Inducible Prostaglandin E Synthase Is Overexpressed in Non-Small Cell Lung Cancer[1]

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ABSTRACT

An inducible microsomal form of human prostaglandin E synthase (mPGES) was recently identified. This enzyme converts the cyclooxygenase (COX) product, prostaglandin (PG) H_2, to PGE_2, a prostanoid that has been implicated in carcinogenesis. Increased amounts of PGE_2 are detected in many types of cancer, but the underlying mechanism is not fully understood. Hence, we compared amounts of mPGES in 19 paired samples (tumor and adjacent normal tissue) of non-small cell lung cancer (NSCLC). By immunoblot analysis, mPGES was overexpressed in about 80% of NSCLCs. Immunohistochemistry localized the expression of mPGES to neoplastic epithelial cells. COX-2 was also commonly up-regulated in these tumors; marked differences in the extent of up-regulation of mPGES and COX-2 were observed in individual tumors. Cell culture was used to define the underlying mechanism(s) that accounts for up-regulation of mPGES in NSCLC. As reported previously for COX-2, levels of mPGES mRNA and protein were increased in NSCLC cell lines containing mutant *Ras* as compared with a nontumorigenic bronchial epithelial cell line. Nuclear run-offs revealed increased rates of *mPGES* transcription in the transformed cell lines. Overexpression of *Ras* caused a severalfold increase in *mPGES* promoter activity in nontransformed cells. Tumor necrosis factor-α induced mPGES and COX-2 in NSCLC cell lines but had no effect on the expression of either enzyme in a nontumorigenic bronchial epithelial cell line. Consistent with prior observations for COX-2, these data suggest that both cellular transformation and cytokines contribute to the up-regulation of mPGES in NSCLC.

INTRODUCTION

Elevated levels of PGE_2[3] have been detected in a variety of common human tumors (1–5). PGE_2 is known to possess properties that promote malignant growth. For example, PGE_2 stimulates angiogenesis (6) and inhibits immune surveillance (7). One strategy for inhibiting carcinogenesis is to prevent the overproduction of PGs in premalignant and malignant tissues. In fact, inhibitors of PGE_2 production such as nonsteroidal anti-inflammatory drugs protect against carcinogenesis (8–10). It is important, therefore, to define the pathways that are dysregulated in tumors leading to increased levels of PGE_2.

The synthesis of PGE_2 from arachidonic acid requires two enzymes that act sequentially. COX catalyzes the synthesis of PGH_2 from arachidonic acid. There are two forms of COX designated COX-1 and COX-2, respectively. COX-1 is constitutively expressed in most tissues (11). By contrast, COX-2 can be induced by cytokines, growth factors, oncogenes, and tumor promoters (12–15). Recently, an inducible mPGES was characterized (16). This enzyme converts COX-derived PGH_2 to PGE_2.

Elevated levels of COX-2 (17, 18) and PGE_2 (1, 2) are found in NSCLC, resulting in local immune suppression, a condition that favors tumor growth. This idea is supported by the findings of Huang *et al.* (18) and Stolina *et al.* (19), who showed that human lung cancer cell-derived PGE_2 caused an imbalance in cytokine production and a reduction in antigen processing by dendritic cells. Moreover, in a murine Lewis lung carcinoma model, treatment with anti-PGE_2 monoclonal antibody retarded the growth rate of tumors, leading to prolonged survival (20).

In this study, we investigated whether mPGES and COX-2 were coordinately overexpressed in NSCLC. Additionally, we determined the effects of cellular transformation and treatment with TNF-α on mPGES expression in cultured lung cells. As reported previously for COX-2, our data suggest that both cellular transformation and cytokines contribute to the observed increase in amounts of mPGES in NSCLC.

MATERIALS AND METHODS

Materials. Rabbit polyclonal antihuman mPGES antiserum and mPGES blocking peptide were from Cayman Chemical (Ann Arbor, MI). Goat antihuman COX-2 antiserum was

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[3] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; mPGES, microsomal prostaglandin E synthase; NSCLC, non-small cell lung cancer; TNF-α, tumor necrosis factor-α.

