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ASTROGLIA INDUCE CYTOTOXIC EFFECTS ON BRAIN TUMORS VIA A NITRIC OXIDE-DEPENDENT PATHWAY BOTH IN VITRO AND IN VIVO

OBJECTIVE: In the central nervous system, astroglia produce nitric oxide (NO) in response to cytokines. We investigated whether cytokine stimulation of astroglia could inhibit brain tumor cell growth in vitro and prolong survival in vivo via an NO-dependent pathway.

METHODS: Astroglia cultures were stimulated with the cytokines lipopolysaccharide and interferon-γ and subsequently seeded with tumor cell lines. Wild-type mice and inducible NO synthase-knockout mice received in vivo cytokine stimulation followed by B16F10 murine melanoma challenge.

RESULTS: Our in vitro studies demonstrate that astroglia stimulated to produce NO by the addition of cytokines dose-dependently inhibit the growth of one primary rat brain tumor cell line (9L) and three primary human brain tumor cell lines (H80, U87, and U373). This inhibition of tumor cell growth is also observed in metastatic cell lines (B16F10 melanoma, Lewis lung carcinoma, and CT26 colon). Cultured astrocytes from inducible NO synthase-knockout mice, which are incapable of induction of NO, are without the enhanced tumoricidal effect. Furthermore, when C57BL/6 mice are primed to produce NO through stereotactic intracranial administration of lipopolysaccharide plus interferon-γ and subsequently challenged with B16F10 murine melanoma, survival is significantly prolonged, with a median survival of 26 days versus 16 days in the control group ($P < 0.001$). The addition of an NO synthase inhibitor (N^G-nitro-L-arginine methyl ester) decreases this beneficial effect (median survival, 21 d).

CONCLUSION: These findings suggest that NO may have an important role as a defense mechanism molecule against brain tumors; stimulation or modification of this mechanism may represent a new approach to the treatment of primary and metastatic brain tumors.

KEY WORDS: Astroglia, Brain tumors, Cytokines, N^G-Nitro-L-arginine methyl ester, Nitric oxide

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Nitric oxide (NO) participates in the physiology of every mammalian organ system and is one of the major defense effectors of systemic macrophages against parasites, bacteria, and tumor cells (5, 55). Although a role for NO in systemic macrophage tumoricidal activity has been established, the possible role of NO in the interaction between astroglia and tumor cells has received little attention. Astroglia are a mixed group of cells that includes astrocytes, oligodendrocytes, and microglia. However, it is primarily the microglia that are considered the central nervous system (CNS) defense effector cells, similar to systemic macrophages, with which they share a common embryological lineage (18, 31). Only a few in vitro studies have examined the tumor cytotoxicity of microglia (31). It has been shown that astrocytes and microglia produce NO in response to cytokines in a manner similar to that of inflammatory macrophages (51, 56). Thus, it seems warranted to investigate the induction of astroglia-based NO production in the CNS against tumors.

The enzyme that generates NO from L-arginine, NO synthase (NOS), exists in mammalian tissue and has three known iso-

forms. Neural and endothelial tissues contain neuronal and endothelial isoforms, respectively; defense effector cells such as macrophages and microglia contain an inducible (iNOS) isoform. Astroglia cell lines incapable of induction of NOS are available for control comparison and are designated as iNOS−/− (27). NO affects tumors in complex and diverse ways, acting as a "double-edged sword" to both promote and limit tumor growth (22, 24, 49). It seems that high levels of NO suppress tumor growth and metastasis (21, 35), whereas low levels may do the opposite (6, 12). Sustained generation of NO occurs after cytokine stimulation of immune cells. The resulting sustained exposure to higher concentrations of NO inhibits a variety of tumor cells and microorganisms (11, 25, 32, 42, 49, 53, 54). NO is believed to be cytostatic/lytic to tumor cells through its inhibition of mitochondrial respiration and deoxyribonucleic acid replication, processes on which tumor cells are inherently dependent for survival (1). In addition, a potential role for NO in tumor cell inhibition has been suggested through its involvement in therapeutic modalities such as radiotherapy (23, 47), immunotherapy (37, 45), and chemotherapy (26, 30). The present experiments explore the possible role of NO in astroglia tumoricidal activity both in vitro and in vivo.

Patients with malignant brain tumors continue to have a poor prognosis despite significant advances in chemotherapy, radiotherapy, and immunotherapy (48). An increased understanding of host CNS defense mechanisms has the potential to improve current therapeutic regimens for patients with brain tumors. We show that NO dose-dependently inhibits a variety of primary and metastatic tumor cell lines. Moreover, induction of astroglia iNOS inhibits both in vitro and in vivo brain tumor growth and prolongs survival of animals with metastatic brain tumors through partially NO-dependent mechanisms.

MATERIALS AND METHODS

Tumor Lines

We obtained B16F10 melanoma, Lewis lung carcinoma, and CT26 colon cancer cell lines from the Division of Cancer Treatment Tumor Repository (National Cancer Institute, Frederick, MD). The 9L rat glioma was obtained in 1985 from Marvin Barker (Brain Tumor Research Center, University of California, San Francisco, CA) and maintained subcutaneously in the flank of male Fischer 344 rats. The human glioma lines H80, U87, and U373 were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (MEM) containing 10% fetal calf serum and penicillin/streptomycin in a 5% humidified 37°C incubator. Cultured tumor monolayers were harvested with trypsin, counted, and resuspended in the medium before use in cytotoxicity experiments or intracranial injection.

