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NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) behaves as a tumor suppressor in lung cancer

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It has been reported that two inducible prostaglandin synthetic enzymes, cylooxygenase-2 (COX-2) and microsomal PGE synthase, are over-expressed in non-small cell lung cancer (NSCLC). Using quantitative reverse transcription–polymerase chain reaction, we analyzed RNA levels of the key prostaglandin catabolic enzyme, NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH), in 19 pairs of NSCLC tumors and adjacent non-malignant tissue from the same patient. We found that 100% of tumor-tissue pairs showed at least a 2-fold decrease and 61% showed a 10-fold decrease. This suggests that the increased expression of COX-2 and PGE synthase in tumors may work in concert with the decreased expression of 15-PGDH to amplify an increase in tissue levels of proliferative PGE_2. To further explore if 15-PGDH is related to tumorigenesis, athymic nude mice were injected with control A549 cells or cells transiently over-expressing wild-type or mutant 15-PGDH (Y151F). It was found that mice injected with control A549 cells or with cells expressing mutant enzyme produced tumors normally. However, mice injected with A549 cells expressing wild-type 15-PGDH had a significant decrease in tumor growth. Examining the effects of 15-PGDH expression on cellular changes in A549 cells, we found that over-expression of 15-PGDH induced apoptosis of A549 cells as evidenced by fragmentation of DNA, activation of pro-caspase 3, cleavage of poly(ADP-ribose) polymerase and decreased expression of Bcl-2. We also found that the expression of 15-PGDH was negatively related to that of pro-adhesive and invasive CD44. Furthermore, the expression of 15-PGDH was found to be stimulated by hyaluronidase. These results suggest that 15-PGDH may decrease the level of proliferative PGE_2, induce apoptosis and function like a tumor suppressor.

Introduction

Lung cancer is the number one killer of all cancers both in men and in women in the US. Despite therapeutic efforts, 5-year survival in lung cancer patients is still <15%. The most common form of lung cancer, non-small cell lung cancer (NSCLC), accounts for 75–80% of the lung cancer cases (1). Adenocarcinoma (AC), a secretory tumor, is the most common NSCLC type among smokers and the only form of lung cancer that non-smokers develop (2). The disease is seldom diagnosed until advanced stages yielding clinical symptoms. Unfortunately, advanced AC almost always has a fatal consequence. The underlying molecular and cellular mechanisms for growth and metastasis of lung cancer and therapies to ameliorate the progression phase of the disease remain to be elucidated.

Accumulating evidence has indicated that aberrant expression of the enzymes involved in arachidonic acid metabolism appears to contribute significantly to the development of lung cancer. Several studies have shown that cyclooxygenases (COXs) particularly inducible COX-2, which catalyzes dioxygenation and cyclization of arachidonic acid to PGH_2, increase in expression in human and animal lung tumor as well as in other tumors (3–6). Similarly, the inducible microsomal PGE_2 (m-PGE_2) synthase, which is preferentially coupled to COX-2 and catalyzes the GSH-dependent isomerization of PGH_2 to PGE_2, was also found to over-express in human NSCLC (7). It appears that the two consecutive enzymes involved in PGE_2 synthesis are both over-expressed in lung tumors. Overproduction of PGE_2 is known to result in local immunosuppression, a condition that favors tumor growth. This idea is supported by the findings of Huang *et al*. (8) and Stolina *et al*. (9), which showed that human lung cancer cell-derived PGE_2 cause 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 (10). Apparently, the level of PGE_2 may play a significant role in lung tumor growth. However, the level of PGE_2 is controlled not only by synthesis but also by degradation, a fact that has been overlooked in studying prostaglandins and cancer.

The first enzyme involved in prostaglandin catabolism is NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This enzyme catalyzes the NAD^+-dependent oxidation of the 15(S)-hydroxyl group of prostaglandins and lipoxins resulting in the production of 15-keto-prostaglandins and 15-keto-lipoxins, which exhibit greatly reduced biological activities (11). Therefore, this enzyme has been considered a key enzyme responsible for the biological inactivation of prostaglandins and lipoxins. The enzyme is widely distributed in various mammalian tissues among which lung is one of the most active tissues (12). The essential role of this enzyme in remodeling the ductus ateriosus was appreciated recently by the generation of knockout mice deficient in 15-PGDH (13). The role of this enzyme in other physiological functions and pathological consequences including cancer remains to be elucidated. It would be interesting to determine if the expression of 15-PGDH is altered during tumor development in the lung.

Abbreviations: COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; m-PGE_2, microsomal PGE_2; NSCLC, non-small cell lung carcinoma; PARP, poly(ADP-ribose) polymerase; PG, prostaglandin; 15-PGDH, hydroxyprostaglandin dehydrogenase; RT–PCR, reverse transcription–polymerase chain reaction.

The first two authors contributed equally to this work.

A549 cells, a human lung AC-derived cell line, have been used widely as a model system to study lung cancer. This cell line is known to increase in expression of COX-2, m-PGE_2 synthase and cytosolic phospholipase A_2 in response to agonists such as interleukin (IL)-1β, tumor necrosis factor α and lipopolysaccharide (8,14). We have found that there is a constitutive expression of 15-PGDH in addition to COXs in this cell line. Previous studies in lung cancer cells indicate that elevated COX-2 expression is associated with the inhibition of apoptosis and increased angiogenesis, tumor invasion and suppression of host immunity (8,9,15–17). Over-expression of COX-2 in A546 cells was shown to induce increased expression of CD44, a cell surface receptor for hyaluronate, which has an important role in regulating tumor growth and metastasis because it mediates cellular adhesion to extracellular matrix (18). Over-expression of COX-2 in other cell lines was also demonstrated to cause up-regulation of anti-apoptotic protein Bcl-2 (17,19). Whether over-expression of 15-PGDH in A549 cells may decrease PGE_2 level, induce apoptosis and inhibit CD44 and Bcl-2 expression remains to be elucidated.

