Microsomal prostaglandin E synthase-1 regulates human glioma cell growth via prostaglandin E2–dependent activation of type II protein kinase A

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Abstract

Dysregulation of enzymes involved in prostaglandin biosynthesis plays a critical role in influencing the biological behavior and clinical outcome of several tumors. In human gliomas, overexpression of cyclooxygenase-2 has been linked to increased aggressiveness and poor prognosis. In contrast, the role of prostaglandin E synthase in influencing the biological behavior of human gliomas has not been established. We report that constitutive expression of the microsomal prostaglandin E synthase-1 (mPGES-1) is associated with increased prostaglandin E2 (PGE2) production and stimulation of growth in the human astroglia cell line U87-MG compared with human primary astrocytes. Consistently, pharmacologic and genetic inhibition of mPGES-1 activity and expression blocked the release of PGE2 from U87-MG cells and decreased their proliferation. Conversely, exogenous PGE2 partially overcame the antiproliferative effects of mPGES-1 inhibition and stimulated U87-MG cell proliferation in the absence of mPGES-1 inhibitors. The EP2/EP4 subtype PGE2 receptors, which are linked to stimulation of adenylate cyclase, were expressed in U87-MG cells to a greater extent than in human astrocytes. PGE2 increased cyclic AMP levels and stimulated protein kinase A (PKA) activity in U87-MG cells. Treatment with a selective type II PKA inhibitor decreased PGE2-induced U87-MG cell proliferation, whereas a selective type I PKA inhibitor had no effect. Taken together, these results are consistent with the hypothesis that mPGES-1 plays a critical role in promoting astroglia cell growth via PGE2–dependent activation of type II PKA. [Mol Cancer Ther 2006;5(7):1817–26]

Introduction

Gliomas are the most common, aggressive, and lethal primary tumors of the central nervous system (1). Despite conventional treatments consisting of surgery, radiation, and chemotherapy, the prognosis of these tumors is extremely poor, and the majority of patients die within 9 to 12 months after diagnosis (2). The failure of conventional treatments to alter the prognosis of these tumors underscores the need for studies aimed at the identification of molecular targets that can be exploited for the development of newer and more efficient therapeutic interventions.

Dysregulation of arachidonic acid metabolism plays an important role in influencing critical aspects of tumorigenesis (3). Recent evidence suggests a crucial role for prostaglandin E2 (PGE2) in the control of growth, survival, and angiogenic potential of tumor cells (4–6). The first step in the biosynthesis of PGE2 is the release of arachidonic acid from membrane phospholipids by phospholipases and its sequential conversion into prostaglandins G2 and H2 by the cyclooxygenase (COX) pathway (7). Prostaglandin H2 is subsequently isomerized to PGE2 by terminal prostaglandin synthase (cPGES) isoforms (7). Different isoforms of COX have been identified. COX-1 is constitutively expressed in most tissues and exerts mainly physiologic homeostatic functions (8). COX-2 is rapidly induced in response to a wide array of stimuli and is constitutively expressed in a variety of human tumors (8–13). Several studies reported constitutive expression of COX-2 in human gliomas and found the degree of its expression to correlate with tumor grade and prognosis (14, 15). Furthermore, pharmacologic inhibition of COX-2 decreases proliferation of glioblastoma multiforme cells in vitro (16, 17). COX-3 has been recently described as a novel splice variant of COX-1, but its relevance to human pathophysiology remains to be defined (18).

Multiple isoforms of terminal prostaglandin E synthases have been identified (19). The cytosolic prostaglandin E synthase (cPGES) isoform is constitutively expressed and functionally coupled to COX-1 (20). Conversely, the microsomal prostaglandin E synthase-1 isoform (mPGES-1) is rapidly induced in response to a wide array of stimuli and is functionally coupled to COX-2 (21). The physiologic
relevance of mPGES-1 expression in vivo is substantiated by recent studies in which mPGES-1-deficient mice display decreased response to inflammation and pain (22). Furthermore, constitutive expression of mPGES-1 is detected in colon, lung, and gastric cancers (23–25), suggesting a potential role for this enzyme in tumorigenesis.

