

Expression of Cyclooxygenase 2 (COX-2) in Human Glioma and *in Vitro* Inhibition by a Specific COX-2 Inhibitor, NS-398¹

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ABSTRACT

The up-regulation of cyclooxygenase 2 (COX-2) expression is a frequent occurrence in a variety of different tumors. In this study, COX-2 protein expression was investigated in 50 glioma and 3 normal brain specimens by immunohistochemistry. Expression of COX-2 protein was observed in all normal brain and glioma specimens by immunohistochemistry, regardless of histological grade. The immunoreactive score was significantly higher in high-grade glioma than low-grade glioma and normal brain specimens. For a subset of these tumors (nine gliomas and three normal brain), Western blot analysis was also performed. COX-2 protein was detected in all specimens by Western blot analysis.

The effect of the specific COX-2 inhibitor NS-398 on monolayer cell cultures and three-dimensional glioma spheroids was investigated using U-87MG and U-251MG human glioblastoma cell lines. The proliferation rate was assessed in monolayer cultures. In addition, a growth assay, a migration assay, an apoptosis assay, and a tumor invasion assay were performed in a three-dimensional spheroid culture system. NS-398 was able to reduce the proliferation of monolayer cell cultures, as well as the growth of spheroids and tumor cell migration, in a dose-dependent manner. There was also a moderate increase in the number of apoptotic cells in the treated spheroids. NS-398 did not have an inhibitory effect on tumor invasion in the coculture spheroid system. Our study provides evidence that COX-2 is up-regulated in the majority of high-grade gliomas and that a potential role of COX-2 inhibitors as an adjuvant therapy for brain tumors may exist.

INTRODUCTION

The COX³ enzyme catalyzes the rate-limiting step in arachidonate metabolism, resulting in PG production. Two COX isoforms have been identified as COX-1 and COX-2 (1). COX-1 is expressed constitutively in several cell types of normal mammalian tissues, where it is involved in the maintenance of tissue homeostasis. In contrast, COX-2 is an inducible enzyme responsible for PG production at sites of inflammation (1, 2). The expression of COX-1 and COX-2 has been reported to be associated with the complex changes observed during a variety of diseases of the brain. Following trauma, an increase in PGs can lead to vascular damage (3). In GBM cells, inhibitors of eicosanoid biosynthesis suppress proliferation and promote astrocytic differentiation (4).

Studies have led to the recognition of the importance of COX-2 in colorectal tumorigenesis (5–9) and in the development of intestinal neoplasms in genetically manipulated animal models (9, 10). COX-2

expression has also been detected in gastric (11), esophageal (12, 13), and lung carcinomas (14, 15) and brain tumors (16).

Recently, NS-398, a selective COX-2 inhibitor has been reported to mediate growth-inhibitory effects in colon (17), esophageal (13) and pancreatic carcinoma cell lines (18). In addition, NS-398 was shown to inhibit proliferation and to induce apoptosis in colorectal and lung carcinoma cell lines (19, 20).

The expression of COX-2 in human glioma and the effects of selective COX-2 inhibitors on the growth of human glioma cell lines have not been analyzed in detail. In this study, we examine the expression of COX-2 protein in human glioma specimens and cell lines. In addition, the effects of the selective COX-2 inhibitor NS-398 on glioma proliferation, migration, apoptosis, and invasion are also examined *in vitro* using a three-dimensional culture assay.

MATERIALS AND METHODS

Tissue Specimens and Cultured Tumor Cell Lines. Fifty patients with glioma (30 males and 20 females) were included in the current study. Twenty-five GBM, 10 AA, and 15 LOW surgical resection specimens were obtained from the Brain Tumor Bank at Brigham Women's Hospital. The mean age was 44.5 years (GBM, 53.9; AA, 45.1; LOW, 34.6). Normal brain tissue was obtained from brain injury patients. Tissue specimens were frozen in liquid nitrogen immediately after surgical removal.

The human glioma cell line U-87MG was obtained from American Type Culture Collection (Rockville, MD). The U-251MG cell line was kindly provided by Dr. D. Bigner (Duke University, Durham, NC). All cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies, Inc., Grand Island, NY).

Western Blot Assay. Frozen tissue samples were homogenized in Tris-HCl buffer (pH 7.4) containing 0.5% NP40 and protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN). Samples containing 100 μ g of protein and SDS-PAGE loading buffer with 5% β -mercaptoethanol were heated for 5 min at 100°C, and loaded on a 12% polyacrylamide gel (Bio-Rad, Hercules, CA). Electrophoretic transfer to nylon membranes (Millipore, Bedford, MA) was followed by immunoblotting with an anti-COX-2 antibody (Transduction Laboratories, Lexington, KY; Ref. 13) or tubulin (Neomarkers, Fremont, CA). This was followed by hybridization with a secondary antibody conjugated with peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). The signal was detected by chemiluminescence using the ECL-Plus detection system (Amersham Pharmacia Biotech).