In the present study, we demonstrate that 15-PGDH is under-expressed in human lung tumors and that over-expression of 15-PGDH in A549 cells results in the inhibition of tumor growth in athymic nude mice. Inhibition of tumor growth by over-expression of 15-PGDH is likely to be attributed to the depletion of PGE_2 by 15-PGDH, the induction of apoptosis, and the inhibition of tumor invasion and metastasis as shown by the reciprocal regulation of the expressions of CD44.

Materials and methods

Materials

The plasmids encoding 15-PGDH cDNA and its mutant cDNA (Y151F) were obtained as reported previously (20,21). The pcDNA3 expression vector was from Invitrogen. AdEasy™ XL Adenoviral Vector System was purchased from Stratagene Co. *Taq* DNA polymerase, all restriction enzymes, geneticin-selective antibiotic (G418) and heat-inactivated fetal bovine serum were from Gibco-BRL. Fast-Link™ DNA Ligation Kit was from Epicentre. The QIAprep Spin Plasmid Miniprep Kit, QIAquick PCR Purification Kit and QIA Quick Gel Extraction Kit were from QIAGEN. Gentamicin and the Lipofectamine 2000 transfection reagent were supplied by Life Technologies. Human NSCLC cell line A549 (AC) was obtained from the American Type Culture Collection. Sodium dodecyl sulfate (SDS), dithiothreitol (DTT), leupeptin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), hyaluronidase and RPMI-1640 were obtained from Sigma Chemical. Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore. Electro-chemiluminescence (ECL^+) plus Western Blotting Detection System RPN 2132 was obtained from Amersham Pharmacia Biotech. Rabbit antiserum against human placental 15-PGDH was generated as described previously (22). Rabbit antisera against human COX-2 C-terminal (NASSSRSGLDDINPTVLLK)-specific sequences were generated using glutathione *S*-transferase fusion protein as antigens (23). Rabbit antisera against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated as reported (24). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was supplied by Transduction Laboratories (Lexington, KY). HRP-labeled goat anti-rabbit IgG was from Zymed. Poly(ADP-ribose) polymerase (PARP) antibody specifically against N-terminal immunogen was from BD Biosciences. Antibodies p53 and caspase-3 were from Santa Cruz Biotechnology. Bcl-2 and CD44 antibodies were from Neomarkers. PGE_2 was supplied by Cayman Chemical. 15(S)-[15-^{3}H]PGE_2 was prepared according to a previously published procedure (25). IL-1β was supplied by PeproTech. Other reagents were obtained from the best commercial sources.

Cell culture

A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 1 mg/100 ml gentamicin in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were plated in 6-well plate (2 ml/well) at ~2 × 10^5 cells/well in duplicate and grown for 24 h before infection or transfection.

Recombinant adenovirus

The wild-type 15-PGDH gene or mutant 15-PGDH (Y151F) gene was cloned into an adenoviral shuttle vector (pshuttle-IRES-hrGFP1) in the AdEasy™ XL Adenoviral Vector System. The preparation of recombinant adenovirus-wild-type 15-PGDH (Ad-Pw) and -mutant 15-PGDH (Ad-Pm) or the control adenovirus (Ad-C) was performed according to the manufacturer's instructions. Adenoviruses were subsequently expanded by sequential rounds of infection on AD-293 cells and purified by the CsCl gradient method. Viral titers were estimated by using two different methods [optical density measurement and tissue culture infectious dose 50 ($TCID_{50}$) method], always run in duplicate, to assure that equal amounts of recombinant and control adenovirus were used in all experiments. The expression of reporter gene, GFP (green fluorescent protein) in the adenoviral shuttle vector, was also used as a reference to control the amount of different viruses used in all experiments.

Viral infection

The cells were infected with adenovirus vectors (Ad-Pw, Ad-Pm or Ad-C) at a multiplicity of infection (MOI) of 2000 viral particles per cell. After infection for 2–3 days, cells were harvested to carry out experiments.

Animal study

All animals were maintained and animal experiments were performed under NIH and institutional guidelines established for the Animal Core Facility at the University of Kentucky Medical Center. A549 tumor cells were infected with Ad-Pw, Ad-Pm or Ad-C at MOI of 1000 viral particles per cell. After infection for 36 h, cells were harvested, washed twice with sterile PBS, counted and re-suspended in PBS. Six-week-old female athymic nude mice were injected subcutaneously in both flanks with infected A549 cells at a density of 5 × 10^6 viable cells/200 μl. Six individual mice were used in each group. Tumor size was measured with a linear caliper twice per week for up to 5 weeks, and the volume was estimated by using the equation $V = (a \times b^2)/2$, where a is the larger dimension and b the perpendicular diameter.

Stable expression of the wild-type 15-PGDH

Human 15-PGDH cDNA was cloned into the mammalian expression vector pcDNA3 at *Bam*HI and *Xho*I sites. The insertion was confirmed by DNA sequencing. To create cell lines stably expressing the wild-type 15-PGDH, pcDNA3 expression vector containing the cDNA of the wild-type 15-PGDH, was transfected into A549 cells using Lipofectamine 2000 transfection reagent according to the manufacturer's directions. To isolate permanent transfectants, G418-resistant cells were selected in complete culture medium containing 500 mg/l G418 as described previously (26). Expression of the wild-type 15-PGDH was monitored by the western blotting analysis and the activity assay of the enzyme.

