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Experimental Study

Inhibition of the arachidonic acid metabolism blocks endothelial cell migration and induces apoptosis*

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Summary

Previous studies have demonstrated that inhibitors of the arachidonic acid metabolism block migration and sensitise human glioma cells to treatment inducing apoptosis. This paradigm may provide a new concept for anti-invasive treatment strategies targeting invasive glioma cells. However, the effect of such treatment on other cellular elements in glial tumours such as endothelial cells is unknown. In this study we have analysed the expression of metabolites of the arachidonic acid pathway in endothelial cells in vitro and in vivo and we have assessed the influence of inhibitors of this pathway on motility, capillary like tube formation, and apoptosis in human endothelial cells.

Human endothelial cells (HUVEC) in culture showed expression for thromboxane synthase and both isoforms of cyclo-oxygenase, COX-1 and COX-2. Immunostaining demonstrated low levels of COX-1 expression in capillaries and larger vessels of normal brain and moderately elevated levels of this enzyme in small vessels of brain tumours of various grades. Both thromboxane synthase and COX-2 expression was limited to endothelial cells found in anaplastic gliomas and glioblastomas. Thromboxane synthase inhibitors strongly decreased endothelial cell migration in HUVEC in vitro and capillary like tube formation was strongly inhibited by the compound at a similar dose range. The non-selective cyclo-oxygenase inhibitor ASA and the selective COX-2 inhibitor sulindac only had a minor effect on endothelial cell migration, however, the COX-2 inhibitor sulindac showed a synergistic effect with the thromboxane synthase inhibitor. Thromboxane synthase inhibitors induced apoptosis in endothelial cells as demonstrated by intracellular histone-complexed DNA fragmentation.

These data suggest that inhibitors of thromboxane synthase influence migration and apoptosis in both human glioma cells and human endothelial cells. An anti-invasive treatment strategy using this class of compounds may therefore not only sensitise glioma cells to conventional treatments inducing apoptosis but may also be supported by an anti-angiogenic effect.

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Keywords: Angiogenesis; thromboxane synthase; cyclooxygenase; migration; apoptosis; glioma; arachidonic acid metabolism.

Introduction

In highly migratory subpopulations of human glioma cells differential mRNA display identified human thromboxane synthase as a strongly overexpressed motility associated gene [18]. Subsequently, we demonstrated that this enzyme is expressed in gliomas in vitro and in vivo and that specific thromboxane synthase inhibitors block migration, which is paralleled by a decrease of thromboxane B_2 (Thx B_2) formation [10]. These data implicate thromboxane synthase as an important regulator of glioma motility. Interestingly, following thromboxane synthase inhibitor treatment the decrease of motility rates is paralleled by increased caspase activity followed by intracellular DNA fragmentation and subsequent apoptotic cell death [36]. Prostanoid synthesis in general appears to be important in pathogenesis and progression of cancer because these metabolites influence cellular behaviour in several ways such as mitotic activity, cellular adhesion, invasion, angiogenesis and apoptosis [29].

Cyclo-oxygenase, which is the rate limiting enzyme in the formation of both prostaglandins and thromboxanes, occurs in two isoforms of which COX-2 is a highly regulated form and frequently overexpressed in transformed cells of various tissue origin [27, 28]. Overexpression of this enzyme increases cell adhesion to

extracellular matrix [31] and increases invasion of human colon cancer cells possibly due to up regulation of metalloproteinases [32]. Furthermore elevated levels of COX-2 are associated with enhanced Bcl-2 expression and resistance to apoptosis, which can be reversed by inhibitors of this enzyme [30]. These data suggest that metabolites of the arachidonic acid synthesis pathway may play a role in the regulation of cell motility and invasion as well as apoptosis.

Thromboxane synthase inhibitors impede the metabolic pathways of cyclic endoperoxides into thromboxane, which indirectly increases the formation of prostaglandins [12]. Inhibitors of this pathway may interfere with migration and render invasive cells susceptible to apoptosis [36]. This mechanism would offer a novel perspective to target widely disseminated glioma cells in infiltrated brain (reviewed in 11). In this study we have investigated the influence of inhibitors of the arachidonic acid metabolism on endothelial cell migration and apoptosis to investigate whether an anti-invasive treatment strategy for invasive glioma cells may be supported by an anti-angiogenic effect.

Material and methods

Cell culture and inhibitors of the arachidonic acid metabolism

HUVEC were cultured on collagen type I precoated surfaces in M199 media containing 2 mM glutamin, 1 mM sodium pyruvate, 60 µg/ml ECGS, with 20% FCS. GP8 rat brain endothelial cells were propagated in Ham's F10 containing 20% FCS. The human glioma cell lines G-44, G-112, and G120 were derived from human glioblastomas [33]. Cell migration, adhesion, matrix interactions, and expression profiles of arachidonic acid metabolites of these cell lines were characterized in these papers [8, 9, 14, 36]. Human astrocytes and fibroblasts were established following a protocol described in paper [34]. Glioma cell lines were grown in MEM containing 10% FCS and human astrocytes and fibroblasts in MEM with 20% FCS.

The specific thromboxane synthase inhibitors furegrelate (Sodium 5-(3′-pyridinylmethyl) benzofuran-2-carboxylate) (Sigma, Deisenhofen, Germany), dazmegrel (3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid) (Pfizer, Karlsruhe, Germany), the non-selective cyclo-oxygenase inhibitor acetyl salicylic acid (ASA) (Bayer AG, Leverkusen, Germany), the specific COX-2 inhibitor sulindac (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were prepared according to the manufacturers instructions for use in tissue culture. The concentration range selected for functional assays was based on data previously reported for glioma cells and other cellular systems in vitro [2, 10, 19].

Cell migration assay

Cell Migration was quantified using a modified monolayer migration assay [3, 8], which measures dispersion of a cell population on surfaces. Ten-well HTC-treated slides (Dynex Technologies, Denkendorf, Germany) were coated with AES (3-aminopropyltriethoxysilane) (Sigma #A-3648) to optimise protein and cell adhesion. Slides were then passively coated with ECM protein solution (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 then incubated for 48 h at 37 °C. The CSM was removed and the circular area occupied by attached cells in each well was imaged using a CCD camera (TK-1280E, JVC) and digitised for quantification with an image analysis system (Quantimed 500, Leica, Hamburg, Germany). Object sizes were measured as the radius in µm of the circular area covered by a cell population. Serial images were captured for up to 48 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 net changes in the geographical distribution of the cell population and do not reflect movement of individual cells.

