Peroxisome proliferator-activated receptor δ as a molecular target to regulate lung cancer cell growth

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Abstract **It has been assumed that prostaglandin (PG)I_2 signaling contributes to the negative growth control of lung cancer cells; however, the mechanism remains unresolved. PGI_2 functions through a cell surface G protein-coupled receptor (prostaglandin I2-binding receptor, IP) and also exerts an effect by interacting with a nuclear hormone receptor, peroxisome proliferator-activated receptor δ (PPARδ). We found that PPARδ was a key molecule of PGI_2 signaling to give negative growth control of lung cancer cells (A549), using carbarprostacyclin, a PGI_2 agonist for IP and PPARδ, and L-165041, a PPARδ agonist. Furthermore, PPARδ-induced cell growth control was reinforced by the inhibition of cyclooxygenase. These results suggest that PPARδ activation under the suppression of PG synthesis is important to regulate lung cancer cell growth.**

Keywords: PPAR-δ; IP; PGI_2; Lung cancer cell; Growth control

1. Introduction

Lung cancer, particularly non-small cell lung cancer (NSCLC), is one of the most common cancers and is the leading cause of cancer death in Western countries as well as Japan [1,2]; of NSCLC, the adenocarcinoma type has the most dominant histology [3]. Recently, it has been suggested that cyclooxygenase-2 (COX-2) is a useful diagnostic marker and target for the prevention and therapy of lung adenocarcinoma [4,5]. The induction of COX-2 is associated with high levels of prostaglandin E_2 (PGE_2) production in lung adenocarcinoma cells and these high levels stimulate cell growth [6]. However, human trials evaluating COX inhibitor and lung cancer chemoprevention remain to be completed, and furthermore, COX-2 inhibition by celecoxib leads to no change in tumor multiplicity and increased lung tumor size in an initiator–promoter model of lung tumorigenesis [7]. These reports suggest that factors other than COX-2 expression are required for the development of lung adenocarcinoma. Thus, determination of these other factors may lead to the establishment of a new prevention and therapy regime against lung cancer.

PGH_2 produced by COX-2 is converted into one of several biologically important prostaglandins (PGs), including PGE_2 and PGI_2, by each specific synthase [8,9], and PGs have wide-ranging effects in regulating aspects of homeostasis and pathogenesis [10]. Different from other PGs, PGI_2 acts as an inhibitory factor against the development of cancers; for example, PGI_2 reduces the growth of established micrometastases [11]. In normal lungs, PGI_2 is one of the most abundant PGs, but the production of PGI_2 by lung adenocarcinoma cells is suppressed to a very low level [12]. In contrast, a high level of PGE_2 linked with the induction of COX-2 is observed in lung adenocarcinoma cells [6]. From these reports, it is speculated that, in addition to the induction of COX-2, the suppression of PGI_2 production contributes to the development of lung adenocarcinoma. The downregulation of PGI_2 synthase is observed in lung adenocarcinoma tissue, and the expression of PGI_2 synthase in lungs leads to the reduced development of lung adenocarcinoma in mice [13,14]. However, it remains unclear which signaling pathway regulated by PGI_2 contributes to the reduction of lung cancer.

Since PGI_2 functions through a G protein-coupled cell surface receptor, termed IP (prostaglandin I_2-binding receptor), and also exerts an effect by interacting with a nuclear hormone receptor, peroxisome proliferator-activated receptor δ (PPARδ) [15,16], we examined which of these two receptors could play an important role in the PGI_2-regulated signaling of lung adenocarcinoama cells (A549). Furthermore, we attempted to clarify why the level of PGI_2 was much lower than that of other PGs in lung adenocarcinoma cells. Finally, as a result of these investigations, we propose that PPARδ is a promising target to regulate lung cancer cell growth.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (St. Louis, MI, USA) unless otherwise stated. Carbaprostacyclin ($cPGI_2$) was from Cayman Chemical Co. (Ann Arbor, MI, USA).

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Abbreviations: PPARδ, peroxisome proliferator-activated receptor δ; PG, prostaglandin; IP, prostaglandin I2-binding receptor; COX, cyclooxygenase; SSAT, spermidine/spermine N^1-acetyltransferase

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2.2. Cell culture and treatment

A human lung adenocarinoma cell line, A549 cell was provided by Riken Cell Bank (Saitama, Japan). This cell was routinely maintained in Dulbecco's modified eagle's medium (DMEM) (Gibco-BRL, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin. For experiments, exponentially growing cells were used in DMEM medium containing 2% FBS (culture medium) unless otherwise stated. Cells were plated on culture plates and cultured for 24 h to permit adherence. After attachment, each agent was added to the culture medium. The control was treated with the vehicle alone.

2.3. PGI_2 and PGE_2 assay

PGI_2 production was determined using culture medium from 48-h cultured A549 cells (6×10^6 cells/100 mm dish). After 48 h culture, the culture medium was collected, and the PGI_2 level was subsequently determined as the main metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, using an ELISA kit (Cayman Chemical Co.). The amount of PGE_2 was measured according to a similar procedure.

2.4. RT-PCR

Total RNA was isolated from cultured A549 cells as described previously [17]. Transcripts were amplified by RT-PCR using primers PPARδ (NCBI reference number 60115372): sense primer (nucleotides 1–18), antisense primer (nucleotides 244–261); IP (NCBI 29825394): sense primer (nucleotides 502–519), antisense primer (nucleotides 735–752); PPARα (NCBI 7549810): sense primer (nucleotides 174–193), antisense primer (nucleotides 313–332); PPARγ (NCBI 30583400): sense primer (nucleotides 63–82), antisense primer (nucleotides 517–536); GAPDH (NCBI 7669491): sense primer (nucleotides 174–193), antisense primer (nucleotides 313–332). GAPDH was used as an internal control. After 35 cycles, PCR products were separated by electrophoresis (1.5% agarose gel) and stained with GelStar (BMA, Rockland, ME, USA). A wide-range DNA ladder (Takara, Shiga, Japan) was used as a marker to size the PCR products. When negative results were observed, fresh polymerase was added after 35 cycles, and the PCR was continued to 70 cycles to confirm the negative result. We also confirmed the performance of PCR for PPARα, using human kidney cDNA as a positive control (data not shown).

