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SHORT REPORT

PPAR-γ ligand promotes the growth of APC-mutated HT-29 human colon cancer cells in vitro and in vivo

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Summary PPAR-γ has been known to induce suppression, differentiation and reversal of malignant changes in colon cancer in vitro. However, there are several reports that PPAR-γ ligands enhance colon polyp development in APC^{min} mice in vivo. These contradictory results have not yet been thoroughly explained. To explain the contradictory results, we analyzed the effects of different concentrations of the PPAR-γ agonist, 15-deoxy-D12, 14-prostaglandin (15-d Δ PGJ2) and pioglitazone, on APC gene-mutated colon cancer cell lines (HT-29). We measured cell growth and suppression by cell count and MTT assay and analyzed the expression of β-catenin and c-Myc protein by Western blot. In addition, we inoculated HT-29 cells into APC^{min} mice to compare tumor size. High concentrations (10–100 μM/L 15-d Δ PGJ2 and pioglitazone) of PPAR-γ ligand suppressed growth, while low concentrations (0.01–1 μM/L 15-d Δ PGJ2 and pioglitazone) of PPAR-γ ligand promoted growth. In particular, the effects of 0.1 μM/L 15-d Δ PGJ2 and pioglitazone on cell growth were statistically significant (P=0.003, P=0.001, respectively). Tumor growth was associated with an increase in β-catenin and c-Myc expression. The growth of xenograft tumors was greater in PPAR-γ ligand-treated mice than in control mice (control vs day 14: P=0.024, control vs day 28: P=0.007). The expression of β-catenin and c-Myc protein were also elevated in PPAR-γ-treated mouse tissues. PPAR-γ ligand can promote the growth of APC-mutated HT-29 colon cancer cells in vitro and in vivo. In addition, the tumor promoting effect seems to be associated with an increase in β-catenin and c-Myc expression. We think that well-controlled clinical trials should be conducted to confirm our results and to verify clinical applications.

Keywords PPAR-γ ligand · APC-mutated · β-Catenin · c-Myc

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Introduction

PPAR-γ (peroxisome proliferator-activated receptor-gamma), a nuclear receptor that plays a major role in the differentiation of adipose cells and insulin sensitivity, is predominantly found in fat tissue and epithelial neoplasms such as colon and breast cancer [1]. PPAR-γ is activated by 15-deoxy-D12, 14-prostaglandin J2 (15-d Δ PGJ2), free fatty acids and anti-diabetic thiazolidinediones such as troglitazone, pioglitazone, and rosiglitazone (BRL49653) [2–4]. Anti-diabetic thiazolidinediones are potent PPAR-γ agonists that induce suppression, differentiation and reversal of malignant changes in colon cancer in vitro [5–7]. Conversely, several reports have indicated that thiazolidinediones enhance colon polyp development in APC^{min} mice in vivo [8–10]. These conflicting results have not yet been thoroughly explained.

The APC^{min} mouse, a model of human familial adenomatous polyposis, develops numerous polyps spontaneously in the intestinal tract because of a mutation in the adenomatous polyposis coli (APC gene). The APC gene mutation is the most common cause of colon cancer related to the Wnt (Wingless type)/β-catenin pathway [11]. The Wnt/β-catenin pathway plays a critical role in the development of colon cancer [12]. We hypothesized that discrepancies between in vitro and in vivo studies could be explained by the dose-dependent action of thiazolidine-

diones, and by the possible association of APC gene mutation and tumorigenesis. We treated APC-mutated colon cancer cells with different concentrations of PPAR-γ ligands, including low dosages, enabling us to determine if a lower concentration of PPAR-γ ligand enhances tumor growth. We tested low doses of PPAR-γ ligand (15-d Δ PGJ2 and pioglitazone) that had never been tested in previous in vitro studies and also conducted an in vivo study of the effects of pioglitazone treatment on tumor growth. Here, we propose a dose-dependent action of PPAR-γ ligands in the proliferation of colon cancer from APC mutations, and we offer an explanation for previously reported contradictory results.

Materials and methods

Cell growth in vitro and MTT assay

We chose the APC-mutated human colon cancer cell line, HT-29, in which a mutant form of the APC gene is expressed as two C-terminal-truncated APC proteins of 100 and 200 kDa [13]. The HT-29 cell line was obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) and cultured in RPMI 1640 supplemented with 10% bovine calf serum (Gibco, USA) in a humidified atmosphere of 50 mL/L CO_2 at 37°C.