Immunohistochemistry. Paraffin tissue sections (4–6 μ m) of tumor and normal brain specimens were used for COX-2 immunohistochemical studies. Slides were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 1% H₂O₂ in PBS for 30 min. Antigen retrieval was performed by incubating slides in 10 mM sodium citrate at 100°C in a microwave for 30 min. A mouse monoclonal antibody against human COX-2 (Transduction Laboratories; Ref. 13) was then applied at a dilution of 1:50 overnight at 4°C. After rinsing with PBS, the biotinylated secondary IgG antibody was applied for 30 min at room temperature. Immunoperoxidase staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA), and sections were counterstained with hematoxylin (Sigma Diagnostics, St. Louis, MO). As an additional negative control, primary antibody was omitted. Tissue from a human colon carcinoma known to overexpress COX-2 was used a positive control. The percentage of positive tumor cells was

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³ The abbreviations used are: COX, cyclooxygenase; GBM, glioblastoma; AA, anaplastic astrocytoma; LOW, low-grade astrocytoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGF, epidermal growth factor; PG, prostaglandin.

determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: 0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), and 2+ (strong). Additionally, an immunoreactive score was calculated by multiplication of the percentage of positive cells and the staining intensity, as proposed by Krajewska *et al.* (21). For heterogeneous staining patterns, each component was scored independently and the results were summed. For example, a specimen containing 25% tumor cells with strong intensity ($1 \times 2+ = 2$), 25% tumor cells with weak intensity ($1 \times 1+ = 1$), and 50% tumor cells without immunoreactivity received a score of $2 + 1 + 0 = 3$.

Cell Proliferation Assay in Monolayer Cell Cultures. The effect of NS-398 (BIOMOL, Plymouth Meeting, PA) on cell growth was determined using a MTT cell proliferation assay. NS-398 was dissolved in 100% DMSO (Sigma Diagnostics) as 1000 \times stock solutions and then diluted further in DMEM for cell culture experiments. The final concentration of DMSO for all experiments was maintained at 0.1%. All drug solutions were prepared fresh on the day of testing. U-87MG and U-251MG cells were seeded at a density of 5×10^3 per well for all cell lines in 96-well plates in DMEM containing 10% fetal bovine serum. After 24 h, fresh medium was added containing NS-398 at concentrations of 0–200 μ M. After a 4-day preincubation, the cell proliferation assay, MTT CellTiter 96 (Promega, Madison, WI), was performed. Absorbance was measured at 560 nm using a microtiter plate reader.

Tumor Spheroids. Tumor spheroids from U-87MG and U-251MG cells were initiated using the agar overlay culture method, as described by Yuhas *et al.* (22). Briefly, spheroids were formed by seeding 2×10^6 cells in 6 ml of media in 25-cm² cell culture flasks base-coated with an agar medium substrate. After 6–7 days in culture, spheroids were formed and selected for additional experiments using a stereo-microscope.

Immunohistochemistry for Ki-67 was carried out on acetone-fixed 6- μ m frozen sections of glioma spheroids using the Vectastain Elite ABC kit. Briefly, the sections were blocked with horse serum for 30 min and incubated with primary antibody against Ki-67 nuclear antigen (1:100; DAKO Corp., Carpinteria, CA) for 1 h at room temperature. Slides were then incubated with biotinylated antirabbit IgG antibody for 30 min and washed. Detection was carried out with avidin-coupled horseradish peroxidase in the presence of chromogen, which resulted in a *dark brown* staining of positive cells. Sections were counterstained with 1% methyl green (Sigma Diagnostics) and mounted in Gelmount (Biomedica, Foster City, CA). Slides where the primary antibody was omitted served as negative controls. The proliferation index (Ki-67 index) was defined as the percentage of positively stained cells from five randomly chosen fields/section, scored under light microscopy at $\times 100$ magnification using the MetaMorph version 4.1 image analysis system (Universal Image Corp., West Chester, PA).

For *in situ* detection of apoptosis, slides from spheroids were fixed in 1% paraformaldehyde with PBS for 10 min at room temperature and quenched in 3.0% hydrogen peroxide in PBS for 5 min at room temperature. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling method (Intergen Co., Purchase, NY), followed by counterstaining with 1% methyl green. Sections of postpartum rat mammary gland (Intergen Co.) served as the positive control, and slides where terminal transferase was omitted served as the negative control. The apoptotic index was defined as the number of positively stained 10 randomly chosen spheroids/fields, as described above for Ki-67.

Growth Assay and Migration Assay in a Three-Dimensional Culture System. For the growth assay, spheroids of U-87MG and U-251MG were divided into five treatment groups (control and 50, 100, 150, 200 μ M NS-398).

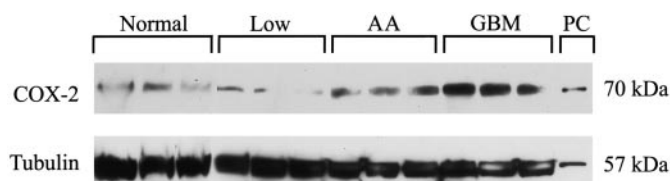


Fig. 1. Western blot analysis for COX-2 protein in glioma tissue specimens. Total protein (100 μ g) was loaded into each lane. The *bottom band* is tubulin, which was used to demonstrate equal loading of protein in each lane. PC, positive control.

