Thromboxane synthase regulates the migratory phenotype of human glioma cells¹

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The capacity of glial tumor cells to migrate and diffusely infiltrate normal brain compromises surgical eradication of the disease. Identification of genes associated with invasion may offer novel strategies for anti-invasive therapies. The gene for TXsyn, an enzyme of the arachidonic acid pathway, has been identified by differential mRNA display as being overexpressed in a glioma cell line selected for migration. In this study TXsyn mRNA expression was found in a large panel of glioma cell lines but not in a strain of human astrocytes. Immunohistochemistry demonstrated TXsyn in the parenchyma of glial tumors and in reactive astrocytes, whereas it could not be detected in quiescent astrocytes and oligodendroglia of normal brain. Glioma cell lines showed a wide range of thromboxane B₂ formation, the relative expression of which correlated with migration rates of these cells. Migration was effectively blocked by specific inhibitors of TXsyn, such as furegrelate and dazmegrel. Other TXsyn inhibitors and cyclooxygenase inhibitors

were less effective. Treatment with specific inhibitors also resulted in a decrease of intercellular adhesion in glioma cells. These data indicate that TXsyn plays a crucial role in the signal transduction of migration in glial tumors and may offer a novel strategy for anti-invasive therapies. *Neuro-Oncology* 1, 3–13, 1999 (Posted to *Neuro-Oncology* [serial online], Doc. 98-14, January 19, 1999. URL <neuro-oncology.mc.duke.edu>)

Local invasion that follows white matter fiber tracts, the perivascular spaces, and within the subependymal zone seems to be an inherent behavior of glial tumor cells. Clearly, invasive behavior requires cellular mechanisms such as substrate adhesion, matrix degradation, cellular detachment from previous attachment sites, and cell locomotion, all of which facilitate single-cell dissemination of a solid tumor (Giese and Westphal, 1996). An increasing number of studies report possible mechanisms of glioma cell invasion in vitro and in vivo. Several cellular and extracellular components—such as matrix protein, glycosaminoglycans, and myelin—have been identified that support adhesion and motility of these tumor cells (Amberger et al., 1994; Giese et al., 1994, 1995, 1996a). Some of the receptors involved have been identified, and we have recently characterized adhesive histioanatomic structures in normal human brain that may serve as routes for glioma cell migration (Giese et al., 1996b, 1998; Paulus et al., 1993). These studies have, however, lead to a substantial number of possible substrates that glioma cells could differentially prefer for dissemination in brain. In addition, there seems to be great redundancy in the receptor systems involved. To date, there is very little information on the downstream signaling cascades triggered by the interaction of extracellular ligands with cellular receptors that regulate the migratory state of glioma cells. Interference with these signaling pathways may offer new targets by which to

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³Abbreviations used are as follows: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein, acetoxymethyl ester; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinase; FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGH₂, prostaglandin H₂ endoperoxide; RT, reverse transcriptase; TXsyn, thromboxane synthase; WHO, World Health Organization.

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interfere with glioma motility, regardless of the matrix substrates and receptor systems involved.

Using differential mRNA display, we have recently identified overexpression of TXsyn³ in a glioma G-112 cell line selected for a highly migratory phenotype (McDonough et al., 1998). TXsyn, an enzyme of the arachidonic acid metabolism, mediates conversion of PGH₂ into TXA₂, which is then converted into a stable but biologically inactive metabolite TXB₂. Both PGH₂ and TXA₂ bind to a common transmembrane receptor that associates with $\alpha_q\beta\gamma$ heterotrimeric G proteins. Binding of both ligands activates phospholipase C β , subsequently leading to a rise in cytosolic Ca⁺⁺ concentration, which activates protein kinase C and the FAK-related protein kinase PYK2 in some cellular systems. Both signaling pathways in platelets eventually lead to adhesion and aggregation (Brass et al., 1997; Negishi et al., 1995). In platelets, these signaling pathways are paralleled by a cascade triggered by $\alpha_{IIb}\beta_3$ integrins, which, when activated in focal adhesion clusters, assemble a signaling complex containing pp125 FAK and possibly PYK2. In this sense, G protein-coupled receptors and integrins may be linked in events leading to activation of Ras, Rho, and Rac, resulting in cytoskeletal rearrangements associated with modulation of cellular adhesion. But in addition, the $\alpha_{IIb}\beta_3$ integrins in focal adhesion clusters may also be activated directly by G protein-coupled receptors through a pathway that still needs to be defined. Phosphorylation of pp125 FAK is inhibited by blocking cyclooxygenase and can be induced by thromboxane agonists (Shattil et al., 1994). To date, it is unknown whether similar pathways are involved in adhesion- and migration-related mechanisms in solid tumors.

