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

Keiko Fukumoto[a], Yoshihisa Yano[b], Nantiga Virgona[c], Hiromi Hagiwara[a,d], Hiromi Sato[a], Hironobu Senba[e], Kazuyuki Suzuki[e], Ryuji Asano[e], Kazuhiko Yamada[a], Tomohiro Yano[a,*]

[a] *Department of Food Science Research for Health, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan*
[b] *Department of Biochemistry, Osaka City University Medical School, Osaka 545-8585, Japan*
[c] *Herbal Medicines Research and Education Center, Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia*
[d] *Japan Human Sciences Foundation, Tokyo 103-0001, Japan*
[e] *Department of Veterinary Pharmacology, Nihon University College of Bioresource Sciences, Kanagawa 252-8510, Japan*

Received 6 April 2005; revised 1 June 2005; accepted 5 June 2005

Available online 16 June 2005

Edited by Varda Rotter

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).

*Corresponding author. Fax: +81 3 3205 6549.
E-mail address: yano@nih.go.jp (T. Yano).

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

0014-5793/$30.00
doi:10.1016/j.febslet.2005.06.004

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.

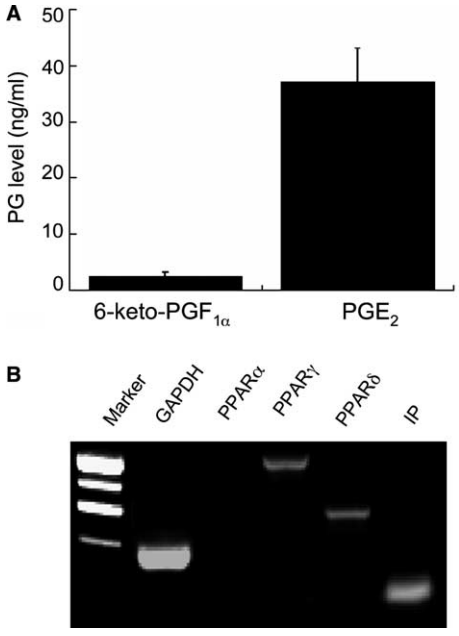

Fig. 1. The production of PGI_2 and PGE_2 (A), and expression pattern of each PPAR subtype and IP in A549 cells. (A) A549 cells were cultured for 48 h, and subsequently each PG level in culture media was determined by ELISA as described in Section 2.3. The amount of PGI_2 was measured as 6-keto-$PGF_{1\alpha}$. 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.

2.11. Polyamine metabolism assay

5×10^6 cells were homogenized in perchloric acid and then centrifuged at $8000 \times g$ for 5 min. Aliquots of the supernatant were then neutralized and dansyl chloride was added. The dansyl derivatives were then extracted with benzene and separated by high performance liquid chromatography [21]. To determine the level of spermidine/spermine N_1-acetyltransferase (SSAT) mRNA, real-time RT-PCR was carried out. cDNA synthesis for SSAT and GAPDH was described above. PCR reactions were carried out using SSAT primers (NCBI reference number 33876789): sense primer (nucleotides 356–375); antisense primer (nucleotides 543–562); GAPDH as above and SYBR green dye. Reactions were performed at 94 °C for 1 min, followed by 40 cycles

