Full Paper

Pathodynamics of Nitric Oxide Production Within Implanted Glioma Studied With an In Vivo Microdialysis Technique and Immunohistochemistry

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Abstract. Nitric oxide (NO) is thought to be a mediator in many of the processes of malignant brain tumor progression. We examined NO production in the brain of normal conscious, freely moving rats with or without implanted C6 glioma. Both nitrite (NO_2^-) and nitrate (NO_3^-) in the dialysates of the two groups were measured using an in vivo microdialysis technique. The mean concentration of NO_2^- in the glioma group was two-times higher than that in the control group ($P < 0.01$). Concentrations of both NO_2^- and NO_3^- in the glioma and control groups decreased following intraperitoneal injection of *N*^G-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NO synthase (NOS). NO production was also significantly suppressed in the glioma group, but not the control group, by intraperitoneal injection of 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT), a selective inhibitor of inducible NOS (iNOS). On immunohistochemical examination, diffuse iNOS-positive cells were located within glioma tissue. ED1-positive cells (microglia/macrophages) were intermingled between glioma cells on double immunostaining. These results indicate that the basal level of NO production in the glioma group is higher than that in the control group and that the increased NO production was continuously induced by iNOS-expressing cells in glioma.

Keywords: glioma, microdialysis, nitric oxide, macrophage, nitric oxide synthase

Introduction

Nitric oxide (NO), a potent signaling molecule produced from L-arginine by NO synthase (NOS), has been implicated in some of the processes involved in brain tumor progression. Three NOS isoforms have been cloned and sequenced: two transiently active calcium-dependent constitutive isoforms, including an endothelial NOS (eNOS) and a neuronal NOS (nNOS); and a continuously active calcium-independent inducible NOS (iNOS). NO is released in short controlled bursts in the activation of eNOS and nNOS isoforms (1). These isoforms act as an important mediator in regulation of local cerebral blood flow (2) and as a neuromodulator to control behavioral activity, influence memory formation (3–5), and intensify responses to painful stimuli (6, 7).

Conversely, large amounts of NO are generated for long periods of time by immune cells such as microglia/macrophages and/or tumor cells which have been induced to express iNOS by stimulation with appropriate cytokine signals (8, 9). However, it is still not well characterized whether NO is continuously produced by tumor cells and/or microglia/macrophages within the glioma or not.

Human malignant gliomas include many microglia/macrophages within the tumor tissue (10). Previous reports have evaluated iNOS expression of tumor cells and/or macrophages in human gliomas (10–13). The sustained exposure to higher concentrations of NO is considered to be related to vascular permeability, vasodilation, angiogenesis and cytotoxicity (9, 14–20). However, it is not still clear whether NO produced in glioma tissue is a positive or negative environmental factor. Furthermore, no equivalent studies have yet been reported concerning the levels of NO and the NOS

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isoform responsible for NO production within malignant gliomas *in vivo*. In the present study, we examined the levels of NO in the brain of animals implanted with C6 glioma cells and the iNOS expression of glioma cells and microglia/macrophages using an *in vivo* brain microdialysis technique and immunohistochemical staining.

Materials and Methods

Animal model

Fifty-two male albino Wistar rats weighing 220–250 g (Kuroda Junkei Dohbutsu, Ltd., Kumamoto) were used. They were divided into two groups, a glioma group ($n = 26$) and a control group ($n = 26$). The housing area was temperature-controlled at 22°C and maintained on a 12-h light/dark cycle. Before surgery, rats were housed individually in clean cages with free access to water and laboratory chow.