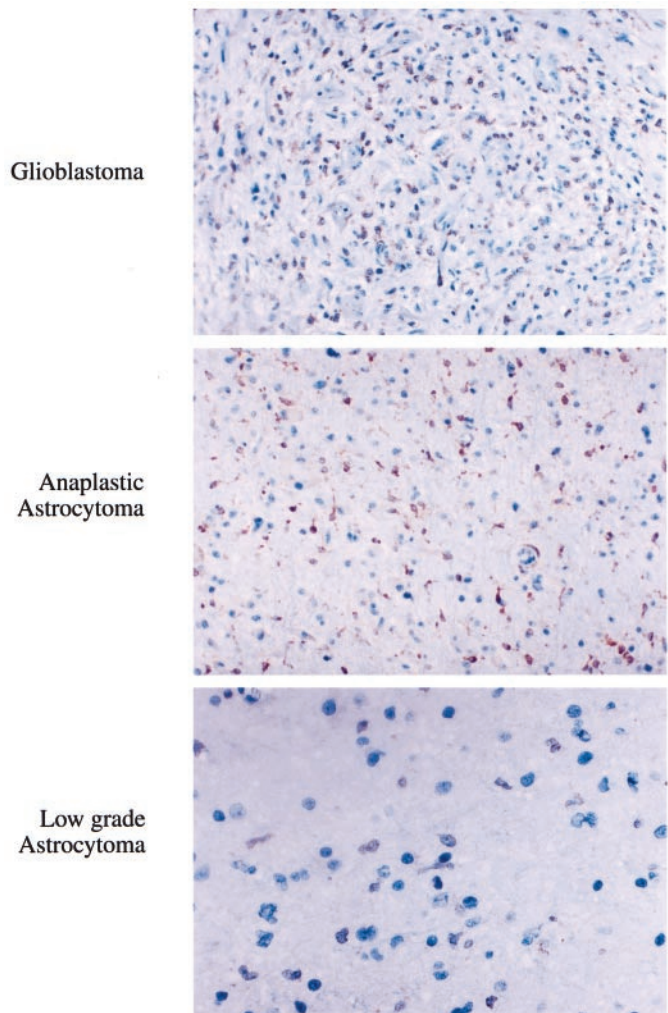


Fig. 2. Pattern of COX-2 expression in human glioma specimens. COX-2-positive staining increased in GBM and AA ($\times 200$) compared with LOW ($\times 400$).

For each group 12 spheroids were transferred individually into 24-well plates using a stereomicroscope. The 24-well plates were base-coated with 0.5 ml of DMEM-agar and filled with 1 ml of DMEM. The diameters of the spheroids were measured daily over a 12-day period using a phase contrast microscope, and the spheroid volumes were calculated. On day 12, spheroids treated with 150 μ M NS-398 were frozen in optimal cutting temperature and immunohistochemistry for Ki-67 and apoptosis (ApoTag kit) was performed as described above.

For the migration assay, spheroids were divided into five groups (control and 50, 100, 150, 200 μ M NS-398). For each group, 12 spheroids were transferred individually into uncoated 24-well plates filled with 1 ml of DMEM. Colony diameter (cellular outgrowth) was measured daily over a 5-day period, and the area was calculated. Immunohistochemistry for Ki-67 was performed on spheroids in glass chamber slides treated with 150 μ M NS-398 for 5 days. All experiments were performed in triplicate.

Preparation and Characteristics of Brain Aggregates for Tumor Invasion Assay. Fetal rat brain cell aggregates were prepared according to the procedure of Bjerkvig *et al.* (23). Fetal rat brain cell aggregates were obtained from 15-day-old fetuses of inbred Sprague Dawley rats (Taconic Farms, Inc., Germantown, NY). Rat brains were extracted under aseptic conditions, and the meningeal coverings were removed. The tissue was cut into small pieces and dissociated by serial trypsination into a single cell suspension using 0.25% trypsin (Life Technologies, Inc.). Brain aggregates were produced by seeding 6×10^6 cells distributed equally into 1 ml of DMEM in a 24-well plate coated with 0.5 ml of a nonadherent agar-medium substrate. Immature brain cell aggregates started to form after 4 days in culture. After 20 days in culture, the

Table 1 Expression of COX-2 in 50 glioma patients, as determined by immunohistochemistry

A. Score of positive tumor cells ^a					
	0	1	2	3	4
GBM	0	0	1	8	16
AA	0	0	4	5	1
LOW	3	3	8	1	0

B. Staining intensity ^b			
	0	1+	2+
GBM	0	9	16
AA	0	1	9
LOW	0	15	0

C. Immunoreactive score ^c									
	0	1	2	3	4	5	6	7	8
GBM	0	0	1	6	2	1	1	7	7
AA	0	0	1	1	2	0	5	0	1
LOW	3	3	8	1	0	0	0	0	0

^a Percentage of positive cells: 0 (0–4%); 1 (5–14%); 2 (25–49%); 3 (50–74%); 4 (75–100%).