Once generated, PGE2 influences tumor cell growth, survival, and metastatic potential via the activation of distinct signaling pathways, which are triggered by its binding to four subtypes of G-protein-coupled receptors (26). Although a large body of evidence shows elevated levels of PGE2 in experimental brain tumor models and in the cerebral fluid and tissue samples of patients with brain tumors (27–30), the contribution of enzymes involved in PGE2 biosynthesis, such as mPGES-1, and the signaling events by which PGE2 controls glioma cell growth remain to be elucidated. In this study, we detect constitutive expression of mPGES-1 in the human astroglioma cell line U87-MG and in tissue samples from human gliomas. Furthermore, we show that overexpression of mPGES-1 is linked to increased generation of PGE2, which in turn regulates U87-MG cell growth via activation of type II protein kinase A (PKA).

Materials and Methods

Reagents
DMEM was purchased from BioWhittaker (Walkersville, MD). Fetal bovine serum was purchased from Hyclone (Logan, UT). SC-58125, MK-886, H-89, and antibodies to types I and II PKA were purchased from Calbiochem (San Diego, CA). Kemptide, forskolin, 3-isobutyl-1-methylxantine, and anti-β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). PGE2 and prostaglandin F2alpha immunoaassays were purchased from Neogen Corp. (Lansing, MI). PGE2, 16,16-dimethyl-PGE2 (dmPGE2), sulprostone, 11-deoxy-PGE1, PGE1-alcohol, butaprost, and antibodies to COX-2, COX-1, mPGES-1, cPGES, EP1, EP2, EP3, and EP4 receptors were purchased from Cayman Corp. (Lansing, MI). Rp-8-CTP-cyclic AMP (cAMP) and Rp-8-Cl-cAMP were purchased from Biolog Life Sciences (Bremen, Germany). The chemiluminescence detection system was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Autoradiography films were obtained from Amersham (Arlington Heights, IL) and ECL plus Western blotting luminol reagent was purchased from Pierce (Rockford, IL).

Cells
U87-MG, U118-MG, and U138-MG astroglialoma cell lines (American Type Culture Collection, Rockville, MD) and human primary cortex astrocytes (Cell Systems, Kirkland, WA) were cultured in DMEM (4,500 mg/L glucose) containing 10% fetal bovine serum and 1% streptomycin/penicillin (Life Technologies, Inc., Grand Island, NY). The T98G cell line (American Type Culture Collection) was cultured in Eagle’s MEM supplemented with 1 mmol/L sodium pyruvate. Cells were maintained in a humidified incubator at 37°C and 5% CO2.

Tissue Samples
Four unscreened human glioma specimens were obtained from the Human Brain and Spinal Fluid Resource Center, VA West Los Angeles Healthcare Center (Los Angeles, CA). Neuropathology reports showed that one tumor was a mixed fibrillary-pilocytic WHO grade 1 astrocytoma, two were grade 2 astrocytomas (glioblastoma multiforme), and one tumor was diagnosed as astrocytoma, although a definitive grading using the WHO criteria was not possible because of postmortem artifacts. The corresponding cerebellar white matter samples were also provided and used for comparison. Specimens were received frozen and were kept at −80°C until further use. The local institution review board approved the use of the received specimens.

Cell Proliferation
Proliferation was assayed by [3H]thymidine incorporation. Briefly, cells were seeded in 24-well plates and allowed to grow overnight at 37°C and 5% CO2. Cells were subjected to serum withdrawal for 24 hours before incubation with the indicated stimuli or inhibitors dissolved in the appropriate vehicle. Equal volumes of vehicle were added to control cultures. In selected experiments, cells grown in culture medium supplemented with 10% fetal bovine serum were used as positive controls. [3H]thymidine (1 µCi) was added to each well during the last 8 hours of incubation. Monolayers were washed twice with ice-cold PBS before incubation in 5% trichloroacetic acid for 30 minutes at 4°C. Cells were then washed with PBS and solubilized with 1 mL of 0.5 N NaOH/0.5% SDS. Incorporation of radioactive was measured by liquid scintillation counting.