Animals

C57BL/6 female mice (wild-type) and female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Non-NOS-inducible (iNOS−/−) mice were bred and genotyped by our laboratory as described previously (13, 27). These animals were allowed free access to Baltimore City water and rodent chow. They were kept in accordance with the policies and principles of laboratory animal care of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

NO Donor Cytotoxicity

Tumor cell sensitivity to NO donors was measured by a cell proliferation assay. The tumor cells were seeded in 24-well plates (without astroglia) at a density of 10,000 cells per well and allowed to attach for 24 hours. After a 5-minute wash in buffer solution, they were exposed to either medium or the NO-donating compounds sodium nitroprusside (SNP; Sigma, St. Louis, MO) (20, 100, or 500 μmol/L) for 10 minutes or NOR-3 (Alexis, San Diego, CA) (5, 20, or 100 μmol/L) for 24 hours. Subsequently, cell growth was determined by the cell proliferation assay on Day 3. Means ± standard error of the mean (SEM) are from four data points from two separate experiments.

Astroglia Cultures

Cultures were prepared from 0- to 3-day-old Sprague-Dawley rats and iNOS−/− mice as described previously (13). The cortex from fetal mice was dissected, and the cells were dissociated by trituration in MEM, 20% horse serum, 25 mmol/L glucose, and 2 mmol/L L-glutamine after 30 minutes of digestion in 0.027% trypsin/saline solution. Cortex from fetal rats was dissociated by trituration in MEM, 10% fetal bovine serum, 10% horse serum, and 2 mmol/L L-glutamine after 30 minutes of digestion in 0.027% trypsin/saline solution. The cells were plated on 75-cm^2 flasks. Rat cultures were then maintained in MEM, 5% horse serum, and 2 mmol/L L-glutamine in an 8% CO_2 humidified 37°C incubator. Maintenance media for mouse cultures contained MEM, 10% horse serum, 25 mmol/L glucose, and 2 mmol/L L-glutamine in an 8% CO_2 humidified 37°C incubator. The growth medium was refreshed twice per week, and after 2 weeks of maturation, the cultures were trypsinized and plated onto 15-cm multiwell plates. After 3 to 5 days, the cells reached confluence, and experiments were conducted.

Stimulated Astroglia Cytotoxicity

Confluent astroglia from rat pups and iNOS−/− mice were stimulated for 48 hours with one of the following: 1) medium alone; 2) lipopolysaccharide (LPS) (100 ng/ml; Alexis) + interferon-γ (IFN-γ) (200 U/ml; Intergen, Purchase, NY); or 3) LPS + IFN-γ + N^G-nitro-L-arginine methyl ester (L-NAME) (500 μmol/L; Sigma). L-NAME is a noncompetitive inhibitor of all NOS isoforms. Tumor cell sensitivity to stimulated as-

troglia was measured by a cell proliferation assay. The number of astroglia was constant and insignificant in comparison to tumor cell number. At the time of medium replacement, tumor cells were seeded over the astroglia at a density of 10,000/well, and 3 days later, cell proliferation was assayed with a Coulter counter. Values are expressed as tumor cells in treatment group/tumor cells in control. Means ± SEM are from four data points from two separate experiments.

In Vivo Mouse Survival

The B16F10 melanoma intracranial tumor model was used (41). Mice were randomized into the following four groups: 1) saline (n = 23); 2) LPS (10 μg/animal) + IFN-γ (1000 U/animal) (n = 20); 3) LPS + IFN-γ + systemic intraperitoneal L-NAME (0.5 mg/animal) × 5 days (n = 24); and 4) iNOS−/− mice treated with LPS (10 μg/animal) + IFN-γ (1000 U/animal) (n = 17). Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl solution. The surgical site was shaved and prepared with 70% ethyl alcohol and Prepodyne solution (West-Agro Chemical, Inc., Kansas City, MO). After a midline incision was made, a 1-mm burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame, and treatment was delivered by a 26-gauge needle to a depth of 3 mm over a period of 3 minutes. The total volume of treatment was 5 μl. The needle was removed, the site was irrigated with sterile 0.9% NaCl solution, and the skin was sutured closed. On postoperative Day 1, the mice were reanesthetized, and B16F10 melanoma cells + treatment (each of the four groups described above) was delivered through the surgical site, with a total volume of 5 μl. Animals were monitored for signs of clinical neurotoxicity, such as hemiparesis or lethargy. Upon becoming moribund or paralyzed, mice were killed, and their brains were placed in formalin. Whenever possible, animals were autopsied to determine the cause of death. The distribution of the intervals until death was estimated by the method of Kaplan and Meier.

iNOS Western Blots

Mice were anesthetized and underwent the procedure for intracranial stereotactic injection as described above. A solution of 5 μl of media containing LPS (10 μg) + IFN-γ (1000 U) was delivered intracranially to a depth of 3 mm over a period of 3 minutes. The needle was removed, the site was irrigated with sterile 0.9% NaCl solution, and the skin was sutured closed. At predetermined time points (0, 6, 12, 24, 48, 72, 96, and 120 h), the animals were reanesthetized and the left parietal lobe was removed for Western blot analysis.

Statistical Analysis

Statview 4.02 (Abacus Concepts, Berkeley, CA) and Graph Pad Prism 2.0 (GraphPad Software, San Diego, CA) statistical packages were used. Data were analyzed by one-way analysis of variance followed by Fisher's protected least significance difference or a Bonferroni post hoc test to test for significance. The Kaplan-Meier method and a log-rank test were used to analyze survival.

