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Patterns of cyclooxygenase-1 and -2 expression in human gliomas in vivo

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Abstract Cyclooxygenases (COX, prostaglandin endoperoxide synthases, PGG/H synthases) are potent mediators of inflammation. While COX-1 is constitutively expressed in a wide range of tissues, COX-2 is cytokine inducible. Although COX-1 expression is observed in normal tissue, enhanced COX-2 expression has been attributed a key role in the development of edema, impeding blood flow and immunomodulation observed in pathologically altered tissues. Here, we have analyzed the expression of COX-1 and COX-2 in 50 gliomas and 10 control brains with no neuropathological alterations by immunohistochemistry; 22 glioblastoma multiforme, 9 anaplastic astrocytomas, 5 protoplasmic astrocytomas, 1 gemistocytic astrocytoma and 13 fibrillary astrocytomas were included in the study. Compared with control brains, accumulation of COX-1 was detected in 20–50% of all cells in both low- and high-grade gliomas. Double-labeling experiments revealed COX-1 expression in subsets of macrophages/microglial cells within the tumor parenchyma and in areas of infiltrative tumor growth. Of the COX-1-positive cells, 90% expressed MHC class II antigens. No COX-1 immunoreactivity was observed in tumor cells. COX-2-positive cells accumulated in tumor cells and in single macrophages/microglial cells in the immediate vicinity of necroses. Further studies are required to determine whether COX-2 is involved in the development of necrosis or, more likely, whether COX-2 is a part of the tumor tissue response to necrosis.

Key words Glioma · Cyclooxygenase · Immunohistochemistry

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Introduction

Cyclooxygenases (COX, prostaglandin endoperoxide synthases, PGH synthases) catalyze the synthesis of the eicosanoid prostaglandin metabolites PGG_2 and PGH_2, which are metabolized to PGE_2, PGD_2 and $PGF_{2\alpha}$, the thromboxane TXA_2, or the functional antagonist of TXA_2, PGI_2 (prostacyclin). Eicosanoids are differential regulators of blood perfusion. PGE_2 and PGE_1 are powerful dilators of the vasculature resulting in a decrease of the peripheral resistance and a consequent decrease of blood pressure. Inversely, $PGF_{2\alpha}$ increases the blood pressure. Apart from affecting the cardiovascular system, differential eicosanoid effects can be observed in the hematopoietic system and a broad range of organs like the kidney, smooth muscle and the gut. Further, eicosanoids are potential modulators of the immune system. PGs of the E series mediate the liberation of histamine from mast cells during anaphylaxis, inhibit the liberation of oxygen radicals from activated leukocytes, the differentiation of B cells and the proliferation of T cells. Macrophage-derived PGE_2 has been shown to inhibit the liberation of lymphokines from activated T cells.

Two COX isoforms have been described [4]. While COX-1 is an enzyme of 66 kDa that is constitutively expressed in a range of tissues, COX-2 is a 70-kDa interferon gamma (IFN-γ)-inducible homologue that shares 61% sequence identity with COX-1. The expression of COX-1 and COX-2 has been reported to be associated with the complex derangements observed during a variety of diseases of the brain. Following trauma, prostaglandins cause vascular damage [2]. Cerebral ischemia leads to upregulation of COX-2 message, protein, and reaction products in the injured hemispheres [14]. In rat experimental allergic encephalomyelitis (EAE), encephalitogenic cells are capable of inducing the expression of COX-2 via the T cell cytokine IFN-g [11]. In glioblastoma cells, inhibitors of eicosanoid biosynthesis suppress proliferation and promote astrocytic differentiation [19]. To evaluate a possible role of cyclooxygenases in brain tumors, we have

Table 1 Summary of glioma patients and immunohistochemical labeling scores for COX-1 and COX-2. Labeling score are given as: *0* no labeled cells, *1* up to 5% labeled cells, *2* up to 20% labeled cells, *3* up to 50% labeled cells, *4* more than 50% labeled cells. Patient age is shown in years (*COX* cyclooxygenase, *GBM* glioblastoma multiforme, *ANA* anaplastic astrocytoma, *PROA* protoplasmic astrocytoma, *FA* fibrillary astrocytoma, *GA* gemistocytic astrocytoma, *GFAP*$^+$ glial fibrillary acidic protein-positive, *M* male, *F* female)