15-PGDH assay

15-PGDH was routinely assayed by measuring the transfer of tritium from 15(S)-[15-^{3}H]PGE_2 to glutamate by coupling 15-PGDH with glutamate dehydrogenase as described previously (25). Briefly, the reaction mixture contained: NH_4Cl, 5 μmol; α-keto-glutarate, 1 μmol; NAD^+, 1 μmol; 15(S)-[15-^{3}H]PGE_2, 1 nmol, 30 000 c.p.m.; glutamate dehydrogenase, 100 μg and crude enzyme extract in a final volume of 1 ml of 50 mM Tris–HCl, pH 7.5. The reaction was allowed to continue for 10 min at 37°C and terminated by the addition of 0.3 ml of 10% aqueous charcoal suspension. The radioactivity in the supernatant after centrifugation (1000 *g*, 5 min) was determined by liquid scintillation counting. Calculation of the amount of PGE_2 oxidized was based on the assumption that no kinetic isotope effect was involved in the oxidation of 15(S)-hydroxyl group of 15(S)-[15-^{3}H]PGE_2 as a substrate.

DNA fragmentation assay

The floating cells and the cells attached to the dish were collected in 1.5-ml microcentrifuge tubes and washed once with 1 ml ice-cold PBS. The cell pellets were re-suspended in the TE lysis buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) containing 0.4% Triton X-100. RNase A (2 mg/ml) and proteinase K (2 mg/ml) were added, respectively, and incubated at 37°C for 1 h or until the solution cleared. Then the sample was analyzed by electrophoresis on a 2% agarose gel. The samples, each equivalent to the same amount of cells, were loaded and visualized with ethidium bromide staining by UV transilluminater.

Western blotting

To determine the expression of various proteins in the lung cancer cells following 15-PGDH over-expression, western blot analysis was performed as described previously (27). Briefly, cells were harvested by trypsinization and lysed in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA,

1 mM DTT, 0.1% SDS, 1% Nonidet P-40, 1 μg/ml leupeptin and soybean trypsin inhibitor, 0.5 mM PMSF) for 30 min on ice. Approximately 50–150 μg of protein extracts were then loaded on a 12% polyacrylamide gel. Next, the separated proteins were electrophoretically blotted from the gel onto PVDF membrane and then blocked with a blocking buffer (5% non-fat dry milk in 1× TBST, i.e. 20 mM Tris–HCl, pH 7.6 containing 0.8% NaCl and 0.1% Tween-20) at room temperature for 1 h. The membranes were incubated with the primary antibodies in blocking buffer, followed by incubation with HRP-labeled secondary antibodies. Bands were visualized using the ECL Western blotting detection system.

Statistical analysis

Each experiment was performed at least three times. The data were expressed as the mean ± SD or SE. Statistical significance was assessed by Student's *t* test. A P value of <0.05 is considered statistically significant.

Quantitative reverse transcription–polymerase chain reaction (RT–PCR) of lung cancer specimens

Lung tumor tissues and matched normal tissues from diagnostic surgical procedures were obtained from the University of Kentucky Hospital and the Cooperative Human Tissue Network without patient identifiers or personal health information after approval from the University of Kentucky Institutional Review Board. Total RNAs were isolated from 19 pairs of lung specimens using RNeasy Maxi kit. The primers (15-PGDH forward: 5′-TCTGTTCATC-CAGTGCGATGT-3′; 15-PGDH backward: 5′-ATAATGATGCCGCCTT-CACCT-3′) were screened in the EMBL gene bank to exclude mispriming to known genes. cDNA was synthesized from 2 μg of total RNA extracted from each sample using SuperScript First-Strand Synthesis System in a 20 μl volume following the instructions supplied by the manufacturer. Quantitative RT–PCR was performed using SYBR Green PCR kit on a Bio-Rad iCycler iQ Real Time PCR Detection System according to the instructions supplied by the manufacturers. Primers were designed that spanned introns to eliminate the possibility of confounding genomic DNA amplification. Briefly, after 10 min hot start at 95°C, the amplification reaction proceeded through 45–50 cycles of 95°C denature for 10 s, 60–64°C annealing for 30 s and a 72°C extension for 30 s. A standard curve against 15-PGDH expression was constructed using normal lung cDNA as the template. Quantification was performed using the iCycler analysis software. A normal curve against 15-PGDH expression was constructed using normal lung RNA as the template. Gene expression was normalized to actin and the ratio of the normalized expression (normal/tumor tissue) was calculated for each pair of specimens.

Results

Gene expression of 15-PGDH in human lung tumors

As shown in Figure 1, RT–PCR analysis of 15-PGDH expression in 19 pairs of human lung tumor tissues and matched normal tissues indicated that the ratio of 15-PGDH to actin mRNA levels in normal tissues was each higher than that in tumor tissues. All tumor-tissue pairs showed at least a 2-fold decrease, 78% tumor-tissue pairs showed at least a 4-fold decrease and 61% of tumor-tissue pairs showed at least a 10-fold decrease.