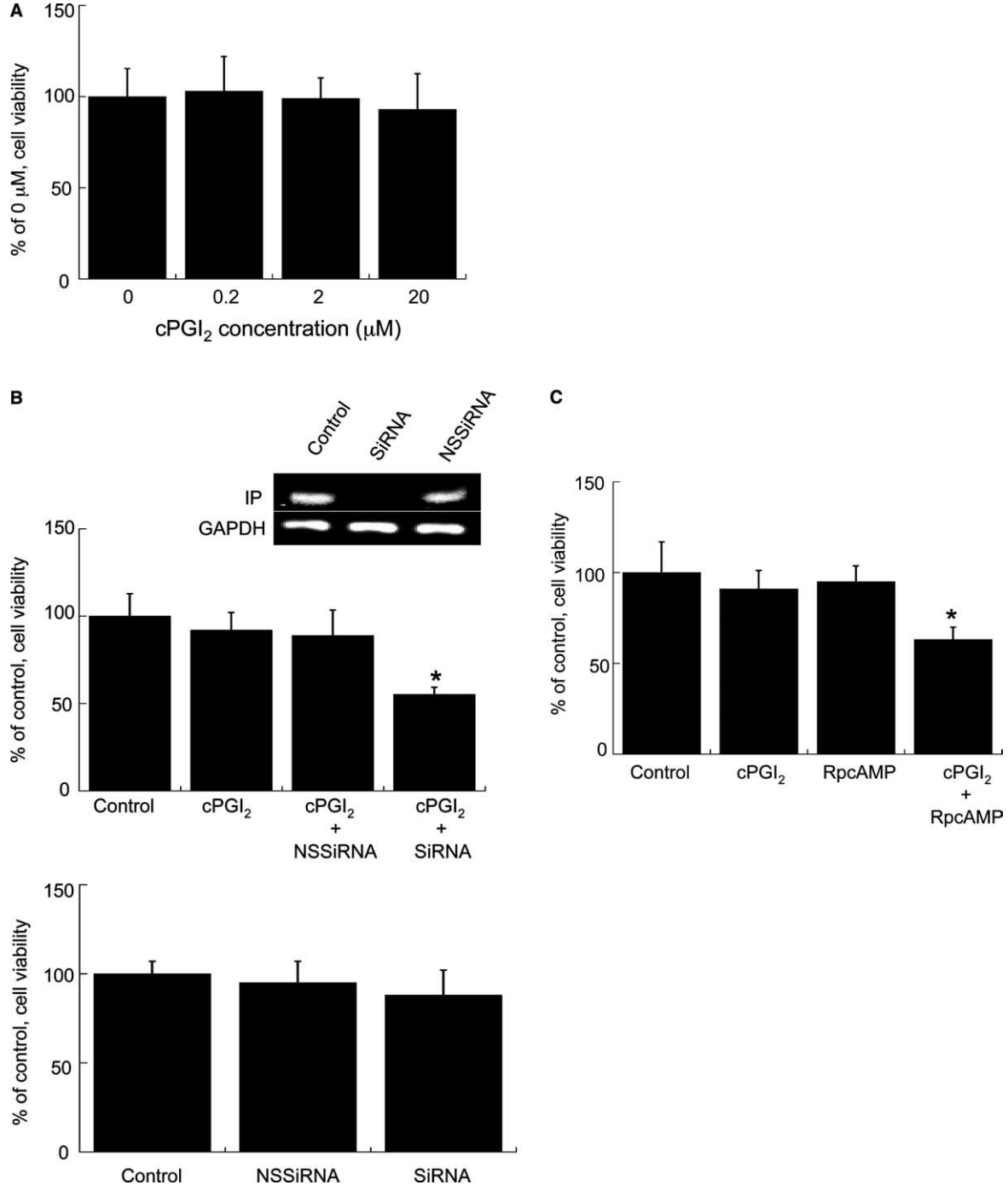

Fig. 2. The effects of $cPGI_2$ on the viability of A549 cells in a dose-dependent manner (A), under IP silencing by SiRNA (B), and in the presence of RpcAMP (C). (A) The cells were treated with $cPGI_2$ at indicated doses for 72 h, and cell viability was determined as described in Section 2.5. Each value indicates the mean of eight samples; vertical lines indicate S.D. (B) Treatment conditions were described in Section 2.6. IP silencing by SiRNA was confirmed by RT-PCR analysis as described in Section 2.4. NSSiRNA, non-specific control SiRNA treatment; SiRNA, SiRNA for IP treatment; $cPGI_2$, $cPGI_2$ treatment; $cPGI_2$ + NSSiRNA, co-treatment of $cPGI_2$ and NSSiRNA; $cPGI_2$ + NSSiRNA, co-treatment of $cPGI_2$ and SiRNA. Each value indicates the mean of five samples; vertical lines indicate S.D. *Significant difference from three other groups. (C) The cells were treated with 20 μM $cPGI_2$ and/or 40 μM RpcAMP for 72 h, and then cell viability was determined as described in Section 2.5. Each value indicates the mean of eight samples; vertical lines indicate S.D. *Significant difference from three other groups.

of 94 °C for 15 s, 60 °C for 5 s and 72 °C for 10 s. The products were detected with the ABI Prism 7700 Sequence Detector (Perkin Elmer Biosystems). The abundance of amplified DNA was determined from the threshold cycle values and normalized to the values for control gene GAPDH to yield the relative abundance. The values of SSAT/GAPDH were normalized to those of the control.

2.12. Statistical analysis

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple-range test. A P value of 0.05 or less was considered significant.

3. Results and discussion

We first determined the production levels of PGI_2 (6-keto-$PGF_{1\alpha}$) and PGE_2 of the A549 cell line to examine its adequacy in exhibiting the same pattern of PG synthesis as that of human lung adenocarcinoma tissues. As shown in Fig. 1A, the production of 6-keto-$PGF_{1\alpha}$ was much lower than that of PGE_2, thus it appears that the PG synthesis pattern of A549 cells is the same as that of human lung adenocarcinoma tissue. Next, we checked the expression patterns of IP and PPAR species in A549 cells. We observed the expression of PPARδ, PPARγ and IP but not PPARα in the cells, indicating that PGI_2 signaling depended on both IP- and PPARδ-regulated signal pathways. In addition, the expression pattern of PPARγ and PPARα in A549 cells was identical to that of other human lung adenocarcinoma cells [22]. Taken together, the expression pattern of IP and PPAR species in A549 cells may be generally observed in human lung adenocarcinoma cells, and A549 cells are suitable to estimate the role of PGI_2 signaling in human lung adenocarcinoma cells.