RESULTS

NO Donors Inhibit Growth of Primary and Metastatic Brain Tumor Cells in Vitro

We tested the ability of two NO donors, SNP and NOR-3, to inhibit the growth of one primary rat (9L gliosarcoma) cell line, three primary human brain tumor cell lines (H80, U87, U373), and three murine tumor cell lines that have the propensity to metastasize to the brain (B16F10 melanoma, Lewis lung carcinoma, and CT26 colon carcinoma). Cell growth was inhibited in all tumor cell lines in a dose-dependent manner ($P < 0.01$ for all comparisons of experimental versus control) with a 50% inhibitory concentration ranging from 50 to 100 μmol/L for SNP and 5 to 20 μmol/L for NOR-3. A dose of 500 μmol/L SNP and 100 μmol/L NOR-3 dramatically inhibited tumor cell growth in all the cell lines (*Fig. 1*).

Stimulated Astroglia Exhibit Enhanced Cytotoxicity toward Tumor Cell Lines via an NO-dependent Mechanism in Vitro

Proliferation of glioma and metastatic tumor lines was significantly inhibited by LPS + IFN-γ treatment of astroglia (*Fig. 2, A* and *B*). Glioma and metastatic tumor growth was reduced approximately 65% ($P < 0.001$). L-NAME (500 μmol/L), a noncompetitive inhibitor of NOS isoforms, markedly reversed ($P < 0.005$) the tumoricidal activity of LPS + IFN-γ, implicating NO as a major tumor growth suppressor. To further evaluate the role of NO in tumor growth suppression, we cultured astroglia from mice lacking the gene for iNOS (27). Tumor growth was not suppressed when astroglia cultures from iNOS−/− mice challenged with LPS (100 ng/ml) and IFN-γ (200 U/ml) were compared with wild-type mice ($P < 0.005$) (*Fig. 2C*). The modest residual growth inhibition in iNOS−/− mice and in rat LPS- and IFN-γ-challenged astroglia treated with L-NAME may be a result of the induction of other effector molecules in addition to NO. LPS or IFN-γ alone did not significantly induce NO production (data not shown).

NO Production Prolongs Survival in Mice Challenged with Intracranial B16F10 Melanoma

We tested whether NO is a principal mediator of CNS host defense against tumors in the mouse B16F10 melanoma intracranial tumor model (15). Stereotactic injection of LPS + IFN-γ into the left parietal cortex induced iNOS protein within 12 hours, followed by a dramatic increase at 48 hours, after which iNOS levels remained stable through 120 hours (*Fig. 3*). Thus, we demonstrated the in vivo capability of these mice for iNOS induction. Twenty-four hours before intracranial tumor challenge, wild-type mice received either LPS + IFN-γ or LPS +

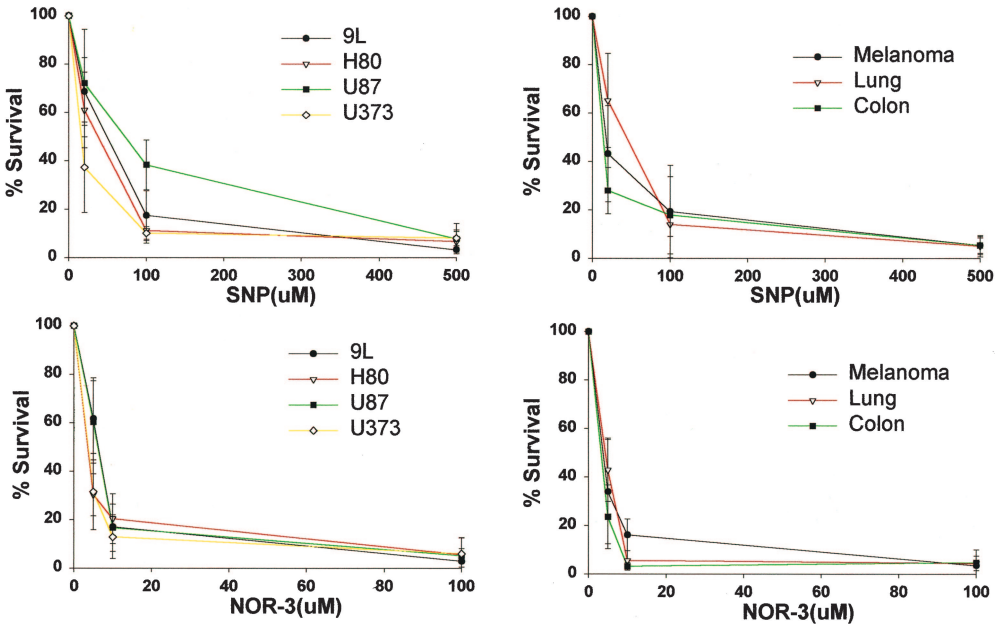

FIGURE 1. *Graphs showing that the NO donors SNP and NOR-3 inhibit glioma and metastatic cell line growth in vitro. Tumor cells from four glioma cell lines (9L, H80, U87, U373) and three metastatic tumor lines (melanoma, lung, colon) were seeded at a density of 10,000 cells per well and exposed to the NO donors SNP or NOR-3 in incremental doses. After 72 hours, cell growth was determined by the cell proliferation assay. Values are expressed as mean ± SEM. Data were analyzed by a one-way analysis of variance followed by Bonferroni post hoc test (P < 0.01 for all comparisons of experimental versus control values).*

IFN-γ + L-NAME. Survival was prolonged by 11.5 days in the LPS + IFN-γ-treated group compared with saline-treated control animals (*Fig. 4*, $P < 0.0001$). L-NAME reduced LPS and IFN-γ enhancement of survival by 5 days. Treatment with LPS and IFN-γ failed to prolong survival in B16F10 melanoma-challenged iNOS−/− mice compared with LPS- and IFN-γ-treated wild-type mice challenged with B16F10 melanoma cells. Thus, induction of iNOS is the major effector of LPS- and IFN-γ-mediated survival.