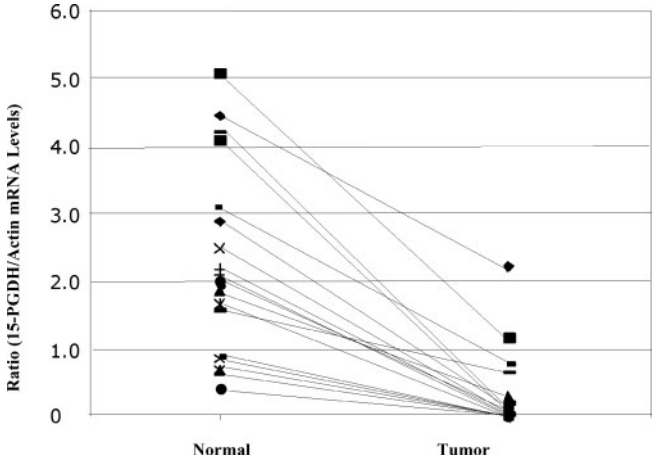

Fig. 1. Expression of 15-PGDH in human lung tumor tissue and matched normal tissue. Total RNAs were isolated from 19 pairs of lung specimens. Quantitative RT–PCR was performed using specific primers for 15-PGDH on a Bio-Rad iCycler iQ Real Time PCR Detection System as described in the Materials and methods section.

Phenotype analysis of 15-PGDH transfectants in athymic nude mice

We investigated if 15-PGDH over-expression would alter the ability of A549 cells to form tumors in athymic nude mice. A549 cells transfected with control adenoviral vector, adenoviral vectors carrying the wild-type 15-PGDH gene or mutant 15-PGDH (Y151F) gene were injected subcutaneously (5×10^6 cells in PBS/animal) into six individual mice.

These cells were ensured to express 15-PGDH with protein (upper panel; PGDH, M-PGDH, VECTOR) and activity (middle panel; PGDH, M-PGDH, VECTOR) before they were injected into animals (Figure 2A). The mice were observed for 5 weeks to monitor tumor growth as shown in Figure 2B. The mice injected with A549 cells transfected with the wild-type 15-PGDH gene (PGDH) grew tumors at a much slower rate than those injected with a mutant gene (M-PGDH). The mice injected with A549 cells harboring the mutant gene grew tumors only slightly slower although not statistically significant than those harboring the control vector (VECTOR). To ensure that over-expression of 15-PGDH in A549 cells resulted in the metabolism of endogenously synthesized PGE2, A549 cells transfected with adenoviral vector, adenoviral vectors carrying wild-type 15-PGDH gene or mutant gene were stimulated with IL-1β to induce endogenous synthesis of PGE_2. Figure 3 shows that cells carrying wild-type 15-PGDH gene (PGDH) secreted almost no PGE_2 in the medium following IL-1β stimulation, whereas cells carrying vector (VECTOR) only secreted as much PGE_2 as the control cells (CONTROL). Interestingly, cells harboring the mutant gene (M-PGDH) secreted attenuated levels of PGE_2 as compared with those carrying vector only.

Ectopic expression of 15-PGDH induces apoptosis

When A549 cells were induced to over-express 15-PGDH by infection with Ad-15-PGDH, these cells were found to undergo apoptosis. Fragmentation of DNA, an index of apoptosis, in A549 cells over-expressing 15-PGDH was readily observed in Figure 4A. However, cells infected with adenovirus alone also showed slight fragmentation of DNA. Since cells undergoing apoptosis execute the death program by activating caspases and cleaving PARP (28), we analyzed cells over-expressing 15-PGDH following infection with Ad-15-PGDH for the activation of caspase-3, a key executioner of apoptosis, and cleavage of PARP. Figure 4B shows that immunoreactive pro-caspase-3 was found to decrease in cells over-expressing 15-PGDH indicating the cleavage and activation of pro-caspase-3 had occurred. At the same time, PARP was shown to undergo cleavage to a smaller fragment in cells over-expressing 15-PGDH. Again, slight cleavage of PARP was also observed in cells infected with adenovirus clone.

Ectopic expression of 15-PGDH results in down-regulation of anti-apoptotic protein Bcl-2 without affecting the expression of pro-apoptotic proteins p53

Bcl-2 has been identified as an apoptosis-suppressing oncoprotein, whereas p53 has been known as an apoptosis-inducing