^b Staining intensity: 0 (negative); 1+ (weak); 2+ (strong).

^c Immunoreactive score: combination of A and B (see "Materials and Methods").

cellular differentiation within the aggregates was completed and the aggregates were used for coculture invasion experiments with tumor spheroids.

Tumor-Cell Invasion Assay. Prior to confrontation, the U87-MG and U-251MG tumor spheroids were treated with 150 μ M NS-398 for 7 days. This was done to obtain effective growth inhibition, based on the results obtained from the proliferation assay. The spheroids were then transferred to 96-well dishes and confronted with fetal rat brain aggregates. The cocultures were continuously exposed to 150 μ M NS-398 during a 4-day period and analyzed using a confocal microscope (LSM410 microscope; Tissue-Tek, Sakura Finetek, Torrance, CA). All experiments were done in triplicate.

Statistical Analysis. All statistical analyses were performed by ANOVA analysis using Statview 5.0.

RESULTS

Expression of COX-2 Protein in Human Glioma Specimens and Cell Lines. To investigate the expression of the COX-2 protein, 50 tissue samples from patients with glioma were examined by immunohistochemistry and a subset by Western blot analysis. Expression of COX-2 protein was detected in all glioma and normal brain specimens

examined by Western blot analysis, regardless of histological grade (Fig. 1). By immunohistochemistry, cytoplasmic COX-2 staining was detected in neurons of histologically normal human brain (data not shown). Granular staining was also observed in the cytoplasm of tumor cells in all histological grades. COX-2-positive staining accumulated in tumor cells, in infiltrative lymphocytes, and in endothelial cells. By immunohistochemistry, the percentage of positive staining cells was significantly higher in high-grade glioma specimens than low-grade glioma specimens (Fig. 2). High-grade glioma specimens had a heterogeneous staining pattern compared with low-grade glioma specimens. Thus, for high-grade specimens the immunoreactive score had a larger range of distribution. The details of expression patterns regarding the percentage of positive cells, staining intensity, and immunoreactive score are shown in Table 1. The immunoreactive score of COX-2 staining in human glioma specimens was related to the WHO classification grade of glioma, with a higher immunoreactive score in more malignant tumors.

In addition, the expression of COX-2 was studied in the human glioma cell lines U-87MG and U-251MG by immunohistochemistry. COX-2 protein was strongly expressed in U-87MG cells, whereas the expression of COX-2 was relatively weak in U-251MG cells (data not shown).

Inhibition of Cell Proliferation by NS-398 in Monolayer Cells.

The effect of the selective COX-2 inhibitor NS-398 on proliferation of the tumor cells was studied by using the MTT cell proliferation assay. Tumor cells were treated with increasing concentrations of NS-398, and the effect on proliferation was determined after 4 days of treatment. In cultures treated with NS-398 there was no increase in the number of nonadherent cells relative to control cultures. NS-398 suppressed the proliferation of the two glioma cell lines examined in a dose-dependent manner (Fig. 3). The IC_{50} for NS-398 was ~ 80 μ M for U-251MG cells and 130 μ M for U-87MG cells. The proliferation assay was done in triplicate.

Inhibition of Growth and Migration by NS-398 in Three-Dimensional Culture System. The effect of the COX-2 inhibitor NS-398 on growth and migration was studied in a three-dimensional culture system. Tumor spheroids of U-87MG and U-251MG cell lines were treated with NS-398 in increasing concentrations, and the effect on growth and migration were examined. NS-398 significantly suppressed both growth and migration in the three-dimensional culture system of both cell lines ($P < 0.0001$). NS-398 had a more potent