Patient	Gender/ age	Diagnosis	WHO	COX-1	COX-2 in GFAP$^+$ cells
1	M/68	GBM	4	3	2
2	M/47	GBM	4	3	1
3	F/66	GBM	4	3	2
4	M/58	GBM	4	3	1
5	F/38	GBM	4	3	2
6	F/53	GBM	4	3	0
7	M/58	GBM	4	3	1
8	M/67	GBM	4	3	0
9	F/60	GBM	4	4	2
10	M/50	GBM	4	3	2
11	F/52	GBM	4	3	1
12	F/79	GBM	4	3	2
13	F/76	GBM	4	3	2
14	M/46	GBM	4	2	0
15	M/70	GBM	4	3	1
16	M/62	GBM	4	3	2
17	M/54	GBM	4	3	0
18	M/54	GBM	4	2	1
19	M/48	GBM	4	3	2
20	M/54	GBM	4	3	0
21	M/47	GBM	4	3	1
22	F/36	GBM	4	3	1
23	F/37	ANA	3	3	2
24	F/35	ANA	3	3	2
25	M/26	ANA	3	3	0
26	M/39	ANA	3	3	1
27	M/21	ANA	3	3	1
28	M/31	ANA	3	3	2
29	F/30	ANA	3	3	0
30	M/40	ANA	3	3	0
31	M/51	ANA	3	3	1
32	M/38	PROA	2	3	1
33	M/22	PROA	2	3	1
34	M/32	PROA	2	3	0
35	F/34	PROA	2	3	0
36	F/33	PROA	2	3	0
37	F/56	GA	2	3	1
38	M/50	FA	2	3	1
39	M/57	FA	2	3	0
40	M/44	FA	2	2	1
41	M/64	FA	2	3	1
42	F/57	FA	2	3	0
43	F/31	FA	2	3	0
44	F/51	FA	2	4	0
45	M/34	FA	2	3	0
46	F/30	FA	2	3	0
47	F/41	FA	2	3	0
48	F/55	FA	2	3	0
49	M/54	FA	2	3	0
50	M/34	FA	2	3	0

now analyzed the expression of COX-1 and COX-2 in a panel of 50 gliomas and 10 control brains by immunohistochemistry; 22 glioblastoma multiforme, 9 anaplastic astrocytomas, 5 protoplasmic astrocytomas, 1 gemistocytic astrocytoma and 13 fibrillary astrocytomas were included in this study.

Materials and methods

Fifty brain tumor specimens (22 glioblastoma multiforme, 9 anaplastic astrocytomas, 5 protoplasmic astrocytomas, 1 gemistocytic astrocytoma and 13 fibrillary astrocytomas) and 10 control brains were analyzed. All tumors were resected at the Department of Neurosurgery in Tuebingen or at the Department of Neurosurgery of the Schildautalklinik, Seesen (Table 1). Control brains were obtained from autopsies at the Department of Pathology in Tuebingen (Table 2). All tissues were fixed in buffered 4% formalin (pH 7.4) and embedded in paraffin by routine methods. Sections, 5 µm thick, were deparaffinized and rehydrated. For antigen retrieval, the sections were immersed in 0.01 M citrate buffer and irradiated in a microwave oven set at 750 W, five cycles of 5 min. Endogenous peroxidase was blocked with 1% H_2O_2 in methanol and the slices were consequently incubated with porcine serum. Monoclonal antibodies against COX-1 and COX-2 (Santa Cruz, Calif.) were diluted in 1% bovine serum albumin (BSA), TBS (TRIS-balanced salt solution, pH 7.5, containing 0.025 M TRIS, 0.15 M NaCl) at a dilution of 1:400. Secondary antibody (biotinylated anti-goat IgG, Dakopatts, Glostrup, Denmark) was diluted at 1:400 in BSA/TBS and applied to the slices for 30 min. Streptavidin-biotin horseradish peroxidase complex (ABC) (Dakopatts) diluted 1:400 was subsequently applied for 30 min. Labeled antigen was visualized with standard diaminobenzidine techniques (Sigma, St. Louis, Mo.).