Tube formation assay

We have used a well established model for in vitro angiogenesis. Previous characterizations of this system have focused on the role of growth factors, adhesion molecules, protease activity, and apoptosis as major determinants of new vessel formation [26]. To quantify the formation of a capillary-like network by endothelial cells cold 24 well plates were coated with 250 µl of Matrigel® (Becton Dickinson, Bedford, MA, USA) and then incubated at 37 °C for 1 hour. 5×10^4 HUVEC were seeded per well in M199 containing 20% FCS in the presence of inhibitors of the arachidonic acid metabolism. The cultures were incubated and images of the cultures at 200 fold magnification were digitised at 24 hours. The extent of the capillary-like network formation was quantified by counts of the number and length of network branches defined as straight cellular segments connecting two cell masses (nodes) [7].

Assessment of cell death

DNA fragmentation

A photometric enzyme-immunoassay was used for quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (Boehringer, Mannheim, Germany) [1]. In this assay the intracellular enrichment of mono- and oligonucleosomes, which occur after induction 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 [36]. 20.000 cells were seeded into 96-well plates and cells were allowed to adhere for 4 hours before treatment. Cultures were rinsed and compounds were added at concentrations indicated in the figures. 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 a Cell Death Detection ELISA^{Plus} (Boehringer Mannheim) according to the manufacturers instructions. A specific enrichment factor of mono- and oligonucleosomes released into the cytoplasm was calculated by absorbance of the treated sample divided by absorbance of the corresponding untreated control. Data reported represent the mean of triplicate determinations.

Trypan blue exclusion

The percentage of dead and viable adherent and detached cells was determined by trypsinization and centrifugation followed by staining with 0.2% trypan blue. Cells were counted using a hemocytometer.

Immunofluorescence microscopy of cultured cells and biopsy specimens

Paraffin sections of surgical specimens from glial tumours 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), or the primary mouse antibody to human thromboxane synthase (Biotechnology Inc., Santa Cruz, CA, USA), followed by overnight incubation at 4 °C. A biotinylated anti-goat or anti-mouse IgG (Vector Laboratories, Burlingame CA, USA) was added at a 1:200 dilution for 1 h, detection was carried out using Vectastatin and DAB Substrate Kit (Vector Laboratories) followed by a counterstain 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.

HUVAC were seeded on glass cover slips and incubated for 24 hours, then fixed in 95% ethanol with 5% glacial acetic acid for 15 minutes at −20 °C and permeabilised with 3% Triton X-100 in PBS for 15 minutes at room temperature. The cover slips were washed in culture medium containing 10% FCS and incubated for 30 minutes at room temperature with anti-Thromboxane synthase antibodies, anti COX-1 antibodies, or anti-COX-2 antibodies. Detection of the primary antibody was achieved with fluoresceinisothiocyanate (FITC)-labelled rabbit anti-mouse or anti-goat antibodies (DAKO, Copenhagen, Denmark).

RT-PCR

Total RNA was isolated from monolayer cultures of HUVAC and quantified by absorbance measurement at 260/280 nm. RT synthesis of cDNA was done using a First Strand Synthesis Kit (Stratagene, La Jolla, Ca, USA). Primers for Thromboxane synthase (CAA GCA GGT GTT GGT TCA GAA and TAA ATG AGC CAG GAG AAG GTC), 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 as described in papers [10, 15]. 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 0.1 µl of Taq polymerase (Perkin Elmer) for Thromboxane synthase and COX-2 and 1 µl of nucleotides in 2 µl of 10× buffer) was allowed to run for 40 cycles (Thromboxane synthase), 40 cycles (COX-1) and 35 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.

Results

Expression of cyclo-oxygenases and thromboxane synthase in biopsies of human glial tumours and in HUVAC

Immunohistochemistry was used to study COX and thromboxane synthase expression in vascular structures of 5 glioblastomas WHO IV, 5 anaplastic astrocytomas WHO III, and 5 astrocytomas WHO II. Capillaries and small vessels in normal white or grey matter contained in tissue volumes removed at tumour surgery stained weakly positive for COX-1 but no immunoreactivity could be detected for COX-2 or thromboxane synthase. In astrocytomas WHO grade II endothelial cells of small vessels within the tumour parenchyma stained positive for COX-1, but not for COX-2 or thromboxane synthase. No relative increase of COX-1 expression in endothelial cells was found in anaplastic astrocytomas WHO grade III or glioblastomas WHO grade IV over low grade tumours (Fig. 1A). Strong expression of COX-2 and thromboxane synthase was observed in tumour vessels of anaplastic astrocytomas WHO grade III and glioblastomas WHO grade IV (Table 1). Endothelial proliferations of glioblastomas expressed both COX-2 and thromboxane synthase. Perivascular tumour cuff like formations frequently showed COX-2 and thromboxane synthase positive tumour cells surrounding a COX-2 positive vascular structure. Small vessels within invaded brain adjacent to the bulk lesion also showed COX-2 expression (Fig. 1B).

RT-PCR of cultured human umbilical vein endothelial cells (HUVAC) demonstrated mRNA expression of COX-1, COX-2, and thromboxane synthase as well as expression of the thromboxane receptor transcript. Immunostaining showed a fibrillary cytoplasmic pattern for COX-1 in >90% of cells. COX-2 protein appeared in a diffuse to perinuclear cytoplasmic distribution, but was limited to approximately 40% of the cell population. A diffuse cytoplasmic staining pattern for thromboxane synthase was found in >90% of cells (Fig. 2).

Inhibition of endothelial cell migration by inhibitors of the arachidonic acid metabolism

The effect of the non-selective cyclo-oxygenase inhibitor acetyl salicylic acid (ASA), the COX-2 selective inhibitor sulindac and the specific thromboxane synthase inhibitors furegrelate and dazmegrel on HUVAC migration was tested in a 24 hour monolayer cell migration assay. The strongest inhibition of cell migration was observed for the thromboxane synthase inhibitor furegrelate, which resulted in a 60% reduction of migration compared to base line migration of mock treated cells. The cyclo-oxygenase inhibitors ASA and sulindac each showed a 20% reduction of cell migration (Fig. 3a). Increasing concentrations of the most effective inhibitor furegrelate resulted in a concentration dependent decrease of cell migration. After a single dose treatment a decrease of migration rates was observed, which did

not recover over the course of a 48 hour assay (Fig. 3b). Because cyclo-oxygenase is the rate-limiting enzyme in the synthesis pathway of thromboxanes COX inhibitors indirectly effect the formation rates of thromboxane A_2 in cells. A combination of COX inhibitors and suboptimal concentrations of the thromboxane synthase inhibitor furegrelate demonstrated that ASA had no additional effect, however, the COX-2 inhibitor sulindac strongly increased the inhibitory effect by suboptimal doses of furegrelate (Fig. 4). Under low serum conditions HUVEV showed a decreased migration rate. Addition of VEGF resulted in a partial restoration of the migratory capacity (Fig. 5a). To test whether the migration arrest inducted by furegrelate can be overcome by VEGF migration experiments were performed in 5% FCS containing media. These experiments demonstrated that under serum deprived conditions VEGF could rescue cells from the furegrelate induced inhibition of migration at low concentrations of the inhibitor, but not at optimal concentrations of the inhibitor (Fig. 5b).

