Inducible Nitric Oxide Synthase Neutralizes Carbamoylating Potential of 1,3-Bis(2-chloroethyl)-1-nitrosourea in C6 Glioma Cells

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ABSTRACT

Expression of iNOS in glioma and other tumors has been extensively documented but the effects of NO derived from iNOS on tumor-killing mechanisms of chemotherapy drugs remain to be fully defined. We note that increased NO synthesis by cytokine exposure or iNOS overexpression neutralized the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), but not cisplatin, in rat C6 glioma cells. Suppression of BCNU cytotoxicity associated with iNOS overexpression could be abolished by pharmacological inhibition of NOS or coexpression of an antisense RNA against iNOS. Both BCNU and CCNU are chloroethylnitrosoureas that kill tumor cells via carbamoylating and alkylating actions. Further studies using compounds that each carry these different activities indicate that iNOS neutralized carbamoylating, but not alkylating, action of chloroethylnitrosoureas. Temozolomide, a novel chemotherapy drug recently available for treating brain tumors, carries only alkylating, but not carbamoylating, action. Overexpression of iNOS in C6 cells failed to neutralize temozolomide cytotoxicity. Results from the present study demonstrate the ability of iNOS-derived NO to confer chemoresistance against the carbamoylating potential of chloroethylnitrosoureas in vitro. Further investigation is needed to test whether iNOS expression, frequently noted in malignant brain tumors, also enhances chemoresistance against chloroethylnitrosoureas in vivo.

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor accounting for more than 40% of neoplasm in the central nervous system (Kleihues et al., 1995). It carries poor prognosis despite multimodality approaches consisting of surgical resection, radiotherapy, and chemotherapy (Fine, 1994). The life expectancy of patients with GBM is usually less than 1 year from the time of diagnosis. To date, the 5-year survival remains at 1% (Davis et al., 1998). Surgery alone gave a median survival length of 14 weeks. Radiotherapy following surgical resection extended this figure to 36 weeks. Combination of surgery, radiotherapy, and chemotherapy prolonged survival to approximately 14 months (Rajkumar et al., 1999). BCNU is the most commonly used adjunct chemotherapy for GBM because of its lipophilic character. Unfortunately, BCNU did not substantially prolong median survival, even though the proportion of patients living more than 18 months increased from 5 to 15% with chemotherapy (Walker et al., 1980; Green et al., 1983; Fine et al., 1993). A recent analysis of results from two Brain Tumor Study Group protocols reaffirms a modest effect of adjunct chemotherapy in increasing long-term survival (DeAngelis et al., 1998). GBM develops chemoresistance against BCNU. Several mechanisms have been proposed that may account for BCNU chemoresistance in GBM. These include an increase in the synthesis of the reduced form of glutathione (Ali-Osman et al., 1990), DNA mismatch repair and O6-alkylguanine-DNA alkyltransferase expression (Friedman et al., 1998). Interventions that enhance chemosensitivity of glioma cells to BCNU may improve clinical outcomes.

Nitric oxide (NO) is a short-lived free radical gas with multiple physiological functions. In pathological states, NO may contribute to microbial killing (MacMicking et al., 1997) and neuronal degeneration (Zhang et al., 1994). NO exhibits tumoricidal activity both in vitro (Stuehr and Nathan, 1989) and in vivo (Farias-Eisner et al., 1994). However, NO may also alter vascular reactivity or promote neovascularization in favor of tumor growth (Andrade et al., 1992). Massive production of NO may be derived from the expression of iNOS (Xie et al., 1992) in response to exogenous stimuli such as cytokine exposure. Lipopolysaccharide, a bacterial endo-

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ABBREVIATIONS: GBM, glioblastoma multiforme; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; NO, nitric oxide; iNOS, inducible nitric oxide synthase; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; NOS, nitric oxide synthase; L-NAME, N \textsubscript{G}-nitro-L-arginine-methyl ester; PBS, phosphate-buffered saline; bp, base pair; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
toxin, in combination with interferon-γ induces iNOS expression in rat C6 glioma cells at both mRNA and protein levels (Feinstein et al., 1994). iNOS expression has also been demonstrated in human glioblastoma cells (Fujisawa et al., 1995) and in a variety of different brain tumors or peritumor areas, with its mRNA levels higher in malignant gliomas than meningiomas (Ellie et al., 1995).