Soft Agar Colony-Forming Assay
U87-MG cells (2 × 103) were plated as a single-cell suspension in DMEM containing 0.5% agar in the presence of various concentrations of MK-886 or vehicle on six-well plates. Cells were incubated at 37°C and 5% CO2. Colonies were inspected under an inverted microscope and counted 3 weeks later.

Measurement of Prostaglandin Levels from Cell Supernatants
Cells were grown in serum-containing tissue culture medium until they reached 80% confluence. Cells were then incubated in serum-free medium for 48 hours at 37°C and 5% CO2. Supernatants were subjected to low-speed centrifugation to remove cell debris, and PGE2 content was measured by enzyme immunoassays as described previously (9). Results are expressed as nanograms of prostaglandins released into the supernatant and normalized for protein concentration, which was measured by the method of Bradford using bovine serum albumin as a standard (31).

Oligonucleotide Synthesis
Phosphorothionate antisense oligonucleotides were synthesized and purified by MWG Biotech (High Point, NC). The sequence of mPGES-1 antisense oligonucleotides synthesized and purified by MWG Biotech (High Point, NC).
(5'-GAGGAAAGACCAGGAACTGCA-3') was derived from previously published studies (23). Phosphorothionate sense oligonucleotides (5'-CTCTCGGCTCCCTAGCTA-3') were used as negative controls. Cellular uptake was monitored using 5'-FITC-labeled oligonucleotides and visualized by fluorescence microscopy.

**Treatment of Cells with Antisense Oligonucleotides**

U87-MG cells were plated at a concentration of 1 x 10^5/mL in 24-well plates. Twenty-four hours later, cells were treated with various concentrations of mPGES-1 antisense or sense oligonucleotides under serum-free conditions using the LipofectAMINE transfection reagent as described previously (32). Proliferation was monitored by [3H]thymidine incorporation at 48 hours following incubation at 37°C and 5% CO2 in serum-free tissue culture medium.

**Western Blot Analyses**

Cell monolayers were scraped off in ice-cold PBS containing 5 mmol/L NaF and 1 mmol/L Na2VO3 and collected by centrifugation at 4°C. Western blot analyses for the detection of COX-2 were done as we described previously (33). To detect mPGES-1 and cPGES-1 protein expression, cells were lysed using the experimental conditions employed for the detection of COX-2 (33). For protein analyses of the EP receptors and PKA isoforms, cells were lysed in radiomunoprecipitation assay buffer. Equal amounts of total proteins were resolved by gel electrophoresis, transferred to nitrocellulose membranes, and subjected to immunoblotting using the following primary antibodies: rabbit polyclonal anti-COX-2 (1:1,000); rabbit polyclonal anti-mPGES-1 (1:1,000); rabbit polyclonal anti-cPGES (1:1,000); rabbit polyclonal anti-EP1, anti-EP2, anti-EP3, and anti-EP4 receptors (1:1,000); chicken polyclonal anti-EP3, and anti-EP4 receptors (1:1,000); chicken polyclonal anti-EP1, anti-EP2, rabbit polyclonal anti-mPGES-1 (1:1,000); rabbit polyclonal anti-cPGES-1 (1:1,000); rabbit polyclonal primary antibodies: rabbit polyclonal anti-COX-2 (1:1,000); for protein analyses of the EPreceptors and PKA isoforms, expression, cells were lysed using the experimental conditions employed for the detection of COX-2 (33).

**Measurement of Intracellular cAMP**

Cells (5 x 10^4) were cultured in 96-well plates in DMEM containing 10% fetal bovine serum. Following overnight incubation at 37°C and 5% CO2, monolayers were washed and preincubated for 30 minutes in serum-free tissue culture medium with 1 mmol/L 3-isobutyl-1-methylxanthine. PGE2 was then added at the indicated concentrations, and cells were incubated for additional 30 minutes at 37°C and 5% CO2. Forskolin (25 μmol/L) was employed as a positive control. After incubation, medium was removed, cells were lysed for 30 minutes at room temperature, and intracellular cAMP was measured in cell lysates as described in the Amersham (Piscataway, NJ) cAMP enzyme immunoassay system protocol 4 (sensitivity, 12 fmol/well or 38.4 pg/mL; curve range, 12.5–3,200 fmol/well). The amount of cAMP produced was normalized to the protein concentration of the lysates. Results are expressed as fmol/well.

