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Cyclo-Oxygenase Inhibitors and Thromboxane Synthase Inhibitors Differentially Regulate Migration Arrest, Growth Inhibition and Apoptosis in Human Glioma Cells

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Summary

We have previously identified thromboxane synthase as an important regulator of glioma cell migration. Inhibitors of this enzyme abrogate cell motility and induce apoptosis. However, the formation rate of thromboxanes is indirectly dependent on the activity of cyclo-oxygenase, which represents the rate-limiting step in the synthesis of prostaglandins and thromboxanes. In this study we have analyzed the expression of COX-1 and COX-2 in glioma cell lines and biopsies of glial tumors. In normal glia no expression of both COX isoforms was present, however, reactive astrocytes and glial tumors of all grades demonstrated expression of both COX-1 and COX-2. In contrast to inhibitors of thromboxane synthase, selective and non-selective cyclo-oxygenase inhibitors did not block cell motility. Specific COX-2 inhibitors resulted in growth inhibition and induction of intracellular DNA fragmentation indicative of apoptotic cell death. Treatment of glioma cells with thromboxane synthase inhibitors had a synergistic effect on induction of apoptosis by camptothecin, whereas COX inhibitors had not. Furthermore, combined treatment using COX-2 inhibitors and specific thromboxane synthase inhibitors did not show a synergistic increase of apoptosis. These data indicate that COX inhibitors and thromboxane synthase inhibitors influence apoptosis in glioma cells through different pathways. We hypothesize that, in contrast to the COX-2 inhibitors, thromboxane synthase inhibitors block the invasive phenotype of glioma cells and therefore increase the pro-apoptotic disposition of the cells and increase the susceptibility to induced apoptosis. This effect may be independent of prostaglandin synthesis controlled by cyclo-oxygenases.

Keywords: Glioma; invasion; migration; apoptosis; cyclooxygenase; thromboxane synthase.

Abbreviations

Abbreviations used are as follows: *COX*, Cyclo-oxygenase; *ELISA*, enzyme-linked immunosorbent assay; *FCS*, fetal calf serum; *GBM*, glioblastoma; *GFAP*, glial fibrillatory acid; *MEM*, minimal essential medium; *MOX*, methoxime; *PCR*, polymerase chain reaction; PBS, phosphate-buffered saline; *PG*, prostaglandins; *SDS*, sodium dodecyl sulfate; Thx, thromboxane; *WHO*, World Health Organization.

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Introduction

The Prostanoid synthesis appears to be important in pathogenesis and progression of cancer because arachidonic acid metabolites affect several important cellular behaviors such as mitogenesis, cellular adhesion, invasion, and apoptosis [5]. In many cancers the levels of prostanoid synthesis exceed that of normal tissues. This has been demonstrated for neoplasms of breast, lung, colon, and neuroepithelial tumors of the central nervous system [4, 6, 8]. Human malignant gliomas show an overall increased formation of prostaglandins (PG) and thromboxane when compared with meningiomas and normal brain. The total synthesis capacity of arachidonic acid metabolites in these tumors, specifically thromboxane B_2 (Thx B_2) and prostaglandin D_2 (PGD_2) determined by high resolution gas chromatography in tumor lysates, correlates to the percentage of cells in S-phase [12]. However, the cellular origin of these metabolites and their functional relevance remain unclear.

Using a monolayer-migration assay we have selected highly migratory glioma cells in vitro. Differential mRNA display identified thromboxane synthase overexpression in highly migratory subpopulations of glioma cells [36]. We could subsequently demonstrate that specific inhibitors of thromboxane synthase block migration of glioma cells in vitro and immunohistochemistry demonstrated expression of thromboxane

synthase in glial tumors [20]. We could further demonstrate that the anti migratory effect of specific thromboxane synthase inhibitors appears to be associated with caspase activation followed by DNA fragmentation and subsequent apoptotic cell death in migration arrested glioma cells [51]. This observation raises the question whether this mechanism could be exploited as an anti-invasive strategy for gliomas. However, it remains unclear whether the decreased formation of thromboxane or an altered balance of thromboxane, prostaglandins, and arachidonic acid mediates this effect. Furthermore, thromboxane synthase and cyclo-oxygenases (COX) may act in conjunction in the regulation of motility and invasion, because the rate-limiting enzyme in the formation of prostanoids is cyclo-oxygenase. Two isoforms have been identified, designated as COX-1 and COX-2, which show differential subcellular distribution. COX-1 is a constitutively expressed isoform present in most tissues, thought to mediate the synthesis of prostanoids required for normal physiological tissue functions. The COX-1 promoter contains no classical TATA box, a feature typical for house keeping genes. In contrast, the COX-2 promoter is induced by several stimuli 10- to 80-fold and contains a TATA motive, numerous *cis*-acting elements, including sites for transcription factors NF-κB, NF-IL6, CRE and is regulated by tyrosine kinase activating growth factors as well as the protein kinase C (PKC) pathway (reviewed in 48). COX-2 is not detectable in most normal tissues but is induced in several malignant tumors [9, 29, 39, 40, 41]. Overexpression of COX-2 in intestinal endothelial cells results in enhanced binding of extracellular matrix, decreased intercellular adhesion, and a decreased susceptibility to apoptosis [49]. Each of these changes may increase the tumorigenic potential of the cells. Not surprisingly, COX-2 overexpression in colon carcinoma cells increases the invasiveness of these cells, which can be reversed by inhibitors of cyclo-oxygenase [50]. Generally, inhibitors of cyclo-oxygenase reduce the incidence of colon and breast cancer. In experimental systems inhibitors of arachidonic acid metabolism protect against mammary, esophageal, oral, and colon cancer [21, 23, 34, 35, 42]. For example, sulindac decreases the number and size of colon polyps in Min mice [7] and in humans with familial ademomatous polyposis [14]. These data suggest that both thromboxane synthase and cyclo-oxygenases play a role in the regulation of progression and invasiveness of neoplasms possibly including gliomas. In this study we investigate the differential role of thromboxane synthase and cyclo-oxygenase in the regulation of motility, proliferation and apoptosis in human gliomas.

Material and Methods

Cells and Cell Culture

Human glioma cell lines (NCE-G-22, G-28, G-44, G-59, G-62, G-112, G-120, G-130, G-168 and U-251) were propagated in minimal essential medium (MEM, Biochem, Berlin, Germany) with 10% fetal calf serum and were passaged using trypsinization at regular intervals depending on growth characteristics. The migration characteristics [15, 16, 19] of the cell lines and the synthesis profiles for thromboxane synthase have been demonstrated earlier [20]. For selected experiments G-44 was used because it demonstrates expression of thromboxane synthase, COX-1 and COX-2 under standard tissue culture conditions. Human astrocytes from normal brain were established as described by Westphal *et al.* 1997 [45]. Astrocytes were maintained in Ham's F12/Dulbeccos MEM (50:50) (Biochem) containing 20% FCS. For astrocyte cultures positive staining (> 95%) for GFAP was confirmed prior to use in experiments. Specific COX-2 inhibitors sulindac (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), piroxicam (Sigma-Aldrich Chemie GmbH), sulindac sulfide and sulindac sulfone (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) and the nonselective inhibitor acetylsalicylic acid (ASA) (Bayer, Leverkusen, Germany), the specific thromboxane synthase inhibitors furegrelate (Sigma, Deisenhofen, Germany), dazmegrel (Pfizer, Karlsruhe, Germany) were prepared according to manufacturer's instructions for use in tissue culture. Camptothecin (Sigma) was prepared as a stock solution at 10 mg/ml in DMSO.

Expression of Cyclo-oxygenase and Prostaglandin Formation

RT-PCR. Total RNA was isolated from monolayer cultures by phase separation using TriStar™ (Hybaid, Heidelberg, Germany) followed by isopropanol precipitation and quantified by absorbance measurement at 260/280 nm. Reverse transcriptase synthesis of cDNA was done using a First Strand Synthesis Kit (Stratagene, La Jolla, Ca, USA). Primers for COX-1 (5′CTT GAC CGC TAC CAG TGT GA 3′ and 5′AGA GGG GAG AAT ACG AGT GT 3′) and COX-2 (5′ATC TAC CCT CCT CAA GTC C 3′ and 5′ATT TCA TCT GCC TGC TCT G 3′) were designed with the help of OLIGO 4.0. The amplification of cDNA (1 μl of RT product, 1 μl of each primer, 0.1 μl of Taq Gold polymerase (Perkin Elmer/Cetus, Foster City, Ca, USA) for COX-1 and Taq polymerase (Perkin Elmer) for COX-2 and 2 μl of nucleotides in 2 μl of 10× buffer) was allowed to run for 40 cycles (COX-1) and 36 cycles (COX-2) (1 min at 56 °C and 2 min at 72 °C). Aliquots of 9 μl were collected and run on a 2% agarose gel, stained with ethidium bromide and photographed under UV illumination. Specific amplification was confirmed by sequencing of PCR products.

Immunohistochemistry. Paraffin sections of surgical specimens from glial tumor were washed, dehydrated and incubated in 0.15% H_2O_2 in PBS for 30 minutes to block endogenous peroxidase activity. Sections were then pre-incubated with 10% horse serum for 60 minutes prior to adding the primary goat polyclonal antibody to human COX-1 or COX-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by overnight incubation at 4 °C. A biotinylated anti-goat IgG (Vector Laboratories, Burlingame CA, USA) was added at a 1:200 dilution for 1 h, detection was carried out using Vectastain and DAB Substrate Kit (Vector Laboratories) followed by counterstaining with hemalum. Sections used for double staining were pre-incubated with 10% horse and 10% swine serum for 60 minutes followed by overnight incubation at 4 °C with the

primary goat polyclonal antibody to COX-1 or COX-2 at a 1:500 dilution (Santa Cruz Biotechnology) and rabbit anti glial fibrillary acidic protein (GFAP, DAKO Diagnostika GmbH, Hamburg, Germany) antibody at 1:250. A biotinylated anti-goat IgG (Vector Laboratories) and an anti rabbit IgG (DAKO) were added for 1 h and incubated with StreptABComplex/AP (DAKO) for 30 minutes and Rabbit PAP (DAKO) for 40 minutes, followed by detection with DAB Substrate Kit (Vector Laboratories) and BCIP/NBT Substrate System (DAKO). For negative controls omission of the specific antibodies and staining with an irrelevant primary polyclonal antibody (anti human immunodeficiency virus (HIV) p24 (DAKO) was used.

