Microsomal Prostaglandin E Synthase-1 Is Overexpressed in Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Elevated levels of prostaglandin E$_2$ (PGE$_2$) occur in head and neck squamous cell carcinoma (HNSCC) and have been associated with a poor prognosis. Recently, an inducible microsomal prostaglandin E synthase-1 (mPGES) was identified. This enzyme converts the cyclooxygenase product prostaglandin H$_2$ (PGH$_2$) to PGE$_2$. Given the apparent significance of PGE$_2$ in carcinogenesis, it is important to elucidate the mechanisms that account for increased amounts of PGE$_2$ in HNSCC. By immunoblot analysis, mPGES was overexpressed in 11 of 14 (79%) cases of HNSCC compared with adjacent normal tissue. Immunohistochemistry localized mPGES expression to neoplastic epithelial cells. Cell culture was used to determine whether cellular transformation was associated with increased amounts of mPGES. Levels of mPGES protein and mRNA were markedly elevated in HNSCC cell lines (1483 and Ca9-22) versus a non-tumorigenic oral epithelial cell line (MSK-Leuk1). Interestingly, treatment of MSK-Leuk1 cells with PGE$_2$ caused both dose- and time-dependent stimulation of cell growth. Each of the four known receptors for PGE$_2$ (E-prostanoid receptor subtypes 1–4) was detected in head and neck squamous mucosa. Taken together, these results suggest that overexpression of mPGES contributes to the increased levels of PGE$_2$ found in HNSCC. Additional studies will be needed to determine whether this enzyme is a bona fide target for anticancer therapy.

INTRODUCTION

Increased levels of PGE$_2$ have been detected in a variety of malignancies including HNSCC (1, 2). Several lines of evidence, beyond the finding of elevated levels of PGE$_2$ in tumors, suggest that PGE$_2$ plays a role in the development and progression of cancer. For example, PGE$_2$ can stimulate cell proliferation and motility while inhibiting apoptosis and immune surveillance (3–10). Importantly, PGE$_2$ can also induce angiogenesis at least, in part, by enhancing the production of proangiogenic factors including vascular endothelial growth factor (11, 12). Consistent with these findings, higher levels of PGE$_2$ in HNSCC specimens correlated significantly with the occurrence of metastatic disease and increased tumor vascularization (2, 13). Recent work in experimental animals has also suggested that PGE$_2$ can promote carcinogenesis. In one study, genetic disruption of the EP$_2$ was found to decrease the number and size of experimental tumors (14). In other studies, treatment with anti-PGE$_2$ monoclonal antibody inhibited the growth of transplantable tumors including HNSCC (10, 15). Given this background, it is important to define the enzymatic pathways that are dysregulated in HNSCC leading to increased amounts of PGE$_2$.

The synthesis of PGE$_2$ from arachidonic acid requires two enzymes that act in sequence. COX catalyzes the conversion of arachidonic acid to PGH$_2$. COX-2, the inducible form of COX, is commonly overexpressed in a variety of solid tumors including HNSCC (16, 17). Recently, an inducible human mPGES was identified and characterized (18). This enzyme converts COX-derived PGH$_2$ to PGE$_2$. Increased levels of mPGES have been detected in several human malignancies (19–21), raising the possibility that aberrant mPGES expression could contribute to increased amounts of PGE$_2$ in HNSCC.

In this study, we found that mPGES was commonly overexpressed in HNSCC. Cell culture was used to determine the effects of cell transformation on mPGES expression. As reported previously for COX-2 (22), cell transformation appears to contribute to the increased amounts of mPGES observed in HNSCC. We also report for the first time that all four PGE$_2$ receptor subtypes are expressed in head and neck squamous mucosa. Taken together, it seems likely that mPGES-derived PGE$_2$ signals through PGE$_2$ receptors and thereby contributes to the progression of HNSCC.