Surgery was performed under aseptic conditions in two stages. Rats in the glioma group were implanted with C6 glioma cells and those in the control group were injected with vehicle. A guide cannula was attached to the skull following the first surgery. The rats were anesthetized (sodium pentobarbital, 40 mg/kg, *i.p.*) and then mounted in a stereotactic apparatus with the skull level between the bregma and lambda suture points. A sterile 23-gauge stainless steel tube was inserted into the left caudate putamen 6 mm from the skull surface through a small hole in the skull. Rats in the tumor and control groups were injected with 10^5 of C6 glioma cells per 10 μ l saline and 10 μ l of saline, respectively. After surgery, the rats were allowed to recover for 10 days in their home cages. Ten days after the first surgery, rats of

the two groups were anesthetized (sodium pentobarbital, 40 mg/kg, *i.p.*) and again mounted in the stereotactic apparatus. The guide cannula (outer diameter: 500 μ m, length: 4 mm; Eicom, Kyoto) was inserted into a site, which was 3-mm deep from the skull surface, through the same hole opened at first surgery. The guide cannula was fixed to the cranial bone with dental cement. During recovery from the second surgery, a stylet was placed into the guide cannula and fixed with a cap nut.

In vivo brain microdialysis

Two days after the second surgery, rats were placed in an acrylic box. A microdialysis probe (straight double-luminal type, outer diameter 220 μ m, length 3 mm, cellulose membrane, cut-off 50,000 Da; A-I-4-03; Eicom) was then inserted through the guide cannula and fixed with a cap nut. The tip of the probe in the glioma group was placed in the center of the implanted glioma. The probe was connected to a microinfusion pump (EP-60, Eicom) by a polyethylene tube (inner diameter: 100 μ m) and perfused with artificial cerebrospinal fluid (1 μ l/min), containing 7.4 g/l NaCl, 0.19 g/l KCl, 0.19 g/l $MgCl_2$, and 0.14 g/l $CaCl_2$ (pH 7.4). During the microdialysis, the animal had free access to food and water.

Measurement of NO production in dialysate

The half-life of NO is very short, being quickly oxidized by O_2 to NO_2^- and NO_3^- *in vivo*. Therefore, the targets for measurement are both NO_2^- and NO_3^- . An *in vivo* microdialysis system (Fig. 1) consists of two constant flow-lines through polyethylene tubes, a reagent solution and a carrier solution. To measure NO_3^- as NO_2^- , the flow injected by autoinjector was passed through a reduction column packed with fine particles of copper-plated cadmium, and NO_3^- was reduced to NO_2^- . The buffered carrier/sample flow was mixed with the reagent flow, and NO_2^- underwent diazotization and coupling in the polyethylene tube at 35°C. The purple azo dye formed through this reaction was quantitated by a flow-through spectrophotometer from the absorbance at 540 nm.

Fig. 1. Designation of the system used for measurement of NO production using an *in vivo* brain microdialysis technique. The system consists of two constant flow-lines through polyethylene tubes, a reagent solution, and a carrier solution. To measure NO_3^- as NO_2^- , the flow injected by autoinjector was passed through a reduction column packed with fine particles of copper-plated cadmium, and NO_3^- was reduced to NO_2^- . The buffered carrier/sample flow was mixed with the reagent flow, and NO_2^- underwent diazotization and coupling in the polyethylene tube at 35°C. The purple azo dye formed through this reaction was quantitated by a flow-through spectrophotometer from the absorbance at 540 nm.

ethylenediamine dihydrochloride, and 1% HCl; Eicom], and a carrier solution (0.3% aqueous NH_4Cl buffer containing 0.07% EDTA, pH 8-8.5; Eicom). Both solutions were degassed before perfusion into the system. The flow rates for the reagent and carrier solutions were maintained at constant rates of 0.1 ml/min and 0.33 ml/min, respectively, by another microinfusion pump (ENO-10, Eicom). The dialysate collected by the microdialysis probe was automatically injected into a carrier flow every 10 min by an autoinjector (AS-10, Eicom). In order to measure NO_3^- with NO_2^- , the flow injected by the autoinjector was passed through a reduction column packed with fine particles of copper-plated cadmium, and NO_3^- was reduced to NO_2^- . The buffered carrier/sample flow was mixed with the reagent flow, and NO_2^- underwent diazotization and coupling in the polyethylene tube at 35°C. The purple azo dye formed through this reaction was quantitated using a flow-through spectrophotometer (NOD-10, Eicom) from the absorbance at 540 nm. The area of the absorbance peak was calculated by an integrator (Chromatocorder 21, Eicom). The actual concentrations of NO_2^- and NO_3^- were calculated by comparing the measured area with the area of the absorbance peak of standard solution [10 μl of solution mixed NO_2^- (0.1 μM) and NO_3^- (0.1 μM)] obtained through the same microdialysis probe.