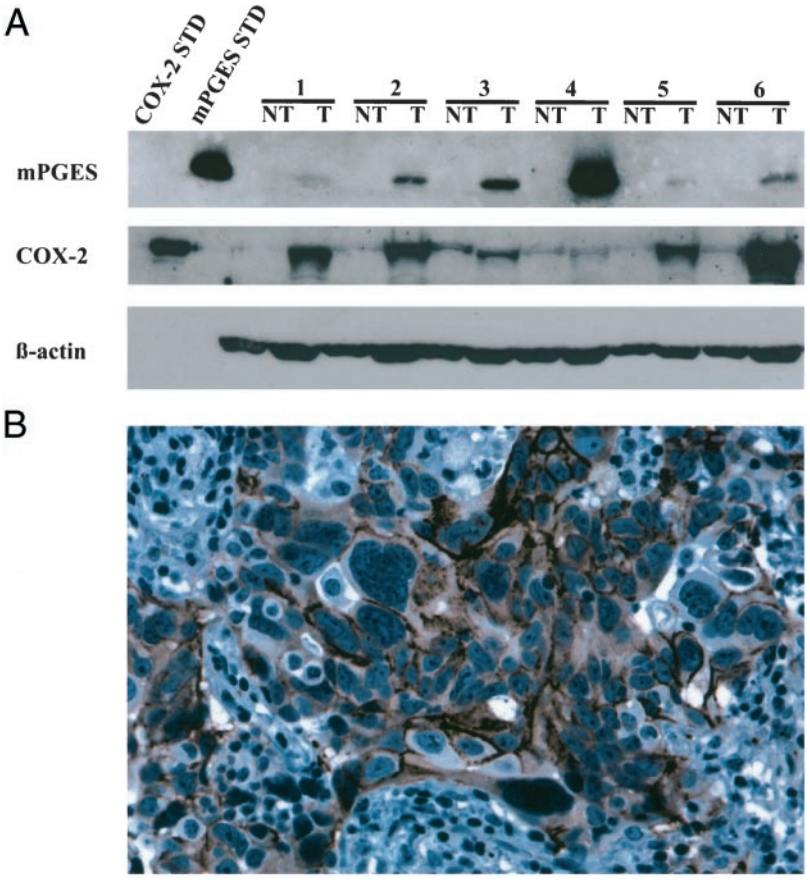

Fig. 1 Levels of mPGES and COX-2 protein are increased in NSCLC. *A*, immunoblot of paired nontumorous (*NT*) and tumorous (*T*) lung tissues from six subjects. Equal concentrations of protein (100 μg/lane) were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was sequentially probed for mPGES, COX-2, and β-actin. Cases 1–4 and 6 represent adenocarcinoma. Case 5 is a squamous cell carcinoma. *B*, this poorly differentiated adenocarcinoma shows diffuse cytoplasmic immunoreactivity with anti-mPGES antibody (×400).

from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human TNF-α, antibody to β-actin, 3,3′-diaminobenzidine, Lowry protein assay kits, and secondary antibody to IgG conjugated to horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO). Biotinylated antirabbit IgG was from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-horseradish peroxidase was from DAKO Corporation (Carpinteria, CA). Superfrost/Plus slides were from Fisher Scientific (Pittsburgh, PA). Western blotting detection reagents (enhanced chemiluminescence) were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). The *Ras* expression vector was a gift of Dr. Geoffrey Cooper (Boston University, Boston, MA).

Patient Samples. Specimens were obtained at the time of surgery from patients with adenocarcinoma or squamous cell carcinoma of the lung. Tissue samples were obtained from a nonnecrotic area of the tumor and from adjacent nontumorous tissue. Samples were immediately stored at −80°C or fixed in neutral buffered formalin until analysis. The study was approved by the Committee on Human Rights in Research at Weill Medical College of Cornell University.