Expression of the thromboxane receptor has been reported in normal glia and there have been single reports on rat glioma cells in culture expressing prostanoid receptors (Inagaki and Wada, 1994). In a study by Castelli et al. (1989), high-resolution gas chromatography-mass spectrometry showed higher TXA₂ synthesis in lysates of malignant gliomas than in meningiomas and normal brain. Furthermore, TXA₂ synthesis seemed to increase with grade of the glial tumor. The authors suggested that expression may be related to invasiveness of these tumors. The method did not determine the cellular origin of prostaglandins and thromboxane, however. To date, no data have demonstrated that mechanisms of glioma invasion may be regulated by prostanoid metabolites. However, levels of thromboxane synthesis correlate with the metastatic potential of malignant cells of different tissue origin (Bryant, 1994; Nanji, 1993). As a possible mechanism, the ability of thromboxanes to stimulate platelet aggregation and to act as a powerful vasoconstrictor have been discussed, which are thought to result in trapping of tumor cell emboli in capillaries (Moncada and Vane, 1979). This mechanistic concept is, however, unrelated to the classic cascade of events that define the process of normal tissue invasion by malignant tumor cells, which includes matrix adhesion, matrix degradation, and cellular migration. Because systemic metastasis of gliomas is extremely rare, trapping of tumor emboli in capillar-

ies most likely does not contribute to glioma dissemination (Pasquier et al., 1980).

In this study we demonstrate that TXsyn and its receptor (TP) are expressed in glioma cell lines and biopsies from glial tumors, but not in quiescent astrocytes or oligodendrocytes of normal brain tissue. The relative formation of TXB₂ in glioma cell lines correlates with migration rates of these cells. TXsyn inhibitors can effectively block migration and may offer a novel strategy for anti-invasive therapies for gliomas.

Materials and Methods

Human glioma cell lines were propagated in MEM (Biochem, Berlin, Germany) with 10% FCS and were passaged using trypsinization at regular intervals, depending on growth characteristics.

Specific TXsyn inhibitors furegrelate (sodium 5-[3'-pyridinylmethyl] benzofuran-2-carboxylate; F-3764, Sigma, Deisenhofen, Germany), dazoxiben (4-[-2-[1H-imidazol-1-yl]ethoxy]benzoic acid hydrochloride; UK 37248, Pfizer, Karlsruhe, Germany), and dazmegrel (3-[1H-imidazol-1-yl-methyl]-2-methyl-1H-indole-1-propanoic acid; Pfizer); and nonselective inhibitors Aspizole (Bayer AG, Leverkusen, Germany), imidazole (I-0125, Sigma), and indomethacin (I-0511, Sigma); a specific TXsyn inhibitor and thromboxane receptor antagonist R 68,070 ((E)-5-[[[(3-pyridinyl) [3-(trifluoromethyl)phenyl]-methylene] amino] oxy] pentanoic acid) or Ridogrel (Janssen, Olen, Belgium) were prepared according to manufacturers' instructions for use in tissue culture. The concentration range selected for functional assays was based on published studies using other cellular systems (Auch-Schwelk et al., 1990; Gorman, 1983; Mohrland et al., 1989; Wynalda et al., 1983). In addition, concentrations used in migration assays were determined to be in a nontoxic dose range by prior growth curve experiments using glioma cell lines.

RT-PCR Analysis of TXsyn RNA Expression

Total RNA was isolated from monolayer cultures and quantified by absorbance measurements at 260/280 nm. RT synthesis of cDNA was done using the First Strand Synthesis Kit (Stratagene, La Jolla, Calif.), and primers for TXsyn (CAA GCA GGT GTT GGT TCA GAA and TAA ATG AGC CAG GAG AAG GTC) and thromboxane receptor (GGC TGT CCT TCC TGC TGA AC and GCT GAG GCG AGG CTG GAG AC) were designed with the help of OLIGO 4.0. Amplification of DNA (1 μ L of RT product, 1 μ L of each primer, 0.2 μ L of Taq polymerase [Perkin Elmer/Cetus, Foster City, Calif.], and 1 μ L of nucleotides in 2 μ L of 10x buffer) was allowed to run for 40 cycles (1 min at 60°C and 2 min at 72°C). Aliquots of 9 μ L were collected and run in 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Specific amplification was confirmed by automated sequencing of PCR products.

Immunohistochemistry and Immunofluorescence

Paraffin sections were washed, dehydrated, and then incubated in 10 mM NaN₃, 0.15% H₂O₂ in PBS for 30

min to block endogenous peroxidase activity. Sections were then pre-incubated with 10% horse serum for 60 min before adding the primary monoclonal antibody to human TXsyn TŮ-300 (BMA, Basel, Switzerland) at a 1:200 dilution followed by overnight incubation at 4°C. A biotinylated anti-mouse IgG (Vector Laboratories, Wiesbaden, Germany) was added at a 1:200 dilution for 2 h, detection was carried out using Vectastain (Vector Laboratories) and DAB Substrate Kit (Vector Laboratories) followed by a hemalum counterstain. For immunofluorescent detection of TXsyn in cultured cells, glioma cells were seeded on glass coverslips, fixed in a 5% acidic acid and 95% ethanol solution, permeabilized with 3% Triton X, and incubated with TŮ-300 for 60 min. The sections were rinsed in MEM, and a secondary antibody conjugated with fluorescein isothiocyanate was added. After 60 min of incubation, the sections were again rinsed and covered with Citifluor (WAK Chemie, Bad Homburg, Germany). The sections were evaluated under 490 nm excitation-520 nm emission fluorescence microscopy.