As mentioned above, PGI_2 functions through a G protein-coupled cell surface receptor, IP and a nuclear hormone receptor, PPARδ. We first examined whether signaling via these two receptors could produce the negative growth control of A549 cells, using an agonist for IP and PPARδ, $cPGI_2$. Although $cPGI_2$ also acts as an agonist for PPARα, the agonist only induces PPARδ activation in A549 cells because the cells do not express PPARα. Although we confirmed that $cPGI_2$ induced the activation of PPARδ under this treatment condition (data not shown), the agonist did not affect the cell growth of A549 cells at the treatment dose, 20 μM (Fig. 2A). A recent report demonstrated that downstream signaling regulated by IP has an antagonistic effect against PPARδ-dependent negative growth signaling in some cells [20]. Based on this report, we

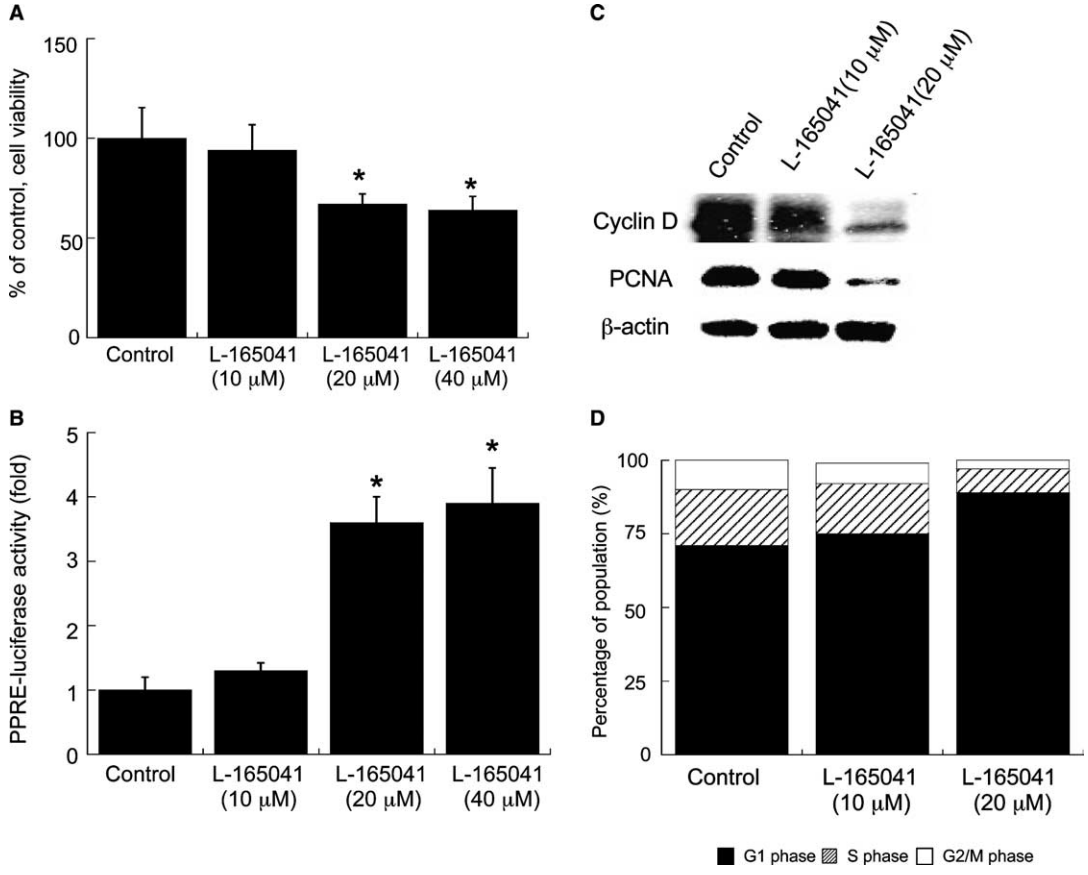

Fig. 3. The effects of L-165041 on cell viability (A), transactivity of PPARδ (B), the levels of molecules related to cell proliferation (C), and cell cycle progression (D) in A549 cells. (A) The cells were treated with L-165041 at the indicated doses for 72 h, and cell viability was determined as described in Section 2.5. Each value indicates the mean of eight samples; vertical lines indicate S.D. *Significant difference from the control and L-165041 (10 μM)-treated group. (B) Treatment and luciferase assay were performed as described in Section 2.7. Each value indicates the mean of five samples; vertical lines indicate S.D. *Significant difference from the control and L-165041 (10 μM)-treated group. (C) The cells were treated with L-165041 at the indicated doses for 48 h, and each protein level was determined by immunoblot analysis as described in Section 2.8. The results shown are representative of three independent experiments. (D) The cells were treated with L-165041 at the indicated doses for 48 h, and cell cycle analysis was performed as described in Section 2.9. Each value represents the mean of three experiments.

