Expression of Cyclooxygenase 2 (COX-2) in Human Glioma and in Vitro Inhibition by a Specific COX-2 Inhibitor, NS-398

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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, Rockville, MD).

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 μg 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 μm) 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
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 × 2+ = 2), 25% tumor cells with weak intensity (1 × 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× 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 × 10^5 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 μM. 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 overlayer 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-cm^2 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 stereomicroscope.

Immunohistochemistry for Ki-67 was carried out on acetone-fixed 6-μm 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 (Biomeda, 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 ×100 magnification using the MetaMorph version 4.1 image analysis system (Universal Image Corp., West Chester, PA).

For in situ determination 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 μM NS-398). 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 μM 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 μM 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 μM 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 trypsinization 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

Fig. 2. Pattern of COX-2 expression in human glioma specimens. COX-2-positive staining increased in GBM and AA (×200) compared with LOW (×400).
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 μM 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 μM 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 IC50 for NS-398 was 80 μM for U-251MG cells and 130 μM 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 suppressed the proliferation of the two glioma cell lines examined in a dose-dependent manner (Fig. 3). The IC50 for NS-398 was 80 μM for U-251MG cells and 130 μM for U-87MG cells. The proliferation assay was done in triplicate.

### Table 1

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<th>A. Score of positive tumor cells&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Percentage of positive cells: 0 (0–4%); 1 (5–14%); 2 (25–49%); 3 (50–74%); 4 (75–100%).

<sup>b</sup> Staining intensity: 0 (negative); 1+ (weak); 2+ (strong).

<sup>c</sup> Immunoreactive score: combination of A and B (see “Materials and Methods”).

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).
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% ± 3.5 versus 28.4% ± 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% ± 2.7 versus 58.0% ± 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 ± 0.45 versus 12.6 ± 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,

<table>
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<th>NS-398 (μM/ml)</th>
<th>Growth assay</th>
<th>Spheroid volume (mm³)</th>
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<th>U-251MG</th>
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* P < 0.0001.
infiltrative lymphocytes, and endothelial cells. As shown by immuno- 
histochemistry, 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 expres-
sion 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, migra-
tion, 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 inhib-
itor 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 
stestinal 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 inflamma-
tory 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 heterozy-
gosity 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 inhib-
ited 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 inva-
sion 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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Expression of Cyclooxygenase 2 (COX-2) in Human Glioma and *in Vitro* Inhibition by a Specific COX-2 Inhibitor, NS-398

Tatsuhiro Joki, Oliver Heese, Demetrios C. Nikas, et al.

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