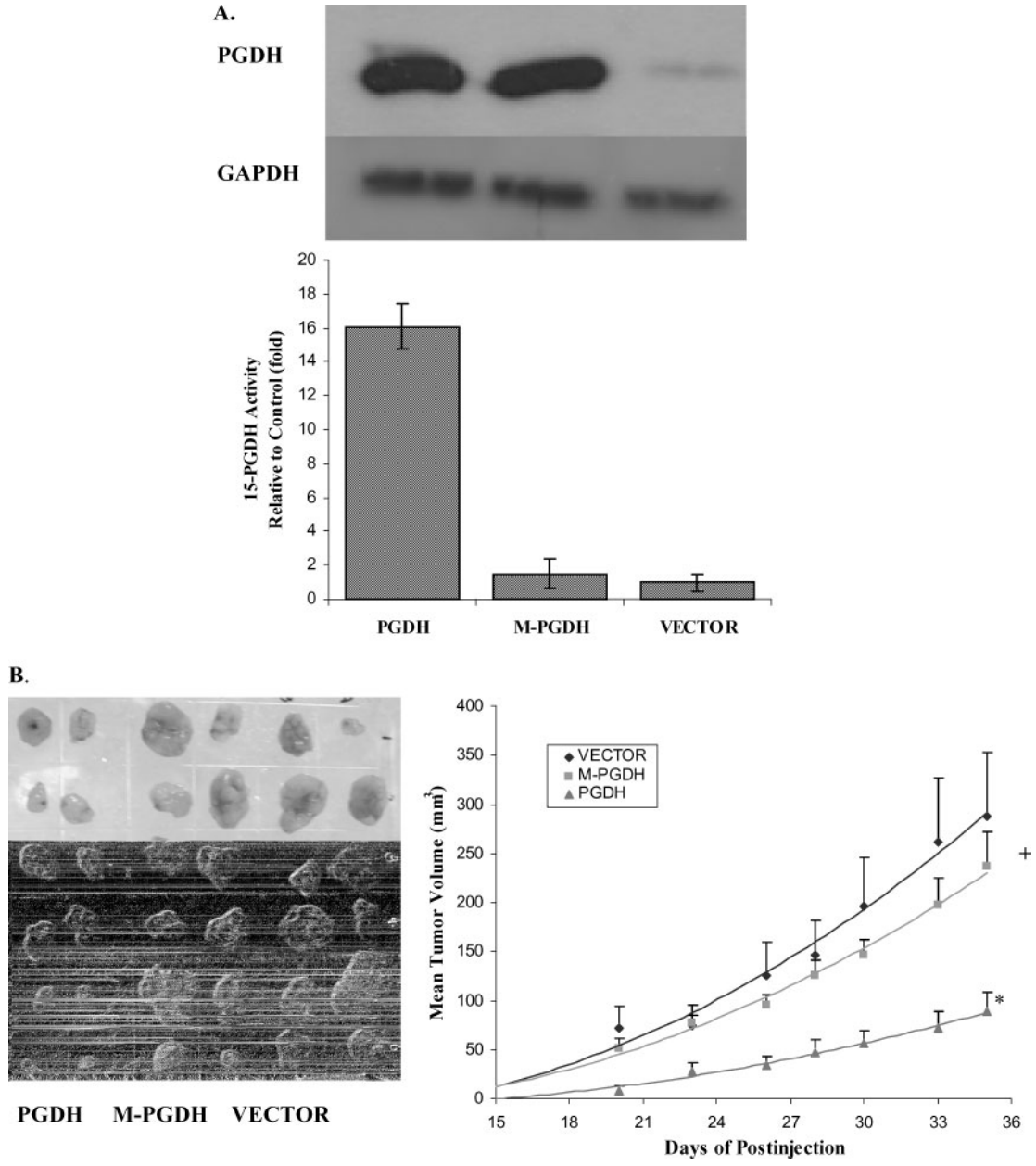

Fig. 2. Suppression of tumorigenesis of A549 lung cancer cells in athymic nude mice by ectopically increased expression of 15-PGDH. (**A**) Western blot (upper panel) and activity analysis (lower panel) of 15-PGDH over-expressed in A549 cells infected with Ad-Pw, Ad-Pm and Ad-C. The construction and infection with recombinant adenoviruses as well as western blot and activity analyses were carried out as described in the Materials and methods section. Each sample was performed in duplicate. The data were expressed as the mean ± SD. (**B**) Six animals per condition as indicated above received injection in both flanks with infected A549 cells at a density of 5×10^6 viable cells per 200 μl as described in the Materials and methods section. Tumor growth shown in the right panel was monitored for 5 weeks, and caliper measurements were conducted twice per week. The left panel shows the tumors removed from the animals at the end of the observation period. The first two left rows display the tumors formed by the cells infected with Ad-Pw. The middle two rows display the tumors formed by the cells infected with Ad-Pm and the last two right rows display the tumors formed by the cells infected with Ad-C. The right panel shows that the tumor volume was plotted against time. Results were expressed as the mean ± SE at each time point. $^{*}P < 0.01$ when compared with Ad-C or Ad-Pm; $^{+}P > 0.05$ when compared with Ad-C.

oncoprotein (29). The effects of over-expression of 15-PGDH on the expression of these apoptosis-related proteins were examined. Western blot analysis indicated that A549 cells infected with Ad-15-PGDH expressed significantly less Bcl-2 protein as compared with cells infected with adenovirus alone or cells without infection as shown in Figure 5. The expression of Bcl-2 protein was decreased down to ~25% of that found in control cells. Interestingly, it appears that the expression of p53 was not significantly affected by the over-expression of 15-PGDH.

Correlation of the expression of 15-PGDH in A549 cells with the expression of adhesion and invasion related proteins, CD44

Cell surface CD44 is the receptor for hyaluronate and may promote tumor invasion (30), whereas cadherins, in particular E-cadherin, may suppress tumor invasion (31). A549 cells, transfected with pcDNA3 encoding the 15-PGDH gene, expressing different levels of 15-PGDH were selected and allowed to become stable clones. Figure 6 shows that five stable clones of A549 cells expressing different levels of

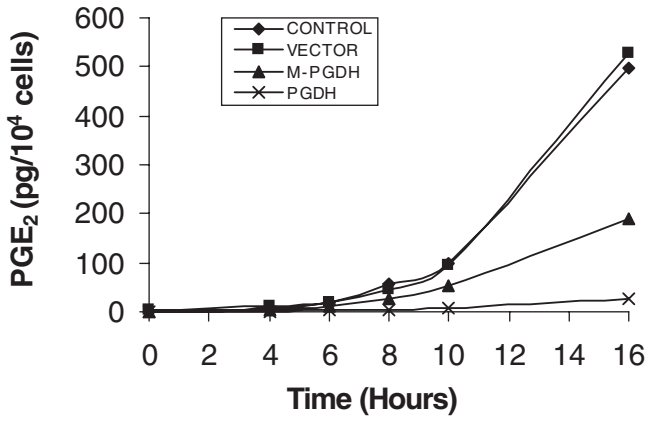

Fig. 3. IL-1β induced release of PGE_2 into the medium by A549 cells infected with recombinant adenoviruses carrying wild-type and mutant 15-PGDH cDNA. A549 cells were infected without adenovirus (■) or with Ad-C (◆), or with Ad-Pm (▲), or with Ad-Pw (x) for 2 days. Cells after harvest were plated and treated with IL-1β (0.5 ng/ml) for the indicated length of time. The medium collected was assayed for PGE_2 by enzyme immunoassay as described in the Materials and methods section.

