Microglia Cyclooxygenase-2 Activity in Experimental Gliomas: Possible Role in Cerebral Edema Formation

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ABSTRACT

Purpose: Cerebral edema is responsible for significant morbidity and mortality in patients harboring malignant gliomas. To examine the role of inflammatory cells in brain edema formation, we studied the expression cyclooxygenase (COX)-2, a key enzyme in arachidonic acid metabolism, by microglia in the C6 rodent glioma model.

Experimental Design: The expression of COX-2 in primary microglia cultures obtained from intracranial rat C6 gliomas was examined using reverse transcription-PCR, Western analysis, and prostaglandin E2 (PGE2) enzyme immunoassay. Blood–tumor barrier permeability was studied in the same tumor model using magnetic resonance imaging.

Results: In contrast to C6 glioma cells, microglia isolated from intracranial C6 tumors produced high levels of PGE2 through a COX-2-dependent pathway. To test whether the observed microglia COX-2 activity played a role in brain edema formation in gliomas, tumor-bearing rats were treated with rofecoxib, a selective COX-2 inhibitor. Rofecoxib was as effective as dexamethasone in decreasing the diffusion of contrast material into the brain parenchyma (P = 0.01, rofecoxib versus control animals), suggesting a reduction in blood–tumor barrier permeability.

Conclusions: These findings suggest that glioma-infiltrating microglia are a major source of PGE2 production through the COX-2 pathway and support the use of COX-2 inhibitors as possible alternatives to glucocorticoids in the treatment of peritumoral edema in patients with malignant brain tumors.

INTRODUCTION

Malignant gliomas account for ~40% of all primary brain neoplasms. Despite aggressive treatment with surgery, radiation, and chemotherapy, most patients harboring these tumors have a <2-year survival after diagnosis. The invasive nature of gliomas not only accounts for local tumor recurrence but also is responsible for breakdown of the blood–brain barrier and cerebral edema formation in patients with malignant gliomas. Because of the associated neurological deficits, control of brain edema is frequently the first line of intervention in the management of these patients. Recently, we demonstrated that dexamethasone, which is routinely used to decrease cerebral edema in patients with malignant brain tumors, suppresses the CNS inflammatory response to gliomas in animal models (1). This observation suggested that in addition to neoplastic cells, tumor-associated inflammatory cells possibly play a role in the peritumoral edema formation in gliomas.

A number of factors has been proposed to contribute to the peritumoral edema formation in gliomas. Among these, the effect of AA metabolites, such as leukotrienes and PGs, on brain vessels has been investigated in detail. Leukotrienes, e.g., have been shown to play a role in the pathogenesis of vasogenic edema surrounding brain tumors. In fact, leukotriene analogues are being investigated to increase the permeability of the BTB to improve drug delivery into malignant gliomas (2). PGs have also been shown to play a role in the pathogenesis of a number of biological processes, such as inflammation, autoimmune diseases, and oncogenesis (3, 4). Select PG, such as PGE2 and PGF2α, can increase the permeability of peripheral microvessels and have been linked to alterations in the blood–brain barrier integrity in CNS inflammation (5–7). Although PGE2 has been detected in gliomas, the exact cellular source of its production in brain tumors remains to be determined. PGE2 can be generated from AA by either of two enzymes: (a) COX-1; or (b) COX-2. In contrast to COX-1, which is expressed constitutively in most tissues, COX-2 is not detected under resting conditions, except in brain tumors. Therefore, COX-2 is not detected under resting conditions, except in tumor-bearing rats treated with rofecoxib, a selective COX-2 inhibitor. Rofecoxib was as effective as dexamethasone in decreasing the diffusion of contrast material into the brain parenchyma (P = 0.01, rofecoxib versus control animals), suggesting a reduction in blood–tumor barrier permeability.

Conclusions: These findings suggest that glioma-infiltrating microglia are a major source of PGE2 production through the COX-2 pathway and support the use of COX-2 inhibitors as possible alternatives to glucocorticoids in the treatment of peritumoral edema in patients with malignant brain tumors.

Received 2/14/02; revised 10/9/02; accepted 10/16/02.