Drug administration

All drugs were administered i.p. during in vivo microdialysis. Four sets of glioma and control groups were employed. Each set of animals was treated with a N^G -nitro-L-arginine methyl ester (L-NAME, 100 mg/kg; RBI, Natick, MA, USA) or 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 0.2 mg/kg; Cayman Chemical, Ann Arbor, MI, USA) or L-arginine (500 mg/kg; Nacalai Tesque, Inc., Kyoto), or both AMT (0.2 mg/kg) and L-arginine (500 mg/kg) at the same time.

Pathological analysis

Paraffin-embedded brain tissues from the glioma group were cut into 4- μm -thick coronal sections, deparaffinized with Histo-clear (National Diagnostics, Atlanta, GA, USA), and dehydrated with graded series of ethanol solutions. These sections were stained with hematoxylin and eosin (HE). For immunohistochemical analyses, deparaffinized and dehydrated sections were immersed (30 min) in methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity, treated (20 min) with phosphate-buffered saline pH 7.4 (PBS) containing 1.5% normal serum and 1% bovine serum albumin, and then incubated overnight at 4°C with anti-iNOS (diluted

1:500) (Calbiochem, San Diego, CA, USA). Sections were washed with PBS and then incubated consecutively with biotinylated anti-rabbit IgG (30 min) and avidin-biotinylated peroxidase complex (30 min) (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized using a solution of 3,3'-diaminobenzidine.

To identify the cell types expressing iNOS, double-immunostaining was performed. After immunostaining for iNOS, the sections were washed with PBS and sequentially incubated overnight at 4°C with ED1 (diluted 1:50) (Chemicon International Inc., Temecula, CA, USA) to detect microglia/macrophages. After washing with PBS, the sections were incubated consecutively with biotinylated anti-mouse IgG (30 min) and avidin-biotinylated alkaline phosphatase complex (30 min) (Vector Laboratories). Alkaline phosphatase activity was visualized using an alkaline phosphatase substrate kit (Vector Laboratories).

Data analyses

Immediately after the insertion to the brain, NO_2^- / NO_3^- levels of the perfusate were high. Six consecutive NO_2^- / NO_3^- data sets were therefore determined over 60 min after the insertion of probe when NO production between glioma and control groups was compared in pretreated rats. The mean value of 3 consecutive outputs of NO_2^- / NO_3^- was employed as the basal level when NO production of pre- and post-treated rats was compared in each group. Statistical analysis of the difference between glioma and control groups was performed by means of Student's *t*-test. The results were considered statistically significant when *P* values were less than 0.01. Data are expressed as the mean $\pm$ S.E.M. All rats were treated in strict compliance with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results

Figure 2A shows the typical chromatographs of a standard solution of mixed NO_2^- (0.1 μM) and NO_3^- (0.1 μM) (a) and samples in the dialysate of the control (b) and glioma (c) groups. Before and after the examinations, the area of the absorbance peak of NO_2^- / NO_3^- was measured for a standard sample.

The concentration of NO_2^- in the glioma group ($0.48 \pm 0.05 \mu\text{M}$) was significantly higher than that in the control group ($0.21 \pm 0.02 \mu\text{M}$, $P < 0.01$, Student's *t*-test, Fig. 2Ba). The concentration of NO_3^- in the glioma group ($3.73 \pm 0.33 \mu\text{M}$) also tended to be higher

Fig. 2. Measurement of NO production in dialysate. A: Typical chromatographs of a standard solution (a: 10 μ l) and samples in the dialysate of the control (b: 10 μ l) and glioma (c: 10 μ l) groups. The two areas of absorbance peak indicate the levels of NO₂⁻ and NO₃⁻, respectively. B: Mean NO₂⁻ (a) and NO₃⁻ (b) concentrations in the glioma (n = 10) and control (n = 10) groups. *: $P < 0.01$

than that in the control group ($3.38 \pm 0.31 \mu\text{M}$), but was not significant (Fig. 2Bb).