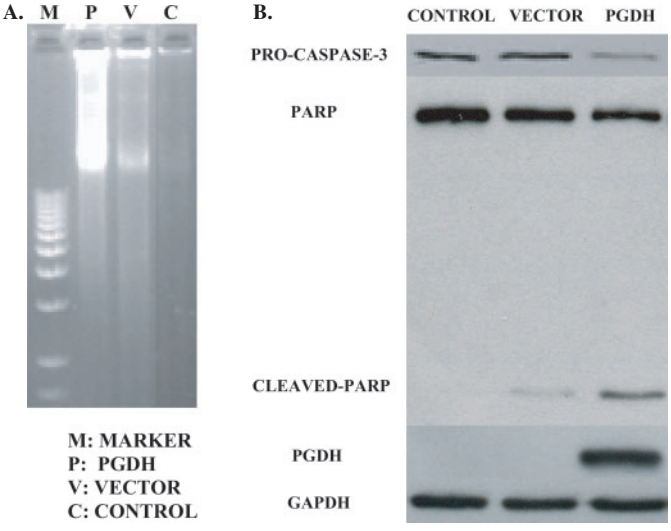

Fig. 4. Induction of apoptosis in A549 cells infected with Ad-15-PGDH. A549 cells were infected with (Vector) or without (Control) adenoviral vector or with adenoviral vector carrying wild-type 15-PGDH gene (PGDH) for 24 h. The medium was then changed to a medium without fetal bovine serum for 48 h. The cells were then collected and subjected to: (**A**) DNA fragmentation assay; (**B**) western blot analysis for pro-caspase-3, PARP, cleaved PARP and 15-PGDH as described in the Materials and methods section. GAPDH was used as a loading control. Cleaved caspase-3, which exhibited a much smaller molecular weight (17 kDa) was not shown because it was not detected well with the antisera we used.

15-PGDH exhibited varied expressions of CD44. The expression of E-cadherin was parallel to that of 15-PGDH, whereas the expression of CD44 was inversely related to the expression of 15-PGDH and E-cadherin. Again, the levels of p53 were found to be independent of the expression of 15-PGDH in the cells.

Hyaluronidase induction of 15-PGDH in A549 cells

Exogenous hyaluronidase is known to be able to chemosensitize tumor cells to cytotoxic drugs (32) and to induce

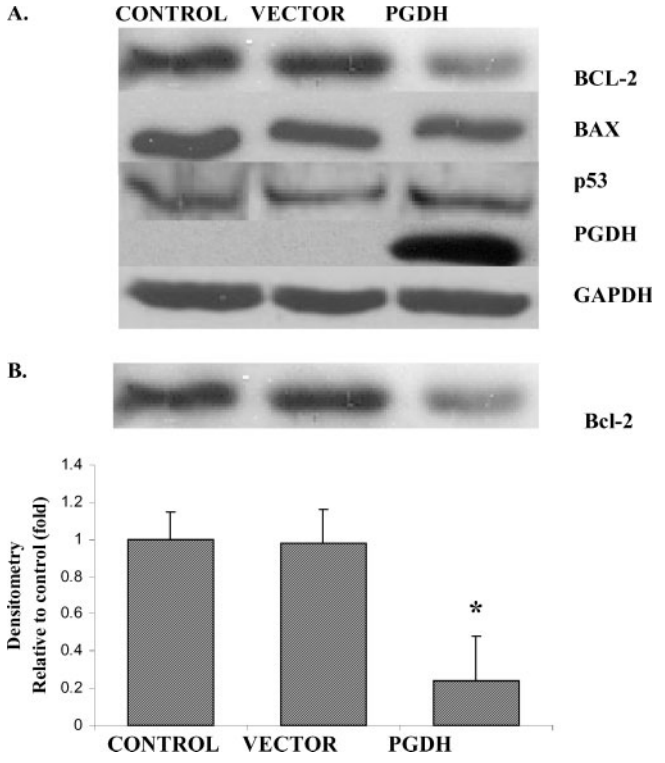

Fig. 5. Over-expression of 15-PGDH results in down-regulation of anti-apoptotic protein Bcl-2 without affecting the expression of pro-apoptotic protein p53 and Bax. A549 cells were treated in the same manner as described in Figure 4. Bcl-2, Bax, p53 and 15-PGDH were detected by western blot as described in the Materials and methods section. A representative figure of three qualitatively similar figures is shown. (**A**) Western blot analysis; (**B**) the blot data on the expression of Bcl-2 were analyzed by densitometry and the results were expressed as the mean ± SD ($n = 3$). $^*P < 0.01$ when compared with the control. GAPDH was served as a loading control.

the expression of an apoptotic oxidoreductase, WWOX (33,34). We examined if hyaluronidase was also able to induce 15-PGDH in A549 cells. When A549 cells were treated with hyaluronidase, 15-PGDH was found to be induced in a dose-dependent manner as shown in Figure 7. Maximal induction was found to be ~200 U/ml.

