S-Nitrosoglutathione and Hypoxia-Inducible Factor–1 Confer Chemoresistance against Carbamoylating Cytotoxicity of BCNU in Rat C6 Glioma Cells

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ABSTRACT: BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea) is the mainstay in glioblastoma multiform chemotherapy with only minimal effects. BCNU may kill tumor cells via carbamoylating cytotoxicity, which irreversibly inhibits glutathione reductase with resultant accumulation of oxidized form of glutathione causing oxidative stress. S-nitrosoglutathione (GSNO) is a product of glutathione and nitric oxide interaction. We report that GSNO formation may underlie carbamoylating chemoresistance mediated by activation of inducible nitric oxide synthase. Transactivation of hypoxia-inducible factor–1 (HIF-1)–responsive genes reduces oxidative stress caused by glutathione depletion. We also noted that preconditioning of C6 glioma cells to induce HIF-1 and its downstream genes confers chemoresistance against carbamoylating cytotoxicity of BCNU.

KEYWORDS: alkylation; carbamoylation; chemoresistance; chemotherapy; hypoxia; iNOS; nitric oxide

S-NITROSOGLUTATHIONE FORMATION UNDERLYING NITRIC OXIDE–DEPENDENT CARBAMOYLATING CHEMORESISTANCE

Glioblastoma multiform (GBM) is the most common type of primary brain tumor accounting for more than 40% of neoplasm in the central nervous system.[1] A combination of surgery, radiotherapy, and chemotherapy results in survival of approximately 14 months.[2] 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is the mainstay in chemotherapy of GBM, in part because of its lipophilic character that allows better

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passage across the blood–brain barrier.[3] Unfortunately, BCNU does not appear to substantially prolong median survival. The mechanism of GBM resistance to BCNU chemotherapy remains to be fully delineated. Variations in multidrug resistance genes,[4] DNA repair activity such as O6-methylguanine–DNA methyltransferase,[5] glutathione ***S***-transferase, and intracellular glutathione contents[6] all have been speculated to cause BCNU chemoresistance.

Nitric oxide (NO) is a free radical gas mediating several physiological functions including modulation of cell viability. NO is synthesized from arginine and oxygen catalyzed by nitric oxide synthases (NOS). Expression of inducible NOS (iNOS) has been demonstrated in human glioma[7,8] and in a variety of brain tumors or peritumor areas, with its mRNA levels higher in malignant glioma than normal brain tissues[9] or meningioma.[10] In addition to its well-established cytotoxicity at higher concentrations, NO may contribute to antioxidant action via its interaction with glutathione to form *S*-nitrosoglutathione (GSNO), an antioxidant that is two orders of magnitude more potent than the reduced form of glutathione (GSH).[11,12] At micromolar concentrations, GSNO is capable of neutralizing oxidative stress exerted by peroxynitrite,[13] a highly reactive species derived from interaction of NO with superoxide anions.[14]

Using a panel of different compounds each carrying alkylating, carbamoylating, or both tumoricidal activities, we have reported previously that overexpression of iNOS conferred chemoresistance against carbamoylating agents, including BCNU, in rat C6 glioma cells.[15,16] Suppression of iNOS expression by an antisense strategy or inclusion of L-NAME, a NOS inhibitor, attenuated BCNU chemoresistance in C6 cells.[15] To further characterize the molecular mechanism underlying this novel *in vitro* effect, we have explored the potential involvement of GSNO mediating the observed iNOS effects. Our findings suggest that GSNO likely plays an important role in this iNOS-induced chemoresistance against carbamoylating agents.[17] Several lines of evidence support this contention. First, among the three NO donors tested, only GSNO conferred BCNU chemoresistance. Exogenous GSNO also enhanced chemoresistance against all the carbamoylating agents tested including cyclohexyl isocyanate and 2-chloroethyl isocyanate, the respective carbamoylating moiety of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and BCNU, but not alkylating agents such as temozolomide. Second, experimental manipulations expected to increase or decrease cellular GSNO contents correspondingly affected carbamoylating chemoresistance. Specifically, chemoresistant C6 glioma cells, such as those exposed to cytokines for iNOS induction or overexpressing iNOS by a gene transfer strategy, contain significantly higher levels of GSNO as detected by immunocytochemistry using a GSNO-specific antibody and by HPLC analysis of medium GSNO contents. Third, copper ions have been shown to modulate GSNO action.[18,19] We found that neocuproine (a Cu^{+} chelator), but not cuprizone (a Cu^{2+} chelator), was effective in blocking the development of chemoresistance against carbamoylating agents induced by iNOS overexpression as well as that induced by exogenous GSNO.