DISCUSSION

Summary of Results

This study provides both in vitro and in vivo evidence that NO plays a role in brain tumoricidal activity. In vitro, cultured astroglia stimulated with LPS and IFN-γ inhibit the growth of primary and metastatic tumor lines, and this inhibition is partially reversed by L-NAME. In addition, when astroglia cultured from iNOS−/− mice are stimulated with LPS and IFN-γ, they fail to inhibit tumor growth. In vivo, when mice are primed to produce NO through the stereotactic intracranial administration of LPS + IFN-γ and subsequently challenged with B16F10 melanoma, their survival is significantly prolonged. Addition of L-NAME, which inhibits the enzyme that catalyzes the synthesis of NO, partially reverses this increase in survival. Mice lacking the *iNOS* gene, which is required for this NO-synthesizing enzyme, failed to show increased survival despite pretreatment with LPS and IFN-γ. The partial reversal of the increase in survival by L-NAME and the fact that some cytotoxicity is observed in the iNOS−/− mice suggest that mechanisms other than NO contribute to the observed effects. These partial effects may include induction of other immune effector molecules and possibly T-cell-mediated immunotoxicity. Overall, our findings indicate that NO is a significant effector molecule for host defense against brain tumors in this experimental model and a potential therapeutic modality for treatment of primary and metastatic brain tumors in humans.

Multifaceted Role of NO in Tumor Biology

Although previous research has documented a role for NO in tumor biology, the primitiveness of our understanding is underscored by other studies that may at times seem contradictory. A large number of studies document that NO production inhibits tumor growth, probably via activation of apoptotic and free radical-induced cell death mechanisms (5, 7). In contrast, NOS inhibitors have been shown to prolong survival in other animal tumor models (19, 44); this effect may be the result of NO-mediated angiogenesis in tumors (22, 50).

NO is a pleiotropic potent molecule that mediates both tumor inhibition and progression (2, 22, 24, 34, 39, 49). The apparently opposing roles of NO relate to local concentration, varying NOS isoforms, and heterogeneity of tumor tissues or cell lines (49, 52). Further complexity is introduced with some tumors, particularly those infiltrated with macrophages, expressing NOS (34, 43, 49, 52). Macrophages produce much higher levels of NOS than do tumor cells and are the main source of NO production in these tumors. The studies of human tumors, particularly breast cancer, show low levels of NOS activity that correlate with tumor grade (43). Depending on the tumor type, NO may inhibit or promote tumor growth. Furthermore, it seems that the resultant effect may be concentration-dependent, with high levels suppressing tumor growth and low levels doing the opposite.

Macrophages serve as the host's surveillance system against tumors. NO produced by systemic macrophages possesses cytostatic/lytic properties (28, 36, 40). The mechanistic basis for the tumoricidal activity of NO includes inhibition of mi-

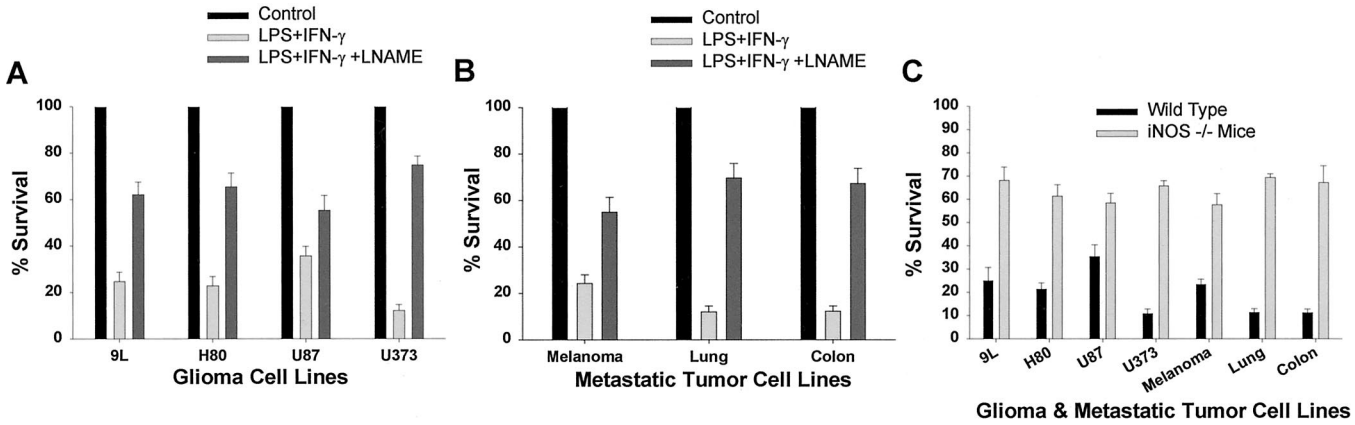

FIGURE 2. *Bar graphs showing that immunologically stimulated astroglia exhibit cytotoxicity toward tumor cell lines via an NO-dependent mechanism in vitro. A, rat astrocytes were stimulated with 100 ng/ml LPS + 200 U/ml IFN-γ in the presence or absence of 500 μmol/L L-NAME, a competitive NOS inhibitor. They were then cultured with one of the four glioma cell lines and assessed for glioma cell survival at 72 hours. Controls were astrocytes stimulated with saline only. B, rat astrocytes were similarly stimulated, cultured with one of the three metastatic tumor cell lines, and assessed for metastatic tumor cell survival at 72 hours. C, astrocytes from wild-type and iNOS−/− mice were similarly-stimulated, cultured with glioma and metastatic tumor cell lines, and assessed for cell survival at 72 hours. Data are mean ± SEM for n ≥ 4 from at least two experiments. For each set of culture data, significance was determined by a one-way analysis of variance, with differences between groups ascertained by Fisher's protected least significance difference post hoc test (P ≤ 0.001 for LPS + IFN-γ versus control; P ≤ 0.005 for LPS + IFN-γ versus LPS + IFN-γ + L-NAME; P ≤ 0.005 for wild-type versus iNOS−/− cultures.*

