Modulation of Cytotoxicity by Nitric Oxide Donors during Treatment of Glioma with Anticancer Drugs

Jeong-Jae Park, M.D., Jong-Soo Kang, M.D., Hyun-Sung Lee, M.D., Jong-Soo Lee, M.D., Young-Ha Lee, M.D., Jin-Young Youm, M.D.

Department of Neurosurgery, San General Hospital, Daejeon, Korea
Departments of Infection Biology, Neurosurgery, College of Medicine, Chungnam National University, Daejeon, Korea

Objective: Nitric oxide(NO) is implicated in a wide range of biological processes in tumors and is produced in glioma. To investigate the role of NO and its interaction with the tumoricidal effects of anticancer drugs, we study the antitumor activities of NO donors, with or without anticancer drugs, in human glioma cell lines.

Methods: U87MG and U373MG cells were treated with the NO donors sodium nitroprusside(SNP) and 5-nitroso-N-acetylpenicillamine(SNAP), alone or in combination with the anticancer drugs 1,3-bis(2-chloroethyl)-1-nitrosourea(BCNU) and cisplatin. Cell viability, cell proliferation, DNA fragmentation, nitrite level, and the expression of Bcl-2 and Bax were determined.

Results: NO was markedly increased after treatment with SNP or SNAP; however, the addition of the anticancer drugs did not significantly affect NO production. NO donors or anticancer drugs reduced glioma cell viability and, in combination, acted synergistically to further decrease cell viability in a dose- and time-dependent manner. Cell proliferation was inhibited and apoptosis was enhanced by combined treatment. Bax expression was increased by combined treatment, whereas Bcl-2 expression was reduced. The antitumor cytotoxicity of NO donors and anticancer drugs differed according to cell type.

Conclusion: BCNU or cisplatin can inhibit cell viability and proliferation of glioma cells and can induce apoptosis. These effects are further enhanced by the addition of a NO donor which modulates the antitumor cytotoxicity of chemotherapy depending on cell type. Further biological, chemical, and toxicological studies of NO are required to clarify its mechanism of action in glioma.

KEY WORDS: Apoptosis, Chemotherapy, Glioma, Nitric oxide.

Introduction

Malignant glioma is one of the most devastating tumors seen in current clinical practice. Its poor prognosis is attributable, at least in part, to its high proliferation rate, resistance to radiotherapy and chemotherapy, and persistent invasiveness into surrounding normal brain parenchyma. Despite multimodal approaches to the treatment of malignant glioma, the mean survival time has remained virtually unchanged for a decade. Therefore, we have sought to increase the efficiency of current treatment modalities for patients with malignant glioma.

Cancer chemotherapy shows relatively high efficacy against malignant glioma, even though antineoplastic drugs usually have little effect on other brain tumors. The mainstay of chemotherapy against brain tumors is 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU or carmustine), which is highly lipid-soluble and readily crosses the blood-brain barrier, and cis-diaminedichloroplatinum (cisplatin), which is thought to act in a manner similar to alkylating agents. Although neither BCNU nor cisplatin appear to substantially prolong survival, recent results show that adjunctive chemotherapy leads to a modest increase in long-term survival. Nitric oxide (NO), a free radical gas molecule, is produced in mammalian cells from L-arginine by a family of enzymes known as NO synthases (NOS). Several recent studies have described the physiological and pathophysiological roles of NO in the central nervous system. The expression of NOS has been demonstrated in human glioma and in human and rodent glioma cell lines. However, there have been few studies on the role of NO in...
cancer chemotherapy, especially in brain tumors. The pathological contribution of NO has generated considerable interest in recent years. Altering NO production and activity could modify tumor cell oncogenesis, tumor blood flow, and the disposition of anticancer drugs in tumor tissues. Until now there have been few reports about NO expression in glioma and its relevance to cancer chemotherapy. To investigate the role of NO and its interaction with the tumor necrosis activity of antineoplastic drugs in glioma, we studied the antitumor activities of NO donors, with or without anticancer drugs, in human glioma cell lines.