Discussion

We have found that the prostaglandin catabolic enzyme 15-PGDH is under-expressed in human lung tumors. This is in contrast to prostaglandin synthetic enzymes, COX-2 and m-PGE_2 synthase, being over-expressed in lung tumors (3–7). These two synthetic enzymes work in concert to generate more PGE_2 than either enzyme alone. Accordingly, enhanced expression of synthetic enzymes coupled with attenuated expression of 15-PGDH may result in accumulation of significantly more PGE_2 in the tumors. In fact, increased amounts of PGE_2 are commonly detected in tumors including NSCLC (35). Correlation of 15-PGDH expression with tumorigenicity was first reported by Celis *et al*. (36). They examined the protein expression profiles of normal bladder urothelium and of 63 transitional cell carcinomas of various histopathological grades and T stages. They found that the loss of 15-PGDH and three other protein biomarkers was associated

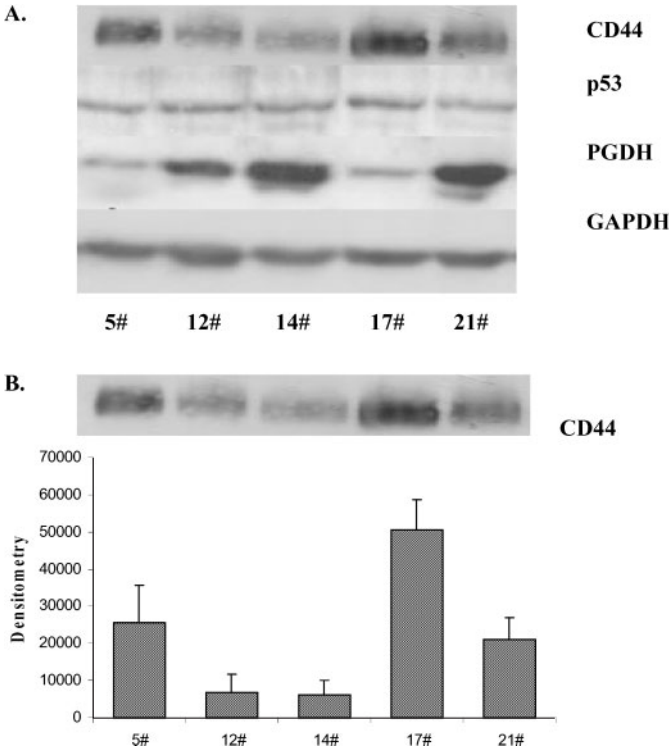

Fig. 6. Correlation of the expression of 15-PGDH in A549 cells with that of adhesion and invasion related protein, CD44. Five stable clones of A549 cells expressing different levels of 15-PGDH were analyzed for the expression of 15-PGDH as well as CD44 by western blot as described in the Materials and methods section. A representative figure of three qualitatively similar figures is shown. (**A**) Western blot analysis; (**B**) the blot data on the expression of CD44 were analyzed by densitometry and the results were expressed as the mean ± SD ($n = 3$). GAPDH was served as a loading control.

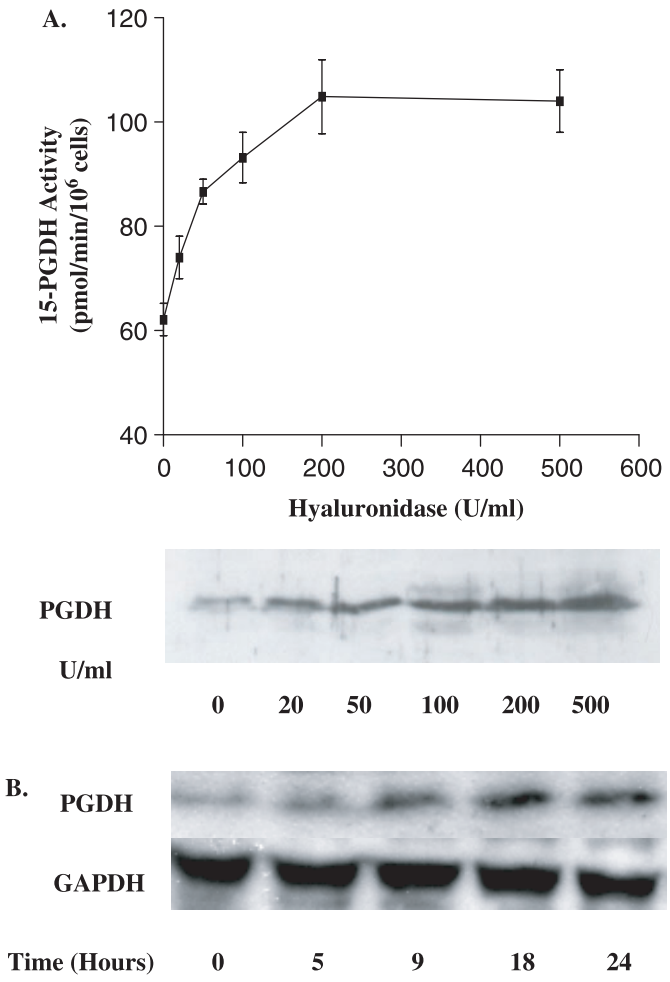

Fig. 7. Dose- and time-dependent induction of 15-PGDH in A549 cells by hyaluronidase. (**A**) A549 cells were treated with the indicated concentrations of hyaluronidase for 24 h. Cells were then washed and sonicated for the determination of the 15-PGDH activity and the protein expression. The data of the activity assay (upper panel) were expressed as the mean ± SD ($n = 3$). The result of the protein expression (middle panel) was shown by a representative western blot. The 15-PGDH activity was assayed by the tritium release method and the expression of 15-PGDH was analyzed by western blot as described in the Materials and methods section. (**B**) A549 cells were treated with 200 U/ml hyaluronidase for the indicated time. The expression of 15-PGDH was analyzed by western blot. GAPDH was served as a loading control.