3 The abbreviations used are: PGE$_2$, prostaglandin E$_2$; HNSCC, head and neck squamous cell carcinoma; mPGES, microsomal prostaglandin E synthase-1; PGH$_2$, prostaglandin H$_2$; EP, E-prostanoid receptor; COX, cyclooxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
MATERIALS AND METHODS

Materials. Rabbit polyclonal antihuman mPGES antiserum was obtained from Cayman Chemical (Ann Arbor, MI). Mouse anti-β-actin antiserum was obtained from Oxford Biomedical Research (Oxford, MI). DMEM:Ham’s F-12, fetal bovine serum, gentamicin, amphotericin B, Taq polymerase, and Trizol reagent were obtained from Invitrogen (Carlsbad, CA). Lowry protein assay kits, biotinylated antirabbit IgG antibody, and 3,3’-diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). Streptavidin-horseradish peroxidase was purchased from DAKO Corp. (Carpinteria, CA). Human EP₁, EP₃, and EP₄ expression vectors were gifts from Dr. Mark Abramovitz (Merck Frosst Centre for Therapeutic Research, Pointe-Claire-Dorval, Quebec, Canada). Human EP₂ expression vector was a gift from Dr. John W. Regan (University of Arizona, Tucson, AZ). Dr. Per-Johan Jakobsson (Karolinska Institute, Stockholm, Sweden) generously provided an expression vector for human mPGES. PCR primers were synthesized by Sigma Genosys (The Woodlands, TX). SYBR Green PCR Master Mix, murine leukemia virus reverse transcriptase, RNase inhibitor, oligo(dT)₁₆, and deoxynucleotide triphosphates were purchased from Applied Biosystems (Foster City, CA). Enhanced chemiluminescence solution was from Perkin-Elmer Life Sciences (Boston, MA). RNeasy Mini-kits were obtained from Qiagen (Valencia, CA).

Patient Samples. Specimens from patients with previously untreated HNSCC were obtained at the time of resection, in accordance with an institutional review board-approved protocol. Tissue specimens from tumor and adjacent normal-appearing mucosa were frozen in liquid nitrogen and stored at −80°C. HNSCC specimens used for immunohistochemistry were obtained from archived, paraffin-embedded tissue blocks from patients with histologically proven HNSCC.

Cell Culture. The 1483 cell line was derived from a T₂N₁M₀, American Joint Committee on Cancer stage III well-differentiated squamous cell carcinoma of the retromolar trigone (23). The Ca9-22 cell line was derived from a patient with squamous cell carcinoma of the gingiva (24). Both 1483 and Ca9-22 cell lines are tumorigenic in immunocompromised mice (23, 24). MSK-LeuK1 was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue. It is nontumorigenic in nude mice and exhibits little anchorage-independent growth (25). 1483 and Ca9-22 cell lines were maintained in DMEM:Ham’s F-12 with 10% fetal bovine serum, 50 μg/ml gentamicin, and 0.25 μg/ml amphotericin B. MSK-LeuK1 cells were maintained in keratinocyte growth medium supplemented with bovine pituitary extract.

Western Blotting. Frozen human tissue was thawed in ice-cold lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin, and 10 μg/ml leupeptin]. Tissues were sonicated for 3 min on ice and then centrifuged at 10,000 x g for 10 min at 4°C to remove particulate matter. The protein concentration of the supernatant was determined using the method of Lowry et al. (26). Cell lysates were prepared as described previously (22). Immunoblot analysis for mPGES and β-actin was performed using methods described in previous studies (19, 20).

Real-Time PCR Analysis of mPGES. Total cellular RNA was isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using 1 μg of RNA per 50 μl of reaction. Reaction mixture contained 1X PCR Buffer II, MgCl₂ (2.5 mM), deoxynucleotide triphosphates (250 μM each), RNase inhibitor (1 unit/μl), murine leukemia virus reverse transcriptase (2.5 units/μl), and oligo(dT)₁₆ (2.5 μM). Samples were incubated for 10 min at room temperature and then cycled at 42°C for 15 min and 95°C for 10 min.

Real-time PCR was performed using SYBR Green PCR Master Mix with 2 μl of cDNA and 200 nM upstream and downstream primer per 20 μl of reaction (iCycler thermal cycler; Bio-Rad Laboratories, Hercules, CA). Each sample was amplified in duplicate in every experiment. Full-length mPGES was excised from the expression vector, gel-purified, and quantified by absorbance at 260 nm. Serial dilutions over a range of 10,000-fold were used to create a standard curve to establish efficiencies of mPGES and β-actin amplification and for mPGES quantification. Primer pairs were as follows: (a)
mPGES, 5′-TGGAGACCATCTACCCCTT-3′ (forward) and 5′-CCACGAGGAAGACCAAGAAC-3′ (reverse); and (b) β-actin, 5′-GGATCATGTTG-3′ (forward) and 5′-GCCAGATTTTCTCCATGTCG-3′ (reverse), encoding products of 99 and 73 bp, respectively. Mean efficiency of mPGES and β-actin amplification was 2.04 and 2.08, respectively. Melt curve analysis and agarose gel electrophoresis confirmed a single PCR product. PCR product was extracted from the gel, and correct sequence identity was confirmed by direct sequencing. Relative mPGES expression was calculated using the following equation: ratio = (E_{mPGES})_{ACT} (control − sample) / (E_{β-actin})_{ACT} (control − sample) (27).