Elisa Detection of Prostaglandins. PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$ were analyzed in cell culture supernatants. The concentration of PGI_2, a short lived eicosanoid, was quantified by measurement of the stable metabolite 6-keto-$PGF_{1\alpha}$, which is produced by a non-enzymatic hydration of PGI_2. 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ were detected by an enzyme immunoassay using alkaline phosphatase-labeled PG conjugates according to manufacturer's instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany). PGD_2, was converted to a stable methoxime derivative. Its concentration was detected by use of Prostaglandin D_2-MOX Enzyme Immunoassay Kit (Cayman Chemicals, Coulter-Immunotech Diagnostics, Krefeld, Germany) with an acetylcholinesterase molecule-labeled PGD_2-MOX tracer. 5×10^5 cells were incubated in a T 25 flask in MEM containing 10% FCS. Cultures were rinsed in serum-free media (Ham's F 12/DMEM (50%/50%, v/v), containing 1 mM glutamine, 2 mM Sodiumpyruvat, 10 µg/µl insulin, 10 µg/µl transferrin, 10^{-8} M selenium, 1 mg/ml BSA-1% linoleic acid complex) and maintained for another 48 h in 7 ml of serum-free media. Supernatants were collected, centrifuged for 10 minutes at 2000 rpm and stored at −80 °C. The concentration of prostaglandins was normalized to the cell number.

Cell Growth. Glioma cells were seeded at 2000 cells per well in a 96 well plate and incubated for 30 minutes on ice to allow attachment. Specific COX-2, non-selective COX inhibitors, and thromboxane synthase inhibitors were added daily and replicate wells were fixed daily in 1% glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany). Cells were stained for 1 h with 50 µl crystal violet, the cell number was determined in triplicate by absorbance measurements at 540 nm of destained nuclei after 10% SDS treatment.

Intracellular DNA Fragmentation

A photometric enzyme-immunoassay was used for quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (Boehringer, Mannheim, Germany) [3]. In this assay the intracellular enrichment of mono- and oligonucleosomes, which occur after activation of endogenous endonucleases is due to the fact that in apoptosis DNA degradation occurs several hours before plasma membrane breakdown. In contrast, necrotic cell death results in early release of fragmented DNA into the culture supernatant. 20.000 cells were seeded into 96-well plates and cells were allowed to adhere for 4 hours before treatment. Cultures were rinsed and inhibitors were added. After incubation (4 to 48 hours) cultures were centrifuged at 200× g and culture supernatants were collected. Cells were lysed and 20 µl of lysate or 20 µl of corresponding supernatant were used in Cell Death Detection ELISA^{Plus} (Boehringer Mannheim) according to manufacturer's instructions. A serial dilution of oligonucleosomal DNA fragments was included as a positive control, which also allows 1 to determine the range of linear detection. A specific enrichment factor of mono- and oligonucleosoms released into the cytoplasm was calculated by absorbance of treated sample divided by absorbance of the corresponding untreated control. Data reported represent the mean of triplicate determination.

Cytotoxicity Assay

Cells were seeded at a density of 2500 cells per 100 µl MEM + 10% FCS in a 96-well plate and incubated for 48 hours. Culture media were changed and inhibitors of arachidonic acid metabolism were added. After 48 hours cells were incubated with 10% (vol/vol) Alamar Blue (Biosource, Nivelles, Belgium) at 37 °C for 4 hours. Fluorescence of metabolized dye in each well, reflecting cellular metabolic reduction, was read on a microplate fluorometer (SLT-Tecan, Crailsheim, Germany) with 530 nm excitation and 590 nm emission filters.

Cell Migration Assay

Cell Migration was quantified using a monolayer migration assay [15, 28] which measures expansion of a cell population on surfaces. Ten-well slides (Dynex Technologies, Denkendorf, Germany) were coated with AES (3-aminopropyltriethoxysilane) (Sigma) to optimize protein and cell adhesion. Slides were then passively coated with merosin (100 µg/ml) and a cell sedimentation manifold (CSM) was placed over the slides containing 50 µl of culture media (Creative Scientific Methods, Mesa, AZ, USA). Cells were seeded in a volume of 1 µl MEM (2000 cells) and slides were incubated for 8 h at 37 °C. The CSM was removed and the circular area occupied by attached cells in each well was imaged using a digital CCD camera (TK-1280E, JVC). Images were digitized for quantification with an image analysis system (Quantimed 500, Leica, Hamburg, Germany). Serial images were captured for up to 24 hours. Quantitative migration scores were calculated as the increase of the radius beyond the initial radius of the object and migration rates were determined by regression analysis. These measurements represent changes in the geographical distribution of the cell population and do not reflect movement of individual cells.

Results

COX Expression and Prostaglandin Formation in Human Glioma Cell Lines

We have previously reported that thromboxane synthase mRNA is expressed in most glioma cell lines and that within a panel of glioma cell lines a wide range of thromboxane formation levels is found. For the cell lines selected for this study G-120 showed the highest thromboxane B_2 levels (3.5 ng/106 cells) followed by G-168 and G-28. G-44 and G-112 showed the lowest levels of thromboxane formation under standard culture conditions. Human astrocytes demonstrated expression of the thromboxane receptor but no detectable mRNA of the enzyme by RT-PCR [20].

The mRNA expression of COX-1 and COX-2 was analyzed by RT-PCR. Within a panel of ten glioma cell lines COX-1 mRNA expression was detectable in 7 and COX-2 expression in 6 cell lines. Human astrocytes were established from normal brain specimens and used in passage numbers < 10. In monolayer

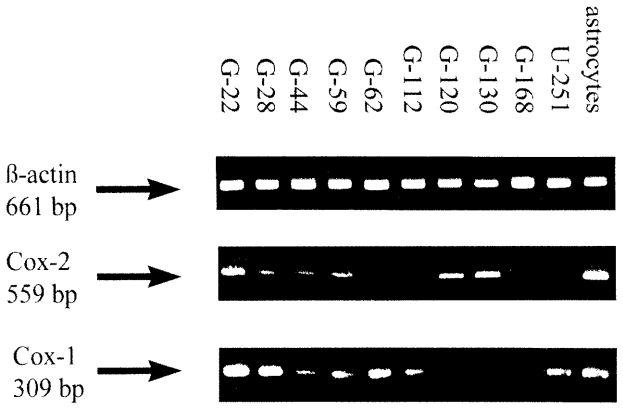

Fig. 1. RT-PCR analysis of cyclo-oxygenases in human glioma cell lines and human astrocytes. COX-2 amplification at 36 cycles, COX-1 at 40 cycles, ß-actin was used as a loading control

tissue culture astrocytes showed mRNA expression of both COX isoforms (Fig. 1). Protein expression of these enzymes was confirmed by Western blot analysis using polyclonal antibodies to COX-1 or COX-2. However, normal astrocytes showed no detectable protein expression for COX-1, for COX-2 expression levels were low compared to positive glioma cell lines (data not shown). In addition the profile of prostaglandin synthesis was studied using ELISA detection of PGE_2, $PGD_{2\text{-Mox}}$, PGF_2, and 6-keto-$PGF_{1\alpha}$ in serum free cell culture supernatants of glioma cell lines. These data demonstrate that no homogeneous pattern of prostaglandin synthesis for glioma cells in culture could be identified. Whereas cell culture supernatants of normal astrocytes generally contained low levels of all prostaglandins analyzed, some glioma cell lines such as G-59 and G-130 showed high prostanoid formation for a single or several metabolites (Fig. 2).

COX I and COX II Expression in Biopsies of Human Glial Tumors

Immunohistochemistry was used to study COX-1 and COX-2 expression in 11 glioblastomas WHO IV, 5 anaplastic astrocytomas WHO III, 5 astrocytomas WHO II, and 4 pilocytic astrocytomas WHO I (Table 1). Immunoreactivity for COX-1 was detected in all grades of glial tumors. COX-1 and GFAP double labeling confirmed expression of this enzyme in GFAP positive glioma cells of all tumor grades (Fig. 3). No detection of COX-1 protein was found in three GBM, two anaplastic astrocytomas and four of low grade gliomas. Protein expression of COX-2 was also found in all tumor grades. Strong immunoreactivity localized to focal clusters confirmed as glioma cells by GFAP double labeling (Fig. 4). The specificity controls for COX immunostaining are demonstrated in Fig. 5. No COX-2 expression was found in one GBM, and two low grade gliomas. No COX-2 negative tumors showed positive reactivity for COX-1. Six COX-1 negative tumors expressed COX-2. No immunoreactivity for both COX-1 and COX-2 in tumor cells was found in three tumor specimens (compare Table 1). These data do not suggest any association of COX-1 or COX-2 with the grade of glial tumors and the morphological differentiation of a tumor was not predictive of COX expression. In addition to expression of COX-1 and COX-2 in tumor cells, the enzymes could also be demonstrated in macrophages, microglial cells, neurons as well as in reactive astrocytes, but not in quiescent glia.