Materials and Methods

Reagents and cell lines
The human glioma cell lines, U87MG and U373MG, were obtained from the American Type Culture Collection (Rockville, MD, USA). NO donors, sodium nitroprusside dihydrate (SNP) and S-nitroso-N-acetylpenicillamine (SNAP), and anticancer drugs, BCNU and cisplatin, were obtained from Sigma-Aldrich Laboratories (Sigma Chemical Co., St. Louis, MO, USA). SNP, SNAP, BCNU, and cisplatin were prepared as 1M, 200mM, 200mM, and 33.3mM stock solutions in dimethyl sulfoxide (DMSO), respectively. Further dilutions were made in culture medium just before use. The final concentration of DMSO in culture medium never exceeded 0.1% (v/v) which was nontoxic to the cells.

Glioma cell culture
U87MG and U373MG were cultured in RPMI 1640 (Gibco BRL Co., Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and antibiotics in a humidified atmosphere of 5% CO₂ at 37°C. After the establishment of monolayer cultures, cells were rinsed with serum-free medium and treated with either NO donors (0.2 and 1mM SNP 0.2 and 1mM SNAP) or anticancer drugs (10 and 50μg/ml BCNU, 2 and 10μg/ml cisplatin). Glioma cells were re-incubated with serum-free medium for 12, 24, and 48h. Control cells were incubated with medium only under the same experimental conditions.

Measurement of NO in culture supernatants
Nitrite (NO⁻) levels, as determined by the Griess assay, were equated with the levels of reactive nitrogen intermediates in the culture supernatants of glioma cells treated with either NO donors or anticancer drugs. One hundred μl of supernatant was mixed with 600μl Griess reagent [1.5% sulfanilamide in 1N HCl and 0.15% N-(1-naphthyl)ethylenediamine in distilled water, 1:1 (v/v)]. After a 30-min incubation at room temperature, the absorbance was read at 540nm, and the nitrite concentration was determined from a standard curve prepared using NaN₃.

Assessment of cell viability
U87MG and U373MG cells (5 × 10⁴ cells/well) were seeded in six-well culture plates, grown to a sub-confluent stage, and exposed to NO donors or anticancer drugs for 12, 24, and 48h. Viable cells were counted by staining with trypan blue dye (0.1% w/v), loading the cells into a hemocytometer, and counting by microscopy.

Microculture tetrazolium (MTT) assay
Cell growth rate was measured by an MTT assay, according to the manufacturer's instructions (Sigma). U87MG and U373MG cells were re-suspended in complete RPMI 1640 containing 10% FBS at 1 × 10⁶ cells/ml. One hundred μl of cell suspension was distributed into each well of a 96-well plate and incubated for 12-18h at 37°C in 5% CO₂. After washing with serum-free medium, NO donors or anticancer drugs were added in serum-free medium, and the cells were re-incubated for 12, 24, and 48h. Fifty μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT formazan, 2mg/ml Sigma) was added to each well, and the cells were incubated for a further 4h. To dissolve the formazan, 100μl DMSO (Sigma) was added, and the absorbance was measured at 540nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). Cell proliferation was checked in three wells for each treatment group; cell proliferation was calculated as the ratio of the absorbance of the treated group divided by the absorbance of the control group, multiplied by 100 to give a percentage proliferation.

Genomic DNA fragmentation assay
To measure apoptosis, DNA was isolated from monolayers of U87MG and U373MG cells, supplemented with either NO donors or anticancer drugs, using a G-DEX™ genomic DNA extraction kit (iNtRON Biotechnology, Seoul, Korea), subjected to electrophoresis through 1.5% agarose gel in Trisborate-EDTA buffer (pH 8.0) at 50 V for 5h, and visualized under UV light after ethidium bromide staining.