**Analysis of EP Receptor Expression.** Expression of EP receptors in oral cavity mucosa was analyzed by reverse transcription-PCR. RNA was prepared from frozen tissue using the RNeasy Mini-kit (Qiagen), and cDNA was generated as described above. Primer pairs used were as follows: (a) EP1, 5′-TGGGCCAGCTTGTCGGTAT-3′ (forward) and 5′-TGGAGACCATCTACCCCTT-3′ (reverse); (b) EP2, 5′-TCCAATGACTC-3′ (forward) and 5′-TCGATAGTGACAG-3′ (reverse); (c) EP3, 5′-CAGCTTATGG-GGATCATGTTG-3′ (forward) and 5′-TCGATAGTGACAG-3′ (reverse); (d) EP4, 5′-CAGCTTATGG-GGATCATGTTG-3′ (forward) and 5′-TCGATAGTGACAG-3′ (reverse); (e) β-actin, 5′-GGTCACCCACACTGTGCCCAT-3′ (forward) and 5′-GGATGCACCAGGACTCCATGC-3′ (reverse). EP receptor cDNAs were used as positive controls. The identity of each PCR product was confirmed by DNA sequencing.

**MTT Assay.** MSK-Leuk1 cells were cultured as described above. Approximately 5 × 10^4 cells in 100 μl of medium were placed in each well of a 96-well plate. After 24 h of incubation at 37°C, cells were treated with varying concentrations of PGE_2_. Treatment medium was changed every 48 h. At the end of the treatment period, cells were incubated for 3 h with MTT (0.5 mg/ml) in 100 μl of medium. DMSO (100 μl) was added to each well, and the cells were incubated at 37°C for 30 min. Absorption at 560 nm was read using a 96-well plate spectrophotometer (SLT Lab Instruments A-5082).

**Statistics.** Comparisons between groups were made using Student’s t test. A difference between groups of P < 0.05 was considered significant.

**RESULTS**

Immunoblot analysis was used to assess amounts of mPGES in 14 paired samples of HNSCC. Overall, increased amounts of mPGES were detected in 11 of 14 (79%) cases of HNSCC (Fig. 1). Expression of mPGES protein was undetectable in most samples of nontumorous tissue. Immunohistochemistry was carried out to determine the cellular source of mPGES. Granular cytoplasmic staining for mPGES was detected in neoplastic epithelial cells in HNSCC (Fig. 2). Immunoreactivity was distributed throughout the tumor. This staining was specific for mPGES because immunoreactivity was lost when the mPGES antiserum was preincubated with a mPGES-blocking peptide.

Based on the discovery that mPGES was commonly overexpressed in neoplastic epithelial cells in HNSCC, we investigated whether increased amounts of mPGES would be detected in cell lines derived from HNSCC. To address this question, levels of mPGES protein and mRNA were compared in tumorigenic cell lines derived from HNSCC (1483 and Ca9-22) versus a nontumorogenic cell line derived from a premalignant oral lesion (MSK-Leuk1). As shown in Fig. 3, A and B, amounts of mPGES protein and mRNA were markedly increased in the transformed cell lines, consistent with the increased levels of mPGES observed in HNSCC.
One potential mechanism by which PGE2, the product of mPGES activity, could impact on tumor growth is by stimulating cell proliferation. Hence, we next investigated the effects of exogenous PGE2 on the growth of MSK-Leuk1 cells. As shown in Fig. 4A, treatment with 500 nM PGE2 stimulated cell growth more effectively than treatment with 100 nM PGE2. Higher concentrations of PGE2 did not lead to a further increase in cell growth. The growth-stimulatory effects of PGE2 were also time dependent. Cell number was enhanced by approximately 40% after treatment with 500 nM PGE2 for 10 days (Fig. 4B).