Inhibition of Migration, Apoptosis, and Cell Growth by Inhibitors of the Arachidonic Acid Metabolism

To study the role of inhibitors of arachidonic acid metabolism in the regulation of cell motility non-selective COX inhibitors, selective COX-2 inhibitors, and specific thromboxane synthase inhibitors were tested in a monolayer migration assay. This assay quantifies the dispersion of a migrating cell population on a permissive matrix substrate (merosin) over 24 hours. Whereas the specific thromboxane synthase inhibitors furegrelate and dazmegrel strongly inhibited cell migration in a dose dependent manner, none of the COX inhibitors significantly influenced glioma cell motility (Fig. 6) regardless of the eicosanoid synthesis pattern of the cell line analyzed (compare Fig. 2). Using a cytotoxicity assay which quantifies the cellular metabolic reduction rate 48 hours after inhibitor treatment it was found that furegrelate showed little impairment of the metabolic reduction at doses that demonstrated strong inhibition of motility, whereas sulindac decreased cellular metabolisms in a dose dependent manner (Fig. 7). When COX-2 inhibitors were tested in seven day growth curve experiments sulindac showed dose dependent growth inhibition. G-59, which showed high levels of eicosanoid formation was relatively more resistant compared to G-44 and G-112 representing a cell line demonstrating generally low levels of prostanoid synthesis (Fig. 8). Similar data were obtained for a second selective COX-2 inhibitor piroxicam. For the non-selective COX inhibitor ASA

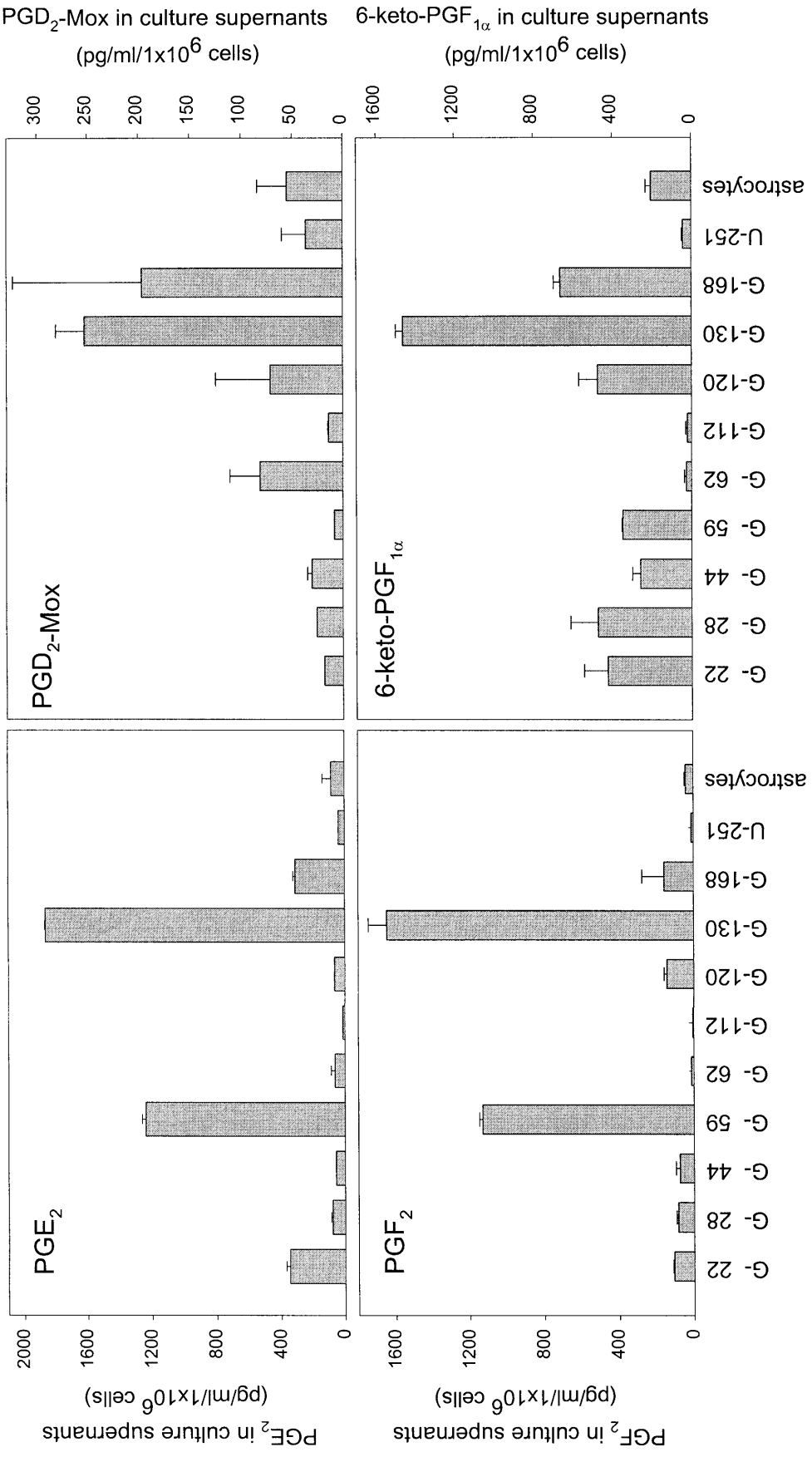

Fig. 2. ELISA detection of PGE_2, $PGD_{2\text{-Mox}}$, PGF_2, and 6-keto-$PGF_{1\alpha}$ in serum free cell culture supernatants of glioma cell lines and normal human astrocytes. The concentration of prostaglandins was normalized to cell number. Values represent determinations in triplicate

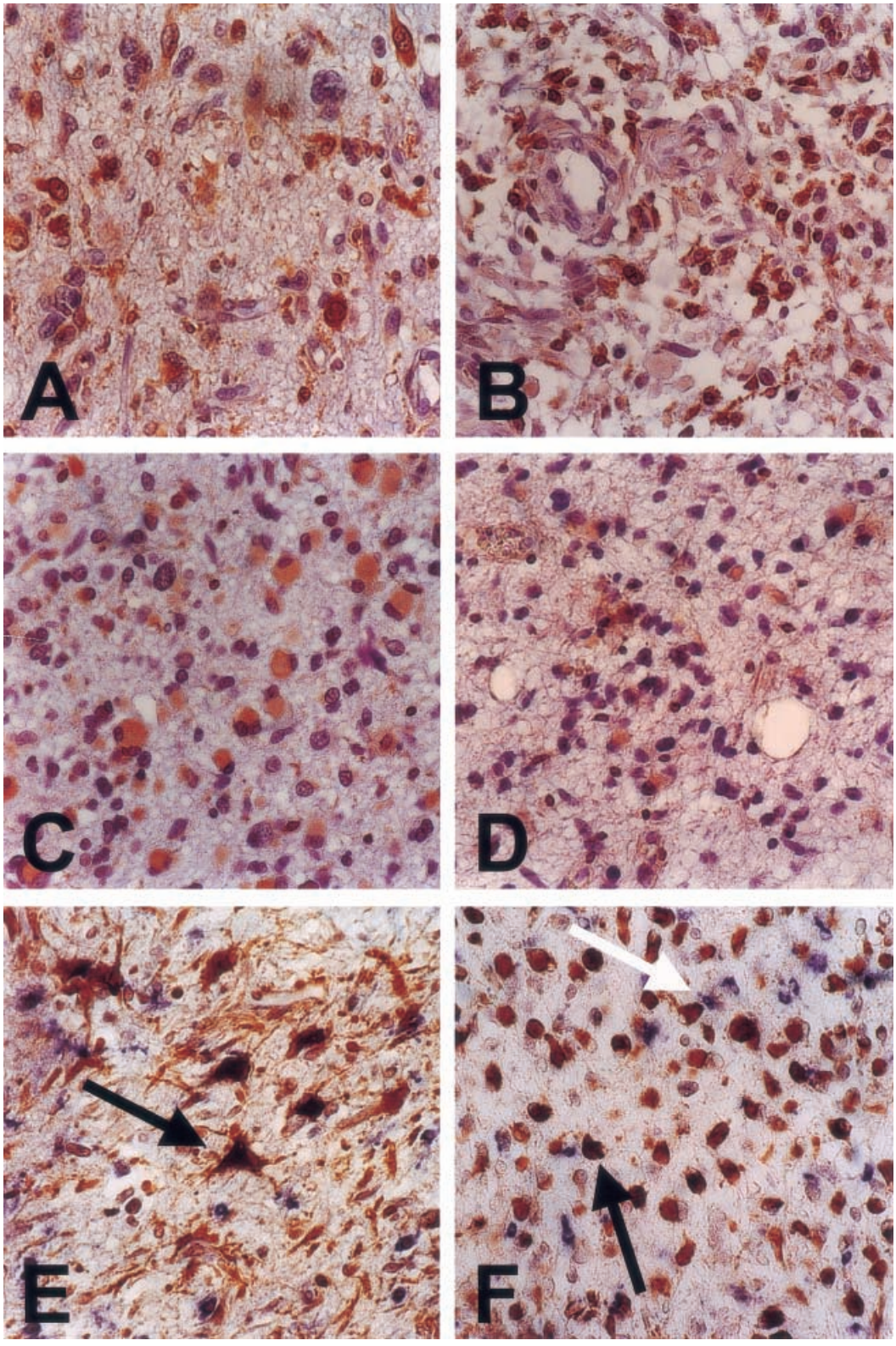

Fig. 3. Immunostaining of glial tumors using a polyclonal COX-1 antibody. (A and B) Glioblastoma multiforme No. 1 and No. 5 (compare Table I), (C) Gemistocytic astrocytoma WHO III No. 5, (D) Astrocytoma WHO II No. 2, (E and F) GFAP (brown) and COX-1 (blue) double labeling of E: glioblastoma specimen No. 1, glioblastoma cells (black arrow) and F: astrocytoma WHO III specimen No. 5, astrocytoma cells (black arrow) and microglia (white arrow). Photographed at 40×