tochondrial respiration, growth inhibition via NOS-dependent depletion of arginine, cell cycle arrest through down-regulation of cyclin D1, and induction of apoptosis by activation of caspases and accumulation of p53 (5, 28). These processes preferentially affect actively dividing tumor cells. A number of in vitro studies document the cytotoxic ability of NO produced by systemic macrophages (4, 10, 16, 17, 20, 23, 29, 31, 35, 38, 46); few reports suggest an important role of NO for systemic tumoricidal activity in vivo (3, 8–10, 14, 33, 54).

NO as a Potential Effector Molecule against Tumors of the CNS

Our present report is the first in vitro and in vivo study of the putative role of NO as a brain tumoricidal effector agent. Although systemic immunological mechanisms to combat tumor cells are well studied, host CNS defense mechanisms remain largely unexplored. Microglia share many properties with systemic macrophages and produce copious amounts of NO in response to cytokine stimulation (31, 51). One recent report documented the cytotoxic effects of NO produced by recruited macrophages in an animal model of hepatoma (33). Our results expand the relevance of these findings to a variety of primary and metastatic CNS tumor lines and suggest that similar mechanisms are operational and possibly widespread in vivo. Furthermore, potential therapies for primary and metastatic brain tumors may exploit mechanisms that promote the production of NO within the CNS, either directly via NO donor agents or indirectly via stimulation of pathways that generate NO.

Potential Application for Human Trials Requires Additional Study

Translation of these findings to human subjects requires additional study. In the treatment paradigm used in these experiments, tumor cells were introduced into a hostile NO-rich environment. The experiments were designed in this manner to provide the best possible opportunity to discover and then elucidate a mechanism of cytotoxic effects on brain tumors that had not been described previously. In human subjects, however, the cellular microenvironment would be vastly different secondary to the tumor. Further animal studies in which NO donors or inducers are introduced after tumor implantation and growth will help clarify these issues. Furthermore, the use of NO donors in human subjects could be constrained by the potential of side effects such as "capillary leak syndrome." These include hypotension, pleural effusion, and renal abnormalities that have been seen in cancer patients and animal models treated with interleukin-2 and attributed to the induction of NO (45).

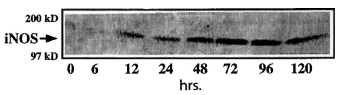

FIGURE 3. *Western blot showing that LPS + IFN-γ induces iNOS protein in mice cortex in vivo. Mice were stereotactically injected with 10 μg LPS + 1000 U IFN-γ in the left parietal cortex. Cortex was assessed for iNOS protein via immunoblotting at various time points (0–120 h) after injection. No iNOS protein is detectable in saline-injected mice and iNOS−/− mice (data not shown).*

CONCLUSION

Our findings suggest that NO may have an important role as a defense mechanism molecule against brain tumors; stimulation or modification of this mechanism may represent a new adjunctive approach to the

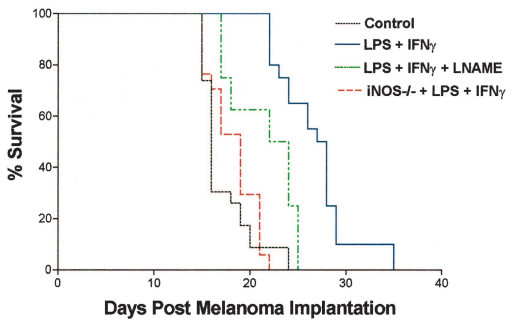

FIGURE 4. *Graph showing that NO production prolongs survival in mice challenged with intracranial B16Fl0 melanoma. Wild-type mice were injected intracranially with control (saline), LPS (10 μg) + IFN-γ (1000 U), or LPS + IFN-γ + L-NAME (0.5 mg intraperitoneally × 5 d). At 24 hours later, they were challenged with tumor cells and treatment (one of the above four treatments) and assessed for survival. iNOS−/− mice were also similarly exposed to LPS + IFN-γ and tumor cells and assessed for survival. Kaplan-Meier survival analysis with a log-rank test was performed to test for significance among treatment groups (P < 0.0001 for LPS + IFN-γ versus control; P < 0.0001 for LPS + IFN-γ + L-NAME versus control; P = 0.3516 for iNOS−/− versus control; P < 0.0001 for LPS + IFN-γ versus LPS + IFN-γ L-NAME).*

treatment of primary and metastatic brain tumors. However, many questions remain unanswered, including a better understanding of why in certain cellular microenvironments NO has been shown to promote tumor growth. Future studies will help clarify the multifaceted role of NO in tumor biology.

DISCLOSURE

Henry Brem is a consultant to Guilford Pharmaceuticals, Inc. The Johns Hopkins University and Henry Brem own Guilford stock, the sale of which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the University in accordance with its conflict-of-interest policies. Under an agreement between the Johns Hopkins University and Guilford Pharmaceuticals, Ted M. Dawson and Valina L. Dawson are entitled to a share of sales royalty received by the University from Guilford. Ted M. Dawson and the University also own Guilford stock, and the University stock is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the University in accordance with its conflict-of-interest policies.