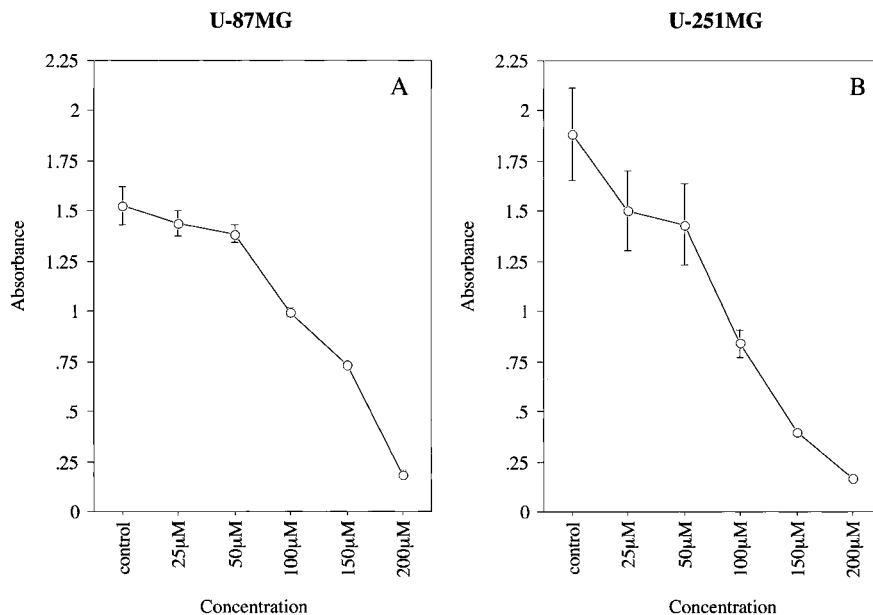


Fig. 3. Monolayer growth inhibition of U-87MG (A) and U-251MG (B) cells after exposure to different concentrations of NS-398 for 4 days. Data points represent the mean from 10 replicate wells; bars, SE. The effect of each treatment was calculated relative to vehicle (0.1% DMSO).

Table 2 Effect of NS-398 on three-dimensional culture systems

Growth assay		
NS-398 ($\mu\text{M}/\text{ml}$)	Spheroid volume (mm^3)	
	U-87MG	U-251MG
Control(0)	29.85 \pm 0.58	1.83 \pm 0.13
50	25.35 \pm 0.59 ^a	1.16 \pm 0.32 ^a
100	15.53 \pm 0.38 ^a	0.92 \pm 0.33 ^a
150	8.43 \pm 0.34 ^a	0.58 \pm 0.45 ^a
200	6.07 \pm 0.18 ^a	0.34 \pm 0.20 ^a
Migration assay		
NS-398 ($\mu\text{M}/\text{ml}$)	Colony outgrowth area (mm^2)	
	U-87MG	U-251MG
Control(0)	15.99 \pm 0.32	3.61 \pm 0.19
50	12.67 \pm 0.35 ^a	2.09 \pm 0.90 ^a
100	9.96 \pm 0.26 ^a	1.48 \pm 0.94 ^a
150	6.46 \pm 0.14 ^a	0.67 \pm 0.36 ^a
200	4.51 \pm 0.18 ^a	0.52 \pm 0.03 ^a

^a $P < 0.0001$.

effect on growth in U-251MG cells than in U-87MG cells. Complete inhibition of spheroid growth was obtained at the 200- μM dose of NS-398 in U-251MG. In contrast, 200 μM NS-398 did not completely inhibit U-87MG growth (Table 2).

To further examine the mechanism that accounts for inhibition of growth and migration, immunohistochemistry in spheroids treated with

150 μM NS-398 for 12 days was performed for Ki-67 and apoptosis (ApoTag kit). Staining revealed a significant reduction in proliferation rate in 150 μM NS-398-treated tumor spheroids; the percentage of positive cells was significantly lower in spheroids treated with NS-398 (64.8% \pm 3.5 versus 28.4% \pm 2.5, $P < 0.001$). Ki-67-positive cells were seen in the outer layer of spheroids; this layer was much thinner in treated spheroids (Fig. 4, A and B). Ki-67 staining was also done on spheroids grown on chamber slides and treated with 150 μM NS-398 for 5 days. Ki-67-positive cells were seen in areas of colony-outgrowth in controls. In the treated spheroids, the percentage of Ki-67-positive cells was significantly less than in controls (84.4% \pm 2.7 versus 58.0% \pm 2.0, $P < 0.0001$; Fig. 4, C and D). In addition, there was a significant increase in the number of apoptotic cells in the treated spheroids (1.7 \pm 0.45 versus 12.6 \pm 1.4, $P < 0.001$; Fig. 4, E and F).

Invasion Assay in Coculture System. Tumor invasion was examined using confocal microscopy 48 and 96 h after the initiation of the cocultures. Tumor cells invaded into the normal rat brain aggregate within 96 h in the absence and presence of NS-398. NS-398 did not have an inhibitory effect on tumor cell invasion in the coculture system (data not shown).

DISCUSSION

This study demonstrates the expression of COX-2 protein in the tumor cells of gliomas and normal brain tissue including, neurons,