Immunoblot analysis
Total cell levels of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax expression were analyzed by immunoblotting. Whole-cell lysates were prepared by lysing the cells in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer [2% (w/v) SDS, 62.5mM Tris-HCl (pH 6.8), 10% glycerol, 50mM dithiothreitol, and 0.01% (w/v) bromophenol blue] containing protease inhibitors (20 μg/ml leupeptin, 10μg/ml pepstatin A, 10μg/ml chymostatin,
2 μg/ml aprotinin, 1mM phenylmethylsulfonyl fluoride) and heating at 98°C for 5 min. Protein concentration was measured by means of the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). SDS-PAGE electrophoresis was performed with 50 μg of each protein on 12% SDS-PAGE. The protein was transferred onto a polyvinylidene difluoride membrane (Bio-Rad) using a semidy transfer method. After transfer, membranes were blocked overnight at 4°C with 1× phosphate-buffered saline-0.1% Tween 20 (TPBS) plus 5% nonfat dry milk, and incubated for 3 h at room temperature with monoclonal antibodies to Bax (1:1000; Cell Signaling Technology Inc., Beverly, MA, USA) and Bcl-2 (1:1000; Cell Signaling Technology). After washing in TPBS, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibodies (Sigma) or goat anti-rabbit antibodies (Sigma) for 1 h at room temperature, then washed four times in TPBS for 10 min. Immunoblotting was carried out by autoradiography using an enhanced chemiluminescence detection kit (Amersham, UK).

Statistical analysis
Results are expressed as mean ± standard deviation (SD). Statistical analyses of the differences in cell viability, cell proliferation, and NO levels were performed using the two-tailed Mann-Whitney U test of the nonparametric-independent method and Student’s t-test. The differences between various groups were considered significant at P < 0.05.

Results

NO production in SNAP- and SNP-treated cells
Table 1 shows that NO concentrations in culture supernatants of medium-treated, control glioma cells were 3.6 ± 0.8 to 5.1 ± 0.5 μM and that the NO level did not differ significantly between U87MG and U373MG cells (P = 0.3938). The NO concentration was markedly increased after treatment with SNAP or SNP in both U87MG and U373MG cells. The NO concentration in cells treated with NO donors and anticancer drugs increased in a time-dependent manner, reaching a peak at 48 h.

There was no significant difference in the NO concentration in cells treated with NO donors alone or in combination with anticancer drugs.

Effect of NO donors or anticancer drugs on glioma cell viability
Fig. 1 shows that NO donors or anticancer drugs decreased cell viability. Cell viability was reduced in a dose- and time-dependent manner in BCNU-treated glioma cells (P < 0.05), but not in cisplatin-treated cells (P = 0.175). The number of vi-

Table 1. Nitrite levels in the supernatants of glioma cells treated with either NO donors or anticancer drugs

<table>
<thead>
<tr>
<th>Group</th>
<th>U87MG (μM)</th>
<th>U373MG (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12h</td>
<td>24h</td>
</tr>
<tr>
<td>Medium(control)</td>
<td>4.9 ± 0.9</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>SNP 0.2 mM</td>
<td>24.7 ± 1.8</td>
<td>28.4 ± 2.8</td>
</tr>
<tr>
<td>+ BCNU 10 μg/ml</td>
<td>23.6 ± 2.8</td>
<td>38.9 ± 1.7</td>
</tr>
<tr>
<td>+ Cisplatin 2 μg/ml</td>
<td>22.9 ± 1.9</td>
<td>28.4 ± 1.4</td>
</tr>
<tr>
<td>SNAP 0.2 mM</td>
<td>32.9 ± 3.8</td>
<td>54.1 ± 5.5</td>
</tr>
<tr>
<td>+ BCNU 10 μg/ml</td>
<td>27.4 ± 2.3</td>
<td>44.9 ± 4.7</td>
</tr>
<tr>
<td>+ Cisplatin 2 μg/ml</td>
<td>25.5 ± 2.6</td>
<td>53.9 ± 5.6</td>
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* Nitrite (NO3−) levels were determined by the Griess reagent. Data shown are mean ± SD of triplicate at each sample. The nitrite concentrations were read off a standard curve prepared using NaNO3.

Fig. 1. Effects of anticancer drugs on the viability of U87MG (A) and U373MG (B) cells. Cells were loaded into a hemocytometer and counted by microscope. C1, control cells; B10 and B50, 10 and 50 μg/ml BCNU; C2 and C10, 2 and 10 μg/ml of cisplatin. Data shown are means ± SD (n=5).
able cells tended to decrease as the concentration of NO donor was increased from 0.2 to 1mM SNP and 0.2 to 1mM SNAP (Fig. 2). Cell viability was further decreased in glioma cells treated with both an NO donor and anticancer drug compared with the viability of cells treated with either agent alone; these changes were more pronounced in SNP-treated U87MG (Fig. 2A) and SNAP-treated U373MG cells (Fig. 2D). BCNU in combination with SNP or SNAP synergistically decreased the viability of both U87MG and U373MG cells (0.015 < P < 0.055) (Fig. 2). Cisplatin did not show any synergy with NO donors (0.054 < P < 0.182), except in U373MG cells treated with cisplatin and SNAP (P=0.038) (Fig. 2D).