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1 Supported in part by the University of Wisconsin Comprehensive Cancer Center and Merck Medical School Grant Program (to B. B.).
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The abbreviations used are: CNS, central nervous system; AA, arachidonic acid; IC, intracranial; EIA, enzyme immunoassay; BTB, blood–tumor barrier; COX, cyclooxygenase; PG, prostaglandin; Gd, gadodiamide; TX, thromboxane; RT-PCR, reverse transcription-PCR; GE, General Electric; PMG, primary microglia; MRI, magnetic resonance imaging; LPS, lipopolysaccharide.

3 The abbreviations used are: CNS, central nervous system; AA, arachidonic acid; IC, intracranial; EIA, enzyme immunoassay; BTB, blood–tumor barrier; COX, cyclooxygenase; PG, prostaglandin; Gd, gadodiamide; TX, thromboxane; RT-PCR, reverse transcription-PCR; GE, General Electric; PMG, primary microglia; MRI, magnetic resonance imaging; LPS, lipopolysaccharide.
hypothesized that microglia may contribute to brain edema formation in gliomas through the expression of COX-2.

In the present study, the role of microglia in peritumoral edema formation was examined by measuring PGE$_2$ production and COX-2 expression in microglia isolated from IC rat C6 gliomas. Microglia, but not C6 glioma cells, produced high levels of PGE$_2$ through a COX-2-dependent pathway. Furthermore, when rats bearing IC C6 gliomas were treated with rofecoxib, a selective COX-2 inhibitor, diffusion of contrast material from tumors to normal brain parenchyma was diminished, suggesting a decrease in BTB permeability. These observations indicate that glioma-infiltrating microglia may be a major source of PGE$_2$ production through the expression of COX-2 and support the use of selective COX-2 inhibitors in the treatment of brain edema in patients harboring brain tumors.

MATERIALS AND METHODS

Glioma Tumor Models. All animals were housed and handled in accordance with the University of Wisconsin Research Animal Resources Center guidelines. To generate tumors, 10$^6$ C6 cells were injected either into the frontal lobes (IC) or flanks (s.c.) of Wistar rats as described previously (11).

Microglia Isolation and Culture. PMG cultures were generated from 14-day-old IC C6 tumors. Tumors were dissected from surrounding brain tissue, minced, triturated, and the trituration process was repeated until all remaining tissue was excluded from analysis.

Flow Cytometry. Characterization of PMG was accomplished by flow cytometry as described before (11). All antibodies and isotype controls were purchased from Pharmingen (San Diego, CA). FITC-conjugated antirat CD45 (clone OX-1), phycoerythrin-conjugated antirat CD11b/c (clone OX-42), and matched isotype controls were used at a dilution of 1:100. After 5 days of culture, PMG were harvested by trypsinization, washed once, and incubated for 24 h after which the nonadherent cells were rinsed, and microglia were allowed to grow for 5 days in the presence of DMEM supplemented with 10% fetal bovine serum.

COX RT-PCR. To study the expression of COX by microglia, RNA from PMG was isolated using the Bio-Rad Aquapure RNA isolation kit. One microgram of total RNA was reverse transcribed using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Ten microliters of this reaction were used for PCR using the Titanium PCR kit (Clontech) and glucose-3-phosphate dehydrogenase control primers according to manufacturer specifications. The COX primers included: (a) COX-1: forward CGA GGA TGT CAT CAA GGA G; reverse TCA GTG AGG CTG TGT TAA CG; and (b) COX-2: forward TCA AGA CAG ATC AGA AGC GA; reverse TAC CTG AGT GTC TTT GAT TG. DNA was denatured for 1 min at 95°C and amplified using 35 cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 1 min with a final 10-min extension at 72°C. DNA bands were resolved on a 1% agarose gel containing ethidium bromide.

PGE$_2$ EIA. Approximately 5 × 10$^4$ PMG or C6 cells were seeded in a 96-well plate. After 3–5 days, the cells were washed and incubated for 24 h with fresh medium with or without MF tricyclic, a COX-2 inhibitor with >500-fold in vitro selectivity for COX-2 than COX-1 (Ref. 15; Merck Frosst, Quebec, Canada). PGE$_2$ levels were measured using the Cayman Chemical PGE$_2$ EIA kit (Ann Arbor, MI) according to manufacturer specifications. For in vivo PGE$_2$ measurements, rats bearing 10-day-old IC C6 tumors were treated with rofecoxib (5 mg/kg/day) or water for 7 days via gavage. After 7 days, tumors were dissected from the brains and processed for PGE$_2$ levels using the EIA kit according to manufacturer specifications with modifications as described by Resnick et al. (16). TXA$_2$ production, another AA metabolite of the COX pathway, was measured by quantifying the levels of TXB$_2$, a stable metabolite of TXA$_2$, using the Cayman Chemical kit.