Cell Culture. NSCLC cell lines harboring oncogenic mutations in *Ras* (A549 and H2122; Ref. 21) and an SV40 immortalized lung epithelial cell line (BEAS-2B) were generous gifts of Dr. Raphael A. Nemenoff (University of Colorado, Denver, CO). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All treatments with TNF-α were carried out in serum-free medium.

Western Blotting. Frozen tissue was thawed in ice-cold lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin]. Tissues were sonicated for 20 s on ice and centrifuged at 10,000 × *g* for 10 min at 4°C to remove the particulate material. The protein concentration of the supernatant was measured using the method of Lowry *et al.* (22). Immunoblot analyses for mPGES and COX-2 were performed using methods described in previous studies (13, 14).

Immunohistochemistry. Neutral buffered formalin-fixed tissue was embedded in paraffin. Tissue sections (4 μm) were prepared using a microtome and mounted on Superfrost/Plus slides. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Antigen retrieval was performed by steaming the sections in 10 mM citric acid (pH 6.0) for 30 min. Subsequently, endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide. The slides were washed three times in PBS and blocked for 20 min with 5% normal goat serum. Tissue sections were then incubated with polyclonal antiserum to mPGES at a 1:1000 dilution (2% BSA in PBS) for 18 h at 4°C. Control sections were incubated with mPGES antiserum preabsorbed with a 100-fold excess of

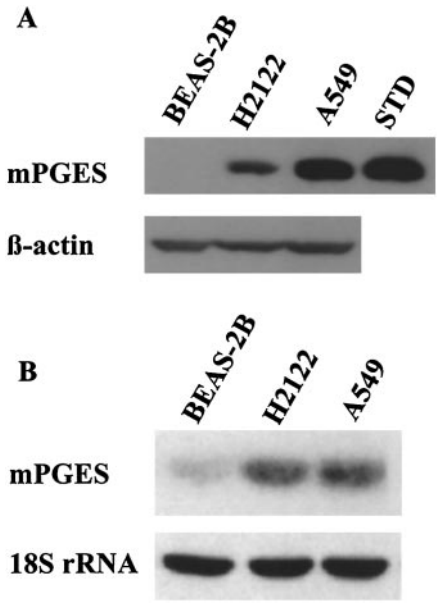

Fig. 2 Transformation is associated with increased amounts of mPGES protein and mPGES mRNA. *A*, cellular lysate protein (100 μg/lane) from BEAS-2B, H2122, and A549 cells was loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was sequentially probed with antibodies specific for mPGES and β-actin. *B*, total cellular RNA was isolated from BEAS-2B, H2122, and A549 cells. Each lane contained 10 μg of RNA. The blot was probed for mPGES mRNA and 18S rRNA. Higher levels of mPGES protein (*A*) and mPGES mRNA (*B*) were detected in NSCLC cell lines (H2122 and A549) than in the immortalized nontumorigenic BEAS-2B cell line.