The mean concentration of NO₂⁻ in both the glioma and control groups decreased significantly 40 and 50 min, respectively, after the administration of L-NAME ($P < 0.01$, Student's *t*-test, Fig. 3a). The mean concentration of NO₃⁻ in both the glioma and control groups decreased significantly 40 min, after the administration of L-NAME ($P < 0.01$, Student's *t*-test, Fig. 3b). When AMT was administered, the mean concentration of NO₂⁻ decreased significantly in the glioma group 70 min after administration ($P < 0.01$, Student's *t*-test), but there was no significant change over 120 min in the control group (Fig. 3c).

The mean concentration of NO₂⁻ in both the glioma and control groups increased significantly 60 and 50 min, respectively, after the administration of L-arginine ($P < 0.01$, Student's *t*-test, Fig. 3d). When both AMT and L-arginine were administered at the same time, the mean concentration of NO₂⁻ in the glioma group did not increase significantly over 120 min, but that in the control group increased significantly 40 min after administration ($P < 0.01$, Student's *t*-test, Fig. 3e).

We microscopically examined whether the tissues in the glioma group were tumorous in HE-stained specimens (Fig. 4A). On immunohistochemical analysis, diffuse iNOS-positive cells were shown within the implanted glioma tissue (Fig. 4B). On double-immunostaining, ED1-positive cells were intermingled between the glioma cells (Fig. 4C), and some of these cells were also concurrently positive for iNOS on higher magnifi-

cation (Fig. 4D).

Discussion

We demonstrated that basal levels of NO₂⁻ and NO₃⁻ in the striatum of normal rats in vivo were 0.21 ± 0.02 and $3.38 \pm 0.31 \mu\text{M}$, respectively. Basal levels of NO₂⁻ and NO₃⁻ in the cerebellum of normal rats under conscious and freely moving conditions have previously been reported by Yamada and Nabeshima (21) to be $0.27 \pm 0.02 \mu\text{M}$ ($5.4 \pm 0.4 \text{ pmol}/20 \mu\text{l}$) and $1.93 \pm 0.12 \mu\text{M}$ ($38.6 \pm 2.4 \text{ pmol}/20 \mu\text{l}$), respectively. The extracellular concentrations of NO₂⁻/NO₃⁻ obtained in our study of the striatum were therefore in accordance with those previously reported in the cerebellum. Basal levels of NO₂⁻ and NO₃⁻ decreased significantly following administration of a non-selective NOS inhibitor, L-NAME. This suggests that the levels of NO represent those produced by NOS in vivo and that the present in vivo assay system can be considered as reliable.

To our knowledge, NO₂⁻ and NO₃⁻ levels within implanted glioma tissue have never been determined in vivo. Our study indicates that NO₂⁻ levels in implanted glioma tissue are about two-times higher than those in normal brain tissue. NO₃⁻ levels within implanted glioma tissue are not significantly higher than those in normal brain tissue, and NO₃⁻ levels are much higher than NO₂⁻ levels within both glioma and control tissue. In vivo NO concentration estimation should be done via analysis of NO₂⁻ in the dialysate without the reduction of NO₃⁻ step, as it has been suggested that inclusion

Fig. 3. Consecutive measurement of NO production on drug administration. a, b: Effect of inhibition of NO production by administration of L-NAME. Panels a and b show the concentrations of NO_2^- and NO_3^- , respectively. Glioma group: $n=10$; control group: $n=10$. c: Effect of inhibition of NO production by AMT. Glioma group: $n=6$; control group: $n=6$. d: Enhanced NO production by administration of L-arginine. Glioma group: $n=5$; control group: $n=6$. e: Effect on NO production by administration of both AMT and L-arginine concurrently. Glioma group: $n=5$, control group: $n=5$. Closed circle: glioma group, open circle: control group; *: $P<0.01$.