Western Blot Analysis

Glioma cell lines were cultured to subconfluence and rinsed with cold PBS twice, then the monolayers were collected by scraping. Cell pellets were lysed in 50 mM HEPES (pH 7.5), 250 mM NaCl, 0.1% NP 40 with 5 µg/mL aprotinin, 5 µg/mL leupeptin, 5 µg/mL pepstatin A. Protein concentrations of the extracts were determined by Bradford staining (BioRad, Hercules, CA). Samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose. The blot was probed with a monoclonal antibody Kon 7 (kindly provided by R. Nüsing, Ph.D., University of Marburg, Germany) (Nüsing et al., 1990) to human TXsyn, which (in contrast to TŮ-300) binds to the enzyme under denaturing conditions. Bands were detected by incubation with a goat anti-mouse secondary antibody followed by visualization with ECL (Amersham International, Buckinghamshire, UK).

TXB₂ ELISA

TXB₂, a stable but biologically inactive metabolite of TXA₂, was analyzed in cell culture supernatants by incubating monolayer cultures to 70% subconfluence in MEM containing 10% FCS. Cultures were rinsed with PBS, fresh serum-free media was added, and cultures were maintained for another 48 h. Supernatants were collected, centrifuged, and stored at -80°C, and cells were trypsinized and counted. TXB₂ was detected by an enzyme immunoassay using peroxidase-labeled TXB₂ conjugates according to manufacturer instructions (Bio-trak RPN 220, Amersham International). TXB₂ levels were reported as nanogram per 10⁶ cells from triplicate determinations.

Cell Adhesion and Cell Migration Assay on Purified Extracellular Matrix

Glioma cells were tested for substrate adhesion using a method modified from that described by Kramer et al. (1989). Briefly, cells were harvested from monolayer

culture and deposited into microtiter wells (5×10⁴ cells/well), which were passively coated with human extracellular matrix proteins. The microtiter plates were incubated for 30 min on ice, then at 37°C for 60 min to allow cell adhesion. The plates were then subjected to vigorous agitation (350 rpm for 6 min on a horizontal rotor), and afterwards nonattached cells were removed by aspiration and rinsing with PBS. Attached cells were fixed and stained using crystal violet (0.1% H₂O). Absorbance of stained nuclei was quantified using spectrometry at 540 nm. Replicates from three wells were averaged. The number of attached cells was reported as absorption units.

Cell migration was quantified using a modified monolayer migration assay (Berens et al., 1994; Giese et al., 1994), which measures expansion of a cell population on surfaces. Ten-well teflon-coated slides (Dynex Technologies, Denkendorf, Germany) were coated with AES (3-aminopropyltriethoxysilane, A-3648, Sigma) to optimize protein and cell adhesion. Slides were then passively coated with a solution of purified merosin (100 µg/mL), an isoform of human laminin that has been demonstrated as a highly permissive substrate for the glioma cell lines used in this study (Giese et al., 1994, 1995). Custom-produced cell sedimentation manifolds (Creative Scientific Methods, Mesa, AZ) were placed over the slides containing 50 µL of culture media. Cells were seeded in a volume of 1 µL MEM (2000 cells), and slides were placed on ice for 60 min to allow faster sedimentation of cells and then incubated for 8 h at 37°C. The cell sedimentation manifold was removed, and fresh MEM with 10% FCS was added. The circular area occupied by attached cells in each well was imaged using a CCD camera (TK-1280E, JVC, Tokyo, Japan) attached to an inverted microscope (Labovet, Leica, Hamburg, Germany) and was digitized with an image analysis system for quantification (Quantimed 500, Leica). Object sizes were measured as the radius in micrometers of the circular area covered by a cell population. Serial images were captured after removal of the cell sedimentation manifolds for up to 48 h. 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 represented net changes in the geographical distribution of the cell population and did not reflect movement of individual cells.

Cell-Cell Adhesion Assay

Intercellular adhesion was tested in this system by seeding trypsinized tumor cells onto a preformed host monolayer of cells that were formed by cells of the same cell line and the same passage number. A serial dilution of cells was seeded into 48-well flat-bottom microtiter plates (maximum 10⁵ cells/well) and allowed to adhere and spread overnight to create a host monolayer. Plates were washed, and fresh MEM containing 10% FCS was added to the wells. An aliquot of glioma cells was harvested from monolayer culture and pre-incubated with the in vivo fluorescent dye BCECF (5 µM BCECF in phenol red-free MEM) (MoBiTec, Eugene, OR) for 15 min at 37°C. Cells were washed in MEM and seeded

Fig. 1. Expression of thromboxane synthase in cultured glioma cells and astrocytes. A. Reverse transcriptase-polymerase chain reaction amplification of β -actin, thromboxane receptor, and thromboxane synthase in normal astrocytes and glioma cell lines. B. SDS-polyacrylamide gel electrophoresis analysis of thromboxane synthase in cellular lysates—obtained glioma cell lines, using monoclonal antibody Kon 7 in a 10% sodium dodecyl sulfate gel.

onto the host layers at a concentration of 10^5 cells/well. After the cultures were exposed to 4°C temperatures for 30 min, plates were incubated for 30 min at 37°C in a humidified atmosphere containing 5% CO_2 . Plates were then agitated on a rotating platform at 250 rpm and washed three times with PBS to remove nonadherent cells. Adherent BCECF-labeled cells were quantified using a Spectra Fluor fluorescent plate reader (Tecan, Crailsheim, Germany). The number of adherent cells was determined based on relative fluorescence calculated from a standard curve. Data were reported as mean determinations in replicates of eight.