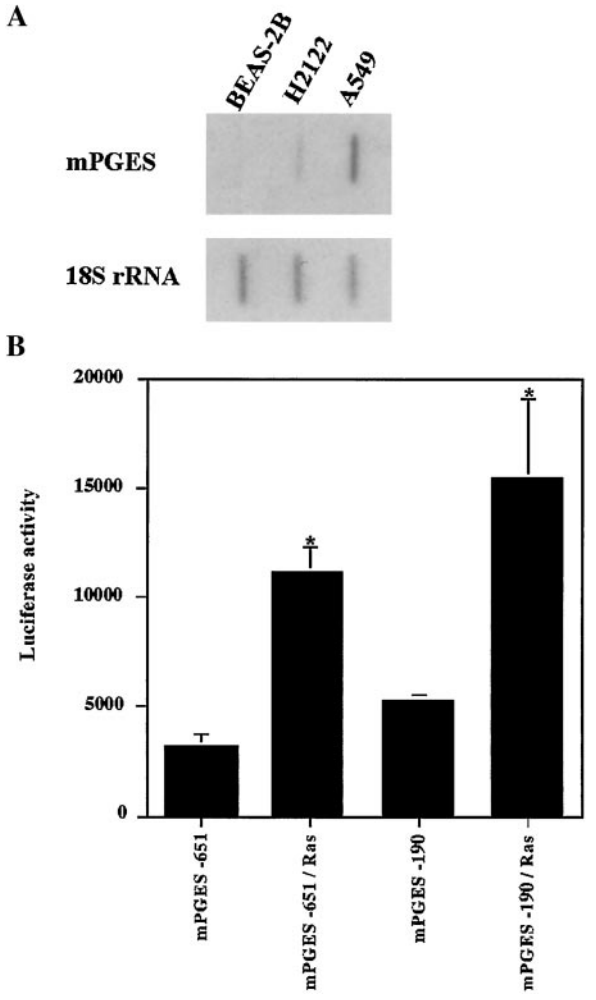

Fig. 3 Transformation of lung epithelial cells is associated with increased rates of mPGES transcription. *A*, nuclei were isolated from BEAS-2B, H2122, and A549 cells. Nuclear run-off analysis was performed as described in "Materials and Methods." The mPGES and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled RNA transcripts from the different cell lines. *B*, BEAS-2B cells were transfected with 0.9 μg of human *mPGES* promoter deletion constructs ligated to luciferase (−651/−20 or −190/−20) and 0.2 μg of pSVβgal. *Bars labeled Ras* represent cells that also received 0.9 μg of expression vector for *Ras*. The total amount of DNA in each reaction was kept constant at 2 μg by using a corresponding empty expression vector. Reporter activities were measured in cellular extract 24 h after transfection. Luciferase activity represents data that have been normalized with β-galactosidase. *Columns*, means; *bars*, SD; $n = 6$. *, $P < 0.001$.

mPGES blocking peptide or with preimmune serum. After being washed three times with PBS, the sections were incubated with biotinylated antirabbit antibody at a 1:500 dilution for 1 h at room temperature. The slides were then washed three times in PBS and labeled using 1:500 streptavidin-horseradish peroxidase for 1 h at room temperature. The reaction was visualized using 3,3′-diaminobenzidine. Subsequently, the slides were rinsed in tap water and counterstained with hematoxylin. The slides were then dehydrated with ethanol, rinsed with xylene, and mounted.

Northern Blotting. Total cellular RNA was isolated from cell monolayers using a RNA isolation kit from Qiagen, Inc. Ten μg of total cellular RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. The blot was probed using methods described previously (13, 14). mPGES and 18S rRNA probes were labeled with [^{32}P]CTP by random priming.

Nuclear Run-off. Nuclei were isolated from 1×10^7 BEAS-2B, H2122, and A549 cells and stored in liquid nitrogen. The transcription assay was performed as described previously (13, 14) using mPGES and 18S rRNA cDNAs that were immobilized onto nitrocellulose.

Transient Transfection Assays. BEAS-2B cells were seeded at a density of 1×10^5 cells/well in 6-well dishes and grown to 50–60% confluence. The −651/−20 and −190/−20 human *mPGES* promoter constructs have been described previously (23). For each well, 2 μg of plasmid DNA were introduced into cells using 20 μg of LipofectAMINE (Life Technologies, Inc.) as per the manufacturer's instructions. After 16 h of incubation, the medium was replaced with culture medium. The activities of luciferase and β-galactosidase were measured in cellular extracts as described previously (24).

Statistics. Comparisons between groups were made by the Student's *t* test. A difference between groups of $P < 0.05$ was considered significant.