Despite these extensive studies on iNOS expression in brain tumors, the interaction of NO derived from iNOS and the tumor-killing effects of chemotherapy drugs has received relatively little attention. Chloroethylnitrosoureas such as BCNU and CCNU kill tumors via carbamoylating and alkylating actions (Wheeler et al., 1974). BCNU decomposes in aqueous solution to form two electrophilic species, namely, 2-chloroethyl diazohydroxide and 2-chloroethyl isocyanate. The former carries alkylating (chloroethylenating) activity, whereas the latter possesses predominantly the carbamoylating potential. 2-Chloroethyl isocyanate also has secondary alkylating activity via the formation of 2-chloroethylamine (Becker and Schirmer, 1995). Like BCNU, CCNU also generates 2-chloroethyl diazohydroxide and 2-chloroethyl diazonium ions upon degradation in aqueous solution. However, the carbamoylating moiety of CCNU is cyclohexyl isocyanate (Wheeler et al., 1974), which, unlike 2-chloroethyl isocyanate, does not carry the aminoethylating activity (Penketh et al., 2000).

In the present study, we report that expression of iNOS substantially suppressed the cytotoxicity of BCNU and CCNU, but not cisplatin, in rat C6 glioma cells. Further identification of the specific cytotoxic action that is sensitive to iNOS expression was accomplished by applying three 1,2-bis(sulfonyl)hydrazine derivatives with carbamoylating or alkylating action (Penketh et al., 2000), temozolomide with alkylating (methylating) potential (Denny et al., 1994), as well as 2-chloroethyl isocyanate and cyclohexyl isocyanate, the respective carbamoylating metabolite of BCNU and CCNU. Results suggest that iNOS-mediated neutralization of chloroethylnitrosourea cytotoxicity is restricted to the carbamoylating action.

Materials and Methods

Reagents. 2-Chloroethyl isocyanate, cyclohexyl isocyanate, cisplatin, and lipopolysaccharide were from Sigma (St. Louis, MO). N^6-Nitro-L-arginine methyl ester (L-NAME) was from Alexis (San Diego, CA). Interferon-γ was purchased from Genzyme (Cambridge, MA). BCNU and CCNU were from Bristol-Myers Squibb Inc. (Princeton, NJ). Temozolomide was a gift from Dr. W. Robert Bishop, Schering-Plough Corporation (Kenilworth, NJ). The three 1,2-bis(sulfonyl)hydrazine derivatives (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine or compound 1, 1,2-bis(methylsulfonyl)-1,2-(2-chloroethyl)aminocarbonyl)-carboxyl)-hydrazine or compound 2, and 1,2-bis(methylsulfonyl)-1,2-[(methyl)amino(carbonyl)-hydrazine or compound 3) were generously provided by Dr. Alan C. Sartorelli in Department of Pharmacology at Yale University (New Haven, CT). The synthesis and characterization of compounds 1, 3, and 5 have been described in details elsewhere (Shyam et al., 1987, 1990, 1993, 1996; Penketh et al., 1994, 2000).

Cell Line, Plasmids, and Transfection. C6 rat glioma cells were purchased from American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection instructions. The plasmid NS05 overexpressing the murine iNOS gene subcloned into pcDNA1.1 was purchased from Oxford Biomedical Research (Oxford, MI). The pcDNA1.1 empty vector served as the control for the NS05 transfection in all experiments. Transient transfection using SuperFect reagent from QIAGEN (Santa Clarita, CA) was performed according to the manufacturer’s instructions. Transfection was conducted for 3 h at 37°C in 5% CO₂, with a DNA to SuperFect ratio of 1:2. One microgram of plasmid DNA was used to transfect C6 cells in each single well of 24-well plates. At the end of 3-h incubation the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and replenished with fresh medium. The transfected iNOS gene was allowed to express at least for 24 h before the transfectants were challenged with various chemotherapy regimens for another 12 to 48 h, depending on the reagents. Therefore, the cell survival assays were conducted within 72 h of iNOS transfection.