In double-labeling experiments, we first labeled a cell type-specific antigen using the alkaline peroxidase-antialkaline peroxidase (APAAP) procedure and then visualized COX-1 or COX-2 using the ABC technique as described above. Briefly, slices were deparaffinized, irradiated in a microwave oven for antigen retrieval and incubated with nonspecific serum as described above. Subsequently, the differentiating antibodies directed against glial fibrillary acidic protein (GFAP) (Boehringer, Mannheim, Germany), leukocyte common antigen (LCA), HLA-DR, -DP, -DQ and CD68 (all Dakopatts) were added to the slices all at a dilution of 1:100 in TBS/BSA. Visualization was achieved by adding rabbit anti-mouse IgG diluted at 1:20 in TBS for 30 min and APAAP

Table 2 Control patients and immunohistochemical labeling scores for COX-1 and COX-2. Labeling score is given as: *0* no labeled cells, *1* up to 5% labeled cells, *2* up to 20% labeled cells, *3* up to 50% labeled cells, *4* more than 50% labeled cells. Patient age is shown in years (*M* male, *F* female)

Patient	Gender/ age	Diagnosis	COX-1	COX-2 in GFAP$^+$ cells
1	M/39	Myocardial infarction	1	0
2	M/26	Cardiac failure	1	0
3	M/32	Opiate abuse	2	0
4	F/39	Pulmonary embolism	1	0
5	F/56	Hemorrhagic shock	1	0
6	M/54	Cardiopulmonary decompensation	2	0
7	F/92	Toxic shock	2	0
8	M/44	Myocardial infarction	1	0
9	M/72	Pulmonary embolism	1	0
10	F/76	Hemorrhagic shock	1	0

complex diluted 1:80 in TBS for 30 min, followed by Fast Blue BB salt chromogen-substrate solution yielding a blue reaction product. To avoid antibody cross-reactivity in double-labeling experiments, slices were irradiated again in a microwave for 20 min in citrate buffer [6]. Complete inhibition of alkaline phosphatase function was achieved as previously described [1]. Then COX-1 and -2 were immunolabeled as described above.

Adjacent sections were stained with irrelevant isotype-matched antibodies (mouse monoclonal anti-desmin, Dakopatts) as a control. All sections were counterstained with hematoxylin.

The level of target antigen expression was quantified as follows: samples with no positive cells were classified as negative (0), samples with singular (up to 5%) positive cells as weakly positive (1), samples with 5–20% positive cells as moderately positive (2), samples with up to 50% positive cells as strongly positive (3), and samples with more than 50% positive cells as very strongly positive (4). Three high-power fields were counted for each sample and antigen. Statistical analysis was performed using the Student's *t*-test.

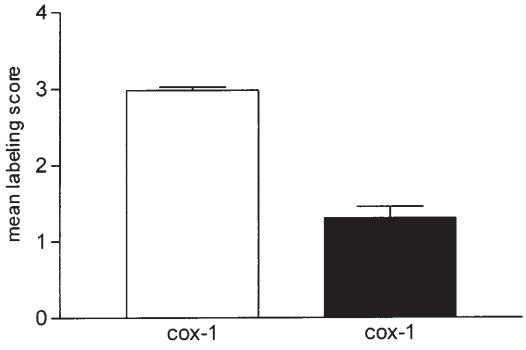


Fig. 1 In gliomas (*open bar*), a significantly ($P < 0.0001$) higher number of COX-1 expressing macrophages/microglia cells (mean score 2.980, SEM = 0.045) are found than in the control tissues (*black bar*; mean score 1.3, SEM = 0.153) (*COX* cyclooxygenase)

Results

In control brains without neuropathological alterations, COX-1 immunoreactivity was detected in macrophages/microglial cells. These cells accounted for 5–10% of all cells (Table 2). Further, singular neurons and few endothelial cells expressed COX-1. COX-2 immunoreactivity was observed in singular neurons located in the neocortex and allocortex. No endothelial or glial cells expressed COX-2.