Inhibition of capillary like network formation by inhibitors of the arachidonic acid metabolism

To demonstrate the role of thromboxane synthase in formation of a capillary network we have measured formation of capillary tubes by endothelial cells on a Matrigel® surface. A mock treated control population demonstrated cell congregation at nodal areas, which interconnected into a web-like network by formation of capillary like tubes between nodal areas. A well struc-

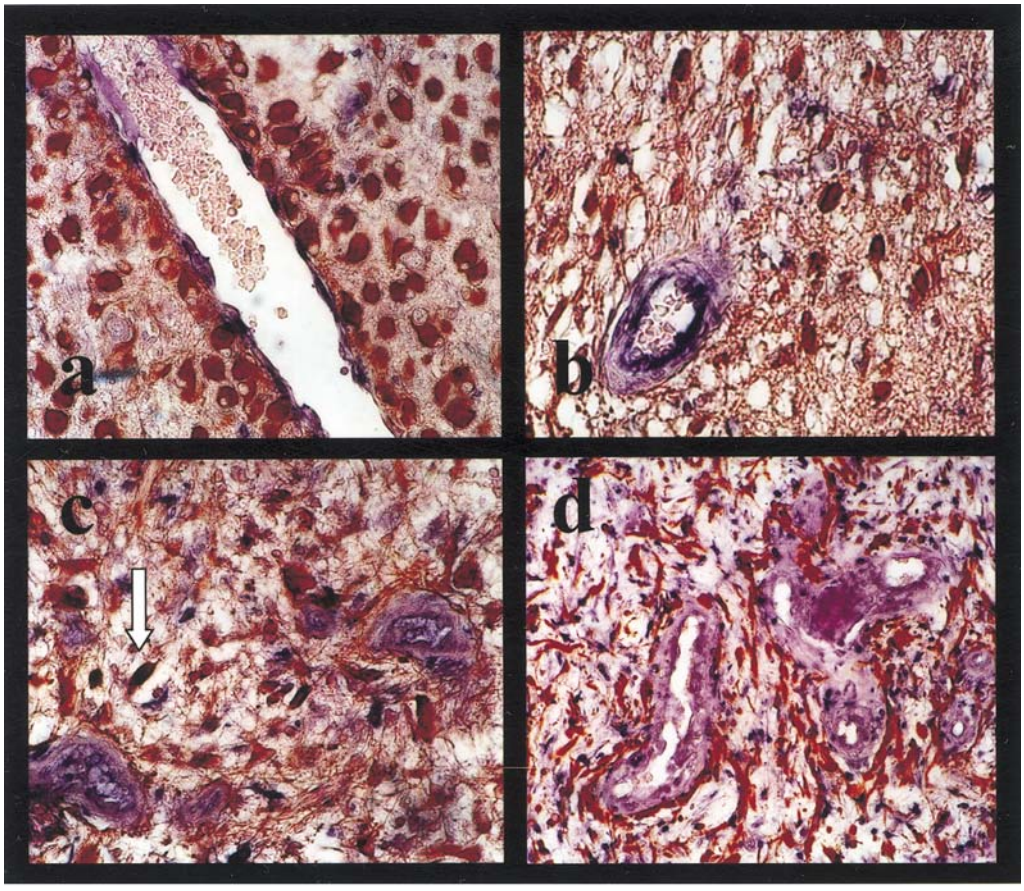

Fig. 1. (A) Immunostaining of glial tumours using double labelling with a monoclonal GFAP antibody (brown) and a polyclonal COX-1 antibody (blue). (a and b) astrocytoma WHO grade II, COX-1 positive endothelial cells and GFAP positive gemistocytic tumour cells; (c) anaplastic astrocytoma WHO grade III, COX-1 positive pathological small blood vessels and COX-1 and GFAP positive tumour cells (arrow); (d) glioblastoma WHO grade IV. (B) Immunostaining of glioblastoma specimens with a monoclonal thromboxane synthase antibody (a) or a polyclonal COX-2 antibody (b, c, and d). Thromboxane synthase and COX-2 positive endothelial proliferations within the tumour parenchyma (a and b), COX-2 positive tumour cuff formation (white arrow) around a vascular structure (c), section of a small vessel in the invasion zone with single COX-2 positive invasive glioblastoma cells (d, white arrow)