**PKA Assay**

Cells were homogenized in a buffer containing 10 mmol/L NaH2PO4 (pH 6.8), 10 mmol/L EDTA, 100 mmol/L NaCl, 0.2% Triton X-100, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 25 μg/mL aprotinin, and 25 μg/mL leupeptin. Homogenates were centrifuged at 18,000 r.p.m. for 20 minutes at 4°C. PKA activity was measured in the cytosol-containing supernatants by the method of Roskoski (34) with modifications. Briefly, cell extracts (20 μg/reaction) were incubated with 100 μmol/L kemptide (PKA-specific substrate) in a reaction mixture containing 50 mmol/L Tris (pH 7.5), 10 mmol/L MgCl, and 0.25 mg/mL bovine serum albumin. Reactions were initiated by the addition of 100 μmol/L ATP and 3,000 Ci/mmol [γ-32P]ATP and carried out for 5 minutes at 30°C. Total PKA activity was measured by the addition of 1 μmol/L cAMP in samples from untreated cells. Reaction mixtures were then spotted onto phosphocellulose P81 paper squares, air-dried, and washed with 1% (v/v) phosphoric acid as described previously (35). Incorporated radioactivity was measured by liquid scintillation counting. PKA activity was assayed in duplicate samples and experiments were repeated at least thrice. Results are expressed as activity ratio, calculated by dividing the average of the test reactions assayed without cAMP by total PKA activity. The activity ratio gives a value between 0 and 1, where 1 indicates full enzyme activity (34).

**Reverse Transcription-PCR**

RNA was isolated using the TRIzol reagent. Total RNA (1 μg) was reverse transcribed using Random Decamers and cDNA was subsequently subjected to PCR amplification using primers specific for EP1, EP2, EP3, and EP4 (36, 37). The sequences were as follows: EP1 sense 5'-TTAACCTGAGCTCCAGAGGATG-3' and antisense 5'-CGCTGACGGTATTGCACACTA-3', EP2 sense 5'-CTTACCTGAGCTTACGAG-3' and antisense 5'-GATGGCCAAAGACCCAAAAGG-3', EP3 sense 5'-GGCGAGTGTTGGCTTTCACT-3' and antisense 5'-GGGTCCAGATCTGGTC-3', and EP4 sense 5'-ATCTTACTTACTCCGGC-3' and 5'-TCTATTTCTTTACTGAGC-3' (MWG Biotech). Amplification conditions were the following: 94°C for 30 seconds and then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds followed by 4°C for 10 minutes. PCR products were resolved in 2% agarose gel (E-gel) for 30 minutes at 60 V and visualized by UV light.

**Statistical Analysis**

Data were analyzed using the InStat software program (GraphPad Software, San Diego, CA). Student's t test or one-way ANOVA was used. All values are expressed as mean ± SD. P ≤ 0.05 was considered statistically significant.

**Results**

**Constitutive Expression of mPGES-1 in Human Glioblastoma Cell Lines and Tumor Tissues**

We did Western blot analyses to investigate the constitutive expression of mPGES-1 protein in human astrogliaoma cell lines and primary astrocytes. The latter were used as a model of normal glia cells. The human astrogliaoma cell line U87-MG, but neither primary astrocytes nor U118-MG cells, overexpressed mPGES-1 under...
constitutive conditions (Fig. 1A). In contrast, cPGES protein expression was detected in U87-MG and U118-MG cells and primary astrocytes (Fig. 1A). We also examined mPGES-1 expression in other human glioma cell lines, including U138-MG and T98G. We detected constitutive expression of mPGES-1 in T98G cells but not in U138-MG cells (data not shown). Next, expression of COX-2 was examined. U87-MG and U118-MG cells constitutively expressed COX-2 (Fig. 1B), whereas astrocytes were negative for COX-2 expression (Fig. 1B). However, challenge of astrocytes with interleukin-1-induced COX-2 and mPGES-1 protein expressions (data not shown). We also examined mPGES-1 expression in tissue samples obtained from four unselected human gliomas. Samples taken from the cerebellar white matter were used as normal brain controls. As shown in Fig. 1C, three of the four tumors were positive for mPGES-1 expression. In contrast, normal brain controls were mPGES-1 negative. Equal loading of protein in each lane was confirmed by stripping and reprobing of the membranes using anti-β-actin antibodies (Fig. 1A–C).