HT-29 cells were treated with different concentrations of 15-d Δ PGJ2 (Sigma, St. Louis, MO; 0.01, 0.1, 1, 10 and 100 μM/L), respectively, and the control cells were treated with an equivalent amount of DMSO. After 7 days of treatment, the cells were counted**.** We applied the same methods for pioglitazone instead of 15-d Δ PGJ2. Pioglitazone was prepared from the respective commercial capsules by solvent extraction followed by re-crystallization or chromatographic purification.

To measure cell viability, HT-29 cells were plated (2×10^3 per well) in 96-well plates and incubated for 7 days. Media containing 0, 0.01, 0.1, 1, 10, or 100 μM/L of 15-d Δ PGJ2 was applied to the cells of the different experimental groups in six parallel wells. After 7 days, 20 μL of 5 mg/mL MTT was added to each well. The cells were then incubated at 37°C for 4-h, after which the MTT medium was removed and 100 μL of DMSO was added. A subsequent color reaction was measured using a spectrometer at a wavelength of 540 nm. The same procedures were repeated for pioglitazone, too.

Tumor growth in vivo

Nude mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and were maintained in a laminar airflow cabinet under pathogen-free conditions and used after 6 weeks. Cultured HT-29 cells (60–70% confluent) were prepared and viable tumor cells (2×10^{6}/0.1 mL in PBS) were implanted subcutaneously into the flank of the mice. Formation of a bulla was an indication of a satisfactory injection. The mice were then assigned to groups (n=14 per group) to normalize the total tumor volume across the groups. Treated nude mice were fed an autoclaved basal diet, which was uniformly mixed with commercially purchased pioglitazone (Actos®, Lilly Korea; 15 mg/kg mouse body weight) and distilled water. Tumor size and volume were assessed every 3 days. The mice were sacrificed and subjected to a necropsy 28 days after initiation of treatment. Tumors were removed and weighed and the presence of tumor was approximated. We calculated the tumor volume according to the formula $(4/3)r_1^2 r_2 (r_1 < r_2)$ and quickly froze the tumor for later protein expression analysis.

Western blot analysis

Thirty micrograms of cellular protein from HT-29 cells were mixed with SDS sample buffer, boiled for 5 min, and subjected to electrophoresis on 10% SDS–polyacrylamide gels. After the transfer of the proteins onto nitrocellulose filters, immunological evaluation was performed using an ECL Western blotting analysis kit (Amersham Corp., Arlington Heights, IL, USA). The primary antibodies used in these experiments were anti-β-catenin and anti-c-Myc mouse monoclonal antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Tissue preparation

Fresh tissue samples from xenografted tumors were collected and immediately snap-frozen in liquid nitrogen followed by storage at −80°C for further analysis. Precautions were taken during sampling to avoid necrotic or hemorrhagic areas to minimize stromal contamination. Frozen tissues were washed in 4°C PBS (pH 7.4) and homogenized in 4–5 volumes (0.3–0.7 ml) of 4°C lysis buffer that contained 50 mM Tris–HCl (pH 7.4), 120 mM NaCl, 0.5% Triton X-100, and 10 μg/ml of phenylmethylsulfonyl fluoride. After sonication for 20 s, the tissue samples were centrifuged at 14,000×g for 15 min at 4°C. The supernatant was then collected and frozen at −80°C until further testing. Protein concentrations were determined using a protein assay kit obtained from Bio Rad Laboratories (Hercules, CA, USA) according to the supplier's instructions.

Statistical analysis

Statistical analyses were carried out with EpiInfo version 3.41 (CDC, USA). Comparisons were made using the