Although no reporter gene for direct quantitation of transfection efficiency is present in the plasmid NS05, our protocols for transient transfection resulted in approximately 30 to 40% of transfection efficiency in C6 cells using another plasmid, pTracer-SV40 (Invitrogen, Carlsbad, CA) with green fluorescence proteins (data not shown). The successful transfection of NS05 to cause iNOS overexpression was always accompanied by increased nitrite contents in the culture medium using the Griess reagent and, in selected experiments, by Western blot analysis. In cotransfection experiments, 3 µg of DNA and 6 µl of SuperFect reagent were used. The amount of different plasmids used was control, 3 µg of empty vector; iNOS overexpression, 1 µg of NS05 and 2 µg of empty vector; and antisense suppression of iNOS overexpression, 1 µg of NS05 and 2 µg of iNOS antisense construct.

For iNOS antisense construct, a primer pair (forward: 5′-CGG-GATCCCTCGGAGTAAAGAAG-3′; reverse: 5′-GGGTTACCTTTACAGGAGTGAAGAC-3′) was designed based on the cDNA sequence of iNOS gene (GenBank accession number M92649) to amplify a 145-bp fragment from mouse genomic DNA via polymerase chain reaction. This 145-bp fragment covers ATG initiation codon of the murine iNOS gene. When subcloned into the expression vector pcDNA3.1 (Invitrogen) in a reverse orientation, this construct can express a 145-bp antisense RNA against iNOS upon transfection into C6 cells.

Western Blotting. Western blot analysis was performed according to the protocols described previously (Xu et al., 1997). To detect the iNOS protein, the primary antibody against mouse macrophage iNOS (Transduction Laboratories, Lexington, KY) was applied at 1:500 dilution in PBS/Tween 20 containing 2.5% bovine serum albumin. The anti-rabbit IgG secondary antibody (NA934; Amersham Pharmacia Biotech, Piscataway, NJ) was applied at 1:500 dilution in PBS/Tween 20 containing 2.5% bovine serum albumin. Nitrite and Cell Death Assays. The amount of NO formed under each experimental paradigm was estimated based on the nitrite level in the medium as determined by the Griess reaction (Green et al., 1982). An aliquot of 100 µl of cell-free supernatant from each sample was mixed with 100 µl of Griess reagent consisting of equal volumes of 1.32% sulfanilamide in 60% acetic acid and 0.1% N-1-naphthylethylenediamine-HCl. The samples were incubated at room temperature for 10 min before the nitrite content was determined by measuring absorbency at wavelength 540 nm. A standard curve was established for each assay with various concentrations of sodium nitrite. To quantitatively assess the extent of cell death, MTT and trypan blue exclusion assays were conducted as previously described (Xu et al., 1998). For propidium iodide (Molecular Probes, Eugene, OR) staining, C6 cells were labeled with 1 µg/ml propidium iodide for 15 min. Cells were examined under a Nikon Diaphot inverted microscope equipped with a 75-W H lamp and a 20X objective. Images were acquired using a XF3A filter (excitation wavelength, 535 ± 35 nm; emission wavelength, 645 ± 90 nm; Omega Optical Inc., Brattleboro, VT) with a charge-coupled device camera (Quantex, Sunnyvale, CA) and digitized using MetaMorph (Universal Image, New York, NY).

Statistical Analysis. Statistical analysis was performed using Student’s unpaired t test between two experimental groups. Multiple groups were analyzed by one-way analysis of variance followed by a
post hoc Bonferroni t test. A p value less than 0.05 was considered significant.