Effect of combined NO donors and anticancer drugs on proliferation of glioma cells

Fig. 3 shows that BCNU decreased the proliferation of U87MG and U373MG cells in a dose-dependent manner (0.01 < P < 0.05); however, the cisplatin dose did not have a significant effect on cell proliferation (0.056 < P < 0.083). Fig. 4 demonstrates that SNAP reduced the proliferation of both U87MG and U373MG cells in a dose-dependent manner (P < 0.05), but SNP had no such effect (P > 0.05). Compared with cells treated with SNP or SNAP alone, U87MG cells treated with SNP/BCNU (Fig. 4A) and U373MG cells treated with SNAP/cisplatin (Fig. 4D) exhibited significant inhibition of cell proliferation. Cell proliferation was inhibited further as the concentration of the anticancer drug increased (Fig. 4).

Synergistic effect of NO donors and anticancer drugs on apoptosis

Genomic DNA fragmentation was seen within 48h in U87MG cells treated with 1mM SNP and 1mM SNAP, but not in 0.2mM SNP- and 0.2mM SNAP-treated cells (Fig. 5A, B). BCNU (10µg/ml) and cisplatin (2µg/ml) induced apoptosis in U87MG cells after 36h (Fig. 6A, B). Combined treatment with SNP or SNAP (0.2mM) plus BCNU (10 and
Fig. 3. Effects of anticancer drugs on the proliferation of U87MG (A) and U373MG (B) cells. Cellular growth rate was measured by the MIT method. Proliferation was calculated as the ratio of the absorbance of the treated group divided by the absorbance of the control, multiplied by 100 to give percent proliferation. Data shown are means ± SD for three separate experiments.

Fig. 4. Effects of NO donors or anticancer drugs on the proliferation of U87MG and U373MG cells. Data shown are means ± SD for three separate experiments.
50μg/ml) induced apoptosis in U87MG cells after 24h (Fig. 7A). U87MG cells treated with SNP or SNAP (0.2mM) plus cisplatin (2 and 10μg/ml) showed DNA fragmentation at the higher concentration of cisplatin after 24h (Fig. 7B). The apoptotic effects of NO donors and anticancer drugs on U373MG cells were similar to those in U87MG cells (data not shown).

**Effect of NO donors and anticancer drugs on expression of Bax and Bcl-2 proteins**

The expression of pro-apoptotic protein Bax was increased in U87MG cells treated with NO donors alone and in combination with anticancer drugs in a time-dependent manner (Fig. 8A). The increase in Bax expression was especially high in SNP/BCNU- and SNAP/cisplatin-treated cells. The expression of anti-apoptotic protein Bcl-2 protein was time-dependently reduced in U87MG cells treated with NO donor alone or in combination with anticancer drugs (Fig. 8B). The expression of Bax and Bcl-2 was similar in U373MG and U87MG cells treated with NO donors or anticancer drugs (data not shown).

**Discussion**

Glioma is the most common primary brain tumor in adults, constituting 40% of all central nervous system tumors, and the limitations of surgery and radiation therapy are well known. Therefore, adjuvant chemotherapy has been suggested as an additional mode of treatment. In the present study, we examined the role of NO and its interaction with the tumoricidal activity of anticancer drugs in human glioma cell lines. BCNU or cisplatin inhibited the viability and proliferation of U87MG and U373MG cells to a limited extent, and these effects were significantly increased by the addition of the NO donors SNP or SNAP. Also, NO donors and anticancer drugs acted synergistically to enhance apoptosis. Our results show that NO modulates antitumor cytotoxicity through apoptosis-related pathways and that its activity depends on cell type and the concentration of NO donors.