Results

TXsyn RNA Expression and TXB_2 Levels in Human Gliomas

To study the expression of TXsyn, total RNA was extracted from nine glioblastoma-derived cell lines and a strain of normal astrocytes that were derived from normal adult human brain (Asch et al., 1986). PCR primers spanning exons 4–6 amplified a 211-bp fragment of expected molecular weight in 9 of 10 glioma cell lines. Thromboxane receptor mRNA was expressed

Fig. 2. Immunofluorescent staining of G-22 (A), G-28 (B), G-96 (C), and G-120 (D) using monoclonal antibody TÛ-300 against thromboxane synthase. Fluorescence microscopy, 490 nm excitation–520 nm emission at $400\times$ magnification.

in all cell lines. Specific amplification was confirmed by sequencing. Normal astrocytes did not express TXsyn mRNA, but did show mRNA expression of the receptor at levels similar to glioma cell lines (Fig. 1A). Western blot analysis showed expression of a 58-kDa protein at varying intensity in all glioma cell lines with the lowest level in G-62 and G-112 cells. This finding corresponds with previously reported detection of purified human TXsyn by monoclonal antibody Kon 7 (Nüsing et al., 1990). Interestingly, G-168 reproducibly showed a high molecular band (82 kDa) in addition to the 58-kDa protein. This high molecular protein was also detected in G-59 cells but at a much lower level of intensity (Fig. 1B). Immunofluorescent staining demonstrated TXsyn in a predominantly cytoplasmatic perinuclear pattern, and some cells showed a membrane-associated distribution with intense staining of areas at the leading edge of the cells (Fig. 2).

To estimate the enzyme activity of TXsyn, we used an ELISA that quantifies the synthesis of the stable but biologically inactive TXA_2 metabolite TXB_2 . A wide range of TXB_2 levels was found in cultured glioma cells. G-112, G-62, and G-44 at the low end of the ELISA detection range demonstrated levels below $0.1 \text{ ng}/10^6$ cells. Most glioma cells showed levels between 0.2 and $0.5 \text{ ng}/10^6$ cells. With $3.6 \text{ ng}/10^6$ cells, G-120 showed the highest levels, a 100-fold increase over G-112. The synthesis of TXB_2 was also analyzed in early cultures of cells that were derived from human glioblastoma biopsies at passages 2 to 5. The supernatants of these cultures contained levels of TXB_2 above those from the established glioma cell lines (Fig. 3). G-112 and G-62, which demonstrated the lowest levels of TXB_2 formation, also showed very low levels of both TXsyn mRNA by RT-PCR and protein by Western blot analysis. They were also found negative for TXsyn by immunofluorescent staining, indicating that the TXB_2 levels are a direct consequence of Txsyn activity rather than being due to secondary modulation by metabolic events.

Fig. 3. Thromboxane B₂ formation in glioma cell lines. Enzyme immunoassay of cell culture supernatants of glioma cell lines and primary cells from glioblastoma specimens. Values are means of triplicate determinations. Bars, SD.

The migration rates of nine glioma cell lines were quantified on a permissive extracellular matrix protein using a monolayer migration assay, and the motility rates were correlated with levels of TXB₂ formation. Cell lines demonstrating high thromboxane levels, arbitrarily chosen as >0.2 ng/10⁶ cells, showed a significantly higher migration rate than cells producing lower levels (<0.2 ng/10⁶) (Fig. 4). However, there was no strict correlation between absolute amounts of TXB₂ synthesis and migration. G-120, which demonstrated very high levels of TXB₂ formation, only ranked third in terms of migration rates.

To study TXsyn and thromboxane receptor expression in glial tumors, total RNA was extracted from snap-frozen biopsy specimens of nine glioblastomas, three low-grade gliomas, and normal adult brain tissue. Normal brain showed minimal mRNA expression of both TXsyn and thromboxane receptor. In contrast, all

glioma biopsies showed high expression of TXsyn mRNA and 8 of 12 expressed thromboxane receptor mRNA (Fig. 5). In addition, the expression and tissue distribution of TXsyn was studied in paraffin sections of gliomas of different tumor grades: 10 glioblastomas WHO IV, 5 anaplastic astrocytomas WHO III, 5 low-grade astrocytomas WHO II, and 5 pilocytic astrocytomas WHO I (Fig. 6). TXsyn expression was found in all grades of glial tumors. Astrocytoma cells of gemistocytic differentiation most prominently expressed TXsyn, regardless of the tumor grade. Generally, expression seemed lower in high-grade tumors, and the lowest expression was found in the highly cellular undifferentiated small-cell glioblastomas, with single cells containing the enzyme scattered throughout the tumor. These tumors also demonstrated the highest rates of mitotic figures. Areas of oligodendrocytic differentiation showed weak staining, indicating that tumors of oligodendrocyte lineage may contain lower levels than astrocytomas. However, there were no pure

Fig. 4. Correlation of glioma migration and levels of thromboxane B₂ synthesis. Cell lines were grouped by levels of synthesis lower or higher than 0.2 ng/10⁶ cells. ●, individual migration rates; ○, mean of grouped cell lines; bars, SD.