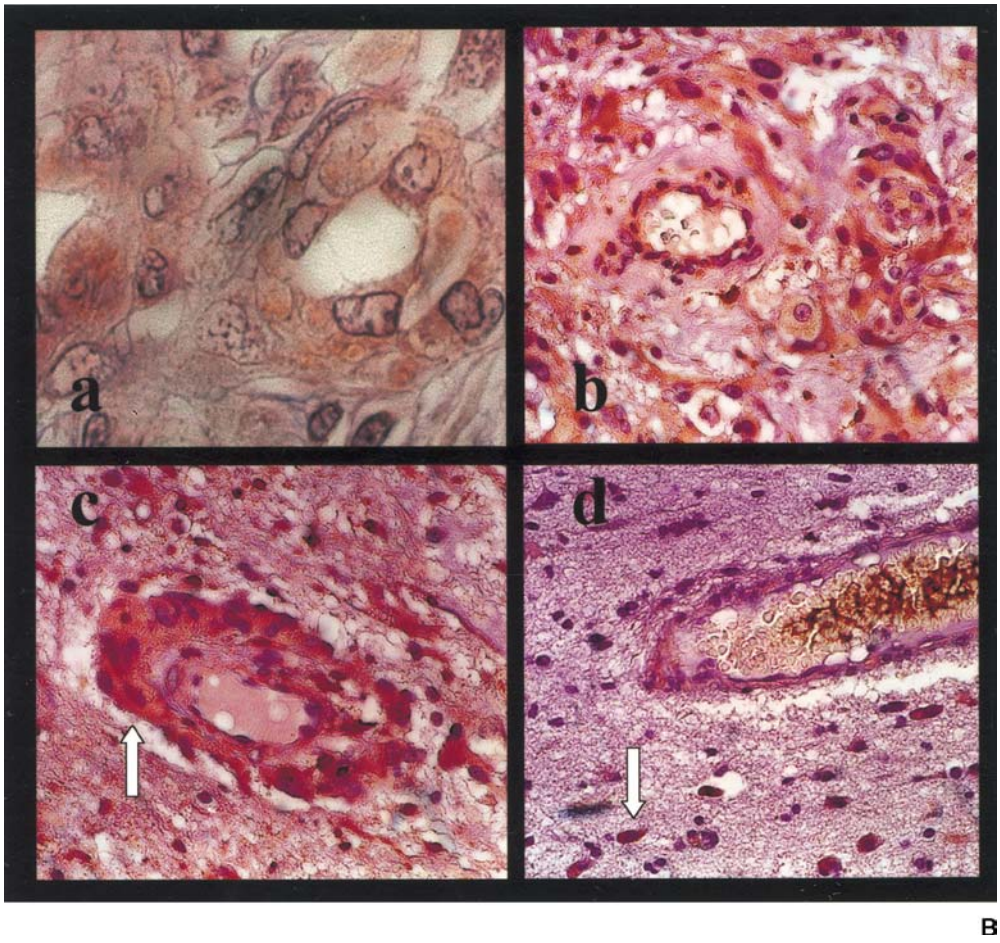

Fig. 1 (*continued*)

Table 1.

Specimen	COX-1	COX-2	ThxSyn
GBM WHO IV No.1	++	+++	+++
GBM WHO IV No.2	++	+++	+++
GBM WHO IV No.3	+	+++	++
GBM WHO IV No.4	++	++	+++
GBM WHO IV No.5	++	+++	+++
AA WHO III No.1	++	+++	++
AA WHO III No.2	++	+++	+++
AA WHO III No.3	(+)	++	++
AA WHO III No.4	++	++	+
AA WHO III No.5	++	+++	++
A WHO II No.1	++	−	−
A WHO II No.2	++	(+)	−
A WHO II No.3	+	−	−
OA WHO II No.4	++	−	−
OA WHO II No.5	++	(+)	−
NB	+	−	−
NB	+	−	−

GBM Glioblastoma; *AA* anaplastic astrocytoma; *A* astrocytoma; *OA* oligoastrocytoma.
Scoring of percent labelled endothelial cells: 0% = −, <10% = +, 10–50% = ++; >50% = +++.

tured network was observed at 24 hours and remained stable over 72 hours. In the presence of increasing concentrations of furegrelate both the number of branches from nodal areas and the length of tube like structures decreased in a dose dependent manner (Fig. 6). In furegrelate treated populations the branches frequently remained unconnected and showed clustering of rounded cells at the terminations.

Inhibition of growth and induction of apoptosis by inhibitors of the arachidonic acid metabolism

When furegrelate treated cell populations were followed over 72 hours by direct cell counts a decrease of the cell number was observed. Trypan blue exclusion demonstrated that after 24 hours the number of cells with a breakdown of membrane transport function began to increase over the mock treated control. At 72 hours after a single dose treatment of 1 mM furegrelate 38% of

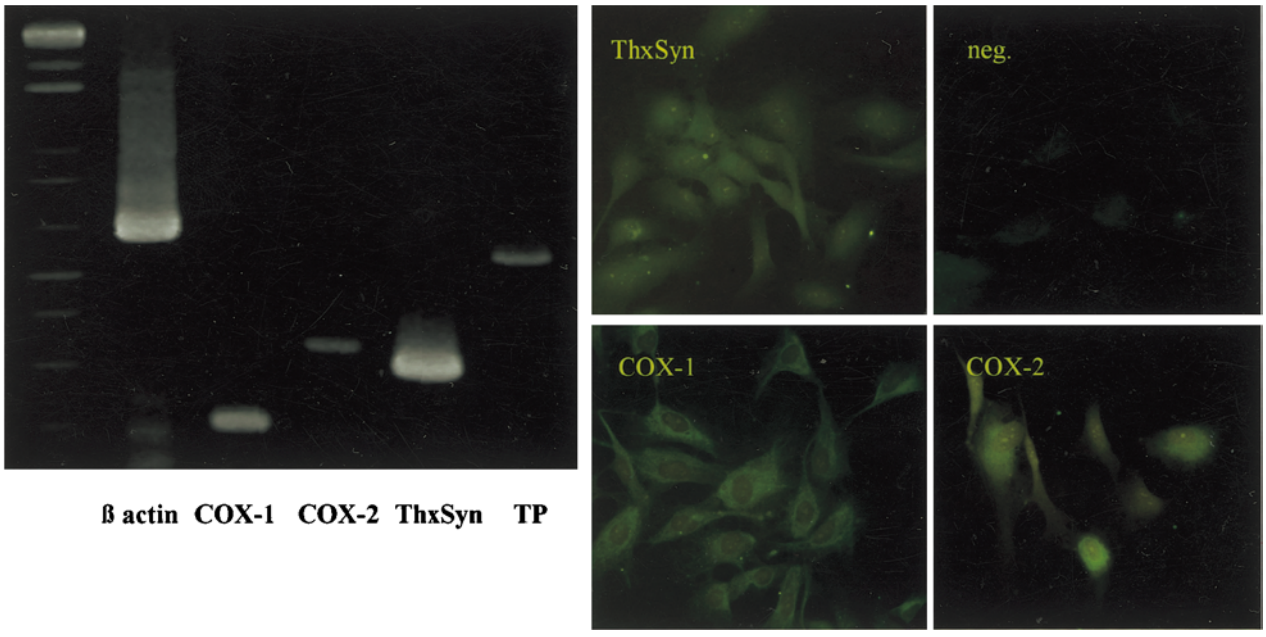

Fig. 2. RT-PCR amplification of COX-1, COX-2, thromboxane synthase (*ThxSyn*), and the thromboxane receptor (*TP*) in HUVAC (left). Immunostaining of cyclo-oxygenases and thromboxane synthase in HUCAC on tissue culture coated plastic surfaces