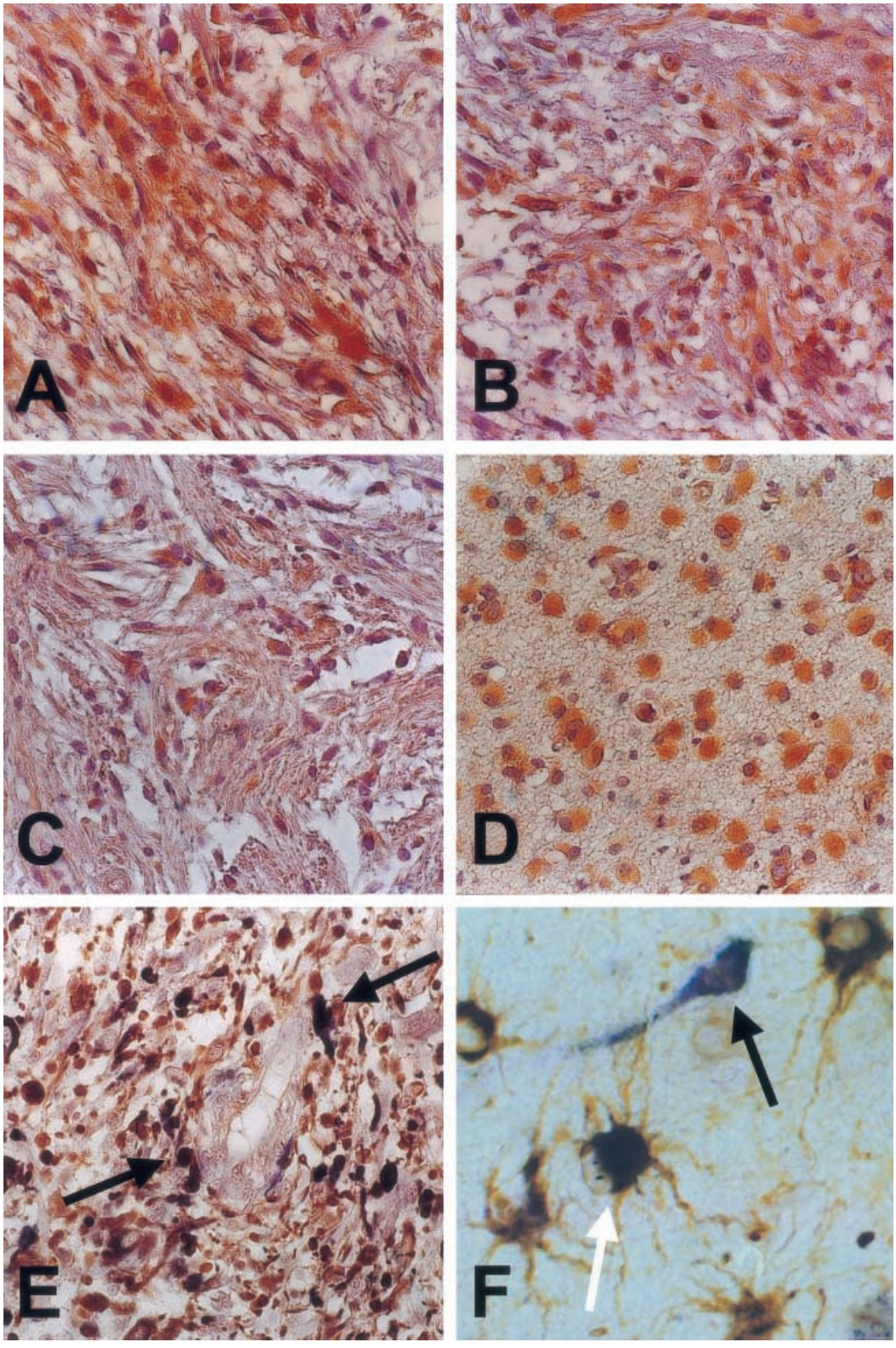

Fig. 4. Immunostaining of glial tumors using a polyclonal COX-2 antibody. (A and B) Glioblastoma multiforme No. 2 and No. 4, (C) Astrocytoma WHO III No. 2, (D) Astrocytoma WHO II No. 4; (E) GFAP (brown) and COX-2 (blue) double labeling of glioblastoma specimen No. 1, glioblastoma cells (black arrows). Photographed at 40×; (F) Double labeling of brain obtained from glioblastoma specimen No. 1, one COX-2 positive neuron (black arrow), COX-2 and GFAP positive reactive astrocytes (white arrow). Photographed at 125×

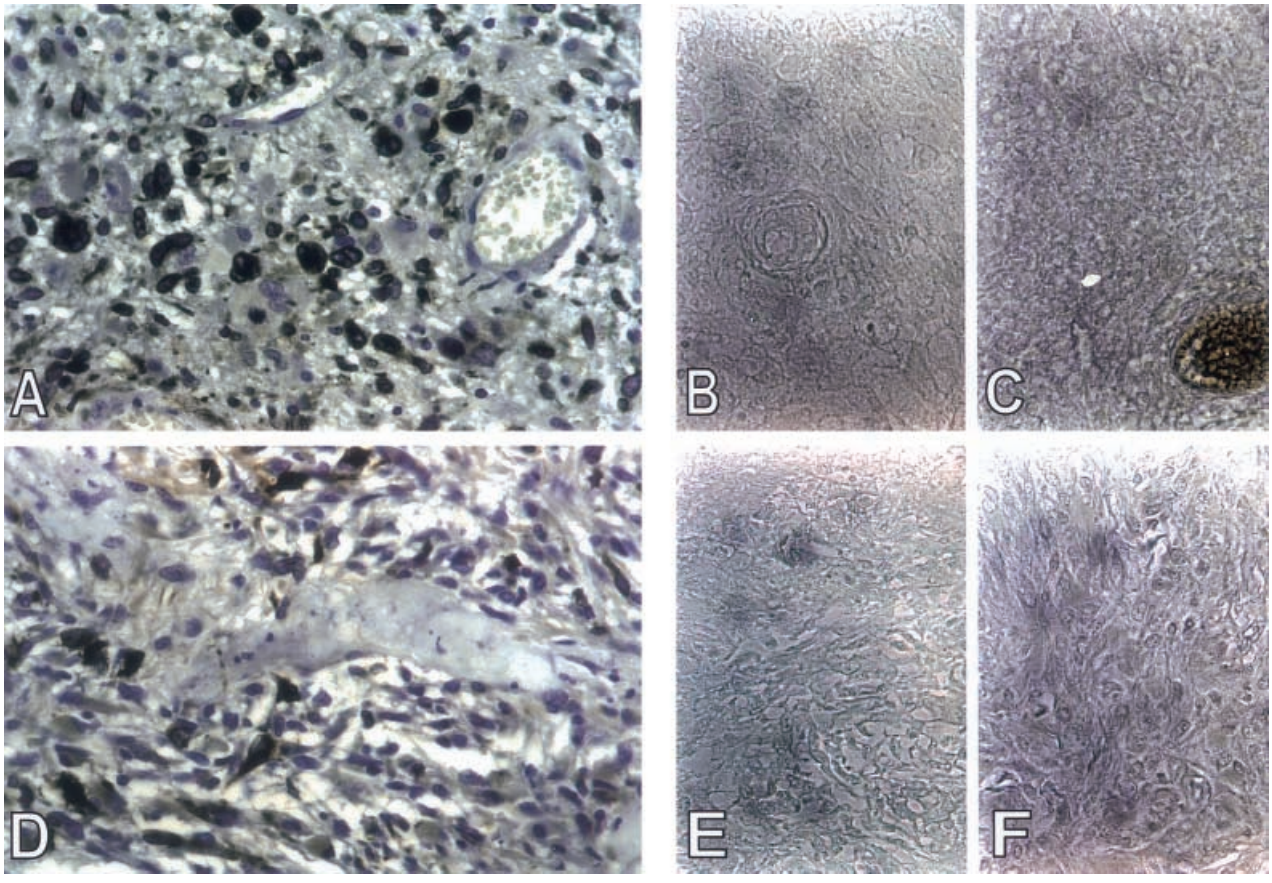

Fig. 5. Specificity control of COX immunostaining in paraffin sections. The glioblastoma specimen No. 10 was immunostained with (A) COX-1 primary antibody followed by a biotinylated secondary antibody, peroxidase detection and a hemalum counterstain. (B) omission of the COX-1 antibody, (C) use of an irrelevant primary (p24) antibody. Glioblastoma specimen No. 11 was immunostained with (D) COX-2 primary antibody, (E) omission of the COX-2 antibody, (F) use of an irrelevant primary (p24) antibody. Photographed at 40×

no significant inhibition of cell growth was observed up to a concentration of 2.7 mM (data not shown). Growth inhibition by the thromboxane synthase inhibitor furegrelate only occurred at high concentrations with a decrease of cell numbers after several days of incubation (Fig. 9). These findings are consistent with our previous data demonstrating that apoptotic cell death occurs several days after pharmacological inhibition of thromboxane synthase [51]. To analyze whether the inhibition of cell growth by COX-2 inhibitors is a consequence of apoptosis, a DNA fragmentation assay, which quantifies mono- and oligonucleosomal DNA fragments in cellular lysates and culture supernatants was used [51]. When G-44 glioma cells were treated with increasing concentrations of COX inhibitors or thromboxane synthase inhibitors a dose dependent increase of intracellular DNA fragmentation was observed for the selective COX-2 inhibitor sulindac and the thromboxane synthase inhibitor furegrelate. Piroxicam was significantly less effective and ASA did not induce intracellular DNA fragmentation. Intracellular DNA fragmentation in apoptosis is an active cellular process that requires protein synthesis and metabolism. Cell death associated with breakdown of membrane function may follow several hours to days after initiation of DNA fragmentation. The absence of DNA fragments detected in the supernatant indicates that subsequent cell death does not occur as a consequence to early loss of membrane function as detected in necrotic cell death. DNA fragmentation is detectable 16 hours after treatment for both inhibitors of COX-2 and thromboxane synthase and increases up to 48 hours (Fig. 10). When the effect of sulindac on DNA fragmentation was evaluated in a larger panel of cell lines it was found that intracellular DNA fragmentation was induced in

Table 1.