Fig. 5. mRNA expression of thromboxane synthase in glial tumors and brain. mRNA expression of β-actin, thromboxane receptor (TP), and thromboxane synthase (Thxsyn) in biopsies of gliomas and normal brain determined by reverse transcriptase-polymerase chain reaction.

Fig. 6. Thromboxane synthase expression in glial tumors. Immunohistochemical demonstration of thromboxane synthase in astrocytic tumors and normal brain. Original magnification of micrographs $\times 123$, hemalum counterstain. A. Labeled and unlabeled glioblastoma cells. B. Labeling of gemistocytic tumor cells in a glioblastoma. C. Anaplastic astrocytoma, staining of gemistocytic cells. D. Anaplastic fibrillary astrocytoma. E. Gemistocytic cells in an astrocytoma, World Health Organization grade II. F. Pilocytic astrocytoma. G. Labeling of endothelial proliferations in a glioblastoma. H. Reactive astrocytes. I and J. Labeling of neurons in the neocortex with thromboxane synthase-negative astrocytes and oligodendrocytes.

oligodendrogliomas included in the present series to verify this hypothesis. In normal brain, astrocytes and oligodendrocytes showed no TXsyn content, but there was positive immunoreactivity in the cytoplasm of neurons. Areas adjacent to tumor or showing single-cell tumor invasion also contained reactive astrocytes, which showed brisk staining for the enzyme. Normal brain capillaries did not contain thromboxane, whereas endothelial proliferations in high-grade tumors showed a weak staining.

Inhibition of glioma migration by inhibitors of arachidonic acid metabolism

Furegrelate, a highly specific TXsyn inhibitor (Gorman, 1983; Mohrland et al., 1989; Wynalda et al., 1983), was tested for its ability to inhibit migration of glioma cells. G-120, which showed the highest levels of TXB₂ forma-

tion, demonstrated significantly higher migration rates than G-112 (Fig. 7A). Increasing concentrations of the inhibitor resulted in dose-dependent inhibition of the migration of G-22, G-28, and G-120, glioma cell lines that synthesize high levels of TXB₂ (0.45, 0.52, and 3.6 ng/10⁶ cells, respectively). In contrast, migration of G-62 and G-112, which demonstrated the lowest levels, were only marginally affected by any concentration of furegrelate (Fig. 7B). Proliferation rates of G-112 and G-120 were not influenced at concentrations of the inhibitor that were used in the migration experiments (data not shown). Other inhibitors of the arachidonic acid metabolism with varying specificity for TXsyn (Fig. 8) were tested for their capacity to abrogate glioma migration. The highly specific inhibitors of TXsyn, furegrelate and dazmegrel, inhibited motility of G-120 cells in a dose-dependent fashion. Another specific inhibitor, the imidazole derivative dazoxiben, was also effective, but only at high concentrations of 1 mM (0.2 mg/mL). Imidazole treatment showed a dose-dependent decrease of glioma migration but the inhibition did not exceed 25%. Ridogrel, a combined inhibitor of TXsyn and antagonist of thromboxane receptor, showed only minimal effect at concentrations that did not alter cell growth. Inhibitors of cyclooxygenase with an indirect inhibitory activity on TXsyn showed significantly less inhibition. While acetylsalicylic acid inhibited migration up to 40%, indomethacin had no effect. Dexamethasone, an inhibitor of phospholipase A2 that mediates the release of arachidonic acid from cellular membranes, also showed no inhibitory effect but rather led to a slight increase in cell migration (Fig. 9).

Inhibition of TXsyn: Proliferation, Cell Adhesion, and Cell Aggregation

Using concentrations of furegrelate between 0.01 and 0.5 mg/mL (0.03–2.0 mM), we observed no significant alteration of cell proliferation in growth experiments for various glioma cell lines (data not shown). Further increases in the concentration of the inhibitor were toxic to glioma cells. Pre-incubation of G-120 cells for 2 h with increasing nontoxic concentrations of furegrelate resulted in a slight increase of cell adhesion to different purified matrix proteins and to surfaces coated with a nonspecific insoluble ligand (Fig. 10A). Attachment using a cell-cell adhesion assay of BCECF-labeled G-120 glioma cells to a preformed monolayer of unlabeled G-120 cells was tested after 2 h of pre-incubation with furegrelate. When plated onto increasing densities of an autologous feeder layer, untreated G-120 cells showed strongly increased adhesion of a density up to 20,000–60,000 cells/well. Higher monolayer densities resulted in less adhesion, and a monolayer of complete confluence (10⁵ cells/well) allowed attachment similar to levels of nonspecific adhesion. Pretreatment with 0.1 mg/mL furegrelate resulted in abrogation of specific cell-cell adhesion (Fig. 10B).