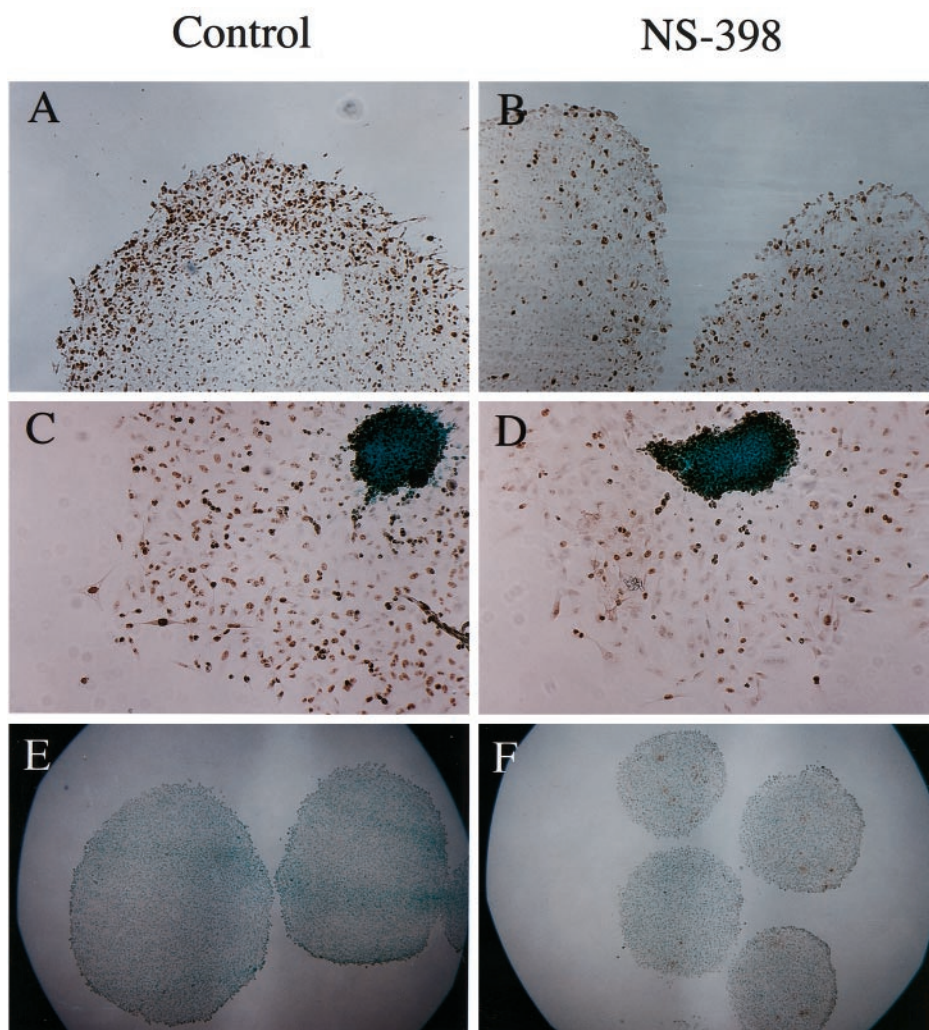


Fig. 4. Photomicrographs using immunohistochemical staining for Ki-67 staining (A–D) and for DNA fragmentation using terminal deoxynucleotidyl transferase-mediated nick end labeling staining (E and F) using the U-87MG human glioma cell line. Cross-section through a control spheroid with a highly proliferative outer layer (A) and NS-398-treated spheroid (B; $\times 100$). Migrating tumor cells in the control group (C) and NS-398 treatment group (D; $\times 100$). Apoptosis in spheroids treated with NS-398 (F) compared with control (E; $\times 40$).

infiltrative lymphocytes, and endothelial cells. As shown by immunohistochemistry, high-grade glioma tissues expressed elevated levels of COX-2 protein when compared with low-grade glioma specimens. There is a significant difference in the immunoreactive score between high-grade glioma and low-grade glioma. Our immunohistochemical analysis showed marked intertumoral heterogeneity in COX-2 expression in high-grade specimens, as previously shown for colon and esophageal cancer (5, 6, 13, 16). Enhanced COX-2 expression in the brain has also been associated with inflammatory disorders (24), trauma (25), ischemia (26), and Alzheimer's disease (27). Previous studies have reported the accumulation of COX-2-positive cells around areas of necrosis (15, 16). This could represent induction of COX-2 in the tumor cells by hypoxia or by factors released at sites of necrosis. In our study, the COX-2-positive cells did not associate with areas of necrosis that are frequently present in high-grade tumors (data not shown).

Increased levels of PGs are, in part, due to COX-2 overexpression. This has been previously reported in colon and human brain tumor tissues as compared with the normal tissue (28–30), suggesting that PGs play a role in tumor development. Thus, NS-398 may lead to a reduction in PGs levels that in turn may inhibit proliferation, migration, and induce apoptosis in the glioma cell lines we examined. A similar effect has been reported in colon (17), esophageal (13), and pancreatic carcinoma cell lines (18), where a selective COX-2 inhibitor was shown to inhibit proliferation and to induce apoptosis (19, 20).

The pathophysiological consequences of COX-2 expression in the brain are not presently known. Overexpression of COX-2 in rat intestinal epithelial cells was found to reduce the rate of apoptosis and increase the expression of the antiapoptotic proto-oncogene *Bcl-2* (31). Moreover, transfection of colon cancer cells with a COX-2 expression vector resulted in an increased metastatic potential of these cells (32).