PGE2 elicits cellular responses via interaction with four cell surface receptors, EP1–4. Reverse transcription-PCR was carried out to determine which receptors were expressed in normal head and neck mucosa. Each of the four known EP receptors was detected (Fig. 5).

**DISCUSSION**

In this study, we found that mPGES was overexpressed in approximately 80% of cases with HNSCC. This finding is consistent with other recent reports describing increased expression of mPGES in cancers of the lung, colon, and endometrium (19–21). It is likely, therefore, that enhanced expression of mPGES in addition to COX-2 contributes to the increased amounts of PGE2 detected in HNSCC. Importantly, multiple lines of evidence suggest that overproduction of PGE2 is mechanistically linked to the progression of HNSCC. Higher levels of PGE2 in human HNSCC specimens correlated significantly with increased tumor vascularization and the occurrence of metastatic disease (2). In a recent study that used an experimental
model of human HNSCC, treatment with a selective COX-2 inhibitor markedly decreased the rate of tumor growth in association with a significant reduction in intratumoral levels of PGE\(_2\) (15). Importantly, treatment with a monoclonal antibody to PGE\(_2\) was as effective as the selective COX-2 inhibitor in reducing tumor growth (15). Although these findings firmly established the significance of PGE\(_2\) as a determinant of tumor growth, the precise mechanism is uncertain. In this study, we demonstrate that PGE\(_2\) can directly stimulate cell growth. Others have shown that PGE\(_2\) can stimulate angiogenesis (11) while enhancing the survival (3) and motility (4) of cancer cells. Immune surveillance is inhibited by PGE\(_2\) (6–10). The relative importance of these different mechanisms is uncertain and may well vary in different tumor types or stages of disease.

PGE\(_2\) exhibits biological activity through binding to G-protein-coupled receptors. Four PGE\(_2\) receptors, EP\(_1\)–EP\(_4\), have been identified. Because of the significance of PGE\(_2\) in carcinogenesis, the potential role of individual receptors is being actively investigated. Different PGE\(_2\) receptors appear to be important in carcinogenesis in different tumor models and tumor types (14, 28–30). We show for the first time that all four EP receptors are expressed in human oral squamous mucosa. Future experiments will be needed to define which EP receptor(s) is important for mediating the growth stimulating effects of PGE\(_2\).

Experiments were also carried out to begin to elucidate the mechanism(s) that accounts for overexpression of mPGES in HNSCC. Markedly increased amounts of both mPGES mRNA and mPGES protein were detected in HNSCC tumor cell lines versus a nontumorigenic cell line derived from a premalignant oral lesion. This result suggests that cellular transformation is one of the mechanisms leading to enhanced expression of mPGES in HNSCC. mPGES can be induced by cytokines including interleukin 1B and tumor necrosis factor \(\alpha\) (18–20, 31, 32). Hence, it seems highly likely that multiple mechanisms will contribute to the overexpression of mPGES in HNSCC. Additional experiments are warranted to define the signaling mechanisms that regulate the expression of mPGES.

The COX enzymes produce PGH\(_2\), which, in turn, is metabolized to a variety of eicosanoids. One of the products, PGE\(_2\), has procarcinogenic properties, but other products, such as prostacyclin, inhibit carcinogenesis (33). Because inhibiting COX suppresses the synthesis of both procarcinogenic and antitumor eicosanoids, more selective forms of treatment may prove useful. Examples of more selective treatment would include a selective inhibitor of PGE\(_2\), synthesis or an EP receptor antagonist. Initial studies have suggested that mPGES represents a potential therapeutic target. More specifically, cells overexpressing mPGES and COX-2 produced more PGE\(_2\), grew faster, and exhibited abnormal morphology compared with cells in which either COX-2 or mPGES was overexpressed (34). To further investigate whether mPGES is a therapeutic target, it will be important to determine whether overexpression or knocking out mPGES affects tumor formation or growth. The availability of selective inhibitors of mPGES would permit complementary experiments to be done. Importantly, our observation that mPGES is commonly overexpressed in HNSCC provides the basis for future studies that will evaluate whether mPGES is a bona fide therapeutic target.

**REFERENCES**


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