Discussion

Prostanoids (prostaglandins and thromboxane) are synthesized in the CNS and are thought to act as neuroac-

Fig. 7. Inhibition of migration by the specific thromboxane synthase inhibitor furegrelate. Migration of glioma cells expressing different levels of thromboxane synthase was studied in the presence of the specific thromboxane synthase inhibitor furegrelate. A. Migration rates of G-120, which demonstrated the highest levels of thromboxane synthase B_2 (TXB₂) expression, and G-112, which showed very low levels of TXB₂ formation in the presence of increasing furegrelate concentrations. B. Degree of inhibition of migration by furegrelate in different glioma cell lines. Values are means of quadruplicate determinations. Bars, SD.

tive substances modulating a wide range of brain functions, such as sleep-wake cycle, body temperature, cerebral blood circulation, neuroendocrine functions, and convulsion (Hayaishi, 1991; Schwarz et al., 1991; Wolfe, 1982). In vitro studies using primary rat astrocytes identified glia cells as a source of brain prostanoid formation. These rat astrocytes have been shown to produce prostaglandins PGD₂, PGE₂, PGF_{2 α} , TXA₂, and TXB₂ (Hertting and Seregi, 1989). Subsequently, the expression of several prostanoid receptors, including the thromboxane receptor, could be demonstrated on those cells, even though there was substantial heterogeneity in astrocytes from different regions of rat brain, regarding their pattern of prostanoid receptor expression (Inagaki and Wada, 1994; Kitanaka et al., 1996). In vivo mapping of cyclooxygenase as the rate-limiting enzyme of prostanoid synthesis has indicated neurons as another important source of prostanoid formation in brain (Breder et al., 1992). However, for TXsyn, the content of enzyme per milligram of normal brain tissue and its specific activity is low when compared with highly expressive tissues such as liver, lung, colon, and adrenal gland (Nüsing and Ullrich, 1990). In this study, we demonstrate that sections of normal human brain, predominantly the cytoplasm of neurons, stained positive for TXsyn. Quiescent astrocytes and oligodendrocytes did not express the enzyme. However, astrocytes with a reactive phenotype (e.g., in areas adjacent to tumor) strongly stained for TXsyn, indicating that astrocytes under in vivo conditions can be induced to express TXsyn associated with a certain functional phenotype. Cultured astrocytes from normal adult human brain express low levels of TXsyn mRNA, but strongly express thromboxane receptor mRNA by RT-PCR.

The ex vivo metabolism of endogenous arachidonic acid has been evaluated by high-resolution gas chromatography (Castelli et al., 1989). Tumor lysates of gliomas showed an overall increased formation of prostanoids, predominantly TXB₂, when compared with meningiomas and normal brain. In the Castelli study, the authors suggested that an increased formation was asso-

ciated with grade of tumor. Subsequently, it was shown that the total synthesis capacity of arachidonic acid metabolites, including TXB₂ and PGD₂, significantly correlates with the percentage of S-phase cells in neuroepithelial tumors (Gaetani et al., 1991), but the cellular origin of prostanoids in gliomas remains unclear. Our results demonstrate expression and synthesis of TXsyn in glioma cells in vitro on the mRNA and the protein levels. Cell lines show a wide range of TXB₂ formation, which tends to be lower than formation rates in primary cells derived from glioblastomas, suggesting that expression of TXsyn mRNA may decrease with time in culture. Biopsies from gliomas also show high levels of mRNA expression of both TXsyn and thromboxane receptor. Immunohistochemistry shows that TXsyn is up regulated in glial tumor cells compared with normal astrocytes and oligodendroglia. In contrast to previous reports analyzing tumor lysates, we did not find the relative intensity of immunoreactivity in paraffin sections for TXsyn to be increased in high-grade tumors, and highly cellular glioblastomas showed the least staining for TXsyn, with only solitary positive cells scattered throughout the tumor. We did observe expression of TXsyn in endothelial proliferations and within cells of hematological origin, which may significantly contribute to the high levels of prostanoids detected in tumor lysates of high-grade tumors known to be highly vascular. However, low-grade gliomas, despite a slow growth rate, are also invasive tumors with a capacity to spread widely into adjacent normal brain. This means that genes associated with glioma invasion would not necessarily be expressed exclusively in high-grade tumors. In fact, accumulating evidence in vitro suggests that an invasive phenotype of gliomas may occur at the expense of the proliferative capacity of the cells. Glioma cells migrating on a permissive substrate show a significantly decreased proliferation rate when compared with nonmigratory cells (Giese et al., 1996b). Similarly, soluble motility factors, such as interleukins, may significantly increase migration, but result in a decreased proliferation rate of glioma cells in culture⁴.