Specimen	COX-1	COX-2	Dominating morphology
Glioblastoma WHO IV No. 1	++	++	astrocytic
Glioblastoma WHO IV No. 2	++	++	astrocytic
Glioblastoma WHO IV No. 3	−	+	astrocytic
Glioblastoma WHO IV No. 4	++	++	astrocytic, pleomorphic
Glioblastoma WHO IV No. 5	++	+	astrocytic, pleomorphic
Glioblastoma WHO IV No. 6	+	+	astrocytic, anaplastic small cells
Glioblastoma WHO IV No. 7	−	+	pleomorphic, anaplastic small cells
Glioblastoma WHO IV No. 8	+	+	astrocytic, anaplastic small cells
Glioblastoma WHO IV No. 9	−	−	astrocytic, pleomorphic
Glioblastoma WHO IV No. 10	++	++	astrocytic, pleomorphic
Glioblastoma WHO IV No. 11	++	+	astrocytic, pleomorphic
Anaplastic astrocytoma WHO III No. 1	+	++	fibrillary
Anaplastic astrocytoma WHO III No. 2	+	+	fibrillary, pilocytic
Anaplastic astrocytoma WHO III No. 3	−	+	fibrillary, gemistocytic, giant cells
Anaplastic astrocytoma WHO III No. 4	−	+	fibrillary
Anaplastic astrocytoma WHO III No. 5	+	+++	astrocytic, gemistocytic, pleomorphic
Astrocytoma WHO II No. 1	−	++	protoplasmatic, gemistocytic
Astrocytoma WHO II No. 2	+	++	fibrillary, gemistocytic, small cells
Astrocytoma WHO II No. 3	−	−	fibrillary, small cells
Astrocytoma WHO II No. 4	++	++	oligodendroglial, gemistocytic
Astrocytoma WHO II No. 5	+	+	fibrillary
Pilocytic astrocytoma WHO I No. 1	−	++	pilocytic
Pilocytic astrocytoma WHO I No. 2	−	−	pilocytic, fibrillary
Pilocytic astrocytoma WHO I No. 3	+	+	pilocytic
Pilocytic astrocytoma WHO I No. 4	+	++	pilocytic

Scoring of relative immunoreactivity:
0% = −; <10% = +; 10–50% = ++; >50% = +++

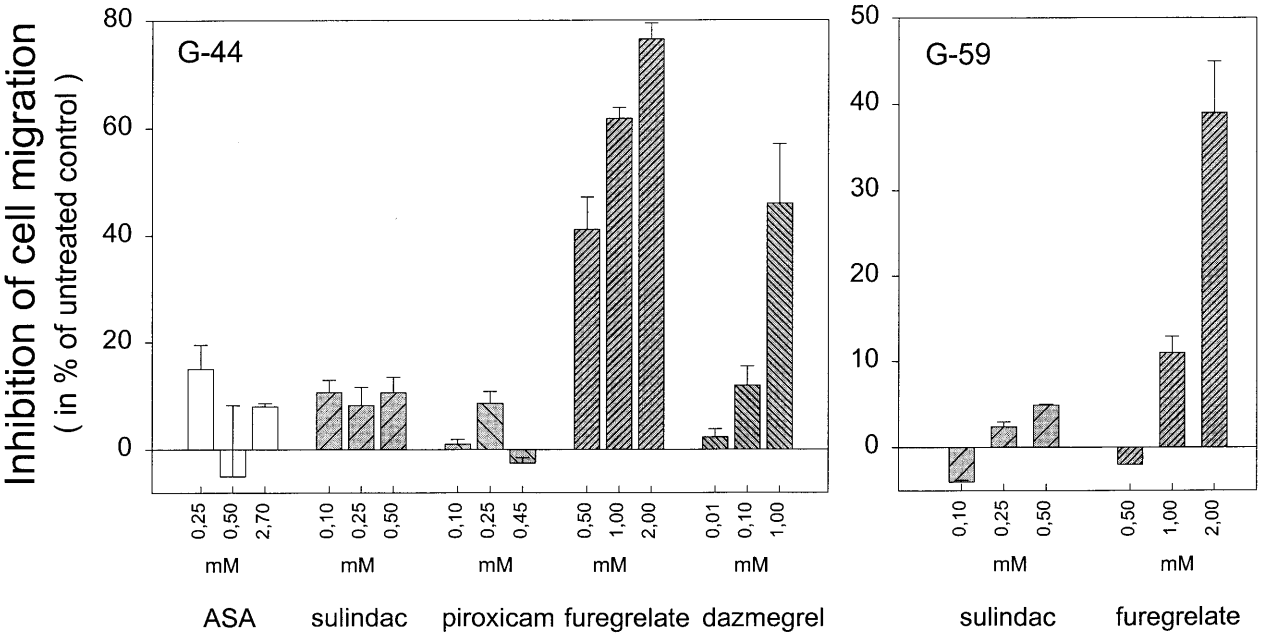

Fig. 6. Inhibition of Monolayer migration by inhibitors of arachidonic acid metabolism. Glioma cells demonstrating different synthesis patterns of eicosanoids were selected for motility analysis. G-44 and G-59 glioma cells were treated with the non-selective COX inhibitor ASA, the selective COX-2 inhibitors sulindac and piroxicam, and the thromboxane synthase inhibitors furegrelate and dazmegrel. Following treatment cell migration was monitored for 24 hours. Values indicate inhibition of the migration of a cell population. Determinations in replicates of at least four; bars, S.D

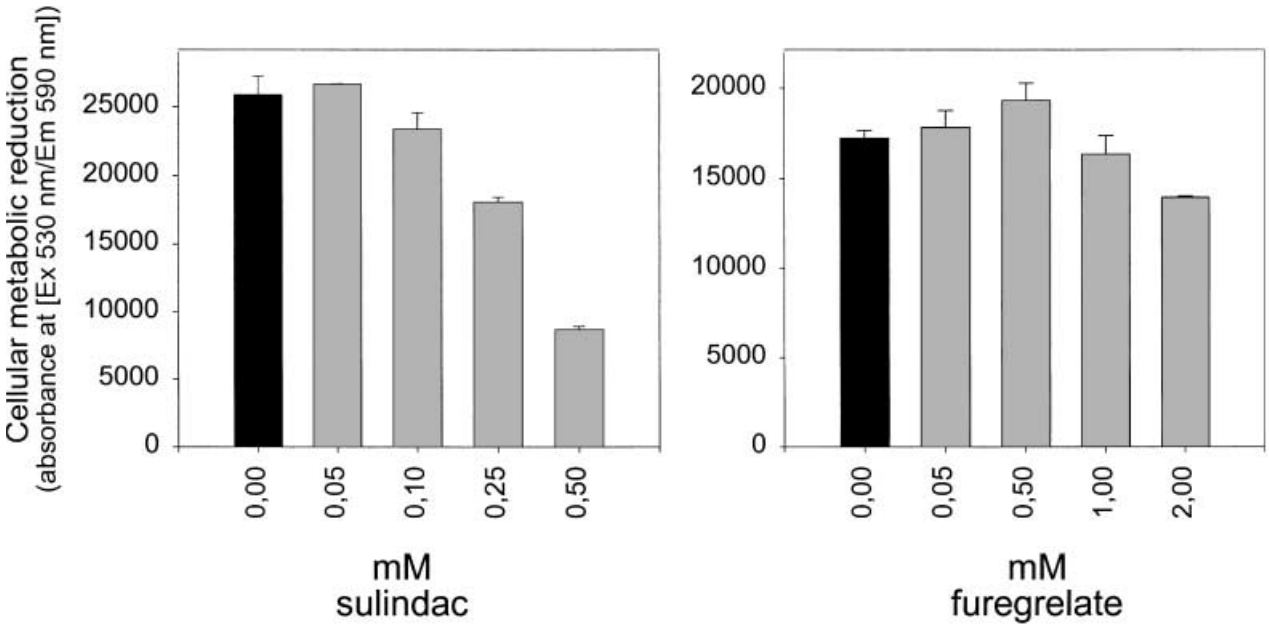

Fig. 7. Cellular metabolic reduction. The cytotoxicity of the COX-2 inhibitor sulindac and the thromboxane synthase inhibitor furegrelate was determined using G-44 glioma cells. Fluorescence of metabolized dye reflecting cellular metabolic reduction, was quantified 48 hours after treatment. Values represent percent absorbance of an untreated control. Determinations in triplicate; bars, S.D

four out of seven glioma cell lines. The synthesis profiles of prostanoids were not predictive for this effect (compare Fig. 2). G-44, G-112, G-120, and G-168 showed varying prostanoid synthesis profiles but showed dose dependent enrichment of intracellular histone complexed DNA fragments after COX-2 inhibition. For a single cell line G-28 treatment concentrations were toxic and resulted in early release of DNA fragments into the supernatant (Fig. 11).

When the effect of sulindac, sulindac sulfone, and sulindac sulfide on DNA fragmentation was compared sulindac and sulindac sulfone were found to have similar effects. Sulindac sulfide treatment resulted in early release of DNA fragments into the culture supernatant indicating a necrotic cell death due to toxicity of the compound (Fig. 12). The activity of thromboxane synthase and the formation of thromboxane is dependent on cyclo-oxygenase as the rate limiting enzyme of this synthesis pathway. To test whether the effect on DNA fragmentation could be increased by a combined treatment of cyclo-oxygenase and thromboxane synthase inhibitors increasing concentrations of sulindac and furegrelate were used. For sulindac and furegrelate no synergistic effect on intracellular DNA fragmentation was found (data not shown). Inhibition of cyclo-oxygenase and also thromboxane synthase may cause an accumulation of arachidonic acid, which is a substrate of these enzymes. Increased concentrations of arachidonic acid may lead to activation of neutral sphingomyelinase activity, which results in the formation of ceramide, a powerful inductor of apoptosis in many cellular systems. To test whether this pathway may be responsible for apoptosis after COX or thromboxane synthase inhibitor treatment increasing concentrations of arachidonic acid were added to the cultures of G-44 glioma cells and DNA fragmentation was measured. No intracellular DNA fragmentation occurred up to a dose of 150 μM. This indicates that induction of apoptosis by both inhibitors is most probably not due to accumulation of arachidonic acid (data not shown).