speculated that the antagonistic effect of IP signaling on PPARδ-dependent signaling might be related to the result shown in Fig. 2A. To determine this possibility, we next examined whether $cPGI_2$ could induce negative growth control in A549 cells by the suppression of IP expression. As shown in Fig. 2B, $cPGI_2$ reduced the cell growth of A549 cells under IP silencing by SiRNA treatment. We also confirmed that SiRNA alone did not affect cell growth (Fig. 2B). These results suggest that IP-regulated signaling interferes with PPARδ-dependent signaling related to cell growth control. IP activation by agonists leads to the increased production of intracellular cAMP via the stimulation of adenylate cyclase [10], therefore we next explored whether cAMP could act as a second messenger contributing to the antagonistic effect of IP. As shown in Fig. 2C, a specific cAMP antagonist, the Rp isomer of adenosine 3′,5′-cyclic monophosphate (RpcAMP), induced negative growth control in A549 cells treated with $cPGI_2$, suggesting that the IP-cAMP pathway serves to protect A549 cells from PPARδ-dependent negative growth control.

To further confirm the contribution of PPARδ-dependent signaling in the negative growth control of A549 cells, we examined the effect of a PPARδ agonist, L-165041, on the growth of A549 cells. As shown in Fig. 3A, L-165041 retarded the growth of A549 cells with statistical significance when treated with either 20 or 40 μM. In addition, the agonist induced PPARδ activation (Fig. 3B). In a recent report, it was shown that L-165041 can act as a PPARγ ligand in addition to PPARδ [23]. However, we previously observed that at doses less than 40 μM, L-165041 did not induce PPARγ activation in cells with only PPARγ expression (data not shown), so we can rule out the possibility that L-165041 stimulated PPARγ under this treatment condition. Taken together, it seems that PPARδ is required for the L-165041-driven negative growth control of A549 cells. Next, we tried to clarify the possible mechanism of PPARδ-regulated cell growth control. As shown in Fig. 3C, L-165041 treatment reduced both the level of cyclin D responsible for G1 to S phase transition and the level of PCNA, a cell proliferation marker, and linked with this event, the treatment induced G1 arrest in A549 cells (Fig. 3D). However, the treatment did not cause apoptosis in A549 cells (data not shown). These results suggest that PPARδ-dependent growth control depends on a cytostatic effect based on G1 arrest but not a cytotoxic effect.