In glioma, there was a marked increase in the number of COX-1-expressing cells. Using the student's *t*-test, we detected a significantly ($P < 0.0001$) higher number of COX-1-expressing macrophages/microglia in gliomas (mean score 2.98, SEM = 0.045) than in control tissue (mean score 1.3, SEM = 0.153; Fig. 1). COX-1 was expressed in 20–50% of all cells in both low- and high-grade gliomas independent of their degree of malignancy (Table 1). COX-1-expressing cells were characterized by morphological hallmarks of both ramified and ameboid macrophages/microglia (Fig. 2a). They were localized in the tumor parenchyma and in areas of infiltrative tumor growth. Cells with membrane-bound and cytoplasmic COX-1 expression could be readily distinguished. These were not confined to distinct areas within the tumor specimens. To characterize the cellular origin of COX-1 expression, we performed double-labeling experiments; 90% of COX-1-positive cells coexpressed MHC class II molecules (Fig. 2b) and about 1% COX-1-positive cells coexpressed LCA. Nearly 70% of COX-1 positive cells surrounding areas of necrosis and approximately 10% of COX-1 positive cells in the tumor parenchyma coexpressed the CD68 antigen. No glial tumor cells were identified to express COX-1.

Single COX-2-positive cells were disseminated throughout the tumor parenchyma of low- and high-grade glioblastomas, including areas of zonal necrosis (Table 1). Most strikingly, we detected an accumulation of COX-2-positive cells in the immediate vicinity of necroses (Fig. 2c). However, COX-2-positive cells did not completely surround entire areas of necroses. Only distinct perinecrotic areas contained COX-2-positive cells. Single endothelial cells also expressed COX-2. Double-labeling experiments revealed that 20–50% of COX-2-positive cells surrounding areas of necrosis expressed GFAP (Fig. 2d). Single perinecrotic COX-2-positive cells expressed CD68. No colocalization of COX-2 and MHC class II antigens was observed.