Next, we investigated whether constitutive expression of mPGES-1 resulted in increased basal PGE2 production. For this purpose, we compared PGE2 released by U87-MG cells with PGE2 released by U118-MG glioma cells and astrocytes (both of which were devoid of mPGES-1 constitutive expression). Prostaglandin F2α was also measured. As shown in Fig. 1D, U87-MG cells released higher amounts of PGE2 than did U118-MG cells or astrocytes (4.6 ± 0.1, 0.82 ± 0.05, and 0.04 ± 0.006 ng/mL). U87-MG and U118-MG glioma cells and astrocytes produced detectable levels of prostaglandin F2α but to a lesser degree when compared with PGE2 (Fig. 1D). Based on these findings, we used U87-MG cells as a representative cell line model to study the effect of mPGES-1 overexpression in regulation of cell growth.

Microsomal PGES-1 Regulates U87-MG Cell Growth

To determine whether constitutive expression of mPGES-1 regulated U87-MG cell growth, [3H]thymidine incorporation was analyzed following treatment with mPGES-1 inhibitors. MK-886, a nonselective mPGES-1 inhibitor that acts on other members of the membrane-associated proteins in eicosanoids and glutathione metabolism family (38), decreased, in a dose-dependent manner, proliferation of U87-MG cells grown in monolayers as well as their ability to form colonies on soft agar (Fig. 2). MK-886 also induced a dose-dependent decrease of arachidonic acid–stimulated PGE2 production (Fig. 2). Moreover, treatment with MK-886 also inhibited T98G cell growth (data not shown).

To rule out that the antiproliferative effects of MK-886 could result from inhibition of additional members of the membrane-associated proteins in eicosanoids and glutathione metabolism family (38), we employed antisense oligonucleotides to selectively block the expression of mPGES-1 and determine the effect of mPGES-1 knockdown on U87-MG growth. Incubation of U87-MG cells with mPGES-1 antisense oligonucleotides caused a concentration- and sequence-dependent inhibition of cell growth. As shown in Fig. 3, mPGES-1 antisense oligonucleotides inhibited U87-MG cell growth (by 51% and 69.5%) at concentrations of 1.5 and 3 μmol/L. In contrast, mPGES-1 sense oligonucleotides at the same concentration had no effect on U87-MG cell proliferation. Treatment with mPGES-1 antisense oligonucleotides also decreased the release of PGE2 from U87-MG cells incubated with 10 μmol/L arachidonic acid (Fig. 3B). Moreover, similar treatment attenuated mPGES-1 protein expression but had no effect on cPGES protein levels (Fig. 3C).
The findings that constitutive expression of mPGES-1 resulted in increased PGE2 production and that inhibition of mPGES-1 decreased cell growth and PGE2 levels prompted us to investigate the possibility that the effects of mPGES-1 on U87-MG cell growth were dependent on the production of PGE2. To test this hypothesis, we first determined whether addition of exogenous PGE2 was able to rescue U87-MG cells from the antiproliferative effects of mPGES-1 inhibition. For these experiments, we employed dmPGE2, a stable analogue of PGE2, which does not undergo the metabolic transformation that limits PGE2 activity during extended incubation intervals in culture. Additionally, we wished to rule out the possibility that other prostanoids derived from PGE2 metabolism could account for the cellular effects of PGE2 studied here. U87-MG cells were treated with 1 μmol/L MK-886 or 1.5 μmol/L mPGES-1 antisense oligonucleotides in the absence or presence of varying concentrations of dmPGE2. We employed low concentrations of PGE2 (0.005 μmol/L) to mimic those released constitutively by U87-MG cells. We also employed higher concentrations of PGE2 in the range of those that have been reported in vivo (27–30). Under these experimental conditions, addition of 0.005, 1, and 10 μmol/L dmPGE2 to U87-MG cells treated with MK-886 restored 46%, 79%, and 81% of growth (Fig. 4A, left). Similarly, addition of 0.005, 1, and 10 μmol/L dmPGE2 to U87-MG cells treated with mPGES-1 antisense oligonucleotides restored 50%, 74%, and 56% of U87-MG cell growth (Fig. 4A, right).