Sensitizing Effect of COX and Thromboxane Synthase Inhibitors to Induced Apoptosis by Camptothecin

Camptothecin is a moderate inducer of apoptosis in G-44 glioma cells at a dose range of 2–8 μg/ml [51]. Increasing doses of camptothecin and sulindac did not result in a synergistic effect on intracellular DNA fragmentation, but rather increased release of DNA fragments into the supernatant indicating additive

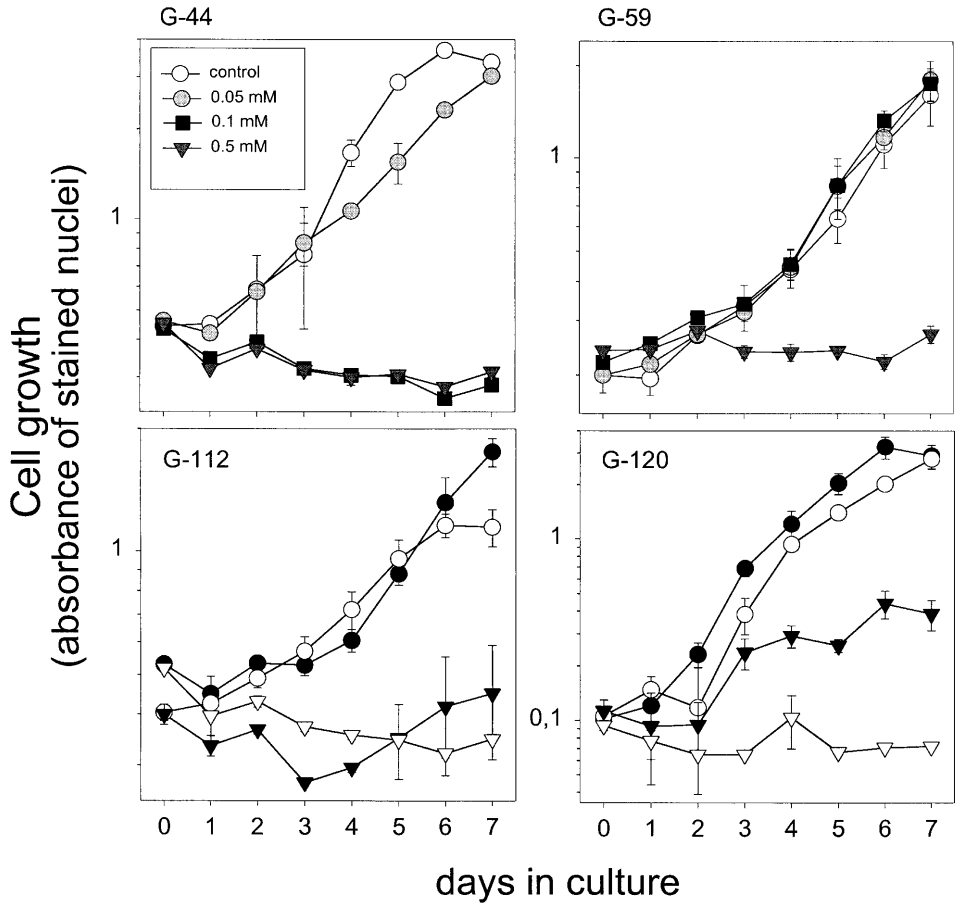

Fig. 8. Growth inhibition by the COX-2 inhibitor sulindac. Glioma cell lines were selected on basis of prostanoid synthesis levels. G-44, G-112, and G-120 generally showed low levels of prostanoid formation, G-59 demonstrated high levels of PGE_2 and PGF_2. Increasing concentrations of sulindac were added to the culture and cell number was determined by absorbance of fixed and stained nuclei. Determinations in triplicate; bar, S.D

toxicity. ASA also showed no synergistic effect. In contrast camptothecin and furegrelate showed a strong synergistic effect increasing intracellular DNA fragmentation (Fig. 13).

Discussion

Prostanoids [prostaglandins (PG) and thromboxanes (Thx)] are synthesized in the central nervous system and are thought to act as neuro-active substances modulating a wide range of brain functions such as sleep-wake cycle, body temperature, cerebral blood circulation, neuro-endocrine functions, and convulsion [24, 43, 47]. In brain expression of COX-1 and COX-2 is regulated in response to various stress factors. Trauma, ischemia, inflammation, and experimental encephalomyelitis may lead to up regulation of COX-2 and subsequent prostanoid synthesis [10, 31, 37, 38]. Deininger *et al.* analyzed the expression of COX-1 and COX-2 in a large series of glial tumors including 22 glioblastomas and found that COX-1 immunoreactivity was restricted to macrophages, microglia, and neurons but no expression was identified in tumor cells [9]. COX-2 immunoreactivity localized to GFAP positive tumor cells surrounding areas of necrosis associated with a tendency to elevated expression in high grade gliomas. This has been confirmed by a recent analysis of 50 human brain tumors, which suggested a tendency of cytoplasmic COX-2 expression to correlate with tumor grade [27]. Deininger *et al.* detected no expression of both COX isoforms in resting astrocytes or oligodendrocytes of normal brain. However, in vitro studies using primary rat astrocytes identified glial cells as a source of brain prostanoid formation and demonstrated PGD_2, PGE_2, $PGF_{2\alpha}$, $ThxA_2$ and $ThxB_2$ expression in these cells [25]. Sub-

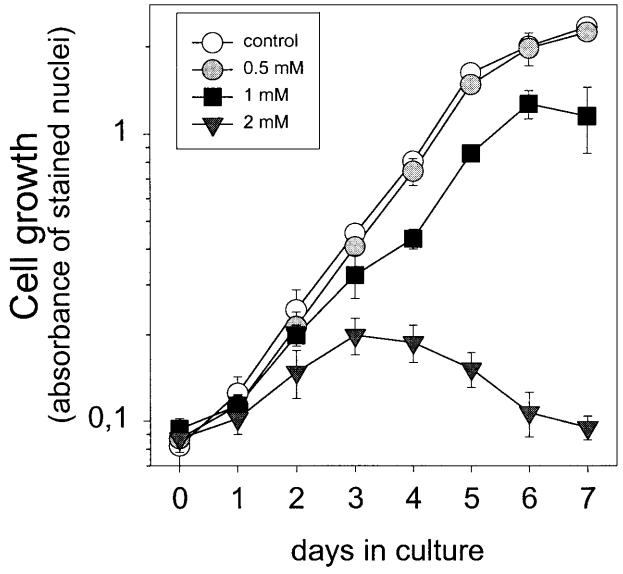

Fig. 9. Growth inhibition by the thromboxane synthase inhibitor furegrelate. G-44 glioma cells were treated with increasing concentrations of furegrelate and replicates were fixed at daily intervals. Determinations in triplicate; bar, S.D

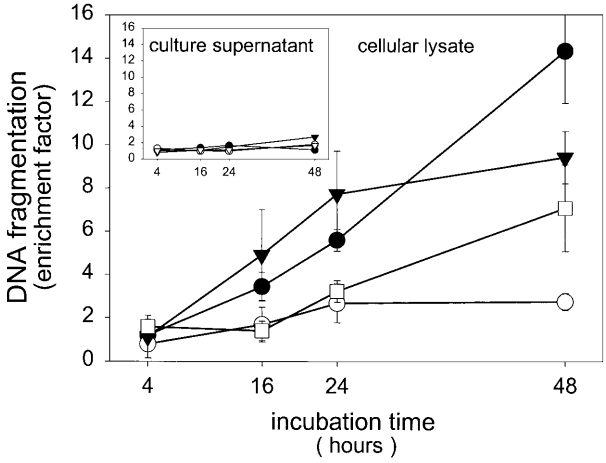

Fig. 10. Induction of apoptosis by COX and thromboxane synthase inhibitors. G-44 glioma cells were treated with 2.7 mM ASA (○), 0.5 mM sulindac (▼), 0.65 mM piroxicam (❑), or 2 mM furegrelate (●). DNA fragmentation was quantified using ELISA detection of enrichment of mono- and oligonucleosoms after 48 hours. Enrichment of intracellular DNA fragments was quantified from cell lysates. Loss of membrane function in necrotic cell death leads to early release of DNA fragments, which was assayed in the culture supernatant (insert). Data are reported as a specific enrichment factor calculated from absorbance values of nucleosoms released in treated samples divided by the corresponding untreated control. Determination in triplicate; bars, S.D