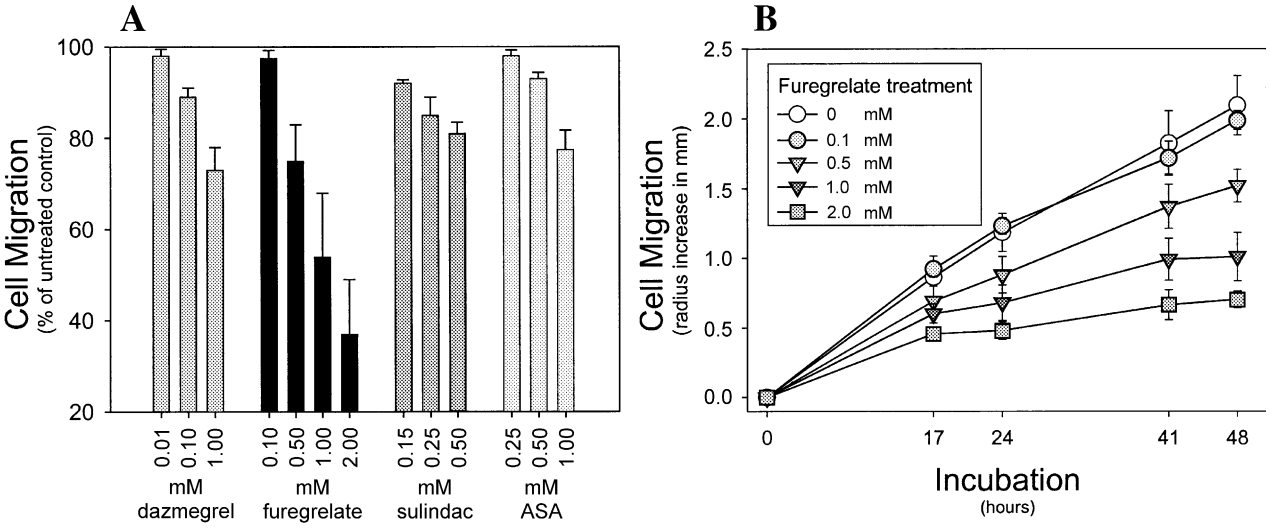

Fig. 3. (A) The effect of inhibitors of thromboxane synthase (dazmegrel, furegrelate), the selective cyclo-oxygenase 2 inhibitor sulindac, and the non-selective cyclo-oxygenase inhibitor acetyl salicylic acid (*ASA*) on HUVAC migration in monolayer. Regression analysis of migration curves from triplicate determinations. Bars, S.D. (B) Migration of HUVAC after a single dose treatment with furegrelate. The plotted values represent triplicate measurements. Bars, S.D

the population showed an impaired cell membrane function (Fig. 7). Morphologically, treated cells showed a condensed cytoplasm with shortened cellular processes and bleb-like structures at the periphery. To test whether cell death in the treated populations was due to an apoptotic cell death an ELISA was used for quantitative in vitro determination of cytoplasmic histone-associated DNA fragments [36]. In this assay, the intracellular enrichment of mono- and oligonucleosomal DNA that occurs after activation of endogenous endonucleases is due to the fact that, in apoptosis, DNA degradation occurs before the physical breakdown of the plasma membrane. A necrotic cell death, in contrast, will lead to early release of DNA fragments into the culture supernatant as a consequence of physical plasma membrane breakdown [1]. These experiments demonstrated that

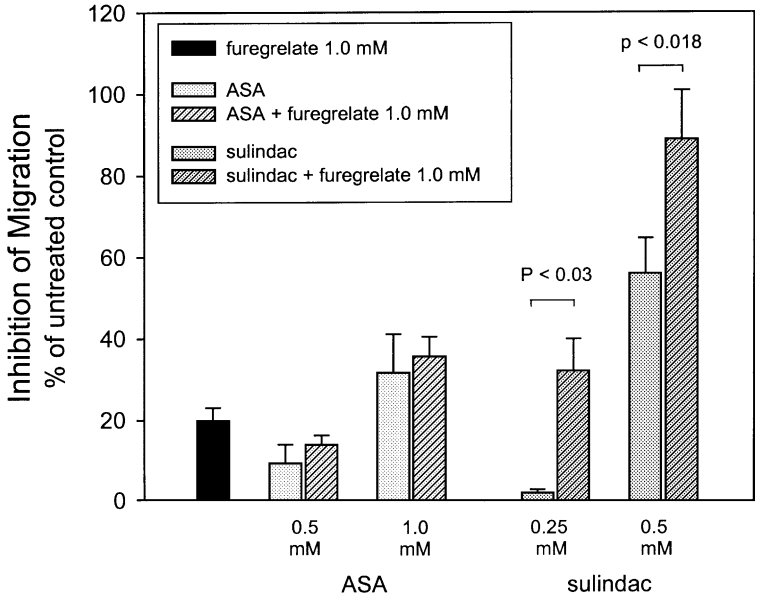

Fig. 4. Combined treatment of cyclo-oxygenase inhibitors and a suboptimal dose of the thromboxane synthase inhibitor furegrelate. Regression analysis of migration curves from triplicate determinations. Bars, S.D

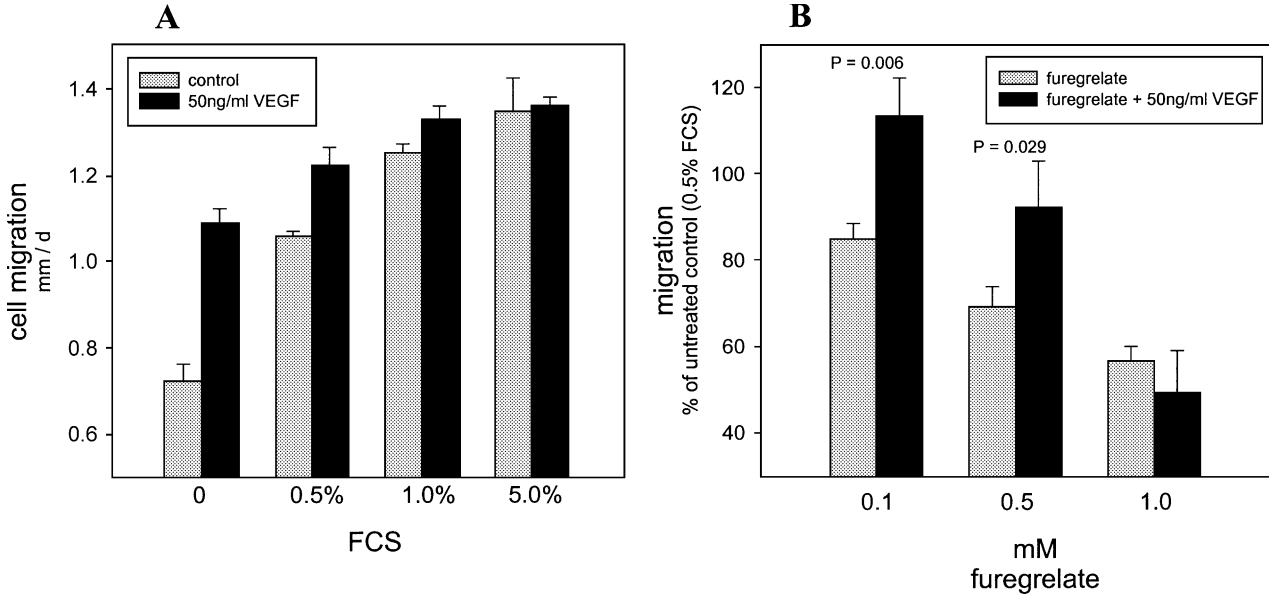

Fig. 5. (A) Monolayer migration of HUVAC under serum deprived conditions and addition of VEGF. (B) Furegrelate arrested HUVAC migration can be rescued by addition of VEGF only at low concentrations of the thromboxane synthase inhibitor. Regression analysis of migration curves from triplicate determinations. Bars, S.D