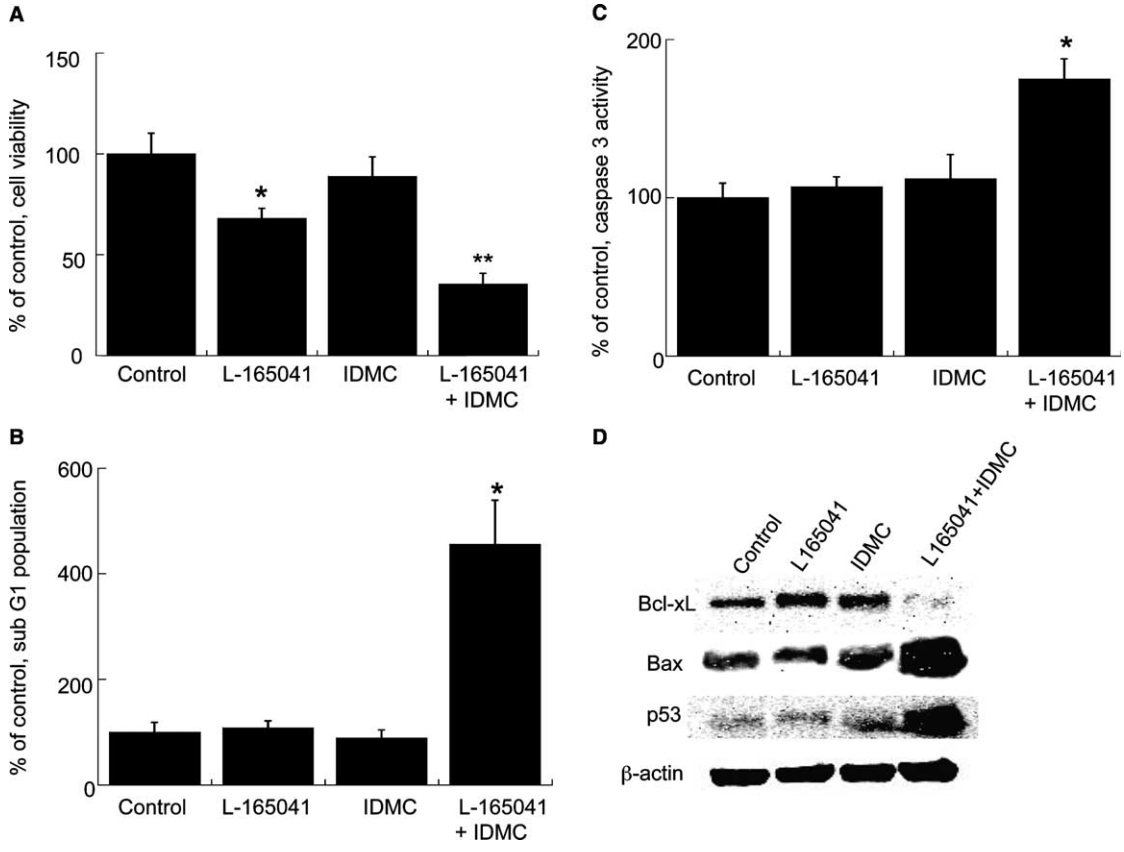

Fig. 4. The effects of indomethacin on cell viability (A), subG1 population (B), caspase3 activity (C), and the levels of molecules related to apoptosis (D) in A549 cells treated with L-165041. IDMC, indomethacin treatment. (A) The cells were treated with 20 μM L-165041 and/or 20 μM indomethacin for 72 h, and then cell viability was determined as described in Section 2.5. Each value indicates the mean of eight samples; vertical lines indicate S.D. *Significant difference from the control and indomethacin-treated group and **significant difference from other three groups. (B) The cells were treated with 20 μM L-165041 and/or 20 μM indomethacin for 72 h, and then the subG1 population was determined by FACS as described in Section 2.9. SubG1 population was calculated from the ratio of the cell number in the subG1 phase to the total cell number of cell cycles (G1, S, and G2/M phases). Each value indicates the mean of three samples; vertical lines indicate S.D. *Significant difference from other three groups. (C) Cell treatment was carried out as described in Fig. 4B. Caspase3 activity was determined as described in Section 2.10. Each value indicates the mean of eight samples; vertical lines indicate S.D. *Significant difference from three other groups. (D) The cells were treated with 20 μM L-165041 and/or 20 μM indomethacin for 48 h, and each protein level was determined by immunoblot analysis as described in Section 2.8. The results shown are representative of three independent experiments.

It has been reported that the induction of COX-2 and subsequent overproduction of PGs except PGI_2 contribute to the appearance of malignant phenotypes in lung adenocarcinoma cells [6,14]. From these reports, it can be assumed that the overproduction of PGs by COX except PGI_2 attenuates the PPARδ-dependent negative growth control of A549 cells, so we examined the effect of L-165041 treatment on the growth of A549 cells under COX inhibition by indomethacin. We also confirmed that COX inhibition in A549 cells reached a plateau (data not shown) under the same indomethacin treatment conditions stated in the Fig. 4 legend. As shown in Fig. 4A, indomethacin treatment significantly reinforced the L-165041-driven negative growth control of A549 cells. Furthermore, we confirmed that reinforcement by indomethacin depended on the induction of apoptosis (Fig. 4B and C). Moreover, we observed that the combination of L-165041 and indomethacin reduced the level of Bcl-xL, an anti-apoptotic molecule and induced Bax, a pro-apoptotic molecule related to the upregulation of p53 (Fig. 4D), indicating that indomethacin caused apoptosis in A549 cells treated with L-165041 by elevating the ratio of Bax to Bcl-xL. Overall, it seems that signaling regulated by PGs except PGI_2 prevents A549 cells from being influenced by PPARδ-dependent negative growth control. Finally, we tried to determine which factors could relate to the induction of apoptosis in A549 cells co-treated with L-1650041 and indomethacin. In previous reports, indomethacin has been shown to exert a chemopreventive action by affecting polyamine metabolism in colon cancers, and reduced intracellular polyamine contents led to the induction of apoptosis [24,25]. We have also reported that indomethacin suppresses the elevation of polyamine at the promotion stage of lung tumorigenesis in mice [26,27]. Furthermore, it has been reported that the activation of PPARδ can induce the transcriptional activation of SSAT, a key polyamine catabolic enzyme [28]. Based on these results, we hypothesized that the reduction of polyamine levels via the induction of SSAT by co-treatment could act as an apoptotic mediator. As shown in Fig. 5A, the co-treatment of L-165041 and indomethacin reduced the total polyamine level with statistical significance compared to the control, L-165041-treated and indomethacin-treated groups, while either L-165041 treatment or indomethacin treatment alone also reduced the polyamine level, but the reduction in level was much less than that exhibited by the co-treated group. In contrast, the difference of SSAT mRNA level in each treatment group showed an opposite tendency with the change in polyamine level (Fig. 5B). These results suggest that the severe reduction of the polyamine level via the induction of SSAT by co-treatment is an important factor to induce apoptosis. This speculation can be supported by the result that the co-treatment-induced cytotoxicity of A549 cells was abrogated by exogenous putrescine (a polyamine) (data not shown).