REFERENCES

1. Albina JE, Reichner JS: Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. **Cancer Metastasis Rev** 17:39–53, 1998.
2. Ambs S, Merriam WG, Ogunfusika MO, Bennett WP, Ishibe N, Hussain SP, Tzeng EE, Geller DA, Billiar TR, Harris CC: p53 and vascular endothelial growth factor regulate tumor growth of NOS_2-expressing human carcinoma cells. **Nat Med** 4:1371–1376, 1998.
3. Baratin M, Ziol M, Romieu R, Kayibanda M, Gouilleux F, Briand P, Leroy P, Haddada H, Renia L, Viguier M, Guillet JG: Regression of primary hepatocarcinoma in cancer-prone transgenic mice by local interferon-γ delivery is associated with macrophages recruitment and nitric oxide production. **Cancer Gene Ther** 8:193–202, 2001.
4. Bhaumik S, Khar A: Induction of nitric oxide production by the peritoneal macrophages after intraperitoneal or subcutaneous transplantation of AK-5 tumor. **Nitric Oxide** 2:467–474, 1998.
5. Bogdan C: Nitric oxide and the immune response. **Nat Immunol** 2:907–916, 2001.
6. Brennan PA, Palacios-Callender M, Zaki GA, Spedding AV, Langdon JD: Type II nitric oxide synthase (NOS_2) expression correlates with lymph node status in oral squamous cell carcinoma. **J Oral Pathol Med** 30:129–134, 2001.
7. Brune B, von Knethen A, Sandau KB: Nitric oxide and its role in apoptosis. **Eur J Pharmacol** 351:261–272, 1998.
8. Cahlin C, Gelin J, Delbro D, Lonnroth C, Doi C, Lundholm K: Effect of cyclooxygenase and nitric oxide synthase inhibitors on tumor growth in mouse tumor models with and without cancer cachexia related to prostanoids. **Cancer Res** 60:1742–1749, 2000.
9. Cao G, Su J, Lu W, Zhang F, Zhao G, Marteralli D, Dong Z: Adenovirus-mediated interferon-β gene therapy suppresses growth and metastasis of human prostate cancer in nude mice. **Cancer Gene Ther** 8:497–505, 2001.
10. Carretero J, Obrador E, Esteve JM, Ortega A, Pellicer JA, Sempere FV, Estrela JM: Tumoricidal activity of endothelial cells: Inhibition of endothelial nitric oxide production abrogates tumor cytotoxicity induced by hepatic sinusoidal endothelium in response to B16 melanoma adhesion in vitro. **J Biol Chem** 276:25775–25782, 2001.
11. Chang CI, Liao JC, Kuo L: Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. **Cancer Res** 61: 1100–1106, 2001.
12. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA: Expression of nitric oxide synthase in human central nervous system tumors. **Cancer Res** 55: 727–730, 1995.
13. Dawson VL, Kizushi VM, Huang PL, Snyder SH, Dawson TM: Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. **J Neurosci** 16:2479–2487, 1996.
14. Eue I: Growth inhibition of human mammary carcinoma by liposomal hexadecylphosphocholine: Participation of activated macrophages in the antitumor mechanism. **Int J Cancer** 92:426–433, 2001.
15. Ewend MG, Williams JA, Tabassi K, Tyler BM, Babel KM, Anderson RC, Pinn ML, Brat DJ, Brem H: Local delivery of chemotherapy and concurrent external beam radiotherapy prolongs survival in metastatic brain tumor models. **Cancer Res** 56:5217–5223, 1996.
16. Fricker SP, Slade E, Powell NA: Macrophage-derived superoxide is not required for nitric oxide mediated tumour cell killing by RAW 264 cells. **Anticancer Res** 19:553–556, 1999.
17. Garban HJ, Bonavida B: Nitric oxide disrupts H_2O_2-dependent activation of nuclear factor κB: Role in sensitization of human tumor cells to tumor necrosis factor-α-induced cytotoxicity. **J Biol Chem** 276:8918–8923, 2001.
18. Gath I, Steppuhn A, Maelicke A, Reinhardt S, Forstermann U: Analysis of NO synthase expression in neuronal, astroglial and fibroblast-like derivatives differentiating from PCC7-Mz1 embryonic carcinoma cells. **Eur J Cell Biol** 78:134–142, 1999.
19. Jadeski LC, Lala PK: Nitric oxide synthase inhibition by N(G)-nitro-L-arginine methyl ester inhibits tumor-induced angiogenesis in mammary tumors. **Am J Pathol** 155:1381–1390, 1999.
20. Jadus MR, Williams CC, Avina MD, Ly M, Kim S, Liu Y, Narasaki R, Lowell CA, Wepsic HT: Macrophages kill T9 glioma tumor cells bearing the membrane isoform of macrophage colony stimulating factor through a phagocytosis-dependent pathway. **J Immunol** 160:361–368, 1998.
21. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S: Roles of nitric oxide in tumor growth. **Proc Natl Acad Sci U S A** 92:4392–4396, 1995.
22. Konopka TE, Barker JE, Bamford TL, Guida E, Anderson RL, Stewart AG: Nitric oxide synthase II gene disruption: Implications for tumor growth and vascular endothelial growth factor production. **Cancer Res** 61:3182–3187, 2001.