To confirm that the proliferative effect of mPGES-1 overexpression was linked to the production of PGE2, we carried out additional studies to investigate whether PGE2 would stimulate cell growth. U87-MG cells were challenged with increasing concentrations of dmPGE2 in serum-free medium and proliferation was monitored at 48 hours by [3H]thymidine incorporation. Challenge with 0.01 to 0.1 μmol/L dmPGE2 failed to stimulate U87-MG cell growth (Fig. 4B) possibly because endogenous PGE2, generated as a result of mPGES-1 overexpression, was sufficient to maintain U87-MG cell growth under basal conditions. However, higher concentrations of dmPGE2 induced a dose-dependent increase in U87-MG cell growth (Fig. 4B). In contrast, stimulation of primary astrocytes with PGE2 caused a 27.9 ± 5.7% increase in proliferation when the prostaglandin was used at a concentration of 20 μmol/L. These findings are consistent with the possibility that the proliferative effects of mPGES-1 are mediated, at least in part, by the production of PGE2.

We also examined the effect of COX-2 inhibition on U87-MG cells. SC-58215, a selective COX-2 inhibitor, induced...
7.3 ± 5.8%, 22 ± 4.1%, and 53 ± 2.0% inhibition of U87-MG cell growth at concentrations of 1, 5, and 10 μmol/L, respectively. Similarly, SC-58215 decreased the release of PGE2 in U87-MG cell supernatants (Fig. 5A). Moreover, dmPGE2, at concentrations of 1 and 10 μmol/L, restored 52% and 63% of U87-MG cell growth from the inhibitory effect of 10 μmol/L SC-58125 (Fig. 5B).

**PGE2 Receptor Expression in U87-MG Cells**

PGE2 exerts its autocrine/paracrine effects by binding to four subtypes of heterotrimeric G-protein-coupled receptors classified as EP1, EP2, EP3, and EP4 (26). We first did reverse transcription-PCR analyses to determine the expression of the EP receptors in U87-MG cells. As shown in Fig. 6A, amplification products for the EP2, EP3, and EP4 receptor subtypes were detected in U87-MG cells. The mRNA for the EP1 receptor subtype, which was barely detectable, migrated below the 154-bp DNA marker (data not shown).

Next, Western blot analyses were done to compare the EP receptor profile of U87-MG cells and primary astrocytes. As shown in Fig. 6B, the EP2 and EP4 receptor subtypes were expressed in U87-MG cells at greater levels compared with primary astrocytes. EP-1 receptor expression was higher in primary astrocytes than in U87-MG cells where it was barely detectable, whereas the EP3 receptor was expressed in U87-MG cells and primary astrocytes. L-161982 (1 μmol/L), a selective antagonist of EP4 receptor (39), decreased U87-MG cell growth by 41%, suggesting the involvement, at least in part, of the EP4 receptor in mediating the effects of PGE2. Sulprostone, a selective EP3 agonist, had no effect on U87-MG cell growth (data not shown).

**Stimulation of the cAMP-PKA Pathway by PGE2**

The EP2 and EP4 receptors are coupled via the heterotrimeric G protein, Gαs, to activation of adenylate cyclase, which leads to generation of cAMP and subsequent activation of PKA, a ubiquitous enzyme that phosphor-ylates target proteins on serine and threonine residues within a specific consensus sequence (40). Therefore, we sought to determine the involvement of cAMP-dependent PKA activation on the proliferative responses of U87-MG cells to PGE2. We first determined whether challenge of U87-MG cells with PGE2 stimulated cAMP production. Cells were preincubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine to prevent the breakdown of cAMP and stimulated with various concentrations of dmPGE2. Dose-dependent increases of cAMP levels were detected in cells stimulated with dmPGE2 (Fig. 7A). Stimulation of U87-MG cells with the EP2/EP4 agonist 11-deoxy-PGE2 also stimulated cAMP production (data not shown).