As mentioned, the exact roles of suppression of PGI_2 synthesis in lung adenocarcinoma cells are unclear. In this study, we observed that, irrespective of the low level of PGI_2, both PGI_2 receptors (PPARδ and IP) were expressed in A549 cells. Thus, if PGI_2 level elevation should occur in the cells, PPARδ and/or IP-dependent signaling would be activated and this activation might influence the growth of cancer cells. We confirmed that the activation of PPARδ but not IP-induced the negative growth control of A549 cells. Since PGI_2 is extremely unstable, endogenously produced PGI_2 in the cells can interact with intracellular PPARδ but not easily

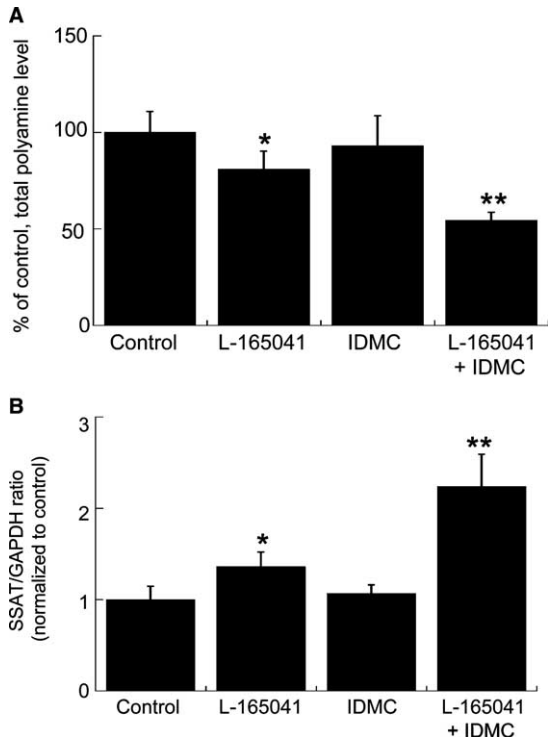

Fig. 5. The combination effect of L-165041 and indomethacin on the levels of total polyamine (A) and SSAT mRNA (B) in A549 cells. (A) The cells were treated with 20 μM L-165041 and/or 20 μM indomethacin for 72 h, and each polyamine (putrescine, spermidine, and spermine) was determined by HPLC as described in Section 2.11. Total polyamine level was expressed as the sum of each polyamine level. Total polyamine level in the control was 21.5 pmol/mg protein. Each value indicates the mean of five samples; vertical lines indicate S.D. *Significant difference from the control and **significant difference from three other groups. IDMC, indomethacin treatment. (B) The cells were treated with 20 μM L-165041 and/or 20 μM indomethacin for 48 h, and SSAT mRNA level was determined by real-time PCR and quantified as the ratio of SSAT/GAPDH. Each value indicates the mean of three samples; vertical lines indicate S.D. *Significant difference from the control and indomethacin-treated group and **significant difference from three other groups.

interact with IP located on the cell surface [20]. Thus, theoretically, it can be assumed that IP-dependent signaling has an antagonistic effect on the PPARδ-induced negative growth of cancer cells, but, due to the instability of PGI_2, the in vivo antagonistic effect may be very weak or actually negative. Taken together, it seems that the suppression of PGI_2 synthesis in lung adenocarcinoma cells serves to protect cancer cells from intracellular PGI_2-PPARδ-mediated negative growth control. As far as we know, this result is the first report to show why the level of PGI_2 is much lower than that of other PGs in lung adenocarcinoma cells.

In this study, we observed that the inhibition of COX reinforced the PPARδ-dependent negative growth control of A549 cells and induced cell apoptosis. This observation can be supported by results that the treatment reduced the level of Bcl-xL and upregulated Bax via the induction of p53. In our previous study, we reported that PGE_2 induces Bcl-xL due to the activation of Src-signal transducers and activators of transcription 3 [29], but this result indicated that the suppression of PGE_2 production by indomethacin treatment alone was insufficient to reduce the level of Bcl-xL. Similarly,