NOS has been detected in human brain tumors, and its production may be associated with pathophy-
siological processes important in tumorigenesis\textsuperscript{23-25}. Broholm et al.\textsuperscript{26} showed increased expression of constitutive NOS(eNOS) in astrocytic tumor cells and tumor endothelial cells, but the highest levels of expression were found in high-grade glioma. Recently, some therapeutic strategies for glioma, involving the manipulation of NO, have been proposed and evaluated experimentally and in preliminary clinical trials\textsuperscript{23,24}. NO is implicated in a wide range of tumor processes: modulation of host tumoricidal activity, including tumor immunity and apoptosis; tumor invasion, metastasis and neovascularization; free radical injury to tumor cells and adjacent normal tissues; dilatation of tumor blood vessels; and alterations in vascular permeability\textsuperscript{25}. In our study, the NO concentration was markedly increased in glioma cells treated with NO donors, SNP or SNAP, in a time-dependent manner, reaching a peak after 48h. This implies that NO is produced in glioma cells after stimulation with NO donors. NO donors inhibited the viability and proliferation of human glioma cells in a dose- and time-dependent manner. Similar results were reported by Lee et al.\textsuperscript{26} and Kurimoto et al.\textsuperscript{27}. However, it is interesting that the patterns of inhibition differed according to the NO donor and the cell type. For example, in U87MG cells, SNP-induced cytotoxicity was enhanced when combined with BCNU, whereas, in U373MG cells, the cytotoxicity of SNAP was significantly increased when combined with cisplatin. One of the reasons is that NO is a lipophilic and highly diffusible solute whose actions are dependent on both its concentration and form within the cell\textsuperscript{22,23,24}.

Malignant glioma is the only brain tumor in which chemotherapy is effective, although the efficacy is limited. Chloroethylnitrosoureas such as BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) kill tumors via carboxylation and alkylation\textsuperscript{24}. In the present study, BCNU or cisplatin inhibited the viability and proliferation of U87MG and U373MG cells, and this inhibition was potentiated by the addition of NO donors. These results indicate that NO may modulate mitosis, anti-proliferative and anti-invasive activities, and apoptosis\textsuperscript{25,26}. The manipulation of NO within tumors, by NOS stimulation or administration of NO donors, may selectively increase the level of anticancer drugs delivered to brain tumors compared with normal areas of the brain\textsuperscript{24}. This offers the opportunity for increasing tumoricidal activity while reducing toxicity in surrounding normal tissue. However, the overexpression of NO may contribute to the development of BCNU resistance\textsuperscript{27}, and NO overexpression has been shown to neutralize the cytotoxicity of BCNU and CCNU\textsuperscript{28,29}. Similarly, in our study, NO production was greater in SNAP-treated cells than in SNP-treated cells, and cytotoxicity was more pronounced in SNP/BCNU-treated cells than in SNAP/BCNU-treated cells.

Microscopically, the hallmarks of apoptosis are cell shrinkage, cytoplasmic and chromatin condensation, cell surface blebbing, and cell fragmentation into apoptotic bodies\textsuperscript{30}. In the present study, apoptosis of glioma cells exposed to SNP, SNAP, BCNU, or cisplatin was identified by DNA fragmentation and gel electrophoresis. Several studies have investigated the mechanism behind NO-mediated apoptosis, and it has been suggested that both cyclic guanosine monophosphate-dependent and -independent pathways are important\textsuperscript{31}. One recent study focused on the involvement of protein kinase C and nuclear factor-κB as mediators of apoptosis\textsuperscript{32}. In another study, the activation of K\textsuperscript{+} channels, leading to excessive K\textsuperscript{+} efflux and cytotoxic K\textsuperscript{+} loss, was suggested as a possible mechanism of NO-mediated apoptosis\textsuperscript{33}. Recently, it was reported that NO has emerged as a bifunctional regulator of apoptosis. Pro-apoptotic and anti-apoptotic responses to NO appear to be specific to the type of cells that are involved\textsuperscript{34}. In many cell types including macrophages, pancreatic islet cells, neuronal cells, and thymocytes, NO activates apoptosis\textsuperscript{35}. On the other hand, NO has been shown to exert anti-apoptotic activity in vascular endothelial cells, hepatocytes, eosinophils, and splenocytes\textsuperscript{36}. The exact mechanism of this bifunctional action of NO is unclear, but different redox states in these cells may be one of the determining factors\textsuperscript{37,38}.