Results

Neutralization of BCNU Cytotoxicity by iNOS Expression. Pretreatment of C6 cells with interferon-γ (200 U/ml) and lipopolysaccharide (1 μg/ml) markedly increased NO production as reflected by elevated nitrite levels in the culture medium (Fig. 1A). This nitrite accumulation is caused by iNOS expression (Feinstein et al., 1994). Neither interferon-γ nor lipopolysaccharide alone was effective in inducing iNOS expression or raising nitrite levels. Interferon-γ (200 U/ml) in combination with 20 ng/ml tumor necrosis factor-α also increased the nitrite content (data not shown). Since massive NO production by iNOS has been shown to be tumoricidal (Stuehr and Nathan, 1989; Farias-Eisner et al., 1994), we tested whether pretreatment with cytokines could potentiate the BCNU tumor-killing effect in C6 cells. Unexpectedly, pretreatment with interferon-γ and lipopolysaccharide substantially suppressed BCNU (100 μg/ml, 12 h) toxicity in C6 cells, leading to a higher cell survival (Fig. 1B). Approximately 60% of C6 glioma cells pretreated with cytokines survived subsequent BCNU treatment compared with a 4% survival in cells without the pretreatment. The unexpected alteration of BCNU toxicity following synergistic exposure to interferon-γ and lipopolysaccharide contradicts the conventional view that NO formed secondary to iNOS expression is cytotoxic (MacMicking et al., 1997). Interferon-γ and lipopolysaccharide are known to have multiple effects besides the induction of iNOS expression. It is therefore not impossible that increased NO production is merely an epiphenomenon that does not play a role in mediating BCNU chemoresistance. To eliminate any compounding effects of cytokine exposure other than iNOS induction, we applied a gene transfer technique to overexpress iNOS in C6 cells. Transfection of C6 cell with NS05, an iNOS-expressing plasmid, resulted in the overexpression of a 130-kD protein that can be recognized by the antibody specific to macrophage iNOS (Fig. 2A, inset) and a substantial increase in the medium nitrite levels (Fig. 2A). Consistent with the results using interferon-γ and lipopolysaccharide, C6 cells transfected with NS05 exhibited resistance to BCNU cytotoxicity based

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Fig. 1. Effects of cytokines on BCNU cytotoxicity in C6 cells. A, medium nitrite levels. C6 cells were exposed to 200 U/ml interferon-γ (IFN), 1 μg/ml lipopolysaccharide (LPS), or both (IFN/LPS) for 24 h. Ctrl, control cells with neither interferon-γ nor lipopolysaccharide pretreatment. ***p < 0.001 between IFN/LPS and other groups. B, extent of cell survival by the MTT assay. Cells under different conditions as shown in A were treated with (■) BCNU (100 μg/ml) for additional 12 h before the MTT assay. The optical density at 540 nm (O.D. = absorbance) from the MTT assay shown on the ordinate serves as an index of the relative mass of viable cells to reflect the extent of cell survival. +++p < 0.001 between IFN/LPS and other BCUU-treated groups. Mean ± S.D. Data shown were triplicate samples representative of three experiments with similar results.

Fig. 2. Effect of iNOS on BCNU cytotoxicity in C6 cells. A, medium nitrite levels. Culture medium from cells transfected with the construct overexpressing iNOS (NS05), the pcDNA1.1 empty vector (pcDNA) or control cells without transfection (Medium) were subjected to Griess reaction to determine nitrite contents. Inset, Western blot showing the overexpression of iNOS after NS05, but not pcDNA, transfection. The arrow indicates the 130-kD iNOS protein. ***p < 0.001 between NS05 and other groups. B, effects of iNOS on BCNU cytotoxicity. Cells were transfected with NS05 (●) or the pcDNA1.1 (○) for 24 h to allow iNOS expression. This was followed by BCNU exposure at indicated concentrations for additional 12 h before the MTT assay. ***p < 0.001 compared with pcDNA1.1-transfectants exposed to the same concentrations of BCNU. Inset, cell survival as determined by the trypan blue exclusion assay. ++p < 0.01 and +p < 0.05 compared with pcDNA1.1-transfectants treated with 100 and 50 μg/ml BCNU, respectively. Mean ± S.D. Data shown were triplicate samples representative of three experiments with similar results.
on both MTT (Fig. 2B) and trypan blue exclusion (Fig. 2B, inset) assays. Collectively, these results suggest that increased iNOS expression by two different strategies neutralized the cell-killing effect of BCNU.