Growth factors, tumor promoters, cytokines, and other inflammatory mediators have been found to induce COX-2 expression (1, 2). EGF is known to up-regulate the expression of the COX-2 enzyme in several cell types, such as human squamous carcinoma cells (33, 34). Among the genetic changes that are commonly observed in GBMs and that seem to be specific for this tumor stage are loss of heterozygosity of chromosome 10 along with EGF receptor amplification (35–37). Therefore, EGF receptor up-regulation may be one of the factors that contributed to the up-regulation of COX-2 we observed. Furthermore, *COX-2* gene expression has been reported to be inhibited by wild-type p53 (38). A recent study suggests an interaction between p53 and COX-2 expression. It shows that wild-type p53 leads to a decrease in COX-2 expression. In contrast, mutant p53 did not reduce COX-2 expression levels. The interactions between p53 and COX-2 may be important for understanding why levels of COX-2 are increasing with progression of tumor grade (39–41). Some groups have reported more p53 mutations in high-grade than low-grade tumors (42). The increase in mutation rate of p53 may contribute to the elevated levels of COX-2 immunostaining we observed.

We have shown that COX-2 levels are frequently elevated in high-grade gliomas. Selective inhibition of COX-2 by NS-398 in human gliomas in two different *in vitro* models reduces growth activity. This inhibition may be due, in part, to a decrease in the proliferation rate of tumor cells and an increase in the number of apoptotic cells. Local invasion into normal brain tissue is an inherent feature of malignant gliomas. NS-398 did not inhibit invasion of glioma cells, supporting the hypothesis that the mechanism of invasion is different from that of cell proliferation and migration (43).

The present study suggests that COX-2 may perform an important function in human glioma tumorigenesis. In addition, nonsteroidal

anti-inflammatory drugs may be an effective therapeutic option for high-grade gliomas.

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REFERENCES

- Smith, W. L., Garavito, M., and DeWitt, D. L. Prostaglandin endoperoxide H synthase (cyclooxygenase)-1 and -2. *J. Biol. Chem.*, *271*: 33157–33160, 1996.
- Eberhart, C. E., and DuBois, R. N. Eicosanoids and the gastrointestinal tract. *Gastroenterology*, *109*: 285–301, 1995.
- Ellis, E., Chao, J., and Heizer, M. L. Brain kininogen following experimental brain injury: evidence for a secondary event. *J. Neurosurg.*, *71*: 437–442, 1989.
- Wilson, D., Anderson, K. M., and Seed, T. M. Ultrastructural evidence for differentiation in a human glioblastoma cell line treated with inhibitors of eicosanoid metabolism. *Neurosurgery*, *27*: 523–531, 1990.
- Sano, H., Kawahito, Y., Wilder, R. L., Hashimoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.*, *55*: 3785–3789, 1995.
- Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, *107*: 1183–1188, 1994.
- Sinicrope, F. A., Lemoine, M., Xi, L., Lynch, P. M., Cleary, K. R., Shen, Y., and Frazier, M. L. Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. *Gastroenterology*, *117*: 350–358, 1999.
- Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, *58*: 409–412, 1998.
- Williams, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. M. Elevated cyclooxygenase-2 levels in min mouse adenomas. *Gastroenterology*, *111*: 1134–1140, 1996.
- Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in *Apc* Δ 716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, *87*: 803–809, 1996.
- Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.*, *57*: 1276–1280, 1997.
- Wilson, K. T., Fu, S., Ramanujam, K. S., and Meltzer, S. J. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res.*, *58*: 2929–2934, 1998.
- Zimmermann, K. C., Sarbia, M., Weber, A. A., Borchard, F., Gabbert, H. E., and Schror, K. Cyclooxygenase-2 expression in human esophageal carcinoma. *Cancer Res.*, *59*: 198–204, 1999.
- Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiura, T., and Takahashi, T. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res.*, *58*: 3761–3764, 1998.
- Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., and Ristimaki, A. Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res.*, *58*: 4997–5001, 1998.
- Deiningner, M. H., Weller, M., Streffer, J., Mittelbronn, M., and Meyermann, R. Patterns of cyclooxygenase-1 and -2 expression in human gliomas *in vivo*. *Acta Neuropathol.*, *98*: 240–244, 1999.
- Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*, *93*: 705–716, 1998.
- Molina, M. A., Sitja-Arnau, M., Lemoine, M. G., Frazier, M. L., and Sinicrope, F. A. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res.*, *59*: 4356–4362, 1999.
- Elder, D. J., Halton, D. E., Hague, A., and Paraskeva, C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin. Cancer Res.*, *3*: 1679–1683, 1997.
- Liu, X. H., Yao, S., Kirschenbaum, A., and Levine, A. C. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res.*, *58*: 4245–4249, 1998.
- Krajewska, M., Krajewski, S., Epstein, J. I., Shabaik, A., Sauvageot, J., Song, K., Kitada, S., and Reed, J. C. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. *Am. J. Pathol.*, *148*: 1567–1576, 1996.
- Yuhas, J., Martinez, A. O., and Ladman, A. J. A simplified method for production and growth of multicellular spheroids. *Cancer Res.*, *37*: 3639–3643, 1977.
- Bjerkvig, R., Steinsvag, S. K., and Laerum, O. D. Reaggregation of fetal rat brain cells in a stationary culture system. I: methodology and cell identification. *In Vitro Cell Dev. Biol.*, *22*: 180–192, 1986.