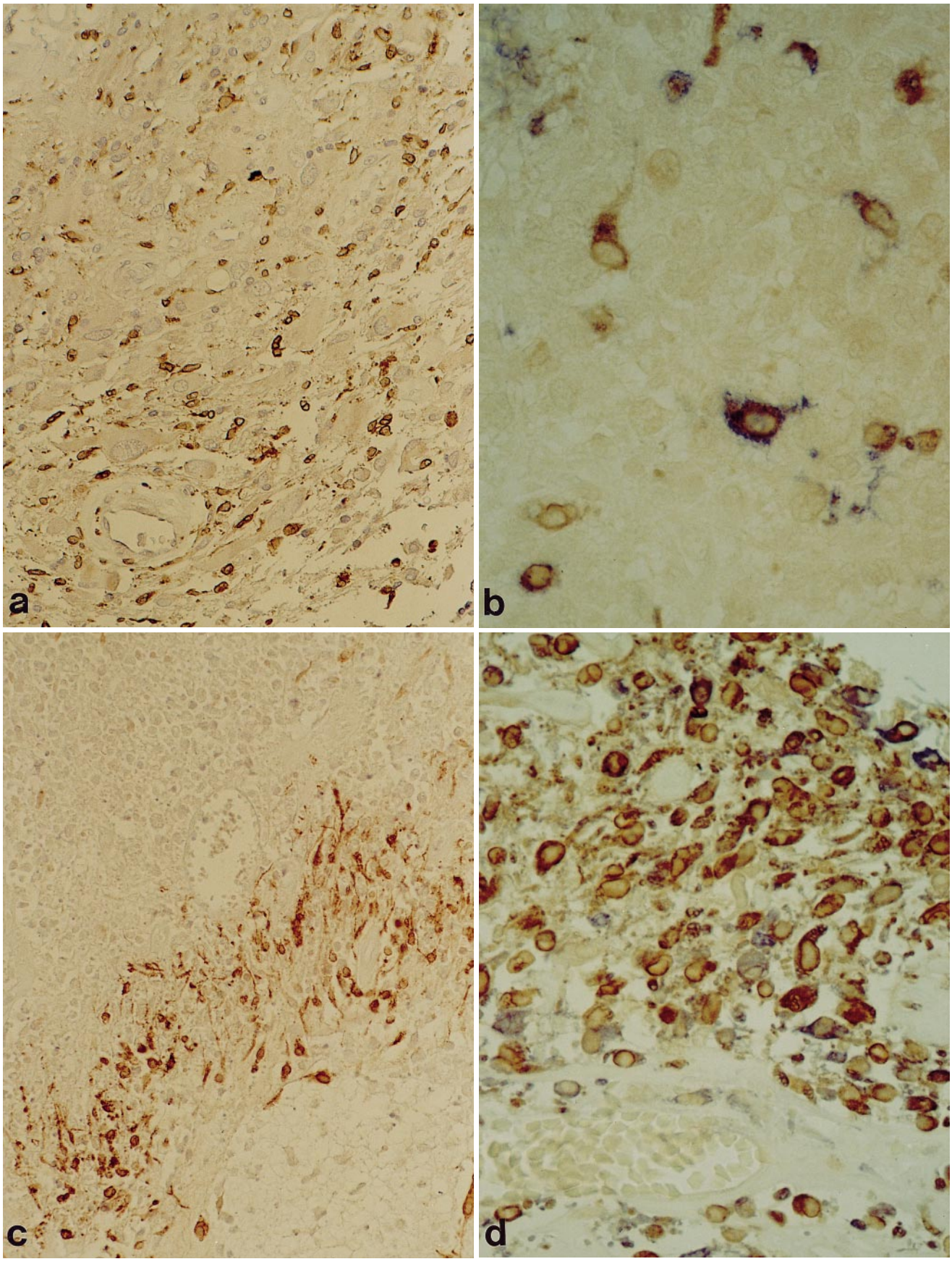
Discussion

In the present study, we have analyzed COX-1 and COX-2 expression in 50 gliomas (22 glioblastoma multiforme, 9 anaplastic astrocytomas, 5 protoplasmic astrocytomas, 1 gemistocytic astrocytoma and 13 fibrillary astrocytomas) and 10 control brains. This is the first description of the differential expression of COX-1 and COX-2 in gliomas. COX-1 expression was limited to macrophages/microglia; 20–50% of all cells were COX-1 positive, independent of the malignancy of the tumors. Accumulation of COX-2 expression was found in glioma cells and in macrophages/microglia in the immediate vicinity of necroses.

High numbers of macrophages/microglial cells in glial tumors of the CNS have been described before, and their participation in the regulation of inflammation has been suggested [13, 16]. Using immunohistochemistry, it has been shown that low-grade malignant gliomas contain around 26% macrophages/microglial cells and glioblastoma multiforme approximately 33% [12].

The pathophysiological role of COX-1 expression has been doubted due to its constitutive expression in a wide range of organs, including the brain [3]. In contrast, en-

Fig. 2a–d Patterns of COX-1 and COX-2 expression in human glioblastoma multiforme. **a** COX-1 expression (*brown color*) is detected in macrophages/microglia in the tumor parenchyma and in areas of infiltrative tumor growth. **b** Double-labeling experiments reveal coexpression of MHC class II molecules (*blue*) in COX-1-positive cells (*brown*). **c** COX-2 expression (*brown*) is found predominantly surrounding areas of necrosis. **d** Double labeling experiments demonstrate GFAP immunoreactivity (*blue*) in 20–50% of COX-2-positive cells (*brown*). **a, b** × 200; **c, d** × 400


a
b
c
d

hanced COX-2 expression in the brain has been associated with inflammatory disorders [5], trauma [10], ischemia [17] and Alzheimer's disease [7]. The pathophysiological consequences of COX-1 and COX-2 expression in the brain have not been resolved. Recent reports suggest the involvement of COX-2 expression in the production of PGE_2, thus causing the febrile reaction in response to proinflammatory stimuli [9]. Further, COX-2 expression is associated with the increased production of vasodilatory prostanoids in the brain [15]. Prostaglandins, in addition, have been shown to promote tumorigenesis by modulating BCL-2 expression in colon cancer cells [18]. These findings are of particular interest, since expression and function of COX-1 and COX-2 can be selectively inhibited by glucocorticoids, non-steroidal anti-inflammatory drugs, and other agents.

The detection of distinct cellular and spatial COX-1 and COX-2 expression patterns in gliomas thus suggests a role in the interactions between tumor and host. Further studies are required to determine whether COX-2 is involved in the development of necrosis or, more likely, whether COX-2 is a part of the tumor tissue response to necrosis.

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