**Alteration of BCNU Toxicity by NOS Inhibition or Antisense iNOS Suppression.** To further confirm a causal role of NO in altering BCNU toxicity, pharmacological and molecular biological approaches were applied to inhibit iNOS activity or suppress its expression, respectively. Inhibition of NO formation by iNOS was achieved by treating C6 cells overexpressing iNOS with L-NAME, a broad-spectrum NOS inhibitor. L-NAME reduced the nitrite levels (Fig. 3A, left) with corresponding restoration of BCNU toxicity (Fig. 3A, right) in a concentration-dependent manner. Nonspecific NOS inhibitors such as l-NAME may exert pharmacological effects other than inhibiting NOS activity. Therefore, we used an antisense strategy to selectively suppress iNOS expression. Cotransfection of glioma cells with NS05 along with another construct expressing a 145-bp antisense RNA against iNOS suppressed iNOS expression and NO production as shown by Western blotting (Fig. 3B, inset) and Griess reaction (Fig. 3B, left), respectively. Quantitative densitometric scanning of the immunoblots indicates a 42 to 56% reduction in iNOS protein expression in cells cotransfected with NS05 and antisense construct compared with those transfected with the NS05 and empty vector. This finding is consistent with a 40% reduction in nitrite levels as determined by the Griess reaction (Fig. 3B, left). Similar to the use of the NOS inhibitor, suppression of iNOS expression with this antisense strategy also restored BCNU cytotoxicity (Fig. 3B, right).

Morphological assessment of the extent of cell death by propidium iodide staining confirmed the findings based on MTT and trypan blue exclusion assays. Following BCNU exposure, propidium iodide-positive cells were less frequently observed in C6 cells transfected with NS05 (Fig. 4D) than those transfected with the empty vector (Fig. 4D). Cotransfection with NS05 and the antisense iNOS construct reversed the effect of iNOS expression on BCNU cytotoxicity (Fig. 4F), leading to more propidium iodide-positive cells. These morphological observations were further substantiated by counting the propidium iodide stains in four randomly selected visual fields for each experimental condition (Fig. 4G). Results demonstrated that NS05 transfectants sustained significantly higher survival under BCNU challenge compared with cells transfected with empty vector pcDNA1.1. Furthermore, cotransfection of C6 cells with NS05 and another construct expressing a 145-bp antisense RNA against iNOS partially restored BCNU cytotoxicity as indicated by the higher death rate compared with those cells overexpressing iNOS. Together, data shown in Figs. 1 through 4 suggest that an increase in the cellular NO content as a result of iNOS expression is accompanied by reduced C6 cell vulnerability to BCNU killing.

**Drug Specificity and iNOS Effect.** We next explored whether iNOS overexpression can alter cytotoxicity of chemotherapeutic agents other than BCNU in C6 cells. CCNU, like BCNU, is a chloroethylnitrosourea with both carbamoylating and alkylating potential (Wheeler et al., 1974). Figure 5A shows that iNOS also suppressed the cytotoxicity of CCNU. Conversely, expression of iNOS did not neutralize cytotoxicity of cisplatin; overexpression of iNOS actually showed a trend in enhancing cisplatin toxicity in C6 cells based on the MTT (Fig. 5B) and trypan blue exclusion (data not shown) assays. Cisplatin carries a tumoricidal mechanism by total platinum binding to nucleic acids and consequently causing DNA/RNA lesions, which is distinctive from the carbamoylating and alkylating actions of chloroethylnitrosoureas. Thus, the observed iNOS effect cannot be generally applied to all the chemotherapy drugs, but is noted in two chloroethylnitrosoureas (BCNU and CCNU).