24. Lacroix, S., and Rivest, S. Effect of acute systemic inflammatory response and cytokines on the transcription of the genes encoding cyclooxygenase enzymes (COX-1 and COX-2) in the rat brain. *J. Neurochem.*, *70*: 452–466, 1998.
25. Miettinen, S., Fusco, F. R., Yrjanheikki, J., Keinänen, R., Hirvonen, T., Roivainen, R., Narhi, M., Hokfelt, T., and Koistinaho, J. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through *N*-methyl-D-aspartic acid-receptors and phospholipase A2. *Proc. Natl. Acad. Sci. USA*, *94*: 6500–6505, 1997.
26. Sairanen, T., Ristimäki, A., Karjalainen-Lindsberg, M. L., Paetau, A., Kaste, M., and Lindsberg, P. J. Cyclooxygenase-2 is induced globally in infarcted human brain. *Ann. Neurol.*, *43*: 738–747, 1998.
27. Lukiw, W. J., and Bazan, N. G. Cyclooxygenase 2 RNA message abundance, stability, and hypervariability in sporadic Alzheimer neocortex. *J. Neurosci. Res.*, *50*: 937–945, 1997.
28. Bennett, A., Tacca, M. D., Stamford, I. F., and Zebro, T. Prostaglandins from tumours of human large bowel. *Br. J. Cancer*, *35*: 881–884, 1977.
29. Maxwell, W. J., Kelleher, D., Keating, J. J., Hogan, F. P., Bloomfield, F. J., MacDonald, G. S., and Keeling, P. W. Enhanced secretion of prostaglandin E2 by tissue-fixed macrophages in colonic carcinoma. *Digestion*, *47*: 160–166, 1990.
30. Kokoglu, E., Tuter, Y., Sandikci, K. S., Yazici, Z., Ulakoglu, E. Z., Sonmez, H., and Ozyurt, E. Prostaglandin E2 levels in human brain tumor tissues and arachidonic acid levels in the plasma membrane of human brain tumors. *Cancer Lett.*, *132*: 17–21, 1998.
31. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, *83*: 493–501, 1995.
32. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. USA*, *94*: 3336–3340, 1997.
33. Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. Retinoids suppress epidermal growth factor-induced transcription of cyclooxygenase-2 in human oral squamous carcinoma cells. *Cancer Res.*, *57*: 2890–2895, 1997.
34. Sato, T., Nakajima, H., Fujio, K., and Mori, Y. Enhancement of prostaglandin E2 production by epidermal growth factor requires the coordinate activation of cytosolic phospholipase A2 and cyclooxygenase-2 in human squamous carcinoma A431 cells. *Prostaglandins*, *53*: 355–369, 1997.
35. von Deimling, A., Louis, D. N., von Ammon, K., Petersen, I., Hoell, T., Chung, R. Y., Martuza, R. L., Schoenfeld, D. A., Yasargil, M. G., and Wiestler, O. D. Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. *J. Neurosurg.*, *77*: 295–301, 1992.
36. Fujimoto, M., Fults, D. W., Thomas, G. A., Nakamura, Y., Heilbrun, M. P., White, R., Story, J. L., Naylor, S. L., Kagan-Hallet, K. S., and Sheridan, P. J. Loss of heterozygosity on chromosome 10 in human glioblastoma multiforme. *Genomics*, *4*: 210–214, 1989.
37. Rasheed, B. K., Fuller, G. N., Friedman, A. H., Bigner, D. D., and Bigner, S. H. Loss of heterozygosity for 10q loci in human gliomas. *Genes Chromosomes Cancer*, *5*: 75–82, 1992.
38. Subbaramaiah, K., Altorki, N., Chung, W. J., Mestre, J. R., Sampat, A., and Dannenberg, A. J. Inhibition of cyclooxygenase-2 gene expression by p53. *J. Biol. Chem.*, *274*: 10911–10915, 1999.
39. Bogler, O., Huang, H. J., Kleihues, P., and Cavenee, W. K. The p53 gene and its role in human brain tumors. *Glia*, *15*: 308–327, 1995.
40. von Deimling, A., Eibl, R. H., Ohgaki, H., Louis, D. N., von Ammon, K., Petersen, I., Kleihues, P., Chung, R. Y., Wiestler, O. D., and Seizinger, B. R. p53 mutations are associated with 17p allelic loss in grade II and grade III astrocytoma. *Cancer Res.*, *52*: 2987–2990, 1992.
41. Lang, F. F., Miller, D. C., Koslow, M., and Newcomb, E. W. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. *J. Neurosurg.*, *81*: 427–436, 1994.
42. Sidransky, K., Mikkelsen, T., Schwechheimer, K. Clonal expansion of p53 mutant cells is associated with brain tumour progression. *Nature (Lond.)*, *355*: 846–847, 1992.
43. Terzis, A. J., Pedersen, P. H., Feuerstein, B. G., Arnold, H., Bjerkvig, R., and Deen, D. F. Effects of DFMO on glioma cell proliferation, migration and invasion in vitro. *J. Neuro-oncol.*, *36*: 113–121, 1998.