Apoptosis and the expression of apoptosis- and oxidative stress-related factors vary greatly among tumor types\textsuperscript{39}. In the present study, the expression of anti-apoptotic protein Bcl-2 was reduced in cells treated with either NO donors or anticancer drugs. On the contrary, the expression of pro-apoptotic protein Bax was increased. Our data, along with those from

\[ \text{Fig. 8. Effects of NO donors or anticancer drugs on the expression of Bax (A) and Bcl-2 (B) in U87MG cells. Protein levels of Bax and Bcl-2 were detected by immunoblotting as described in Materials and Methods.} \]
previous studies, indicate that glioma cells upregulate Bax and downregulate Bcl-2, which is important in NO-induced apoptosis. However, the precise mechanism of how NO changes Bax and Bcl-2 gene expression is unknown. Some investigators have focused on protein p53 as a link between NO and Bax/Bcl-2 genes, as NO is known to induce the accumulation of p53. NO is a multi-faceted molecule with dichotomous regulatory roles in many areas of biology. We have shown that anticancer drugs for glioma can inhibit cell viability and proliferation and induce apoptosis and that these effects are further enhanced by the addition of NO donors. The effect of NO donors on cytotoxicity appears to be cell-type specific. Further studies of the biological, chemical, and toxicological mechanisms of NO in glioma are required to clarify its mechanism of action.

Conclusion

Nitric oxide (NO) is implicated in a wide variety of biological processes in neoplasms and is produced in glioma cells. The correlation between NO and its relevance to cancer chemotherapy in glioma has received little attention. Glioma U87MG and U373MG cells were treated with the NO donors SNP and SNAP, alone or in combination with the anticancer drugs BCNU and cisplatin. After the addition of an NO donor or anticancer drug alone, cell viability was decreased in U87MG and U373MG cells, and the inhibition was synergistically enhanced for treatments with an NO donor plus an anticancer drug, in a dose- and time-dependent manner. Cell proliferation was also significantly inhibited in the combined treatment with an NO donor plus an anticancer drug. Treatment with anticancer drugs alone induced apoptosis, and cells exposed to an NO donor plus an anticancer drug exhibited further increased Bax expression and decreased Bcl-2 expression compared with cells treated with an anticancer drug alone. In this study, we have shown that BCNU or cisplatin can inhibit cell viability and proliferation of glioma cells and can induce apoptosis. These effects are further enhanced by the addition of an NO donor which modulates the antitumor cytotoxicity of chemotherapy depending on cell type. Further biological, chemical, and toxicological studies of NO are required to clarify its mechanism of action in glioma.

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References


Commentary

I read with great interest the article: Modulation of cytotoxicity by nitric donors during treatment of glioma with anticancer drugs. Nitric oxide (NO) is derived from inducible nitric oxide synthase (iNOS) and a short-lived free radical serving a number of physiological functions including neurotransmission, vasodilation, and macrophage cytotoxicity. NO released from macrophage, natural killer cells and microglia in response to cytokines in glioma has the host tumoricidal agents.1, 2 However, NO may enhance vasodilation and promote neovascularization, thereby facilitating tumor growth.3-5. The correlation between NO and cytotoxicity of chemotherapeutic reagents in glioma have been less well characterized.

Authors demonstrated that cytotoxicity of BCNU or cisplatin was enhanced by the addition of a NO donor which modulates the antitumor cytotoxicity of chemotherapy depending on cell type. They explained that NO modulates antitumor cytotoxicity through the apoptosis-related pathways. These data show us possibilities of clinical trial for patients with glioma.

Yang et al reported that overexpression of NO neutralized the cytotoxicity of BCNU and CCNU in rat C6 glioma cells.6 Whereas authors explained that greater NO production in SNP-treated cells than in SNP-treated cells has less cytotoxicity, they could not show the which level of NO expression may have cytotoxicity or tumorigenicity. Further study of the NO level for the cytotoxicity against glioma cells will be required to clarify the potential for clinical implication.

Kyoung Gi Cho, M.D.
Ajou University Hospital College of Medicine

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