Fig. 8. Inhibitors of thromboxane and prostaglandin synthesis. Several pharmacologically active substances directly or indirectly interfere with thromboxane synthase (TP) receptor-mediated cellular response, most of which have been investigated in platelet activity. Dexamethasone inhibits PLA₂ activity, reducing the concentration of free arachidonic acid (AA). Inhibitors of cyclooxygenase (COX), such as acetylsalicylic acid (ASA) and indomethacin, have been demonstrated to reduce thromboxane synthase A₂ (Tx_A₂) levels. Selective inhibitors of thromboxane synthase (furegrelate, dazoxiben, dazmegrel) directly block Tx_A₂ synthesis, but do not affect PGH₂ production and may even result in increased formation of other prostaglandins and prostacyclins. Thromboxane receptor antagonists specifically impede the action of both Tx_A₂ and PGH₂ on their receptor, while leaving the normal pattern of thromboxane and prostaglandin formation unaltered. A combination of Tx_{syn} inhibitors with receptor antagonism reduces the rate of Tx_A₂ synthesis and blocks PGH₂ binding to the thromboxane prostanoid (TP) receptor (Gresele et al., 1991; Schrör, 1995; Vermeylen and Deckmyn, 1992).

There are few reports on the functional role of prostanoids in gliomas. The prostaglandins PGD₂ and PGE₂ and synthetic analogues are effective and long-acting inhibitors of ³H-thymidine incorporation in glioma cell lines, causing a G2 arrest (Conde et al., 1991; Westphal et al., 1986). Inhibitors of cyclooxygenase, such as indomethacin and ketoprofen, slow the growth of glioma spheroid cultures (Gati et al., 1990). Acetylsalicylic acid also significantly suppressed growth of rat glioma cell in vitro and in vivo (Aas et al., 1995). Apart from these investigations concerned with cell proliferation, no functional data illustrate a role of prostanoids in motility and invasion of glial tumors. We have recently shown that a human glioma cell line selected for migration in vitro differentially expressed 15 mRNA populations by differential mRNA display when compared with the nonselected parental cell line. One of the over-expressed messages in the highly motile cell population was identified as human Tx_{syn} (McDonough et al., 1998). In this study, we demonstrated that the formation of Tx_B₂, reflecting Tx_{syn} activity, is positively correlated with motility in gliomas and that specific inhibitors of Tx_{syn} and inhibitors of cyclooxygenase can inhibit

Fig. 9. Inhibition of G-120 glioma cell migration by specific and nonspecific thromboxane inhibitors. Values are determined in replicates of four. ASA, acetylsalicylic acid; bars, SD.

migration of these cells, with specific inhibitors being the more effective compounds. Interestingly, furegrelate, a highly specific inhibitor, was more effective in blocking migration of cell lines that showed high formation rates of Tx_B₂. G-112, and G-62, which showed the lowest levels of Tx_B₂ formation and low amounts of the enzyme by Western blot analysis, were only marginally affected. These data suggest that locomotive activity may be directly linked to the rate of thromboxane formation by Tx_{syn}. To our knowledge, this is the first report that provides evidence for a role of Tx_{syn} in the migration mechanisms of solid tumors. In vivo invasion is, however, a complex multistep process that involves adhesion to extracellular matrix components, a protease activity that degrades matrix and creates an extracellular space into which tumor cells may then migrate by mechanisms of active cellular locomotion. It is likely that Tx_{syn} may only regulate some aspects of this cascade of events. For example, there is good experimental evidence for the regulation of cell adhesion (a precondition for cellular locomotion) in other cellular systems. Binding of both Tx_A₂ and PGH₂ to the G-protein-linked thromboxane receptor triggers a second messenger cascade,

Fig. 10. Inhibition of cell-matrix adhesion and intercellular adhesion by furegrelate. A. Adhesion of G-120 glioma cells to surfaces passively coated with purified extracellular matrix proteins and nonspecifically coated surfaces (bovine serum albumin) after pretreatment with furegrelate at concentrations indicated. Values are means of triplicate determinations. Bars, SD. B. Adhesion of BCECF-labeled G-120 glioma cells to a host layer of autologous cells at increasing density, ○, 0.1 mg/mL furegrelate pretreatment; ●, solvent-treated control. Values are means of eight determinations. Bars, SD.

leading to activation of protein kinase C and a FAK-associated kinase PYK2, resulting in phosphorylation events and biochemical activation of tyrosine kinase pp125 FAK (Fig. 11). Integrins serve as receptors, anchoring the cell to a matrix substrate, but are also important mediators of signal transduction pathways (Luna and Hitt, 1992). This signaling also includes activation of pp125 FAK when integrins are assembled into focal adhesion clusters early after cell adhesion (Huang et al., 1993). While co-activation of pp125 FAK by the thromboxane receptor has only been studied for $\alpha_{IIb}\beta_3$ integrins in platelet adhesion, the assembly of a signaling complex involving pp125 FAK by several integrins seems to be a more general phenomenon (Kornberg et al., 1991). We previously demonstrated that adhesion of glioma cells to matrix proteins of different permissiveness results in profound changes of pp125 FAK expression. Glioma cells seeded on vitronectin, a substrate that is nonpermissive for migration of these cells, resulted in strong staining for FAK localized to the periphery of the membrane, reminiscent of point contacts. On laminin,