RESULTS

mPGES Is Overexpressed in NSCLC. Immunoblot analysis for mPGES was performed on 19 cases of NSCLC. Overall, increased expression of mPGES was detected in 15 of 19 (79%) cases of NSCLC. Overexpression of mPGES was detected in both adenocarcinomas (13 of 16 cases) and squamous cell carcinomas (2 of 3 cases) of the lung (Fig. 1*A*). COX-2 was also detected in 84% (16 of 19) of these tumors (Fig. 1*A*). Interestingly, marked differences in the extent of up-regulation of mPGES and COX-2 were observed. In case 4, for example, mPGES was dramatically up-regulated, whereas low levels of COX-2 were detected. This pattern was reversed in case 6. This difference in the relative magnitude of overexpression of mPGES and COX-2 suggests that the regulation of the two enzymes is different. COX-2 is expressed in neoplastic epithelial cells in NSCLC (17, 18). Immunohistochemistry was performed to evaluate the cellular source of mPGES in 10 cases of NSCLC. mPGES was detected in 8 of 10 cases and localized to the cytoplasm of tumor cells (Fig. 1*B*). This staining was specific for mPGES because immunoreactivity was lost when the antiserum to mPGES was preincubated with a mPGES blocking peptide. Neither mPGES nor COX-2 was detected in normal bronchial mucosa.

Transcription of mPGES Is Enhanced in NSCLC Cells. Previously, COX-2 was reported to be overexpressed in NSCLC cell lines that harbor oncogenic *Ras* (H2122 and A549) as compared with nontransformed bronchial epithelial cells (BEAS-2B; Ref. 25). We used the same cell system to determine whether *mPGES* gene expression was also enhanced in NSCLC cells. As shown in Fig. 2, *A* and *B*, levels of mPGES protein and mRNA were increased in the transformed cell lines (H2122 and A549). Differences in levels of mRNA could reflect altered rates of transcription. To investigate this possibility, nuclear run-offs were performed. Higher rates of synthesis of nascent mPGES mRNA were observed in the two transformed cell lines containing mutant *Ras* (Fig. 3*A*), a finding consistent with the differences observed by Northern blotting.

To further investigate the potential importance of *Ras* in modulating the expression of mPGES, transient transfections were performed using a *Ras* expression vector cotransfected with *mPGES*-luciferase reporter constructs. As shown in Fig. 3*B*, *Ras* up-regulated *mPGES* promoter activity in nontransformed BEAS-2B cells. This effect was observed with both the −651/−20 and −190/−20 *mPGES* promoter constructs.

TNF-α Induces mPGES and COX-2 in NSCLC Cells. Cytokines could also modulate the expression of mPGES and COX-2 in tumors. With this in mind, we investigated the effects of TNF-α on the expression of mPGES and COX-2 in cultured cells. Treatment of H2122 cells with TNF-α induced both mPGES and COX-2, but the magnitude of induction was somewhat less for mPGES than it was for COX-2 (Fig. 4*A*). Induction of both mPGES and COX-2 was observed for at least 24 h after treatment with TNF-α. It is possible that transformed cells respond differently than nontransformed cells to TNF-α. To evaluate this question, we compared the effects of TNF-α on the expression of mPGES and COX-2 in H2122 and A549 cells as compared with BEAS-2B cells. Interestingly, TNF-α induced both mPGES and COX-2 in H2122 and A549 cells, whereas it had no effect on either enzyme in immortalized nontransformed BEAS-2B cells.