COX Western Blotting. IC and s.c. C6 tumors were carefully dissected from surrounding tissue and snap frozen in liquid nitrogen. Samples were then homogenized in harvesting buffer [25 mM Tris, 2 mM EDTA, 6.25 units/ml aprotinin, 1.3 mg/ml leupeptin, 13 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, and 20 mg/ml phenylmethylsulfonyl fluoride]. Protein concentrations were defined spectrophotometrically using a BSA protein assay (Pierce, Rockford, IL). Total protein (7 μg for COX-1 and 25 μg for COX-2 blots) and appropriate COX controls (Cayman Chemical) were separated on 10% SDS-PAGE under nonreducing conditions after a 2-min boil in Laemmli Buffer. Gels were then electroblotted onto Immobilon-P polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). After blocking for nonspecific binding with 2% BSA in TBS + 0.15% Tween 20, membranes were incubated with rabbit anti-COX-1 (1:5000) and rabbit anti-COX-2 (1:6000, both from Cayman Chemical) for 1 h at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Sigma Chemical) for 1 h at room temperature. After washing, signal was visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

To examine COX expression by microglia, 5-day-old PMG cultures were used for Western analysis. PMG cultures were incubated in fresh medium 24 h before protein isolation, and ~5 × 10$^5$ cells were harvested by trypsinization and washed in ice-cold PBS. To stimulate the expression of COX-2, some cells were incubated with Escherichia coli LPS (10 ng/ml) and IFN-γ (100 units/ml) during this 24-h period. COX detection was performed as described above. Cultured C6 cells were used as control.

BTB Permeability. Disruption of the BTB was studied in vivo using contrast-enhanced magnetic resonance described previously by Bitzer et al. (17). Rats bearing 10-day-old IC C6
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Fig. 1 The expression of COX by C6 tumors. Rat C6 glioma cells were implanted IC or s.c. (SC) in Wistar rats. After 2–3 weeks, tumors were dissected from surrounding tissue and examined for COX-1 and COX-2 protein. An equal amount of protein (7 μg for COX-1 and 25 μg for COX-2 blots) was separated on 10% SDS-PAGE. Normal (NL) brain and purified COX proteins were used as controls. The expression of COX proteins was dependent on the location of tumor propagation. IC but not s.c. tumors were strongly positive for COX-2, suggesting that brain-specific cells or factors were responsible for most of COX-2 expression in IC tumors. Representative blots from three separate experiments are shown.

Fig. 2 Morphological (left) and flow cytometric (right) characteristics of microglia isolated from rat brain tumors. Fourteen-day-old IC C6 tumors were digested enzymatically and separated using Percoll centrifugation. The 35/70 fraction, which contained microglia, was then plated in culture media and grown for 5 days. Microglia demonstrated a variety of shapes, including amoeboid, spindle, ramified, and spherical morphologies, while maintaining their flow cytometric labeling pattern (window R2).

RESULTS

COX-2 Expression in C6 Gliomas. To examine the role of non-neoplastic cells in peritumoral edema formation in brain tumors, the expression of COX-2 was examined in the C6 glioma model. Rat C6 glioma cells, which express very low levels of COX-2 in vitro (18), were implanted in two separate locations, and the expression of COX-1 and COX-2 was examined by Western analysis (Fig. 1). The expression of COX-1 and COX-2 varied with the site of tumor implantation. Although COX-2 expression was low in s.c. tumors, IC C6 gliomas demonstrated a significant up-regulation of this protein. The expression of COX-1, however, was opposite to COX-2 and higher in s.c. tumors. These findings suggested that either unknown microenvironmental factors stimulated COX-2 expression by C6 cells in the brain, or most likely, other non-neoplastic CNS cells contributed to the COX-2 expression in IC tumors. Because microglia have been shown to express COX-2 in a number of CNS pathologies (19, 20), and because they are absent in s.c. tumors (21), we hypothesized that microglia accounted for the observed COX-2 up-regulation in IC tumors. To support this, microglia were isolated from IC C6 tumors.