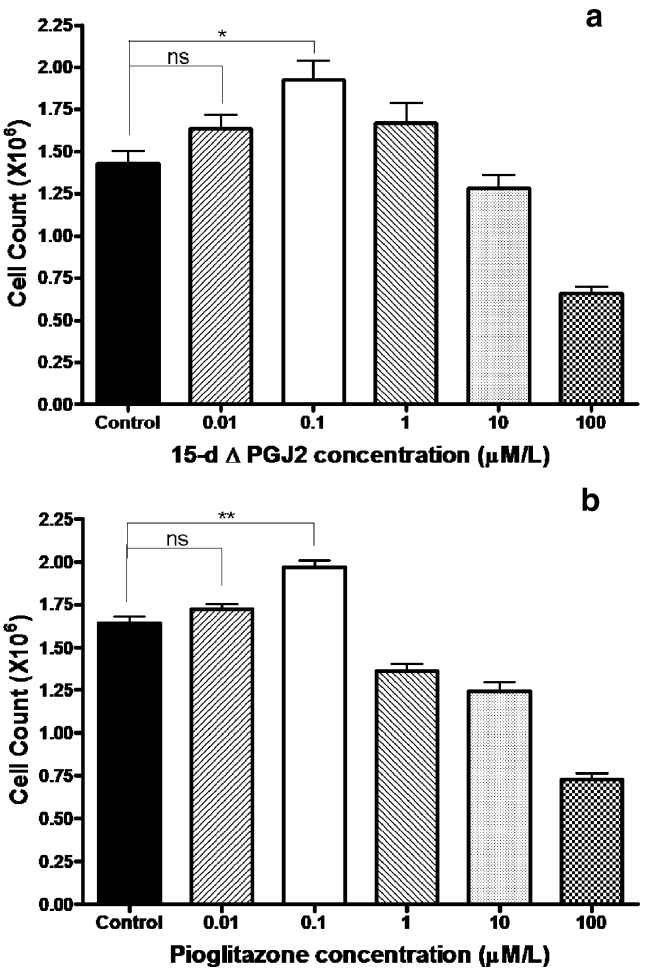

Fig. 1 Cell count in APC-mutated HT-29 cells. **a** Treated with 0, 0.01, 0.1, 1, 10 and 100 μM/L 15-d Δ PGJ2 for 7 days (*asterisk*: P=0.003, *ns*: statistically non significant), **b** Treated with 0, 0.01, 0.1, 1, 10 and 100 μM/L pioglitazone for 7 days (*double asterisk*: P=0.001, *ns*: statistically non-significant)

Mann–Whitney U test. All P values are two-tailed, and the level of the significance was set at 0.05.

Results

Low concentrations (0.01–1 μmol/L) of 15-d Δ PGJ2 and pioglitazone promote HT-29 cell proliferation

To determine the dose-dependent behavior of 15-d Δ PGJ2 and pioglitazone, we treated HT-29 colon cancer cells with different doses of 15-d Δ PGJ2 and pioglitazone respectively. The cell survival rates of HT-29 treated with 0, 0.01, 0.1, 1, 10, or 100 μM/L 15-d Δ PGJ2 for 7 days were 100, 130, 163, 125, 81, and 36%, respectively. In particular, the 0.1 μM/L 15-d Δ PGJ2-treated group showed statistically significant tumor growth promotion (P=0.003; Fig. 1a). The cell survival rates of HT-29 treated with 0, 0.01, 0.1, 1, 10, or 100 μM/L pioglitazone for 7 days were 100, 105, 121, 83, 76, and 44%, respectively. The 0.1 μM/L pioglitazone-treated group also showed statistically significant tumor growth promotion (P=0.001; Fig. 1b).

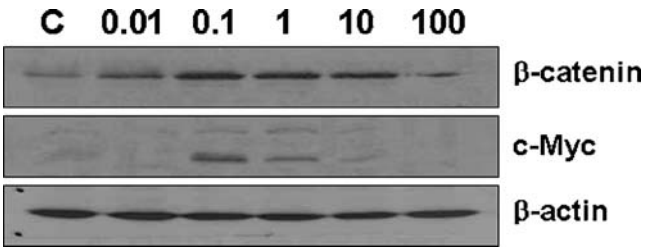

Fig. 2 Expression of β-catenin and c-Myc in HT-29 cells after treatment with different doses of 15-d Δ PGJ2 (*c*: control, *0.01*: 0.01 μμM/L, *0.1*: 0.1 μM/L, *1*: 1 μM/L, *10*: 10 μM/L, *100*: 100 μM/L)

15-d Δ PGJ2 increases expression of Wnt downstream proteins (β-catenin and c-Myc) in HT-29 cells

The growth effect of tumors in the APC-mutated HT-29 colon cell line is believed to be associated with the Wnt-signaling transduction pathway. We tested the expression level of the Wnt downstream proteins, β-catenin and c-Myc, to explore this association. β-catenin and c-Myc levels increased with increasing concentrations of 15-d Δ PGJ2 (0.01, 0.1 and 1 μM/L; Fig. 2). Low dose of pioglitazone (0.01 and 0.1 μM/L) also increased the expression of β-catenin and c-Myc in Western blot (data are not shown).