Fig. 4. Immunohistochemical staining of implanted C6 glioma tissue. A: Low magnification of HE-stained specimen shows progressive C6 glioma in the rat brain. Scale bar = 480 μm . B: Higher magnification shows many diffuse iNOS-expressing cells (brown). Scale bar = 30 μm . C: ED-1-positive cells (blue) intermingled between glioma cells on double immunostaining. Scale bar = 120 μm . D: Some ED-1-positive cells (blue) are also concurrently positive for iNOS (brown) on high magnification. Scale bar = 60 μm .

of NO_3^- in the determination of *in vivo* NO causes an overestimation of approximately 500% compared to the value obtained when NO was measured by the hemoglobin reaction (22). In recent studies, it was suggested that increased plasma $\text{NO}_2^-/\text{NO}_3^-$ levels were correlated to tumor volume in patients with hepatocellular carcinoma (23) and to tumor stage in patients with gastric cancer (24). Furthermore, the presence of relatively high levels of NO have been reported in both cerebrospinal fluid and tumor cyst fluid of a patient with glioblastoma (13). In this study, it has clearly been demonstrated that NO concentration in glioma tissue is higher than that in normal brain tissue.

The first report on NOS activity and localization in human tumors was a study on gynecological cancer (25). NOS activity has also been observed in other solid human tumor tissues, including head and neck cancers (26), gastric cancer (27) and brain tumor (12). In particular, some previous reports have evaluated the expres-

sion of iNOS in human brain tumor (11, 28), which generated large amounts of NO for long periods of time by macrophages and/or some tumor cells which have been induced to express iNOS specifically among the three NOS isoforms (9). In the present study, the administration by L-NAME significantly inhibited intratumoral NO production, while the selective iNOS inhibitor (AMT) significantly suppressed NO production within glioma tissue but not within normal tissue. Furthermore, the enhanced effect of NO production by L-arginine was suppressed by AMT in the glioma group but not in the control group. This suggests that NO is mainly induced by eNOS and/or nNOS within tissues of the normal group, whereas iNOS in tumor tissue has an important role in the generation of high NO levels in the glioma group.

A recent study has shown that the action of NO as a cytotoxic substrate is modulated by reactive oxygen species such as superoxide anion radicals and hydrogen

peroxide (14, 29). Murine macrophages can also be induced to express iNOS and generate cytotoxic amounts of NO in vitro (9, 30) and in vivo (31). Although NO₂⁻ levels of the implanted glioma tissue were about two-times higher than those of normal brain tissue in the present study, the concentrations ($0.48 \pm 0.05 \mu\text{M}$) are much lower than those in media for suppression of C6 glioma cells in vitro ($20 \mu\text{M}$), which were reported in another study (32). According to the previous and present results, we suggest that intratumoral NO production may be insufficient to produce a cytotoxic effect against tumor cells.

Although the role of NO in tumor biology is still unknown, knowledge is accumulating about the role of NO and NOS in growth and microcirculation of solid tumors. Previously, C6 glioma cells were reported to express iNOS, which may contribute to tumor vessel dilatation and blood flow (17, 33–35). Furthermore, acute administration of NOS antagonists in animals with implanted tumor cells resulted in a decrease of tumor blood flow and perfusion (36), and caused hypoxia (37). High NOS-expressing tumors are also known to be highly vascularized, suggesting a close relationship between NO production and angiogenesis in vivo (17). A recent experimental study showed that NO can induce an increase of vascular endothelial growth factor gene expression in a human glioblastoma cell line (15). Using in vivo brain microdialysis, we showed that high levels of NO production were induced by iNOS in glioma tissue. On immunohistochemical examination, iNOS-positive cells were found diffusely within glioma tissue. Microglia/macrophages were intermingled between iNOS-positive glioma cells and were also concurrently positive for iNOS. Microglia/macrophages may be also related to continuous NO production within glioma. These previous and present findings provide evidence suggesting that endogenous NO production in solid tumors favors angiogenesis and therefore promotes tumor growth. Intratumoral NO production may promote tumor progression rather than exhibiting any cytotoxic effects. Long-term delivery of a selective iNOS inhibitor may provide a suitable therapeutic strategy for inhibiting the progression of glioma.

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