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Diminished lipocalin-type prostaglandin D_2 synthase expression in human lung tumors

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ABSTRACT

Previously, we demonstrated that lipocalin-type prostaglandin D_2 synthase (L-PGDS) induces apoptosis and prevents cell cycle progression in several cell types. In this study we determined the expression of L-PGDS in a variety of human lung tumor types. While L-PGDS expression was evident in the surrounding margins, we observed significantly decreased protein and gene expression in the tumor tissue. Using RT-PCR we demonstrated that L-PGDS gene expression decreased proportionately with tumor progression. In addition, we demonstrated that exogenously added L-PGDS could suppress the hyperproliferation and PDGF-stimulated migration of A549 cells, a cultured carcinomic human alveolar basal epithelial cell line. We conclude that L-PGDS may play a key role in modulating lung cancer growth and may offer a novel diagnostic and therapeutic approach for treatment.

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1. Introduction

The importance of cyclooxygenase-2 (COX-2) derived prostaglandins (PGs) in tumor progression has been realized for several years [1,2]. Both epidemiological and clinical studies indicate that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a decreased incidence of certain malignancies, including lung cancer [3]. The predominance of COX activity in cell lines derived from human non-small cell carcinomas of the lung suggests that PG biosynthesis may be characteristic of certain types of lung cancer cells, particularly adenocarcinoma, large cell undifferentiated carcinoma, and possibly adenosquamous carcinoma [4].

Lipocalin-type prostaglandin D_2 synthase (L-PGDS) is a unique protein originally associated with the regulation of the sleep–wake cycle and sensitivity to tactile pain [5,6]. Several more recent findings have demonstrated that L-PGDS also has important vascular functions [7–11] as well as associations to cancer [12–26]. Our laboratory has uncovered several findings related to L-PGDS-mediated cell proliferation, apoptosis and migration that would also suggest a possible role in cancer progression [27–29].

PGD_2, the enzymatic product of L-PGDS, has been linked to the inhibition of ovarian cancer [30,31], leukemia cell proliferation [32], and reduced colitis-associated colorectal cancer [33]. In addition, PGD_2 induces various transduction pathways and activates the function of SOX9 [34], a transcription factor of which methylation has been linked to lung cancer [35]. Moreover, PGD_2 is spontaneously converted to 15-deoxy $\Delta^{12,14}$ PGJ_2 (15d-PGJ_2), a natural ligand for PPARγ, which plays an important role in the death of malignant T lymphocytes (Jurkat cells) [36]. PPARγ expressed in lung cancer cells can be activated by other various ligands and can inhibit lung cancer cell growth through the induction of apoptosis [37]. 15d-PGJ_2 has also been shown to inhibit the growth of A549 and H460 non-small-cell lung cancer cell lines and xenograft tumors, and can enhance the anti-tumor action of docetaxel in lung tumor cells by both PPARγ-dependent and -independent mechanisms mediated by the induction of apoptosis [38]. Furthermore, growth suppression of PPARγ expressing prostate tumor cells by PGD_2 metabolites is likely to be an endogenous mechanism involved in tumor suppression that potentially contributes to the indolence and long latency period observed in this disease [13].

In this study we examined the expression of L-PGDS in lung tumors and the surrounding margin. We monitored L-PGDS gene expression in lung tumors at various stages of progression using quantitative RT-PCR and investigated the effects of exogenously added L-PGDS on proliferation and PDGF-stimulated migration of A549 cells, a cultured carcinomic human alveolar basal epithelial cell line.

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2. Materials and methods

2.1. Materials

Cell culture reagents, including fetal bovine serum and platelet derived growth factor-BB (PDGF), were all purchased from Life Technologies (Grand Island, NY). SDS/polyacrylamide gel electrophoresis and western blot reagents were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). Western blots were visualized with enhanced chemiluminescence reagent purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Type-1 collagenase was from Worthington Biochemical Co. (Freehold, NJ). All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

2.2. Lung tissue samples and arrays

The Normal Lung Tissue Array (NC04-01) and Lung Adenocarcinoma Tissue Array (CS04-08) were purchased from CYBRDI Inc. (Rockville, MD). TissueScan Real Time Cancer Expression Panel (HLRT 101) was purchased from Origene (Rockville, MD). In addition, we procured tumor and surrounding margin from eight lung adenocarcinomas to help support the commercial tissue array data.