We then measured PKA activation in lysates of untreated or dmPGE2-treated cells by monitoring the incorporation of 32P into kemptide, a synthetic PKA substrate. Challenge of U87-MG cells with dmPGE2 induced a dose-dependent increase of PKA activity (Fig. 7B). The maximal increases of PKA activity were detected in cells stimulated with 1 to 20 μmol/L dmPGE2.

**Involvement of PKA in PGE2-Dependent U87-MG Cell Proliferation**

As dmPGE2 stimulated U87-MG cell growth at concentrations that activated PKA, we investigated whether inhibition of PKA would inhibit the proliferative response of U87-MG cells to exogenous dmPGE2. [3H]thymidine uptake into U87-MG cells incubated with the selective PKA inhibitor H-89 (10 μmol/L) was measured following stimulation with 10 μmol/L dmPGE2 for 48 hours. Treatment of U87-MG cells with H-89 markedly decreased basal and PGE2-stimulated cell growth by 33% and 67%, respectively (Fig. 8A).

PKA exists in two isoforms, types I and II (41). Therefore, we sought to determine which PKA isoform mediated the proliferative responses of glioma cells to PGE2. We first did...
Western blot analyses to examine the expression of PKA isoforms in U87-MG cells. As shown in Fig. 8B, types I and II PKA were both expressed in U87-MG cells. We then employed Rp-8-Cl-cAMP or Rp-8-CTP-cAMP, two cAMP analogues that inhibit types I and II PKA, respectively. As shown in Fig. 8C, preincubation with Rp-8-CTP-cAMP reduced [3H]thymidine uptake into U87-MG cells stimulated with 10 μmol/L dmPGE2 for 48 hours by 53%, whereas preincubation with Rp-8-Cl-cAMP had no effect.

**Discussion**

To our knowledge, the current study presents the first evidence that constitutive expression of mPGES-1 is functionally linked to regulation of growth in the human glioma cell line U87-MG. Recent studies have shown that, at least in certain tumors, mPGES-1 is coexpressed with COX-2 (24, 25). Similarly, U87-MG cells constitutively expressed COX-2 in addition to mPGES-1. Although both enzymes participated in the production of PGE2, mPGES-1 activity was required for the increased production of PGE2 detected in U87-MG cells under steady-state conditions. Consistent with this possibility, a greater production of PGE2 was detected in the mPGES-1/COX-2-expressing U87-MG cells compared with the COX-2-positive U118 glioma cells, which were devoid of mPGES-1 expression. Whether the coexpression of mPGES-1 and COX-2 confers a more aggressive phenotype in glioma cells remains to be determined.

The present study also provides evidence for a role of PGE2 in mediating the effect of mPGES-1 in U87-MG cell growth, although additional PGE2-independent mechanisms may exist. First, U87-MG cells released significant amounts of PGE2. Second, genetic and pharmacologic manipulation of mPGES-1 expression and activity resulted in decreased production of PGE2 and inhibition of cell growth. Third, addition of exogenous PGE2 overcame the antiproliferative effects of mPGES-1 inhibition. Thus, PGE2 released as a consequence of mPGES-1 overexpression, functioned as an autocrine mediator to promote U87-MG...
cell growth. However, our finding that addition of exogenous PGE2, in the absence of mPGES-1 inhibitors, stimulated U87-MG cell proliferation, suggests that PGE2 operates in a paracrine fashion to amplify the proliferative response of glioma cells. Consistent with previous published studies (16), inhibition of COX-2 also decreased U87-MG cell growth. Our findings that exogenous PGE2 partially rescued U87-MG cells from the antiproliferative effects of COX-2-inhibition indicate that the effects of COX-2 on U87-MG cells are mediated, at least in part, by PGE2. Increased levels of PGE2, in the range of those used in the present study, have been detected in experimental brain tumor models and in the cerebral fluid and tissue samples of patients with brain tumors (27–30, 42). Because PGE2 is short-lived and acts locally on cell targets, it is possible that the concentrations of PGE2 in the tumor area are higher than those measured in circulation. Interestingly, we detected an increased responsiveness of U87-MG cells to the growth-stimulatory effects of PGE2 compared with astrocytes. The molecular basis for the increased responsiveness of U87-MG cells to PGE2 remains to be investigated. These investigations in turn might provide additional clues to the contribution of PGE2 to the pathophysiology of brain tumor growth and development.