Microglia Characterization. Microglia isolated from IC C6 tumors demonstrated a variety of morphological characteristics, including amoeboid, spindle, ramified, and spherical shapes typical of microglia isolated from other CNS models (Fig. 2). The purity of PMG was further assessed by flow cytometry as described previously by Sedgwick et al. (14) in CNS inflammation and by us (11) in glioma models. The intensity of CD45 staining of CD11b/c-positive cells was used to distinguish microglia from neoplastic and other inflammatory cells. Thus, microglia were identified as CD45-positive, CD11b/c-positive cells, whereas C6 cells were negative for both antigens. Using these criteria, PMG were found to be 95–97% pure (Fig. 2).

COX-2 Expression by PMG. RT-PCR demonstrated that PMG expressed COX-1 and COX-2 at baseline and after activation with LPS/IFN-γ (Fig. 3, top). To assess the relative expression of each protein, Western analysis was performed on freshly isolated PMG. In contrast to C6 cells, which were negative for COX-2, PMG cultures expressed low levels of COX-2 at baseline (Fig. 3, middle). Furthermore, when PMG were activated with LPS and IFN-γ, the expression of COX-2 significantly increased. COX-1 expression, on the other hand, remained unchanged after microglia stimulation. To confirm the contribution of COX-2 to PG biosynthesis in microglia derived from gliomas, supernatants from 5-day-old PMG were collected and assayed for PGE₂ (Fig. 3, bottom). As compared with cultured C6 cells, which only secreted 400 pg of PGE₂/ml/24 h, microglia were found to be a major source of PGE₂ production in vitro. Furthermore, complete inhibition of this PGE₂ production by low doses of selective COX-2 inhibitors, such as MF tricyclic (Fig. 3, bottom) and rofecoxib (data not shown), strongly suggested that most of the microglia PGE₂ production occurred through the COX-2 pathway. To look at other downstream COX products, TXA₂ levels were also measured and found to be very low in both PMG and C6 culture supernatants (<100 ng/ml/24 h).

Effect of COX-2 Inhibition on BTB Permeability. To test whether COX-2 expression plays a role in peritumoral edema formation, we studied the permeability of the BTB in glioma-bearing rats. Ten days after IC C6 implantation, animals were treated with a 7-day course of rofecoxib, dexamethasone (positive control), and vehicle (negative control). BTB permeability was measured using Radworks V5.1 software (Applicare, GE). The purity of PMG was further assessed by flow cytometry as described previously by Sedgwick et al. (14) in CNS inflammation and by us (11) in glioma models. The intensity of CD45 staining of CD11b/c-positive cells was used to distinguish microglia from neoplastic and other inflammatory cells. Thus, microglia were identified as CD45-positive, CD11b/c-positive cells, whereas C6 cells were negative for both antigens. Using these criteria, PMG were found to be 95–97% pure (Fig. 2).
ability was examined by studying the spread of Gd from tumor tissue into the adjacent brain parenchyma on serial MRIs (Fig. 4). This technique has been used previously to assess BTB breakdown in human brain tumors (17). As expected, in vehicle-treated animals, Gd freely diffused into the brain parenchyma within 3 h of infusion. Rofecoxib, which inhibited in vivo PGE$_2$ production by 80% (72 versus 405 pg of PGE$_2$/mg tumor protein), was as effective as dexamethasone in restricting Gd diffusion into the brain parenchyma (Fig. 4). These observations strongly suggested that the BTB became less permeable after COX-2 inhibition.

**DISCUSSION**

This is the first study suggesting the possible role of microglia in peritumoral edema formation in brain tumors. As CNS macrophages, microglia are potent immune effector cells of the brain. When activated, microglia release reactive oxygen intermediates and become capable of phagocytosis, antigen presentation, and lymphocyte activation (22). Microglia and macrophages also represent a major component of infiltrating cells in malignant brain tumors (12, 13) and can account for $\approx$20% of cells in IC rat glioma models (11). Considering that microglia have been shown to express COX-2 under pathological conditions, we hypothesized that they may also play a role in peritumoral edema formation by releasing AA metabolites in gliomas.

