbates the effects, while treatment with anti-TGF-β1 diminishes NO production at low

FIG. 3. The concentration of nitric oxide produce during therapeutic approaches will be critical for biological response. Elevation of NO and targeting of NOS can produce antitumorigenic effects as long as NO levels are in a therapeutic range. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

irradiated cell fraction (98). NO and TGF- β 1 were also involved in bystander effects of irradiated T98G cells on vicinal normal human fibroblasts AG01522 cells, causing DNA damages through increased ROS levels (62, 99).

Considering the cytotoxic nature of high NO levels, an alternative strategy of treatment with NO donors has also been evaluated as a method to increase the efficacy of radio- and chemotherapies in glioma. SNAP at 100 μ M showed sensitizer enhancement ratio (ratio of the isoeffective radiation dose between presence and absence of inhibitor) of 1.8 for human glioma cell line T98G (60). More recent studies showed that SNAP along with other NO donors includes GSNO and diethylamine NONOate (DEA/NO), potentiate cytotoxicity of alkylating agents, synergistically reducing colony formation in glioma cells (136). These data may also explain why there is some evidence that NOS2-produced NO may contribute to TLR9-mediated radiosensitization (62). Combinations of IR and a synthetic unmethylated-CpG-containing oligodeoxynucleotide (CpG ODN107) upregulated TLR9 expression (62). TLR9 elevation leads to elevated NF- κ B and NO production in U87 glioma cells, resulting in cell cycle arrest. In a human glioma xenograft model, combined treatment of CpG ODN107 and local IR reduces tumor growth by up to 67% (62). Together the data suggest that high levels of NO produced by NO donors and perhaps some cases of NOS elevation can increase the efficacy of chemo- and radiotherapies.

Conclusions

Given the expression of NOS and the extensive roles of NO in glioma, modulation of NO levels is an expected therapeutic strategy. However, current publications can diverge on conclusions regarding whether the overall effect of NO and NOS is pro- or antitumorigenic, likely due to differences in multiple variables. These variables include the levels of NO or NOS produced, the stage of tumorigenesis, the differentiation state of the neoplastic cells, and the type of NOS and the cell type within the tumor where that NOS is preferentially expressed. However, we believe that the data lead to the general consensus that targeting endogenous NOS to reduce physiological levels of NO is antitumorigenic, whereas elevation of NO to superphysiologic levels can promote cell death to also yield antitumorigenic effects (summarized in Fig. 3).

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Abbreviations Used

BTIC = brain tumor initiating cell
EGFRvIII = epidermal growth factor receptor variant III
EPC = endothelial progenitor cell
GBM = glioblastoma
GSNO = S-nitrosoglutathione
HIF = hypoxia inducible factor
IR = irradiation
IL = interleukin
L-NAME = Ng-nitro-L-arginine methyl ester
LPS = lipopolysaccharide
MR = magnetic resonance
NO = nitric oxide
NOS = nitric oxide synthase
PBN = N-tert-butyl- α -phenylnitron
PDGF = platelet-derived growth factor
PDGF β = platelet-derived growth factor beta
SNAP = S-nitroso-N-acetylpenicillamine
TGF- β = transforming growth factor- β
VEGF = vascular endothelial growth factor