with the progression of human bladder transitional cell carcinomas. Gee *et al*. (37) also observed down-regulation of 15-PGDH and up-regulation of COX-2 in invasive transitional cell carcinomas. Our finding that 15-PGDH is under-expressed in lung tumors is consistent with a very recent report that 15-PGDH is down-regulated in lung tumors. Heighway *et al*. (38) reported a cDNA microarray study in which they compared gene expression in 39 pairs of NSCLC tumors and matched normal lung tissue. The 15-PGDH gene was under-represented in lung tumors at least 2-fold in 27 of these pairs (69%) and under-represented at least 4-fold in 20 of lung tumor normal-tissue pairs (51%). The potential under-expression of 15-PGDH in tumors other than lung and bladder remains to be determined.

How 15-PGDH expression alters the growth characteristic of A549 cells is not known. PGE_2 has been shown to cause local immunosuppression (39), to induce Bcl-2 expression (40) and to stimulate cell proliferation (41), conditions that favor tumor growth. Accordingly, removal of PGE_2 by 15-PGDH-induced rapid degradation of PGE_2 should retard cell proliferation and tumor growth. We have employed both an animal model and a cellular model to investigate this hypothesis. A549 cells infected with Ad-15-PGDH or Ad-15-PGDH (Y151F) or Ad alone were used to induce tumors in athymic nude mice. Both activity assay and western blot analysis were run to confirm that 15-PGDH, either wild-type or inactive mutant, was over-expressed in A549 cells carrying Ad-15-PGDH or Ad-15-PGDH (Y151F) as compared with those carrying Ad alone. The expression of wild-type 15-PGDH but not mutant 15-PGDH appears to impose the cells to a clear growth disadvantage. This may be attributed to cells over-expressing 15-PGDH catabolize any cell-derived PGE_2. This is supported by a separate study shown in Figure 3 in which A549 cells expressing 15-PGDH release little PGE_2 following stimulation by IL-1β, whereas cells expressing 15-PGDH mutant release significantly more PGE_2 under the same conditions. However, A549 cells expressing the 15-PGDH mutant release less PGE_2 than those control cells. This is primarily due to the fact that 15-PGDH protein but not activity inhibits the IL-1β induced expression of COX-2 to some extent (unpublished data). This is also reflected in animal studies in which A549 cells expressing the 15-PGDH mutant generated tumors slightly slower than those infected with adenovirus alone although they were

not statistically significant. We also examined how over-expression of 15-PGDH affected the growth behavior of A549 cells. When A549 cells over-expressed 15-PGDH by infection with Ad-15-PGDH, these cells became apoptotic as shown by DNA fragmentation, activation of pro-caspase-3 and cleavage of PARP. These cells also exhibited decreased expression of anti-apoptotic protein Bcl-2 without altering the expression of apoptotic proteins p53 and BAX indicating that the apoptosis induced by over-expression of 15-PGDH is independent of the p53 pathway. Bcl-2 is known to prevent the release of apoptosis-inducing factor (42) and cytochrome *c* from the mitochondria (43), which is assumed to be a key event during apoptosis. Over-expression of 15-PGDH down-regulates the expression of the Bcl-2 protein indicating a role for the Bcl-2 in mediating or triggering the event of apoptosis.

Significant evidence has indicated that elevated expression of COX-2 has been shown to increase tumor invasiveness and enhance metastatic potential (44). This has been attributed to the increased generation of PGE_2, a downstream metabolite of COX-2. PGE_2 has been shown to increase CD44 and MMP-2 expression in A549 cells (45). CD44 is a cell surface receptor for hyaluronate, a major glycosaminoglycan component of the extracellular matrix. Adhesion to the extracellular matrix, a critical step in the metastatic process, has been found to be CD44-dependent in several malignancies (46,47). Expression of 15-PGDH in A549 cells appeared to cause down-regulation of CD44. Furthermore, hyaluronidase, an enzyme that degrades hyaluronan, was also found to induce 15-PGDH. Therefore, 15-PGDH expression, which induces down-regulation of CD44 may play a significant role in the retardation of tumor growth and metastasis. These results support the hypothesis that degradation of PGE_2 as a result of 15-PGDH over-expression may suppress the growth, invasion and metastasis of cancer cells.

Among the characteristic features of tumor suppressor genes is the inactivation of both alleles. Tumor suppressor alleles are usually silenced by point mutations, complete or partial deletions, or promoter methylation. In this report, we provide evidence showing that 15-PGDH behaves as a tumor suppressor gene when ectopically expressed in A549 cells with low levels of endogenous expression and activity. We demonstrated that tumorigenicity of A549 cells was significantly reduced by ectopically expressed catalytically active 15-PGDH but not inactive mutant enzyme. Although it is not clear if the endogenous low activity or expression of 15-PGDH in A549 lung cancer cells is due to point mutations or deletion or promoter methylation, it has been reported in other cancer cell lines that deletion of the 15-PGDH gene is observed. Two C-terminal truncated isoforms, one missing exon VI and the other missing exons V and VI, of 15-PGDH have been reported in the human promyelocytic HL-60 cell line (48) and in the human thyroid cancer TT cell line (49). Both of these isoforms are catalytically inactive because of the lack of active site domain in the molecules. Other point mutants and methylated promoter in the 15-PGDH gene have not been described. Obviously, much needs to be done to establish if 15-PGDH is a tumor suppressor gene.

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