23. Kurimoto M, Endo S, Hirashima Y, Hamada H, Ogiichi T, Takaku A: Growth inhibition and radiosensitization of cultured glioma cells by nitric oxide generating agents. **J Neurooncol** 42:35–44, 1999.
24. Lala PK, Orucevic A: Role of nitric oxide in tumor progression: Lessons from experimental tumors. **Cancer Metastasis Rev** 17:91–106, 1998.
25. Li LM, Kilbourn RG, Adams J, Fidler IJ: Role of nitric oxide in lysis of tumor cells by cytokine-activated endothelial cells. **Cancer Res** 51:2531–2535, 1991.
26. Lind DS, Kontaridis MI, Edwards PD, Josephs MD, Moldawer LL, Copeland EM III: Nitric oxide contributes to adriamycin's antitumor effect. **J Surg Res** 69:283–287, 1997.
27. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, Chen H, Mudgett JS: Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. **Cell** 81:641–650, 1995.
28. MacMicking J, Xie QW, Nathan C: Nitric oxide and macrophage function. **Annu Rev Immunol** 15:323–350, 1997.
29. Mateo RB, Reichner JS, Albina JE: NO is not sufficient to explain maximal cytotoxicity of tumoricidal macrophages against an NO-sensitive cell line. **J Leukoc Biol** 60:245–252, 1996.
30. Mullins DW, Burger CJ, Elgert KD: Paclitaxel enhances macrophage IL-12 production in tumor-bearing hosts through nitric oxide. **J Immunol** 162: 6811–6818, 1999.
31. Murata J, Ricciardi-Castagnoli P, Dessous L'Eglise Mange P, Martin F, Juillerat-Jeanneret L: Microglial cells induce cytotoxic effects toward colon carcinoma cells: Measurement of tumor cytotoxicity with a γ-glutamyl transpeptidase assay. **Int J Cancer** 70:169–174, 1997.
32. Naama HA, McCarter MD, Mack VE, Evoy DA, Hill AD, Shou J, Daly JM: Suppression of macrophage nitric oxide production by melanoma: Mediation by a melanoma-derived product. **Melanoma Res** 11:229–238, 2001.
33. Nishikawa M, Sato EF, Kuroki T, Utsumi K, Inoue M: Macrophage-derived nitric oxide induces apoptosis of rat hepatoma cells in vivo. **Hepatology** 28:1474–1480, 1998.
34. Radomski MW, Jenkins DC, Holmes L, Moncada S: Human colorectal adenocarcinoma cells: Differential nitric oxide synthesis determines their ability to aggregate platelets. **Cancer Res** 51:6073–6078, 1991.
35. Shi Q, Huang S, Jiang W, Kutach LS, Ananthaswamy HN, Xie K: Direct correlation between nitric oxide synthase II inducibility and metastatic ability of UV-2237 murine fibrosarcoma cells carrying mutant p53. **Cancer Res** 59:2072–2075, 1999.
36. Sotomayor EM, DiNapoli MR, Calderon C, Colsky A, Fu YX, Lopez DM: Decreased macrophage-mediated cytotoxicity in mammary-tumor-bearing mice is related to alteration of nitric-oxide production and/or release. **Int J Cancer** 60:660–667, 1995.
37. Sun H, Gutierrez P, Jackson MJ, Kundu N, Fulton AM: Essential role of nitric oxide and interferon-γ for tumor immunotherapy with interleukin-10. **J Immunother** 23:208–214, 2000.
38. Sveinbjornsson B, Olsen R, Seternes OM, Seljelid R: Macrophage cytotoxicity against murine meth A sarcoma involves nitric oxide-mediated apoptosis. **Biochem Biophys Res Commun** 223:643–649, 1996.
39. Tamir S, Tannenbaum SR: The role of nitric oxide (NO) in the carcinogenic process. **Biochim Biophys Acta** 1288:F31–F36, 1996.
40. Thomas C, Nijenhuis AM, Dontje B, Daemen T, Scherphof GL: Tumoricidal response of liver macrophages isolated from rats bearing liver metastases of colon adenocarcinoma. **J Leukoc Biol** 57:617–623, 1995.
41. Thompson RC, Pardoll DM, Jaffee EM, Ewend MG, Thomas MC, Tyler BM, Brem H: Systemic and local paracrine cytokine therapies using transduced tumor cells are synergistic in treating intracranial tumors. **J Immunother Emphasis Tumor Immunol** 19:405–413, 1996.
42. Thomsen LL, Miles DW: Role of nitric oxide in tumour progression: Lessons from human tumours. **Cancer Metastasis Rev** 17:107–118, 1998.
43. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S: Nitric oxide synthase activity in human breast cancer. **Br J Cancer** 72:41–44, 1995.
44. Thomsen LL, Scott JM, Topley P, Knowles RG, Keerie AJ, Frend AJ: Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: Studies with 1400W, a novel inhibitor. **Cancer Res** 57:3300–3304, 1997.
45. Timoshenko AV, Lan Y, Gabius HJ, Lala PK: Immunotherapy of C3H/HeJ mammary adenocarcinoma with interleukin-2, mistletoe lectin or their combination: Effects on tumour growth, capillary leakage and nitric oxide (NO) production. **Eur J Cancer** 37:1910–1920, 2001.
46. Tonetti M, Millo E, Sturla L, Bisso A, De Flora A: Effects of the murine L929 and L1210 cell lines on nitric oxide and TNF-α production by RAW 264.7 murine macrophages. **Biochem Biophys Res Commun** 230:636–640, 1997.
47. Verovski VN, Van den Berge DL, Soete GA, Bols BL, Storme GA: Intrinsic radiosensitivity of human pancreatic tumour cells and the radiosensitising potency of the nitric oxide donor sodium nitroprusside. **Br J Cancer** 74:1734–1742, 1996.
48. Walter KA, Tamargo RJ, Olivi A, Burger PC, Brem H: Intratumoral chemotherapy. **Neurosurgery** 37:1128–1145, 1995.
49. Wang B, Xiong Q, Shi Q, Le X, Abbruzzese JL, Xie K: Intact nitric oxide synthase II gene is required for interferon-β-mediated suppression of growth and metastasis of pancreatic adenocarcinoma. **Cancer Res** 61:71–75, 2001.
50. Wink DA, Vodovotz Y, Cook JA, Krishna MC, Kim S, Coffin D, DeGraff W, Deluca AM, Liebmann J, Mitchell JB: The role of nitric oxide chemistry in cancer treatment. **Biochemistry (Mosc)** 63:802–809, 1998.
51. Wood PL, Choksi S, Bocchini V: Inducible microglial nitric oxide synthase: A large membrane pool. **Neuroreport** 5:977–980, 1994.
52. Xie K, Fidler IJ: Therapy of cancer metastasis by activation of the inducible nitric oxide synthase. **Cancer Metastasis Rev** 17:55–75, 1998.
53. Xie K, Huang S, Dong Z, Juang SH, Gutman M, Xie QW, Nathan C, Fidler IJ: Transfection with the inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis by K-1735 murine melanoma cells. **J Exp Med** 181:1333–1343, 1995.
54. Yim CY, Bastian NR, Smith JC, Hibbs JB Jr, Samlowski WE: Macrophage nitric oxide synthesis delays progression of ultraviolet light-induced murine skin cancers. **Cancer Res** 53:5507–5511, 1993.
55. Yun HY, Dawson VL, Dawson TM: Nitric oxide in health and disease of the nervous system. **Mol Psychiatry** 2:300–310, 1997.
56. Zielasek J, Hartung HP: Molecular mechanisms of microglial activation. **Adv Neuroimmunol** 6:191–222, 1996.