Microglia COX-2 activity was demonstrated by COX-2 protein expression and COX-2-dependent PGE$_2$ production in freshly isolated microglia from IC tumors. Because cultured C6 glioma cells and s.c. C6 tumors expressed very low levels of COX-2, these results implied that microglia, which are only present in IC tumors, were a major source of PG production in this glioma model. To examine the function of COX-2 activity in vivo, rats bearing IC gliomas were treated with rofecoxib, a selective COX-2 inhibitor. Rofecoxib inhibited PGE$_2$ production by $\approx$80% in vivo and abolished the diffusion of contrast material from the tumors into the brain parenchyma. This decrease in the BTB permeability was similar to that achieved by dexamethasone, which is clinically used to reduce brain edema in patients with malignant brain tumors. These observations collectively support the role of microglia as a mediator of brain edema formation in gliomas.

Because the discovery of the COX-2 and development of
potent inhibitors to this enzyme, the role of PGs in the development and growth of malignant tumors has received increased attention. COX-2 expression has been detected in a number of cancers, namely: (a) colorectal; (b) esophageal; (c) lung; (d) breast; and (e) gliomas. Masferrer et al. (23) studied the expression of COX-2 in 150 archival specimens of breast, colon, prostate, and lung carcinomas and found strong staining for COX-2 in 85–100% of these tissues but weak expression in normal colonic epithelium. The expression of COX-2 has also been studied in gliomas. Using immunohistochemical techniques, Deininger et al. (24) detected the COX-2 protein in tumor and endothelial cells in human and experimental gliomas. The expression of COX-2 by gliomas has also been confirmed by others (25, 26) and reported to be related to the degree of malignancy of these tumors. Our observations strongly suggest microglia to be yet another source of PGE2 in brain tumors. Considering that PGs can regulate many physiological processes, such as immunomodulation, angiogenesis, cell growth, and differentiation (27, 28), these observations also underscore the possible role of microglia in glioma biology.

Although the exact mechanism by which selective inhibition of COX-2 decreased Gd diffusion in this glioma model is unclear, our observations suggest microglia to play an important function in this process. Considering that COX-2-dependent PGE2 production has been shown to permeabilize brain microvessels (6), microglia PGE2 may directly enhance the endothelial cell permeability within tumors, thus increasing the diffusion of Gd into adjacent brain parenchyma. Furthermore, given the role of COX-2 in tumor angiogenesis (23), its expression by microglia may be important in new vessel formation with leaky capillaries. Alternatively, the effect of COX-2 inhibition on attenuating the BTB permeability may be completely independent of microglia function and a direct effect on other COX-2-expressing cells, such as endothelial cells (24, 26). Our observations, however, argue against this possibility. The fact that IC C6 tumors had higher amounts of COX-2 protein as compared with the s.c. tumors strongly suggested that CNS-specific cells and not C6 or endothelial cells, which are present in tumors in both locations, were responsible for most of the COX-2 expression in this glioma model. Irrespective of the mechanism, our findings support the use of COX-2 inhibitors in the management of cerebral edema in brain tumor patients.

Although microglia infiltration in most human gliomas is not as strong as the rat model used in this study, our observations have direct clinical relevance. Exacerbation of cerebral edema is commonly observed in patients undergoing resection of brain tumors. Considering that microglia are activated in response to CNS trauma (29), our observations suggest that microglia COX-2 activity may be a major contributor to post-surgical brain edema in neurosurgical patients. Another condition that is associated with intractable brain edema is radiation necrosis, which can occur after irradiation of brain tumors. Histologically, a significant macrophage response is seen in the CNS in this condition. Again, our observations suggest that these macrophages may be responsible for the observed brain edema, which is occasionally intractable to dexamethasone therapy.

In summary, we have demonstrated that glioma-infiltrating microglia produce high levels of PGE2 through the expression of COX-2. Considering the role of PGE2 in angiogenesis and brain inflammation, these observations strongly support the use of selective COX-2 inhibitors as a possible alternative to glucocorticoids in the treatment of brain edema in glioma patients.

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