although a previous report suggested that the inhibition of COX by indomethacin is sufficient to induce p53-Bax signaling in cancer cells [30], the inhibition of COX alone in this study could not cause signal induction. However, the combination of indomethacin treatment and PPARδ activation induced apoptosis in A549 cells due to the reduction of Bcl-xL and upregulation of p53-Bax signaling. These results indicate that common factors regulated by the inhibition of PG synthesis and PPARδ activation are required for the induction of apoptosis in A549 cells. It has been reported that the activation of PPARδ stimulates polyamine catabolism via the induction of SSAT, leading to a decreased polyamine level in cancer cells [31] and that indomethacin treatment reduced the polyamine level in tumor tissues [28]. In addition to these reports, the cellular polyamine level is considered a key determinant to induce a cytostatic or cytotoxic effect in cells [32,33]. Overall, it is likely that the cellular polyamine level in A549 cells acts as a determinant to induce cytostatic or cytotoxic effects as observed in this study. Our evidence shows that, in A549 cells, a moderate reduction of the polyamine level by L-165401 led to the induction of a cytostatic effect but not a cytotoxic effect, whereas severe reduction of the level by the L-165401/indomethacin combination caused a cytotoxic effect. From these reports and our results, it is concluded that the activation of PPARδ under the suppression of PG synthesis is a promising strategy to regulate lung cancer cell growth.

Acknowledgments: We thank Dr. Hatae (National Cardiovascular Center Research Institute of Japan) for supplying the triplicated PPRE-firefly luciferase reporter vector and Miss. Satoh for her technical assistance. This study was supported in part by a Grant-in-Aid for Cancer Research (16-1) from the Ministry of Health, Labor and Welfare of Japan.