Fig. 11. G-protein-receptor signaling and integrin-mediated adhesion and cytoskeletal reorganization. Agonist binding of G protein-coupled receptors in different cellular systems activates phospholipase C β leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to yield 1,4,5-IP₃ and diacylglycerol (DG), which raise cytosolic Ca²⁺ concentration by releasing sequestered Ca²⁺ from the dense tubular system resulting in both activation of protein kinase C (PKC) and activation of the FAK-related protein kinase PYK2. Ligand binding of integrins and activation of PKC results in a net increase in phosphorylation of FAK on tyrosine residues. Phosphorylated PYK2 can serve as center of a signaling complex associating with Grb2 and SOS triggering Src activation followed by Syk, Vac, and Rac activation which is thought to be involved in cytoskeletal rearrangements during adhesion. The same signaling cascade is activated by FAK phosphorylation subsequent to integrin ligand binding. This model is mainly based on observations in platelet activation. It remains unclear whether these mechanisms are paralleled in adhesion and motility of solid tumors.

which strongly promotes migration of the same cells, the entire cell membrane perimeter appeared devoid of staining (Giese et al., 1996b). Migration of gliomas on such extracellular matrix substrates or through artificial basement membrane is tightly controlled by integrin receptors in vitro (Bernstein et al., 1991; Giese et al., 1996c; Paulus and Tonn, 1994). Furthermore, Paulus et al. (1996) demonstrated that transfection of glioma cells with anti-sense β_1 integrin resulted in a more delineated growth pattern of intracranial C6 gliomas and suggested that β_1 integrins may be required for diffuse brain invasion in vivo. These observations demonstrate that glioma motility is dependent on integrin receptors and that pp125 FAK activation is very likely involved in downstream signaling after integrin activation by extracellular ligand binding in glioma cells. When a concentration of a specific thromboxane inhibitor that resulted in abrogation of glioma migration was tested in a cell-substratum adhesion assay, adhesion of these cells was slightly increased independent of the matrix substrate, including surfaces nonspecifically coated with bovine serum albumin. Inhibition of migration also occurred regardless of a matrix substrate. These data suggest that the signaling pathway regulated by TXsyn may modulate adhesion and migration phenomena at a more gen-

eral level rather than activating or inactivating a specific integrin receptor complex. This may also offer a broader perspective for an anti-invasive strategy, since recent data suggest that gliomas express a multitude of adhesion receptors that apparently enable these tumors to use a wide range of extracellular substrates as routes for dissemination within normal brain (Giese et al., 1998; Paulus et al., 1993).

One of the functions of a thromboxane inhibitor characterized in great detail is its inhibition of platelet aggregation mediated by the $\alpha_{IIb}\beta_3$ integrin. Treatment with the inhibitor also resulted in a dramatic decrease in intercellular adhesion of glioma cells. Although we have demonstrated expression of the β_3 integrin subunit mRNA in these cells, expression of functional $\alpha_{IIb}\beta_3$ integrins mediating cell-cell adhesion has not been characterized (Giese et al., 1994). However, experimental evidence positively correlate intercellular adhesion force with the migration of glioma cells on a host layer and with the dissemination of these cells out of three-dimensional tumor cell aggregates (Giese et al., manuscript in preparation, 1999). We hypothesize that regulation of glioma migration by TXsyn may also be possible through modulation of intercellular adhesion, but it remains unclear whether this phenomenon is mediated by an integrin receptor or an adhesion molecule of a different receptor family.

In platelets and the blood vessel wall, thromboxane and some prostaglandins show antagonistic functions. For example, thromboxane and PGH_2 activate platelet aggregation and mediate vasoconstriction, whereas prostacyclin and PGD_2 powerfully inhibit platelets and vasodilatation. PGE_2 activates platelets like TXA_2 but seems to be a vasodilator. TXsyn inhibitors impede the metabolic pathways of cyclic endoperoxides into thromboxane which indirectly increases the formation of PGD_2 , PGE_2 , PGF_{α_2} , and PGI_2 . Thromboxane

inhibitor administered to normal humans increases PGI_2 formation two- to threefold, shifting the pathway of arachidonic acid metabolites (Gresele et al., 1991). At present, it is unknown whether thromboxane alone or a balance of thromboxanes, prostaglandins, and prostacyclin are part of the regulation of the migratory and invasive phenotype of gliomas, similar to prostanoid functions in other cellular systems. Clearly, the signaling cascade triggered by binding of thromboxane to a G-protein-linked receptor results in activation of protein kinase C, activation of the Ras/Raf pathway and mitogen-activated protein kinase (MAPK) pathways. Other receptors related to adhesion and migration events, such as integrins, the thrombin receptor, and the prostacyclin receptor, interfere with this system. This complex system offers a multiplicity of possible entry points for factors co-regulating signaling by thromboxane. This may explain why our data show a good association between levels of thromboxane formation and migration rates but no strict linear dependency. Other factors involved in this signaling pathway and relevant to glioma biology await further study.

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