increasing concentrations of furegrelate up to a concentration of 2 mM resulted in an increase of intracellular DNA fragmentation in the absence of DNA release into the supernatant, indicating an intact membrane barrier function in cells undergoing fragmentation of their chromatin. Time course experiments demonstrated that fragmentation was increased over the control population as early as 4 hours after a single dose treatment with 1 mM furegrelate (Fig. 8). No DNA fragments appeared in the culture supernatants within the 48 hour course of these experiments. DNA fragmentation in HUVEC tended to occur at relatively higher concentrations compared to glioma cells in the absence of toxicity of the compound. However, HUVEC and GP8 rat brain endothelial cells showed intracellular DNA fragmentation after furegrelate treatment, whereas this thromboxane synthase inhibitor induced no apoptotic intracellular DNA fragmentation in normal human astrocytes or human fibroblasts (Fig. 9). When compared to the thromboxane synthase inhibitors furegrelate and dazmegrel, the non-

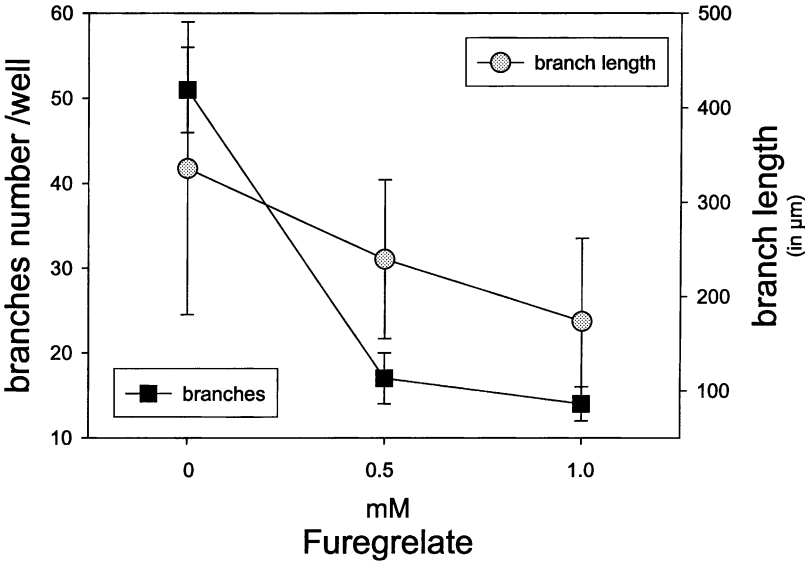

Fig. 6. HUVAC tube formation assay in the presence of increasing concentrations of the thromboxane synthase inhibitor furegrelate. Number of tube like structures (branches) and mean length of branches connecting nodal areas of congregated endothelial cells. Values represent the mean of triplicate determinations; bars, S.D

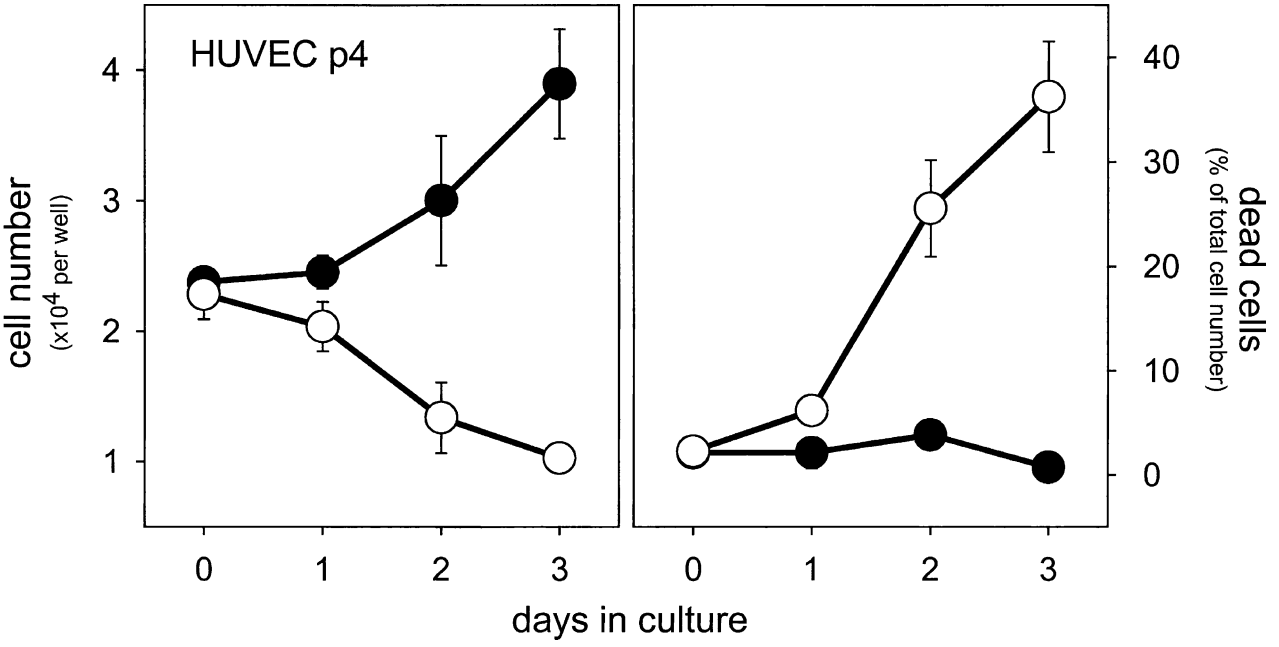

Fig. 7. Assessment of cell number (direct counts) and impairment of cellular membrane function (trypan blue exclusion test) after a single suboptimal dose treatment of 1 mM furegrelate. After 24 hour incubation an increase of cells with impaired membrane transport function is observed. Values represent the mean of triplicate determinations; bars, S.D

selective COX inhibitor ASA and the selective COX-2 inhibitor sulindac showed no significant induction of DNA fragmentation in HUVAC (Fig. 10).