**iNOS-Dependent Neutralization of Carbamoylating Action of Chloroethylnitrosoureas.** Chloroethylnitrosoureas kill tumor cells by multiple mechanisms, including carbamoylation and alklylation. Alkylating action includes chloroethylation, methylation, and aminoethylation. In an attempt to further identify which of these mechanisms is
sensitive to iNOS, we tested a number of compounds each carrying different tumoricidal activities. Table 1 summarizes the tumor-killing effects possessed by these different reagents. Briefly, compound 1 exerts alkylating (chloroethylating) action but lacks carbamoylating potential (Penketh et al., 2000). Similar to compound 1, temozolomide kills tumor cells via alkylation (methylation) without carbamoylating potential. Compound 3 and 2-chloroethyl isocyanate possess both carbamoylating and alkylating (aminoethylating) actions. Compound 5 and cyclohexyl isocyanate are both pure carbamoylating agents without alkylating activity.

Results shown in Figs. 6 and 7 support the contention that iNOS expression reduces the cytotoxicity of carbamoylating action only. The cytotoxicity of compound 5 (a pure carbamoylating agent) at concentrations above 0.6 mM was substantially inhibited by the expression of iNOS (Fig. 6A). Induction of iNOS subsequent to cytokines exposure also resulted in protective effect against compound 5 (0.8 mM, 24 h) based on trypan blue exclusion assay (data not shown). Transfection of iNOS exerted similar protective effect, albeit to a lesser extent, against toxicity of compound 3, which carries both carbamoylating and alkylating (aminoethylating) actions. Compound 5 and cyclohexyl isocyanate are both pure carbamoylating agents without alkylating activity.

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clohexyl isocyanate and 2-chloroethyl isocyanate, resulting in higher cell survival in NS05-transfectants.

**iNOS Effects on Agents with Alkylating Action.** Alkylating activities, including chloroethylation, methylation, and aminoethylation, represent another major category of tumor-killing effect possessed by various chemotherapeutic drugs. Thus, we next tested whether expression of iNOS can alter the cytotoxicity of alkylating agents. Results shown in Fig. 8, A and B, respectively, illustrated that iNOS did not suppress the cytotoxicity of compound 1 (a chloroethylating agent) or temozolomide (a methylating agent) based on the MTT assay (Fig. 8). Similar results were observed by using the trypan blue exclusion assay (data not shown). Expression of iNOS indeed slightly potentiated cytotoxicity of both alkylating agents, although statistical significance was not achieved in this study. Together, we conclude that the tumoricidal mechanism of BCNU that can be blunted by iNOS expression is its carbamoylating, but not chloroethylating action. Since no pure aminoethylating agents are available, we were not able to determine the iNOS effect on this type of alkylating activity.

**Discussion**

Induction of iNOS has been noted in brain tumors (Fujisawa et al., 1995; Hara et al., 1996) and the extent of iNOS
expression correlates with the degree of malignancy (Ellie et al., 1995). NO has been shown to regulate vascular reactivity (Swaroop et al., 1998), permeability (Nakano et al., 1996), and angiogenesis (Andrade et al., 1992). These effects may contribute to invasion of brain tumors via enhancing blood flow, thereby facilitating nutrient supply and removal of metabolic wastes. In this regard, NO may promote tumor growth. On the other hand, NO has also been shown to be tumoricidal (Stuehr and Nathan, 1989; Farias-Eisner et al., 1994). Extensive studies have been conducted to clarify the roles of NO in cell death. However, very little attention has been focused on NO effects in altering the cytotoxicity of chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells. To address this issue, we investigated the iNOS effects on several chemotherapeutic agents toward tumor cells.