References

[1] Landis, S.H., Murry, T., Bolden, S. and Wingo, P. (1998) Cancer statistics, 1998. CA Cancer J. Clin. 48, 6–29.
[2] Yoshimura, K., Yamashita, N. and Ishikawa, H. (1980) Clinical statistics for lung neoplasms in Japan according to their histological types. Nihon-Rinsho 38, 2581–2591.
[3] Bos, J.L. (1989) *ras* oncogenes in human cancer: a review. Cancer Res. 49, 4682–4689.
[4] Campa, D., Zienolddiny, S., Maggini, V., Skaug, V., Haugen, A. and Canzian, F. (2004) Association of a common polymorphism in the cyclooxygenase-2 gene with risk of non-small cell lung cancer. Carcinogenesis 25, 229–235.
[5] Shaik, M.S., Chatterjee, A. and Singh, M. (2004) Effect of a selective cyclooxygenase-2 inhibitor, nimesulide, on the growth of lung tumors and their expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-gamma. Clin. Cancer Res. 10, 1521–1529.
[6] Heasley, L.E., Thaler, S., Nicks, M., Price, B., Skorecki, K. and Nemenoff, R.A. (1997) Induction of cytosolic phospholipase A2 by oncogenic Ras in human non-small cell lung cancer. J. Biol. Chem. 272, 14501–14504.
[7] Kisley, L.R., Barrett, B.S., Dwyer-Nield, L.D., Bauer, A.K., Thompson, D.C. and Malkinson, A.M. (2002) Celecoxib reduces pulmonary inflammation but not lung tumorigenesis in mice. Carcinogenesis 23, 1653–1660.
[8] Vane, J.R. and Botting, R.M. (1995) Pharmacodynamic profile of prostacyclin. Am. J. Cardiol. 75, 3A–10A.
[9] Tanabe, T. and Ullrich, V. (1995) Prostacyclin and thromboxane synthases. J. Lipid Mediat. Cell Signal. 12, 243–255.
[10] Narumiya, S., Sugimoto, Y. and Ushikubi, F. (1999) Prostanoid receptors: structures, properties, and functions. Physiol. Rev. 79, 1193–1226.
[11] Schirner, M. and Schneider, M.R. (1997) Inhibition of metastasis by cicaprost in rats with established SMT2A mammary carcinoma growth. Cancer Detect. Prev. 21, 44–50.
[12] Hubbard, W.C., Alley, M.C., Gray, G.N., Green, K.C., McLemore, T.L. and Boyd, M.R. (1989) Evidence for prostanoid biosynthesis as a biochemical feature of certain subclasses of non-small cell carcinomas of the lung as determined in established cell lines derived from human lung tumors. Cancer Res. 49, 826–832.
[13] Nana-Sinkam, P., Golpon, H., Keith, R.L., Oyer, R.J., Sotto-Santiago, S., Moore, M.D., Franklin, W., Nemenoff, R.A. and Geraci, M.W. (2004) Prostacyclin in human non-small cell lung cancers. Chest 125, 141S.
[14] Keith, R.L., Miller, Y.E., Hudish, T.M., Girod, C.E., Sotto-Santiago, S., Franklin, W.A., Nemenoff, R.A., March, T.H., Nana-Sinkam, S.P. and Geraci, M.W. (2004) Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. Cancer Res. 64, 5897–5904.
[15] Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A. and Narumiya, S. (1994) cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla. J. Biol. Chem. 269, 9986–9992.
[16] Lim, H. and Dey, S.K. (2002) A novel pathway of prostacyclin signaling–hanging out with nuclear receptors. Endocrinology 143, 3207–3210.
[17] Yano, T., Zissel, G., Muller-Qernheim, J., Shin, S.J., Satoh, H. and Ichikawa, T. (2002) Prostaglandin E_2 reinforces the activation of Ras signal pathway in lung adenocarcinoma cells via EP3. FEBS Lett. 518, 154–158.
[18] Fujimoto, E., Satoh, H., Negishi, E., Ueno, K., Nagashima, Y., Hagiwara, K., Yamasaki, H. and Yano, T. (2004) Negative growth control of renal cell carcinoma cell by connexin 32: possible involvement of Her-2. Mol. Carcinogen. 40, 135–142.
[19] Juge-Aubry, C., Pernin, A., Favez, T., Burger, A.G., Wahlim, W., Meier, C.A. and Desvergne, B. (1997) DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements: importance of the 5′-flanking region. J. Biol. Chem. 272, 25252–25259.
[20] Hatae, T., Wada, M., Yokoyama, C., Shimonishi, M. and Yanabe, T. (2001) Prostacyclin-dependent apoptosis mediated by PPAR delta. J. Biol. Chem. 276, 46260–46267.
[21] Seiler, N. and Knodgen, B. (1980) High-performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. J. Chromatogr. 221, 227–235.
[22] Tsubouchi, Y., Sano, H., Kawahito, Y., Mukai, S., Yamada, R., Kohno, M., Inoue, K., Hla, T. and Kondo, M. (2000) Inhibition of human lung cancer cell growth by the peroxisome proliferator-activated receptor-gamma agonists through induction of apoptosis. Biochem. Biophys. Res. Commun. 270, 400–405.
[23] Wurch, T., Junquero, D., Delhon, A. and Pauwels, J. (2002) Pharmacological analysis of wild-type alpha, gamma and deltasubtypes of the human peroxisome proliferators-activated receptor. Nanunyn Schmiedebergs Arch. Pharmacol. 365, 133–140.
[24] Turchanowa, L., Dauletbacv, N., Milovic, V. and Stein, J. (2001) Nonsteroidal anti-inflammatory drugs stimulate spermidine/spermine acetyltransferase and deplete polyamine content in colon cancer cells. Eur. J. Clin. Invest. 31, 887–893.
[25] Li, L., Rao, J., Bass, B.L. and Wang, J.Y. (2001) NF-kappaB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. Am. J. Physiol. 280, G992–G1004.
[26] Yano, Y., Yano, T., Uchida, M., Murakami, A., Ogita, M., Ichikawa, T., Otani, S. and Hagiwara, K. (1997) The inhibitory effect of vitamin E on pulmonary polyamine biosynthesis, cell proliferation and carcinogenesis in mice. Biochim. Biophys. Acta 1356, 35–42.
[27] Yano, T., Yano, Y., Uchida, M., Murakami, A., Hagiwara, K., Otani, S. and Ichikawa, T. (1997) The modulation effect of vitamin E on prostaglandin E_2 level and ornithine decarboxylase activity at the promotion phase of lung tumorigenesis in mice. Biochem. Pharmacol. 53, 1757–1759.
[28] Porter, C.W., Bernacki, R.J., Miller, J. and Bergeron, R.J. (1993) Antitumor activity of N1,N11-bis(ethyl)norspermine against human melanoma xenografts and possible biochemical correlates of drug action. Cancer Res. 53, 581–586.

[29] Yamaki, T., Endoh, K., Miyahara, M., Nagamine, I., Huong, N.T.T., Sakurai, H., Pokorny, J. and Yano, T. (2004) Prostaglandin E_2 activates Src signaling in lung adenocarcinoma cell via EP_3. Cancer Lett. 214, 115–120.
[30] Zhang, L., Yu, J., Park, B.H., Kinzler, K.W. and Vogelstein, B. (2000) Role of BAX in the apoptotic response to anticancer agents. Science 290, 989–992.
[31] Babbar, N., Ignatenko, N.A., Casero Jr., R.A. and Gerner, E.W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. J. Biol. Chem. 278, 47762–47775.
[32] Scorcioni, F., Corti, A., Davalli, P., Astaneolle, S. and Bettuzzi, S. (2001) Manipulation of the expression of regulatory genes of polyamine metabolism results in specific alterations of the cell-cycle progression. Biochem. J. 354, 217–223.
[33] Vujeic, S., Halmekyto, M., Diegelman, P., Gan, G., Kramer, D.L., Janne, J. and Porter, C.W. (2000) Effects of conditional overexpression of spermidine/spermine N_1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. J. Biol. Chem. 275, 38319–38328.