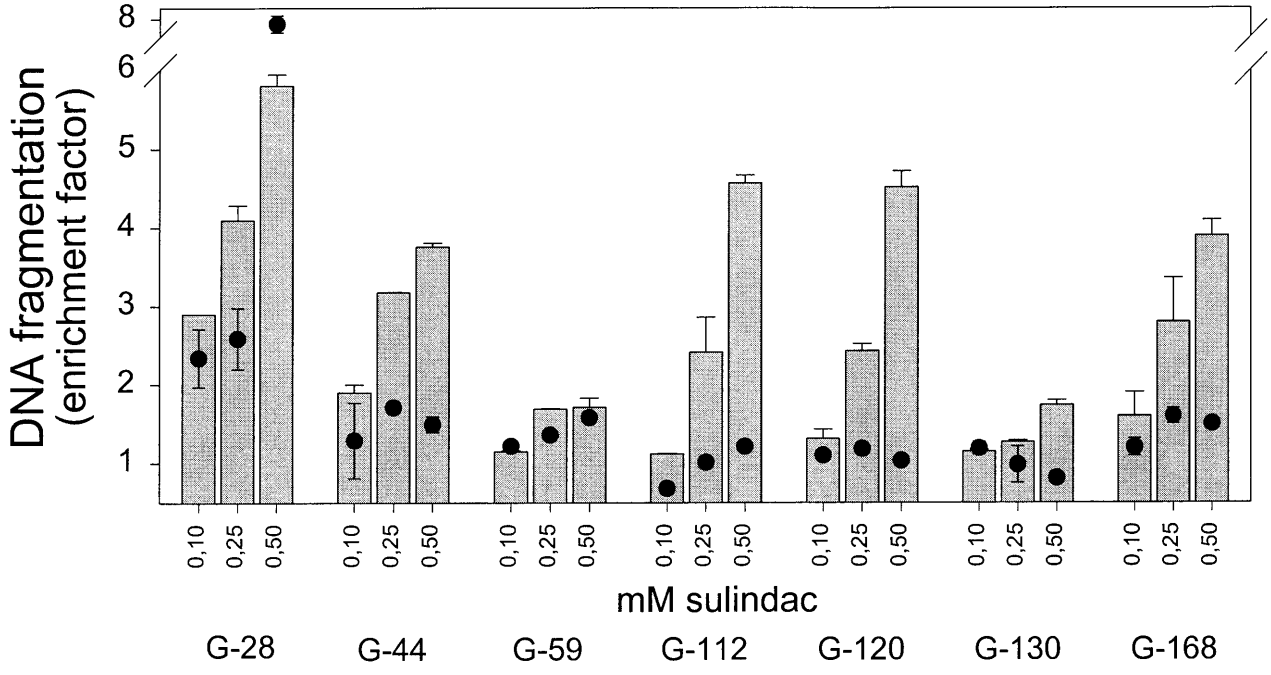

Fig. 11. Induction of apoptosis by sulindac. The susceptibility to induction of apoptosis was tested for a panel of glioma cells. Cells were treated with 0.5 mM sulindac and incubated for 48 hours. DNA fragmentation was quantified using ELISA detection of mono- and oligonucleosoms in cellular lysates (bars) and in the culture supernatant (●). Early release of fragmented DNA into the supernatant indicates a necrotic cell death (for example G-28). Data are reported as a specific enrichment factor calculated from absorbance values of nucleosoms released in treated samples divided by the corresponding untreated control

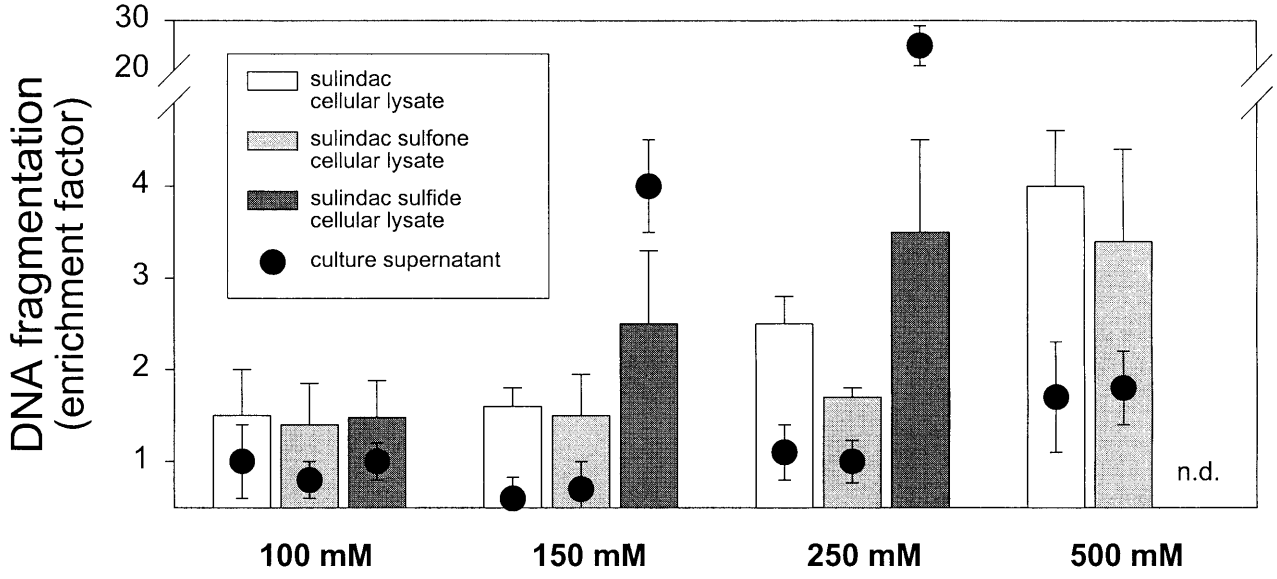

Fig. 12. Induction of DNA fragmentation by sulindac and the sulfone and sulfide derivatives. G-44 glioma cells were treated for 48 hours and DNA fragmentation was quantified in cellular lysates (*bars*) and in the culture supernatant (●). Data are reported as a specific enrichment factor calculated from absorbance values of nucleosoms released in treated samples divided by the corresponding untreated control

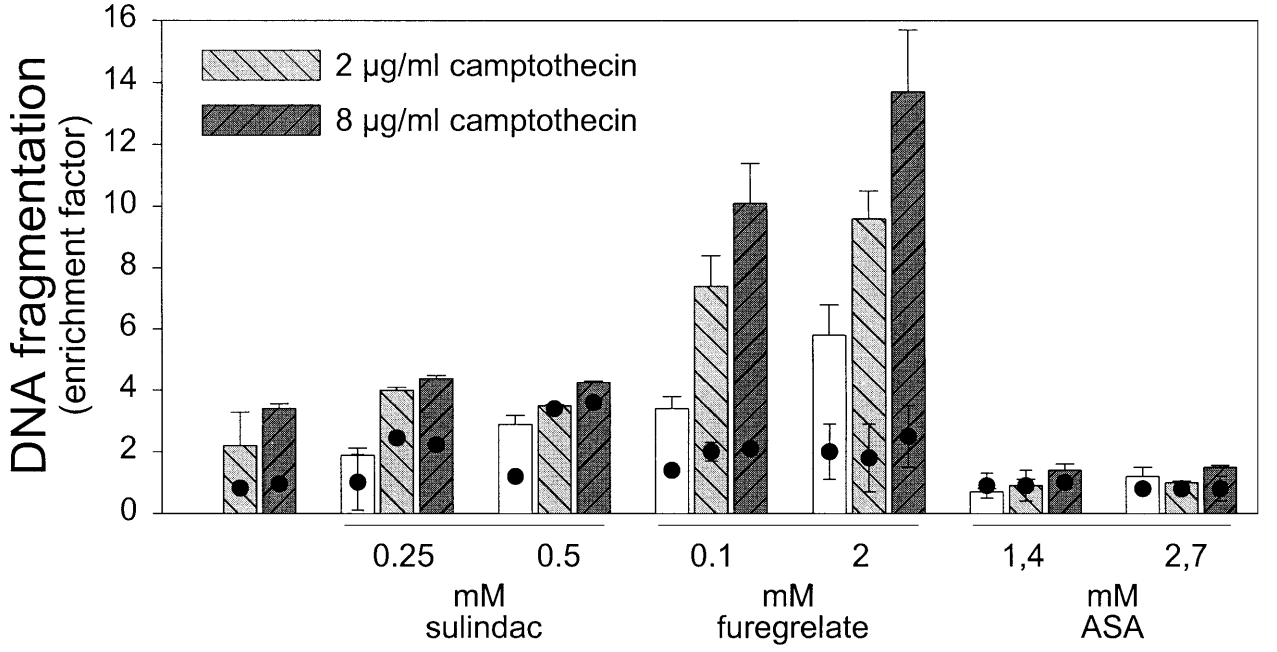

Fig. 13. Sensitizing effect of COX and thromboxane synthase inhibitors to induction of apoptosis by camptothecin. Within a panel of glioma cell lines G-44 was found to be relatively sensitive to induction of apoptosis by camptothecin. Cultures were treated with increasing doses of sulindac, furegrelate, and ASA and 2 µg/ml or 8 µg/ml of camptothecin. DNA fragmentation was quantified in cellular lysates (*bars*) and in the culture supernatant (●). Data are reported as specific enrichment factors calculated from absorbance values of nucleosoms released in treated samples divided by the corresponding untreated control

sequently the expression of several prostanoid receptors could also be demonstrated for rat glia cells [26, 30]. In this study we demonstrated that the majority of glioma cell lines in culture expresses mRNA for both isoforms COX-1 and COX-2. However, the profiles of prostaglandin formation detected in culture supernatants vary considerably under standard tissue culture conditions. Astrocytes in culture also express

mRNA for both COX isoforms and reactive astrocytes in brain tissue also stained positive for COX-2 in contrast to their quiescent counterparts. These data suggest that human glial cells may be induced to express both COX-1 and COX-2 in vitro and in vivo. Double labeling with COX and GFAP antibodies demonstrated the presence of both COX isoforms in glial tumor cells of all tumor grades. However, COX-1 labeling tended to be less intense. These findings in part contrast the study by Deininger *et al.*, who also found COX-2 expression but no COX-1 expression in astrocytic elements and tumor cells [9]. This difference may be due to the use of polyclonal antibodies in our study.