2.5. Assessment of cell viability

Cell viability was examined using a WST-1 assay kit (Quick Cell Proliferation Assay Kit, MBL, Nagoya, Japan), according to the manufacturer's instructions.

2.6. SiRNA design, preparation and transfection

The design of short interfering RNA (siRNA) was carried out using an on-line design system for siRNA (Qiagen, Germantown, MD). siRNAs were synthesized in high performance purity grade by Qiagen. The sense and anti-sense strands of IP siRNA were: sense: 5′-AACGUCGUCCAAAGCAGAAGCdTdT-3′; anti-sense: 5′-GCUUCUGCUUUGGACGACGUUdTdT-3′. The sense and anti-sense strands of non-specific control siRNA were: sense: 5′-UUCUCCGAACGUGUCACGUdTdT-3′; anti-sense: 5′-ACGUGACACGUUCGGAGAAdTdT-3′. SiRNA was transfected into A549 cells using RNAiFect Transfection Reagent (Invitrogen) as previously reported [18]. At 12 h after transfection, the cells were incubated for 72 h in culture medium containing 20 μM $cPGI_2$ or the vehicle, and the IP expression was subsequently determined by RT-PCR. Cell viability was estimated by WST-1 assay.

2.7. Luciferase assay

A synthetic, triplicated PPAR-responsive element (PPRE)-firefly luciferase reporter vector, containing three copies of PPRE for hydroxymethylglutaryl-CoA reductase [19], was constructed as previously reported [20]. The triplicated PPRE were subcloned into the *Mlu*I–*Xho*I site of pGL3-promoter vector (Promega, Madison, WI, USA). A549 cells (5×10^4 cells/well in 12-well plates) were cotransfected with the triplicated PPRE-firefly luciferase reporter vector and renilla luciferase expression vector using Gene Jamar Transfect Reagent (Stratagene, LaJolla, CA, USA). After 24 h of transfection, the cells were washed with PBS, incubated with FBS-free DMEM containing 20 μM $cPGI_2$ or L-165046 at the indicated doses for 24 h at 37 °C. After incubation, the cells were harvested and the firefly luciferase and renilla luciferase activities were quantified. The firefly luciferase activity of the extract was normalized with the renilla luciferase activity. We also confirmed that the above treatment did not affect the basic activity of the pGL3-promoter vector alone (data not shown).

2.8. Immunoblot analysis

The cells were lysed in 1 ml of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCL, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM DTT). The lysates were separated on 10 or 15% SDS–PAGE, transferred to a nitrocellulose membrane and subjected to immunoblotting with anti-proliferating cell nuclear antigen (PCNA), anti-cyclin D (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p53, anti-p21, anti-Bcl-xL and anti-β-actin (BD Sciences, Tokyo, Japan). Detection was accomplished using the ECL system (Amersham, Piscataway, NJ) and a cooled CCD camera-linked Cool Saver system (Atto, Tokyo, Japan). Molecular sizing was estimated using a Rainbow Molecular Weight Marker (Amersham). Protein concentrations were determined using a DC Protein Assay kit (Bio-rad, Tokyo, Japan).

2.9. Cell cycle analysis

Cells were suspended in PBS containing 70% ethanol, and kept at 4 °C for 30 min. Before analysis, cells were incubated for 30 min in propidium iodide (PI) solution containing 0.05 mg/ml PI, 1 mM EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A in PBS. The suspension was then passed through a nylon mesh filter and analyzed on a Becton–Dickinson FACScan.

2.10. Apoptosis assay

To estimate apoptosis quantitatively, its induction was determined by the ratio of subG1 population to the total cells in the cell cycle measured as above, and caspase 3 activity was measured using a Caspase-3/CPP32 Assay Kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions.

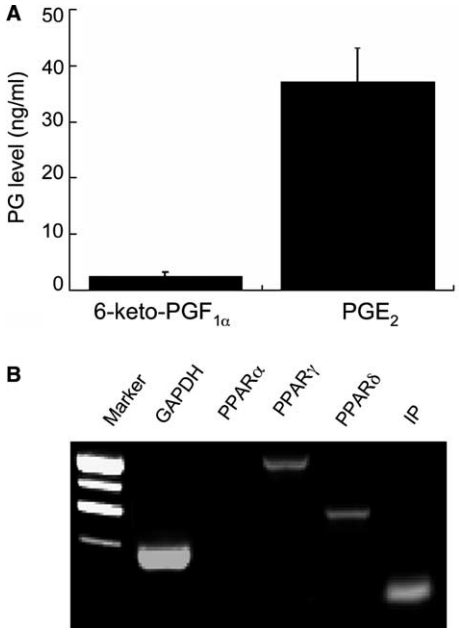

Fig. 1. The production of PGI_2 and PGE_2 (A), and expression pattern of each PPAR subtype and IP in A549 cells. (A) A549 cells were cultured for 48 h, and subsequently each PG level in culture media was determined by ELISA as described in Section 2.3. The amount of PGI_2 was measured as 6-keto-$PGF_{1\alpha}$. Each value indicates the mean of five samples; vertical lines indicate S.D. (B) RT-PCR analyses were performed as described in Section 2.4. The results shown are representative of three independent experiments.