Having established a link among mPGES-1 constitutive expression, PGE2 production, and stimulation of cell growth, we sought to elucidate the underlying signaling mechanisms by which PGE2 influenced glioma cell growth. The cAMP/PKA pathway was investigated, because activation of this signaling pathway occurs through ligation of G-protein-coupled receptors, including EP2 and EP4 receptor subtypes, the predominant receptor subtypes expressed in U87-MG cells. Our results indicated that PGE2 activated the cAMP/PKA pathway in glioma cells. Moreover, a selective type II PKA inhibitor decreased the proliferative responses of U87-MG cells when exposed to PGE2. These results suggested that type II PKA was involved in mediating the effects of PGE2 on U87-MG cell growth. Inhibition of PKA also decreased growth of unstimulated U87-MG. This effect likely results from inhibition of basal PKA activation, which could be increased as a result of autocrine stimulation by endogenous PGE2. Consistent with this possibility, we detected higher constitutive levels of PKA activation in U87-MG cells compared with primary astrocytes (data not shown).
An interesting finding of our study was that PGE$_2$ at concentrations similar to those generated endogenously, failed to activate PKA. These findings suggest the possibility that exogenous and endogenous PGE$_2$ operate via distinct signaling pathways to regulate U87-MG cell growth. Other pathways, in addition to PKA, can be activated by PGE$_2$ as a result of its binding to the EP4 and EP2 receptors as indicated by recent studies (43, 44). The participation of other signaling pathways and the elucidation of the downstream effectors of type II PKA that mediate PGE$_2$-induced proliferation in U87-MG cells are currently under investigation in our laboratory.

Elegant studies showed that the expression of certain EP receptors influence growth, invasiveness, and tumor-induced angiogenesis (45, 46). In this study, we found that a selective EP4 receptor antagonist partially decreased U87-MG cell growth. Because of the lack of available EP2 selective antagonists, we were unable to determine the effects of blockade of the EP2 receptor on U87-MG cell growth. However, 11-deoxy-PGE$_2$, an EP2/EP4 agonist, stimulated U87-MG cell growth. This suggests that, as shown in other cellular systems (47, 48), the EP2 and EP4 receptors cooperate in transmitting PGE$_2$-dependent signals that lead to stimulation of cell growth.

In previous studies, we showed that exogenous arachidonic acid limits the growth of human gliomas by inducing apoptosis (49). Thus, it is possible that under conditions in which tumor cells are exposed to excess of arachidonic acid, as may occur in vivo during tumor-associated inflammation, overexpression of mPGES-1 functions as a protective mechanism to convert arachidonic acid-dependent proapoptotic signals into PGE$_2$-dependent proliferative signals. Therefore, the antiproliferative effects of mPGES-1 inhibition could also result from accumulation of unesterified arachidonic acid, in addition to decreased production of PGE$_2$, as suggested by other studies (50).

In summary, our studies show that constitutive expression of mPGES-1 is linked to increased production of PGE$_2$, which in turn acts in an autocrine and paracrine manner to stimulate U87-MG cell growth. These findings are consistent with the hypothesis that mPGES-1 plays a role in the regulation of U87-MG cell growth. It is important to note that the effects and mechanisms of mPGES-1 overexpression were studied only in the U87-MG cell line. Therefore, future studies are needed to determine the universality of our findings as well as the physiologic relevance of mPGES-1 overexpression in human gliomas. These studies may in turn reveal important information on the role of mPGES-1 and PGE$_2$ in gliomagenesis.

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