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COMMENTS

The authors have demonstrated that resident glial cells can be induced to produce nitric oxide (NO) and that this can have a cytotoxic effect on tumor cells. These experiments include the use of an NO inhibitor as well as a knockout model to support the conclusions. An inducible factor that is derived from normal glia and that helps to control brain tumors would be a significant contribution to potential therapy.

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In this article, the authors describe the in vitro and in vivo effects of NO on glioma cell line proliferation. Astroglial cultures were exposed to lipopolysaccharide and interferon-γ with concordant upregulation of inducible NO synthetase. Subsequent seeding of tumor cell lines provided a platform for testing the effect of NO in vitro. The authors observed a dose-dependent inhibition of tumor cell growth that was not

seen when cell lines were seeded with astroglial cultures derived from inducible NO synthetase knockout mice. These results were replicated with metastatic cell lines in vitro and in vivo when mice were pretreated with lipopolysaccharide and interferon-γ.

This study is a good beginning to what will likely be an interesting avenue of investigation. Future studies should attempt to extend these results to more relevant models, such as ex vivo primary human astrocyte cultures combined with autologous low passage primary human cell cultures. In addition, future studies directed at crossing inducible NO synthetase knockout mice with transgenic models of glioma may delineate more clearly NO-mediated inhibition of tumor growth. Results from experiments using highly passaged cell lines unfortunately do not always translate effectively to clinical treatment paradigms.

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The authors investigated an interesting phenomenon—whether glia can act as defense effector cells when stimulated by cytokines—and the mechanisms by which this defense occurs. They postulated that NO plays a key role in this equation and investigated its effects both in vitro and in vivo. The authors found that when astroglia are stimulated to produce NO by the cytokines lipopolysaccharide and interferon-γ, they inhibit the growth of a variety of tumor cells in vitro. In glial cells that are incapable of NO induction, this effect is not observed. In the in vivo experiments, mice primed to produce NO through stereotactic intracranial administration of lipopolysaccharide plus interferon-γ, and subsequently challenged with B16F10 melanoma, demonstrated prolonged survival. Knockout mice lacking the gene for inducible NO failed to show this effect. Furthermore, N^G-nitro-L-arginine methyl ester, an inhibitor of the enzyme that catalyzes the synthesis of NO, partially reverses the increase in survival.

This study provides both in vitro and in vivo evidence that NO participates in brain tumoricidal activity. Some of this effect may be the result of a nonspecific activation of the immune system, which is further discussed in the article. One concern we have with this article is that the authors refer to glia as defense effector cells, similar to macrophages, but they may mean microglia. It is not possible from this article to separate whether it is indeed the microglia, which are not considered to be true glial cells, or the astroglia, which are responsible for this effect. However, the results do suggest that NO participates in the host defense against brain tumors and may be a target for future therapeutic intervention.

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This report illustrates that there are potentially tumoricidal defense mechanisms present in the brain, one of which is associated with the production of NO by either microglia or astroglia. Like several other defense mechanisms, this seems to be ineffective or not sufficiently activated in the context of intracerebral malignancy. Therapeutic approaches that are based on the activation or the support of natural defense mechanisms are always attractive, but the authors are right in their caution in that it may be very difficult to selectively activate NO production for therapeutic purposes in the brain. In particular, the pleiotropic nature of NO makes it a biological agent of the same difficult category as transforming growth factor β. Also, it is certainly interesting that survival was prolonged in an intracranial model of melanoma, but that unfortunately lacks the infiltrative nature and disseminated growth pattern of a glioma. It will be interesting to see from future experiments with glioma models how far the survival benefit of intracranial stimulation of NO production can be extrapolated from metastasis to glioma. It also remains to be seen how tolerable an increase in NO in the whole brain will be, because that is what would be required for treatment of a disease involving the whole cerebral compartment.

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