In the present study C6 cells were treated with BCNU at 50 and 100 μg/ml, corresponding to approximately 235 and 470 μM, respectively, for 12 h before cell death assays. At concentrations lower than 50 μg/ml, BCNU treatment for 12 h did not exert significant cytotoxicity toward C6 glioma cells. The half-maximal lethal dosage of BCNU varies from 2 to 60 μg/ml, depending on the cell types and the duration of BCNU treatment, in most cases 3 to 6 days (Carmichael et al., 1988; Heim et al., 2000). Several reasons have prompted us to select the higher dosages of BCNU with shorter periods of treatment in the present study. First, the optimal transfection efficiency was achieved with C6 cells seeded at approximately 40 to 60% confluence before the day of transfection. Cell densities either too low or too high result in poor transfection efficiency and consequently compromise the extent of iNOS expression. Since the cells usually reach 90% confluence 24 h post-transfection before the application of BCNU, overconfluence may occur as a result of additional 3 to 6 days of incubation with BCNU at lower dosages. Furthermore, the transient gene transfer strategy only allows iNOS to express for 72 h following transfection, with declined expression level thereafter. Thus, lower dosage of BCNU for longer time of incubation does not provide an optimal experimental paradigm to maintain proper iNOS expression in the present study. An alternative approach is to generate stable C6 cell lines overexpressing iNOS. Although transient transfection alone caused only 10% of cell death throughout the first 24-h period, permanent transfection of iNOS or long-term exposure to interferon-γ and lipopolysaccharide for iNOS induction in C6 cells may cause more significant apoptosis or affect cellular proliferation, further complicating the interpretation of experimental results. For the rationale given above, we selected the paradigms of 12-h treatments with 50 and 100 μg/ml BCNU coupled with transient iNOS gene transfer in an attempt to characterize the NO effect on various chemotherapy reagents. Since other reagents tested in this report, such as isocyanates or sulfonyl hydrazine derivatives, exhibited appreciable cytotoxicity only at higher concentrations (mM ranges) during the shorter period of treatment (24–48 h), we also have to conduct the experiments with these reagents at dosages higher than those administered in vivo. This experimental paradigm thus raises the concern as to whether the observed iNOS effects on carbamoylating action of chloroethylnitrosoureas faithfully reflect the clinically relevant in vivo conditions. The best approach to demonstrate clinical relevance of our finding is to conduct animal studies entailing tumor implantation coupled with molecular biological and pharmacological approaches that alter NO contents. Although another in vitro cell viability assay, clonogenic assay, is generally considered a better strategy mimicking the in vivo effects of chemotherapy drugs, it is technically very difficult to manipulate iNOS expression without altering the long-term cellular viability as described above. Overall, although animal studies are needed for establishing clinical relevance of our findings, our studies nevertheless disclose a novel in vitro effect of iNOS in neutralizing the immediate cytotoxic carbamoylation of chloroethylnitrosoureas, but not their alkylating cytostatic effect in C6 glioma cells. This effect may at least in part underlie the molecular mechanism of glioma chemoresistance to BCNU.

Cytokines have been shown to induce iNOS expression in human glioblastoma cells (Fujisawa et al., 1995). Inflammation that develops following surgery and/or radiation may lead to cytokine induction of iNOS expression. BCNU is a common adjunct therapy following surgical resection and radiation in the treatment of GBM. It is interesting to note
that L-NAME, an NOS inhibitor, can restore cell-killing effect of BCNU in C6 glioma cells. Although it remains uncertain as to whether iNOS expression in GBM may alter the efficacy of chloroethylnitrosourea in vivo, our finding raises the possibility that pharmacological modulation of iNOS activity to reduce cellular NO content may potentially reduce chemoresistance of glioma cells to BCNU therapy. Several iNOS inhibitors, both selective (e.g., aminoguanidine [Corbett and McDaniel, 1996] and N-iminoethyl-L-lysine [Salvemini et al., 1995]) and nonselective (e.g., L-NAME) are available. Application of NOS inhibitors in the management of various neurological disorders, including migraine (Lassen et al., 1997) is within reach in clinical settings. Therapeutic agents, including anti-inflammatory agents may also enhance the tumor-killing efficacy of BCNU by preventing iNOS induction in glioma.

In conclusion, results reported here demonstrate that increased NO synthesis derived from iNOS overexpression suppressed BCNU/CCNU cytotoxicity by inhibiting their carbamoylating potential in C6 glioma cells. Neither chloroethylnitrosourea nor methylating action of alkylating agents was neutralized by iNOS. The present study thus identifies a novel in vitro effect of NO in neutralizing the carbamoylating potential of chloroethylnitrosoureas, which continue to be an important group of chemotherapy drugs in the treatment of GBM.

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References


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