Discussion

Neovascularisation is a tightly regulated process involving endothelial cell proliferation, migration, and tube differentiation, which is a prerequisite for local expansion of tumour colonies beyond the size (0.125 mm^2) restricted by oxygen and nutrient diffusion [6]. Angiogenesis is the process by which new capillaries sprout from pre-existing blood vessels, which is distinct from vasculogenesis in that it entails endothelial cell proliferation and migration, rather than the differentiation of endothelial cell from stem cells [25]. This process begins with the degradation of the basement membrane by activated endothelial cells, which requires

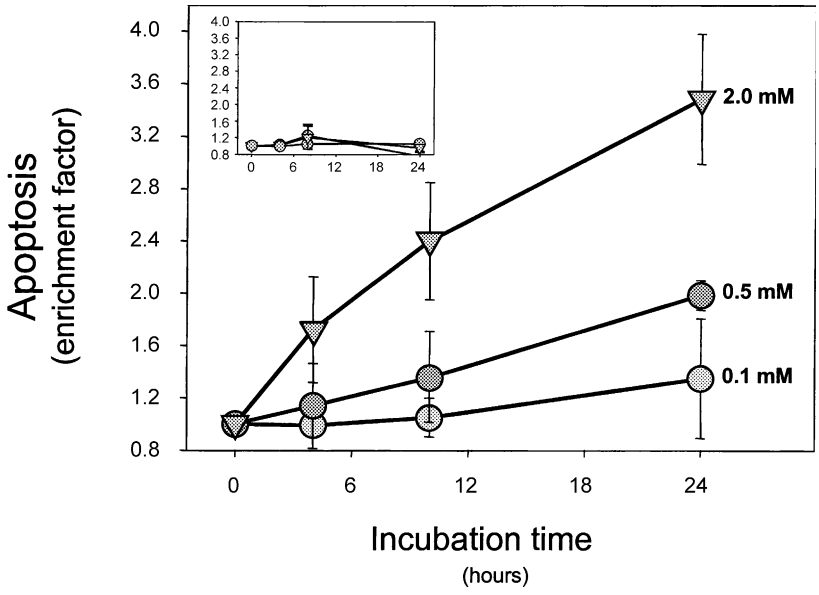

Fig. 8. Effect of thromboxane synthase inhibitor treatment on DNA fragmentation in HUVAC. Increasing concentrations of the inhibitor result in a dose dependent increase of intracellular DNA fragmentation in the absence of nucleosomal DNA released into the culture supernatant of the treated cell populations (insert). The plotted values represent triplicate measurements. Bars, S.D

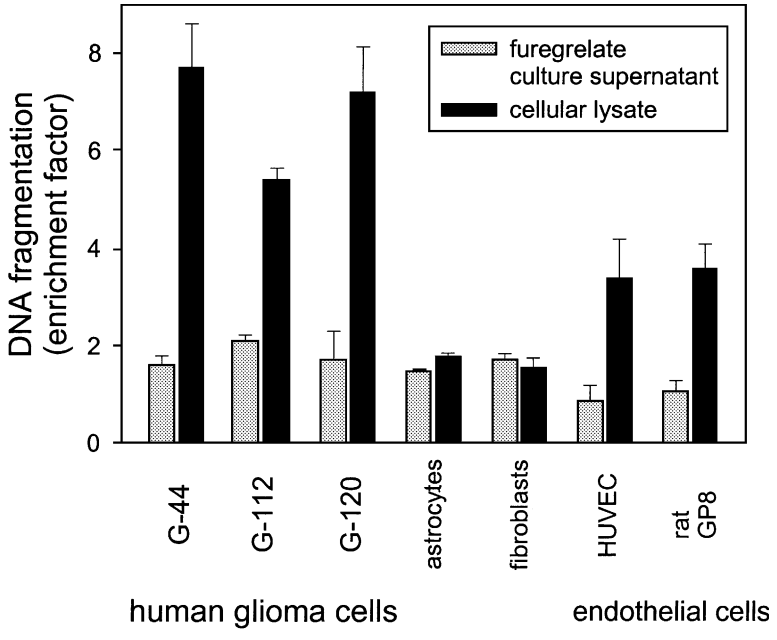

Fig. 9. Induction of apoptotic intracellular DNA fragmentation by the thromboxane synthase inhibitor furegrelate in glioblastoma derived cell lines, normal human astrocytes and fibroblasts, and endothelial cells (HUVAC, rat GP8 brain endothelial cells). The plotted values represent triplicate measurements. Bars, S.D

migration and proliferation, leading to the formation of solid endothelial cell sprouts into the stroma. Then, vascular loops and capillary tubes are formed, tight junctions develop and a new basement membrane is laid. The mechanisms underlying angiogenesis in malignant gliomas have yet to be identified, but rapid growth of malignant gliomas causes focal ischemia and hypoxia, which induces angiogenesis mediated by VEGF activity [24]. VEGF has been demonstrated to increase the synthesis of arachidonic acid metabolites. For example thromboxane A_2 levels rise three- to fivefold in endothelial cells after treatment with exogenous VEGF [22]. Thromboxane A_2 agonists stimulate endothelial migration and antagonists of the thromboxane receptor can reduce VEGF stimulated endothelial cell migration [22]. Generally, mobilization of arachidonic acid and subsequent formation of bio-active eicosanoids through cyclooxygenase, lipoxygenase, or the P450 epoxygenase pathways seem to be key elements in the cellular signalling of angiogenesis [21]. Inhibition of the cyclooxygenase activity by non-steroidal anti-inflammatory drugs has been shown to reduce angiogenesis in vivo [23]. More recently, selective COX-2 inhibitors have been shown to inhibit endothelial cell migration and