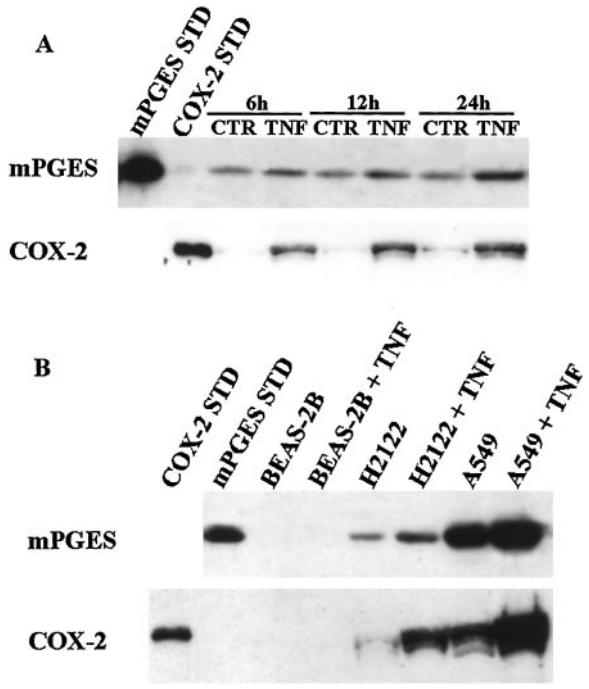

Fig. 4 TNF-α induces mPGES and COX-2 in NSCLC cell lines. *A*, H2122 cells were treated with vehicle (*CTR*) or TNF-α (10 ng/ml) for 6, 12, and 24 h. *B*, BEAS-2B, H2122, and A549 cells were treated with vehicle or TNF-α (10 ng/ml) for 24 h. In *A* and *B*, cellular lysate protein (100 μg/lane) was loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed sequentially with antibodies specific for mPGES and COX-2.

DISCUSSION

Increased amounts of PGE_2 are commonly detected in tumors including NSCLC (1–5). On the basis of the results of this study, it seems likely that up-regulation of both COX-2 and mPGES contributes to enhanced production of PGE_2 in NSCLC. Significantly, this is the first report of mPGES being overexpressed in any form of neoplasia. It will be of considerable interest, therefore, to determine whether mPGES is overexpressed in other common malignancies in which increased levels of PGE_2 are detected (3–5, 26). Additional studies are also needed to evaluate whether mPGES is up-regulated in premalignant tissues as reported previously for COX-2 (27, 28). Elevated levels of PGE_2 are found in inflamed tissues (29), and chronic inflammation predisposes to cancer (30). Consequently, it will be worthwhile to determine whether increased expression of mPGES occurs in chronic inflammatory diseases such as ulcerative colitis or Crohn's disease.

Experiments were performed to try to elucidate the mechanisms that account for overexpression of mPGES in NSCLC. Cellular transformation enhanced *mPGES* transcription. This finding is consistent with a previous report (13) of increased *COX-2* gene expression in transformed cells. We also found that

transformed cells were more sensitive than nontransformed cells to TNF-α-mediated induction of mPGES and COX-2. These findings strongly suggest, therefore, that multiple mechanisms contribute to enhanced expression of mPGES as well as COX-2 in NSCLC.

Both COX-2 and mPGES were commonly up-regulated in NSCLC, but the relative degree of overexpression varied, suggesting that the regulation of the two enzymes is not identical. In support of this idea, TNF-α caused greater induction of COX-2 than mPGES in cultured cells. In contrast, *Ras* stimulated *mPGES* promoter activity, a finding reported previously for COX-2 (13). Other oncogenes including *wnt-1*, *v-src*, and HER-2/*neu* enhance the transcription of *COX-2*, but their effects on *mPGES* remain unknown (31, 32). Clearly, additional studies are warranted to further delineate similarities and differences in the regulation of mPGES and COX-2. In the case of COX-2, regulation is both cell type and inducer specific (33). The same is likely to be true for mPGES.

Several lines of evidence from different experimental systems suggest that enhanced expression of mPGES could be important in tumorigenesis. Murakami *et al.* (34) showed that cells coexpressing COX-2 and mPGES produced more PGE_2, grew faster, and exhibited aberrant morphology compared with cells in which either COX-2 or mPGES was overexpressed. In another recent study (35), mice that were deficient in the PGE receptor subtype EP1 developed fewer preneoplastic lesions in the colon than wild-type mice. Newly developed selective COX-2 inhibitors prevent the synthesis of PGE_2 and possess anticancer properties (36). Further investigation is required to determine whether mPGES represents a pharmacological target for preventing or treating cancer.

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