2.3. Immunohistochemistry

Sections were deparafinized in a xylene/ethanol series; antigen unmasked in 10 mM citric acid and blocked in 10% goat serum in PBS. The primary antibody was an L-PGDS monoclonal raised in rat (Cayman, Ann Arbor, MI) diluted 1:1000 in 1.5% goat serum and incubated overnight at 4 °C. The secondary antibody was a FITC-labeled goat anti-rat (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:20 in 10% goat serum. Sections were visualized with a Nikon Eclipse TE 300 fluorescent microscope. Control slides were incubated with nonspecific primary anti-sera or in some cases without the primary antibody. In no case did controls show a positive signal.

2.4. Western blotting

Typically, 50 μg of whole cell protein lysate was mixed with Laemmli sample buffer containing 0.1% bromophenol blue; 1.0 M NaH_2PO_4, pH 7.0; 50% glycerol and 10% SDS, boiled for 5 min and loaded on an SDS 10% polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), probed with the proper primary antibodies followed by 1:2000 diluted secondary antibody and detected with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ) and subsequent autoradiography. The intensity of the signal was quantitated by densitometric analysis using SigmaGel 1.0 software (Jandel/Systat Inc., SanJose, CA).

2.5. Real-time PCR

RNA was isolated from confluent cells or lung tissue with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNAs were prepared from 2.5 μg RNA of each sample using the Transcriptor High Fidelity cDNA Synthesis Kit for RT-PCR (Roche, Indianapolis, IN) according to the manufacturer's protocol. Primers for targets L-PGDS or the reference GAPDH were designed with Beacon Designer software using sequences found in the NCBI gene bank based on the criteria that they were at least 20 nucleotides in length had a T_m of approximately 60 °C, and an amplicon length of between 75 and 200 bp. L-PGDS: Forward 5′-GAA GAA GGC GGC GTT GTC-3′, Reverse 5′-GAG GAA GGT GGA GGT CAG G-3′; GAPDH: Forward 5′-GCT CTC TGC TCC TCC TGT TC-3′, Reverse 5′-GAC TCC GAC CTT CAC CTT CC-3′. Quantitative RT-PCR was performed in duplicate on a LightCycler 480 using the Syber-Green I Master Mix (Roche). A final reaction volume of 20 μl containing 2 μl cDNA, 10 μl MasterMix, 1 μl of each primer (0.5 μM), and 6 μl of PCR grade water was used. The reaction was performed with a denaturation step of 95 °C for 5 min followed by 40 cycles at 95 °C for 45 s, 61 °C for 1 min, and 72 °C for 1 min. For the creation of standard curves, cDNA from a test sample was diluted 1:3, followed by a 5-fold dilution to yield a final 1:375 dilution. All reactions were run with a negative control and subjected to melting curve analysis. Fold changes in gene expression were calculated using the Pfaffl method.

2.6. Cell culture

A549, a human non-small cell carcinoma line that exhibits type II-like alveolar epithelial characteristics was obtained from the American Type Culture Collection, and cultured in RPMI and supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and grown in a humidified atmosphere of 5% CO_2 at 37 °C.

2.7. Cell proliferation assay

Cell proliferation was measured with the use of a CellTiter 96 AQeous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were plated in 96-well plates at a density of 500 cells/well in 200 μl of medium with 5% FBS. The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time, after which 20 μl of combined MTS/PMS solution (Promega) was added per well. After a 2 h incubation at 37 °C, absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader. Data represent the average absorbance of three experiments each performed in triplicate.

2.8. Cell migration assay

Migration assays were performed using 24-well cell culture inserts with 8.0 μm polyethylene terephthalate cyclopore membranes (Falcon) as detailed in Lundberg et al. [39]. The underside of the membrane was coated with 100 μl rat tail collagen type I (50 μg/ml) for 18–20 h, washed and air-dried before each experiment. Cells were trypsinized and re-suspended in RPMI. Then 2×10^4 cells/250 μl were loaded into the cell culture inserts. The inserts were then added to the wells of 24-well plates which were filled with PDGF-BB diluted in RPMI with 0.1% BSA, and where indicated, L-PGDS was also added to this medium below the inserts. The chambers were then incubated at 37 °C for 5 h to allow for cell migration. Afterwards, cells were completely removed from the upper side of the membrane with a cotton swab and the remaining migrated cells fixed and stained with Diff-Quik® solution (Dade Behring, Newark, DE). Results are reported as the mean +/− SEM of five different fields, from three experiments, counted at 200× magnification.

2.9. Statistical analysis

Experimental results are reported as means ± SEM. Data was analyzed by one-way ANOVA for comparisons of multiple data sets and by Student's *t*-test for comparison of two data sets using SigmaStat 3.5 (Systat), with statistical significance set at $P < 0.05$.

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