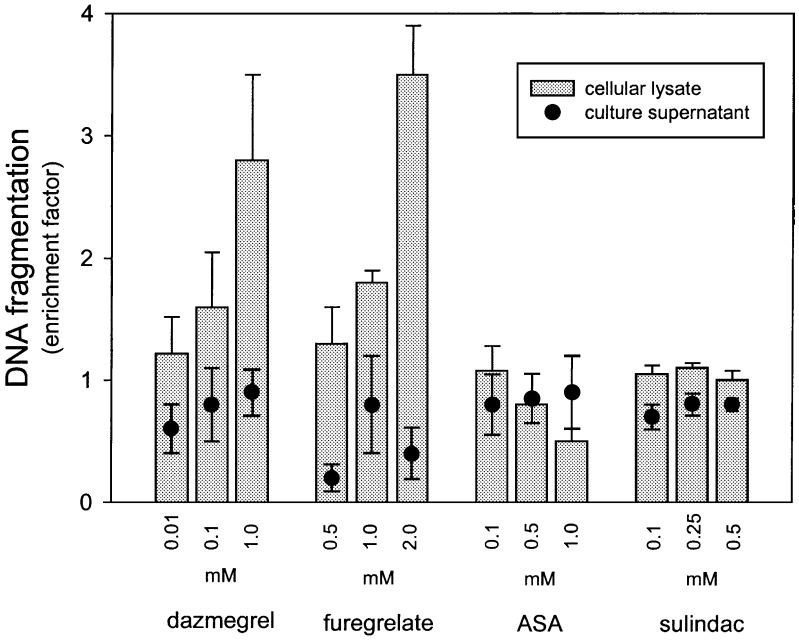

Fig. 10. Induction of intracellular DNA fragmentation by inhibitors of thromboxane synthase (dazmegrel, furegrelate), the specific cyclo-oxygenase 2 inhibitor sulindac, and the non-specific cyclooxygenase inhibitor acetyl salicylic acid (*ASA*) in HUVAC. The plotted values represent triplicate measurements. Bars, S.D

angiogenesis [4]. However, some products of the cyclo-oxygenase pathway such as PGE_1 and PGE_2 may promote angiogenesis [37], whereas products of PGD_2 may induce endothelial cell apoptosis and inhibit angiogenesis [35]. Therefore, the profile of downstream COX metabolites rather that the levels of COX protein or COX activity may be relevant in the regulation of angiogenesis. In this study we have demonstrated that non-selective inhibitors of cyclo-oxygenase and selective COX-2 inhibition only had a minor effect on endothelial cell motility. The specific thromboxane synthase inhibitor furegrelate strongly decreased endothelial cell migration. VEGF treatment, which increases thromboxane A_2 in endothelial cells resulted in increased migratory activity of HUVAC, but could only antagonize the effect of the thromboxane synthase inhibitor at low concentrations of the inhibitor. The thromboxane synthase inhibitor induced arrest of endothelial cell migration could be enhanced by co-treatment with a selective COX-2 inhibitor, which indirectly decreases the formation of thromboxane A_2 by a decrease of the substrate of thromboxane synthase PGH_2. This suggests that the downstream metabolite thromboxane A_2, rather that a balance of angiogenesis promoting or inhibiting prostaglandins, may be a predominant mediator of endothelial cell migration.

In malignant gliomas both isoforms of cyclo-oxygenase, COX-1 and COX-2, are expressed [5, 13, 15] as well as the downstream thromboxane synthase [10]. We have previously demonstrated overexpression of thromboxane synthase in glioma cell subpopulations selected for migration [18] and that thromboxane synthase inhibitors block motility of malignant glioma cells in vitro [10]. In glioma cells migration arrest leads to an increase of apoptotic cells in the treated cell population and also has a sensitising effect to treatments with other agents inducing apoptosis [36]. This paradigm is intriguing because there is increasing evidence that invasive glioma cells may be protected from apoptosis and therefore, be intrinsically resistant to many current treatment strategies [16, 17]. For invasive glioma cells thromboxane synthase may represent a conversion point of signalling pathways that may allow invasive cells to be rendered susceptible to induced apoptosis. Interestingly, this paradigm may not be limited to neopalstic cells. HUVAC in vitro like vascular structures within malignant gliomas in vivo express cyclo-oxygenases and thromboxane synthase as well as the thromboxane A_2 receptor. Our results demonstrate that thromboxane synthase inhibitors also lead to migration arrest in human vascular endothelial cells and rat brain endothelial cells (data not shown), which is paralleled by a decreased formation of capillary like tubes, indicating that interference with thromboxane A_2 formation may disrupt essential mechanisms in angiogenesis. This may be a result of both inhibition of migration as well as induction of apoptosis in endothelial cells. Compared to glioma cells both human vascular endothelial cells and the rat brain derived endothelial cells GP8 were more resistant to thromboxane synthase inhibitor induced apoptosis. However, normal human astrocytes and fibroblasts did

not show apoptotic DNA fragmentation at concentrations used in these experiments. These data suggest that inhibitors of thromboxane synthase influence migration and apoptosis in human glioma cells and human endothelial cells by similar mechanisms. The pharmacokinetics of furegrelate the thromboxane synthase inhibitor, which showed the strongest inhibition of endothelial migration and induction of apoptosis in this study, has been studied up to an oral dose of 1600 mg/d in human volunteers with no adverse effects [20]. We could recently demonstrate that intralesional infusion of furegrelate leads to a greater than 70% volume decrease of intracranial tumours in a glioma mouse model. Interestingly, this effect was associated with a significant decrease of tumour microvasculature. These experiments showed no evidence of local or systemic toxicity and no intracranial haemorrhage as a potential consequence of inhibition of platelet aggregation (Schmidt *et al.* manuscript in preparation). An anti-invasive treatment strategy using this class of compounds may therefore not only sensitise glioma cells to conventional treatments inducing apoptosis but may also support a potential treatment effect by an anti-angiogenic effect.

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Comments

Doctors Jantke *et al.* previously studied the effect of arachidonic acid metabolism blockers on migration and induction of apoptosis in cells from established human glioma cell lines. In this study they have demonstrated in a nice series of experiments that thromboxane synthase and COX-2 expression was a feature of endothelial cells in anaplastic gliomas and glioblastomas and that thromboxane synthase inhibitors decreased endothelial cell migration and capillary tube like formation. These compounds also induced apoptosis in endothelial cells. The studies are well conceived and, performed; they expand on the previous work by the authors and provide additional insights into potential arachidonic acid related angiogenesis in gliomas.

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As COX-2 and thromboxane synthase are strongly upregulated in anaplastic astrocytoma and glioblastoma endothelial cell proliferations as compared to normal brain endothelia, the authors studied in vitro, with anti-invasive therapy of gliomas in mind, as a special clinical application, whether a non-selective COX inhibitor (ASA), a specific COX-2 inhibitor (sulindac) and two specific thromboxane synthase inhibitors (furegrelate, dazmegrel) would affect the migration, capillary like tube formation and apoptosis of cultured human umbilical endothelial cells (HUVEC) that expressed COX-2 and thromboxane synthase at RT-PCR as well as rat brain endothelial cells (GP8), as compared to human astrocytes and fibroblasts. The combination of sulindac and furegrelate was most efficient against HUVEC migration and in inducing apoptosis.

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