Cellular proteins undergoing carbamoylation may lose their biological functions. Carbamoylation may render those enzymes critically involved in maintaining cellular redox homeostasis, such as glutathione reductase, irreversibly nonfunctional, thereby leading to accumulation of GSSG.[20–22] In this respect, carbamoylation may be considered as a chemical-induced oxidative stress that can be antagonized by antioxidants. Recently, GSNO has been reported to act as an antioxidant two orders of

magnitude more potent than GSH, capable of protecting brain dopaminergic neurons against iron-induced oxidative stress.[13,23] Heightened iNOS expression has been detected in malignant glioblastomas as compared with normal brain tissues[9] or meningiomas.[10] Surgical procedures or radiation therapy may also result in inflammatory responses leading to enhanced iNOS expression. Formation of GSNO therefore may occur in malignant brain tumors before the initiation of BCNU therapy, thereby causing chemoresistance against carbamoylating agents. Interestingly, we have observed that *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), another NO-releasing nitrosothiol, was effective in increasing GSNO formation and neutralizing BCNU cytotoxicity under a pretreatment condition, but not in a cotreatment paradigm. Similarly, overexpression of iNOS also has to occur before application of BCNU to develop NO-mediated chemoresistance.[15,17] Further investigation is required to examine whether GSNO metabolism in malignant brain tumors is distinct from normal brain tissues or meningiomas.

PUTATIVE ROLES OF HYPOXIA-INDUCIBLE FACTOR–1 IN GBM CHEMORESISTANCE

Hypoxia-inducible factor–1 (HIF-1), a heterodimeric protein complex consisting of alpha (HIF-1α) and beta (HIF-1β or ARNT; aryl hydrocarbon receptor nuclear translocator) subunits, is a key regulator of mammalian oxygen homeostasis. HIF-1α expression is tightly regulated by the cellular oxygen tension,[24,25] whereas the expression of HIF-1β is oxygen independent. The activity of this basic helix-loop-helix transcription factor is increased in most cells in response to low oxygen tension.[26,27] In addition to tissue hypoxia, several reagents including cobalt chloride and iron chelator desferrioxamine (DFO) are also known to induce HIF-1.[28] Recently, a growing body of evidence has also shown HIF-1 activation in response to hypoxia in tumors.[29,30] HIF-1 appears to play a key role in cancer growth by transactivating genes such as erythropoietin (EPO),[31] vascular endothelial growth factor (VEGF),[32] and iNOS[33] that may confer cytoprotective as well as angiogenic effects. Indeed, differential regulation of VEGF, HIF-1α, and angiopoietin-1, -2, and -4 by hypoxia and ionizing radiation was observed in human glioblastoma.[34] Clinical studies revealed strong nuclear expression of HIF-1α protein in the majority of glioblastomas and anaplastic astrocytomas, particularly surrounding areas of necrosis in glioblastomas.[35] Upregulation of HIF-1α mRNA was also detected with a significant increase in glioblastomas compared with lower grade tumors.[35]

We have recently demonstrated that preconditioning of C6 glioma cells with reagents mimicking hypoxia and hence capable of HIF-1 induction, namely, cobalt chloride and DFO, enhanced carbamoylating but not alkylating chemoresistance against BCNU.[33] Expression of HIF-1α protein and HIF-1 DNA binding activity were induced by cobalt chloride pretreatment based on Western blotting and electrophoretic mobility shift assay, respectively.[33] Downregulation of cobalt-mediated HIF-1 activation, either by coincubation with cadmium ions or transfection with HIF-specific oligodeoxynucleotide (ODN) decoy or an antisense phosphorothioate ODN against HIF-1α, abolished at least in part the carbamoylating chemoresistance associated with cobalt preconditioning, suggesting a putative role of HIF-1 implicated in the observed chemoresistance.

Hypoxia has been shown to increase chemoresistance against BCNU in human glioma cell lines.[36] The expression of the drug resistance genes was, however, unchanged under this condition, suggesting alternative mechanisms that may exist in hypoxia-induced chemoresistance. We have provided experimental evidence supporting the contention that HIF-1 induction under hypoxia may contribute to acquired chemoresistance against BCNU through inhibition of its carbamoylating cytotoxicity. The molecular mechanisms underlying HIF-1–mediated chemoresistance against carbamoylating cytotoxicity of chloroethylnitrosoureas remain unclear but may involve the transcriptional activation of genes downstream of HIF-1. Genes that are up-regulated by microenvironmental hypoxia through activation of HIF include glucose transporters, glycolytic enzymes, and angiogenic growth factors such as VEGF and EPO.[28,37,38] Transactivation of these genes may contribute to HIF-1–dependent protection against oxidative stress caused by glutathione depletion in primary cortical neurons.[39] Thus, carbamoylating action constitutes a chemical-induced oxidative stress that may be neutralized in a hypoxic microenvironment with resultant HIF-1 activation. We have provided direct experimental evidence supporting an important role of HIF-1 in the observed preconditioning effects.

CONCLUSIONS

Results from these studies suggest that GSNO formation as a result of iNOS expression as well as induction of HIF-1 and its target genes may represent important mechanisms underlying the development of chemoresistance, at least *in vitro*, against carbamoylating agents in glioma cells that are independent of the well-known angiogenesis actions of NO and HIF-1.[40] Such effects are also distinct from other known mechanisms of resistance to chemotherapeutic agents, such as the induction of O6-alkylguanine-DNA alkyltransferase[41] and DNA mismatch repair,[42] which are more likely to render GBM resistant to the alkylating action of chloroethylnitrosoureas. Pharmacological modulation of GSNO formation and/or HIF-1 activation may open a novel avenue to reduce chemoresistance against BCNU in GBM.

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