There are few reports on the functional role of prostanoids in gliomas. In glioblastoma cells eicosanoid synthesis inhibitors may suppress proliferation and induce astrocytic differentiation [46]. Inhibitors of cyclo-oxygenase such as indomethacin and ketoprofen have been demonstrated to slow growth of glioma spheroid cultures [13] and acetylsalicylic acid significantly slowed growth of rat glioma cell in vitro and in vivo [1]. As a first report establishing a functional link for prostanoid expression and migration of glial tumor cells we demonstrated overexpression of thromboxane synthase in highly motile glioma cells by differential mRNA display [36]. Interestingly, the levels of $ThxB_2$ formation correlated with glioma migration rates and inhibitors of this enzyme block glioma migration [20]. We could further demonstrate that the anti-migratory effect of specific thromboxane synthase inhibitors appears to be associated with caspase 3 activation followed by DNA fragmentation and subsequent apoptotic cell death in treated glioma cells. Thromboxane synthase inhibitor induced DNA fragmentation could be blocked by selective caspase 9 inhibitors suggesting involvement of a mitochondrial pathway of apoptosis [51]. These data have implicated thromboxane synthase as an intersection point of diverging signaling cascades regulating motility and apoptosis in glioma cells. However, the synthesis of thromboxanes is functionally dependent on COX activity, which represents the rate-limiting enzyme of this pathway. Cyclo-oxygenases catalyze the synthesis of eicosanoids metabolites PGG_2 and PGH_2, which are further metabolized to PGE_2, PGD_2, $PGF_{2\alpha}$, $ThxA_2$, and PGI_2. In platelets and blood vessels thromboxane and some prostaglandins show antagonistic functions. Thromboxane synthase inhibitors impede the metabolic pathways of cyclic endoperoxides into thromboxane, which indirectly increases the formation of PGD_2, PGE_2, and $PGF_{2\alpha}$, and PGI_2 [22]. In this sense cyclo-oxygenase and thromboxane synthase functions may be interdependent (Fig. 14). Therefore it is possible that a balance of thromboxanes, prostaglandins, and prostacyclin are part of the regulation of the migratory phenotype in gliomas. However, in contrast to the thromboxane synthase inhibitors furegrelate and dazmegrel, selective and non-selective inhibitors of COX-2 had no effect on glioma migration when tested in a 24 hour monolayer migration assay. Quantification of intracellular histone complexed DNA fragmentation after inhibitor treatment demonstrated that both selective COX-2 and thromboxane synthase inhibitors induce apoptosis but not the non-selective COX inhibitor acetylsalicylic acid. In a different study another COX-2 inhibitor NS-398 also demonstrated strong growth inhibition of glioma cells, but was a moderate inducer of apoptosis with no inhibition of invasion in a three-dimensional assay system [27]. Analysis of our panel of glioma cell lines showed that the susceptibility to COX-2 inhibitor induced DNA fragmentation is independent of the prostaglandin synthesis profile. It is unlikely that induction of DNA fragmentation by both COX and thromboxane synthase inhibitors is a consequence of accumulation of arachidonic acid, because direct addition of arachidonic acid to the culture media did not induce apoptosis. Induction of apoptosis by COX and thromboxane synthase inhibitors may not be a result of a common pathway. This is indicated by the fact that the thromboxane synthase inhibitor furegrelate showed a synergistic effect on DNA fragmentation in combined treatment with camptothecin. In contrast, treatment with sulindac or acetylsalicylic acid did not increase the susceptibility to camptothecin induced apoptosis. Furthermore, co-treatment of COX and thromboxane synthase inhibitors did not result in increased DNA fragmentation over thromboxane synthase inhibitors alone, suggesting different mechanisms by which COX and thromboxane synthase inhibitors induce apoptosis in glioma cells. This is further supported by the observation that within the pathway of arachidonic acid metabolism thromboxane synthase and not cyclo-oxygenase predominantly controls cell migration.

Cell migration and apoptosis seem to be interrelated cellular phenomena, because both phenomena are functionally dependent on the proliferative activity of a cell population [11]. The pro-apoptotic disposition of a cell is positively correlated with the proliferative

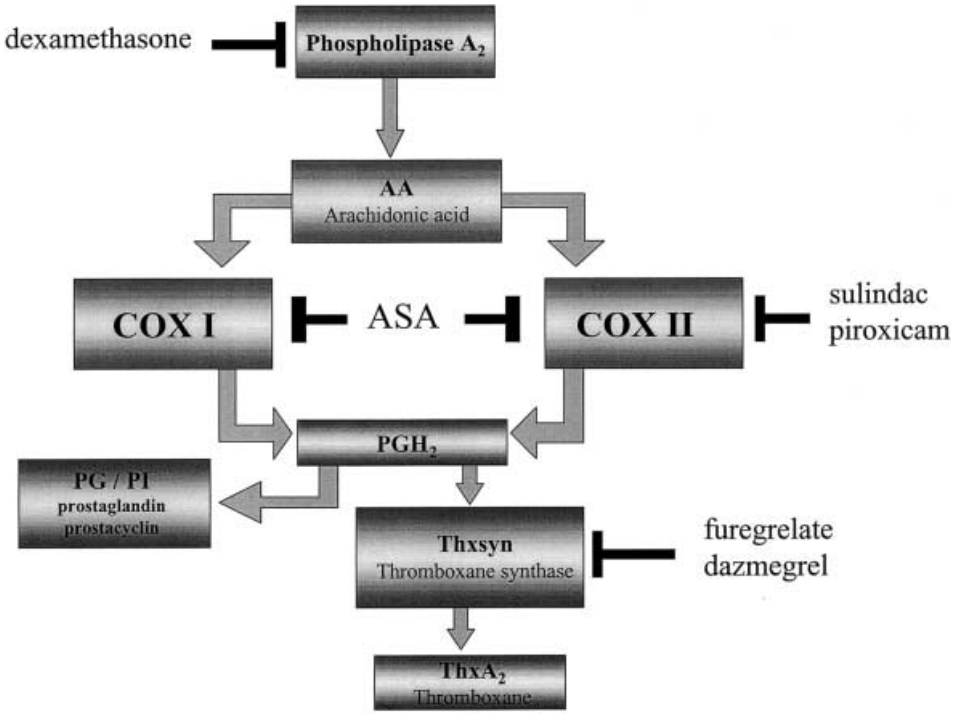

Fig. 14. Synthesis pathway of arachidonic acid metabolism and inhibitors of cyclo-oxygenase and thromboxane synthase. Dexamethasone inhibits Phospholipase A_2, which mediates release of arachidonic acid from cellular membranes. Arachidonic acid (*AA*) is a substrate for both COX-1 and its inducible isoform COX-2. Acetylsalicylic acid (*ASA*) is a non-selective inhibitor of both cyclo-oxygenase isoforms. Sulindac and piroxicam are selective inhibitors of COX-2. Inhibitors of COX block the formation of prostaglandin H_2 endoperoxide (PGH_2), which is a substrate for the synthesis of prostaglandins and prostacyclins. PGH_2 is also converted by thromboxane synthase (*Thxsyn*) to thromboxane A_2 ($ThxA_2$). Specific thromboxane synthase inhibitors decrease thromboxane synthase function with no effect on cyclo-oxygenase. Cyclo-oxygenase inhibitors influence the formation rate of thromboxane through decrease of PGH_2 and increase the buildup of arachidonic acid, activating alternative pathways

activity of a cell population [2, 44]. On the other hand there is increasing evidence that migration and proliferation may be antagonistic cellular behaviors [16, 18, 19]. This may have important implications for the treatment of invasive cells. Invasion is a complex process, generally perceived as a multi-step process, which initially requires receptor mediated *adhesion* of tumor cells to matrix proteins, followed by a second phase of matrix *degradation* by tumor secreted proteases. This creates an intercellular space into which invading cells can *migrate* by an active mechanism that requires membrane synthesis, receptor turnover, and rearrangement of cytoskeletal elements (reviewed in 17). If cell migration contributes to invasiveness in vivo and migration and proliferation are in fact antagonistic cellular programs in invasive glioma cells this would suggest that the invasive phenotype may not only be less proliferative but may also protect this fraction of tumor cell population against induction of apoptosis. This would render invasive cells relatively resistant to irradiation and chemotherapy [17]. In a recent study Mariani *et al.* have demonstrated by a microarray expression analysis that in glioma cells stimulated to migrate cassettes of genes associated with cellular proliferation and apoptosis get down-regulated, whereas a number of anti-apoptotic genes are up-regulated [33]. These data have been extended by data that have identified death associated protein 3 (DAP-3), which protects glioma cells from camptothecin induced apoptosis, in glioma cells that were laser capture micro-dissected from the invasive edge of a glioblastoma [32]. Antisense DAP-3 treatment rendered glioma cells susceptible to apoptosis and decreased cellular migration. This line of evidence suggests that invasive glioma cells in vivo overexpress genes associated with migration and also show a less apoptotic disposition compared to the proliferating tumor mass. Thromboxane synthase inhibitors decrease cell migration and may thus shift the cellular program of migratory cells to a phenotype that renders the cells suscep-

tible to apoptosis. We hypothesize that, in contrast to COX-2 inhibitors, thromboxane synthase inhibitors may block the invasive phenotype of glioma cells and therefore increase the pro-apoptotic disposition. This increased the susceptibility to drug induced apoptosis, which offers an interesting perspective to specifically sensitize invasive glioma cells to conventional treatments inducing apoptosis.

Acknowledgments

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Comment

Migration and resistance to apoptosis are a characteristic phenotype of gliomas. Understanding of underlying mechanisms are certainly important.

In the present manuscript the authors compare the effects of inhibition of thromboxane synthetase and cyclo-oxygenase synthetase on migration, proliferation and apoptosis of glioma cell lines. The expression of the respective enzymes is also documented in human astrocytomas to demonstrate biological relevance of this process in cancer. Previously, the authors had shown that inhibition of thromboxane synthetase abrogates cell migration and induces apoptosis. Here, the authors focus on the effects of inhibition of cyclo-oxygenase synthetase activity, since this represents the rate limiting step in the formation of thromboxanes. Inhibition of cyclo-oxygenase synthetase did not have an effect on migration and induction of apoptosis, in contrast to inhibitors of thromboxane synthetase. The authors conclude that the respective inhibitors act through different pathways. They hypothesize that inhibition of the invasive phenotype increases susceptibility to undergo apoptosis.

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