Pioglitazone treatment induces tumor cell growth in vivo

To determine whether PPAR-γ ligand also could affect HT-29 cells in vivo, we used a nude mouse xenograft model. Tumor volume was increased in the pioglitazone-treated group compared to the control group (control vs day 14: P=0.024, control vs day 28: P=0.007; Fig. 3).

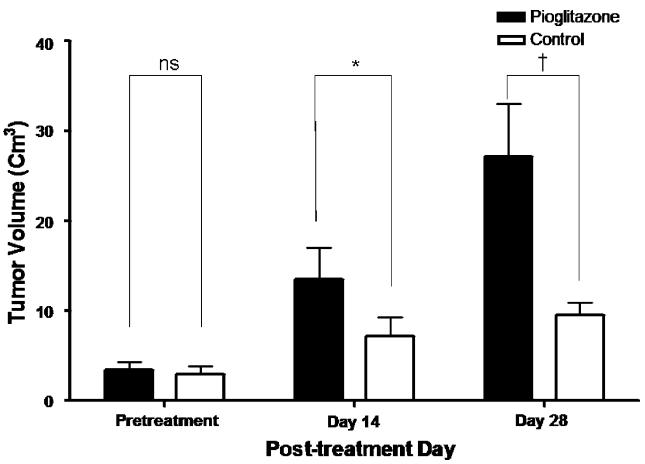

Fig. 3 The difference in xenografted tumor volume between the control group and the pioglitazone-treated group (ns: statistically non significant, *asterisk*: P=0.024, *cross*: P=0.007)

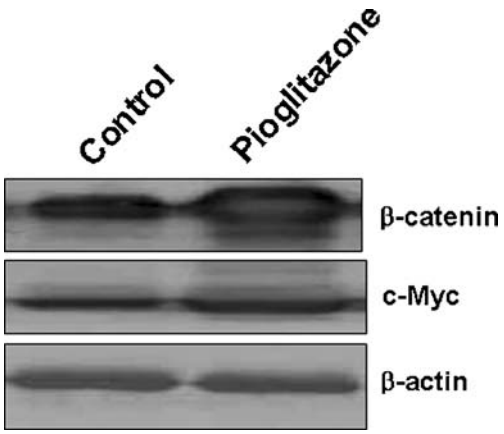

Fig. 4 Expression of β-catenin and c-Myc in HT-29 xenografted tumor tissues in the control and pioglitazone-treated groups

Pioglitazone treatment increases expression of β-catenin and c-Myc in xenografted tumor tissues

The growth effects of tumors in xenograft models may also be associated with Wnt-signaling transduction pathways. As in the in vitro study, western blot analyses revealed that levels of β-catenin and c-Myc were increased in the pioglitazone-treated group compared to the control group (Fig. 4).

The Ki-67 labeling index is increased in pioglitazone-treated xenografted tumor tissues

Tumor mass was evaluated to assess the difference in tumor proliferation between the control and treatment groups. Based on H&E stain, there were no differences between the control and treatment groups. Irregular glandular proliferation and tumor necrosis were observed in both groups. However, the Ki-67 labeling index was higher in the treatment group than in the control group (Fig. 5).

Discussion

Colorectal cancer is one of the most common non-smoking-related causes of cancer deaths in the developed world. The 20-fold difference in incidence worldwide might be explained by environmental influences [14]. Despite the epidemiological evidence of a positive correlation between dietary fat intake and incidence of colon cancer, the causal relationship continues to be questioned. To prove this causal relationship, a spontaneous tumor mouse model, like the APC^{min}, mouse is used. The mutation in APC^{min} mouse is analogous to that found in human FAP kindred. Using this mouse model, Wasan et al. [15] showed that colon tumors increase by 125% in heterozygote APC^{min} mice on high (15%) fat diets. Interestingly, numerous free fatty acids, which are metabolized from fat, are known to be physiologic PPAR-γ ligands [16].

Pioglitazone, a thiazolidinedione that can directly bind PPAR-γ receptor [4], is used in a clinical setting to reduce insulin resistance and hyperglycemia in obese patients with type 2 diabetes. Fujita et al. [17] reported that plasma levels of pioglitazone range from 0.4 to 1.2 μM/L after 18 h of

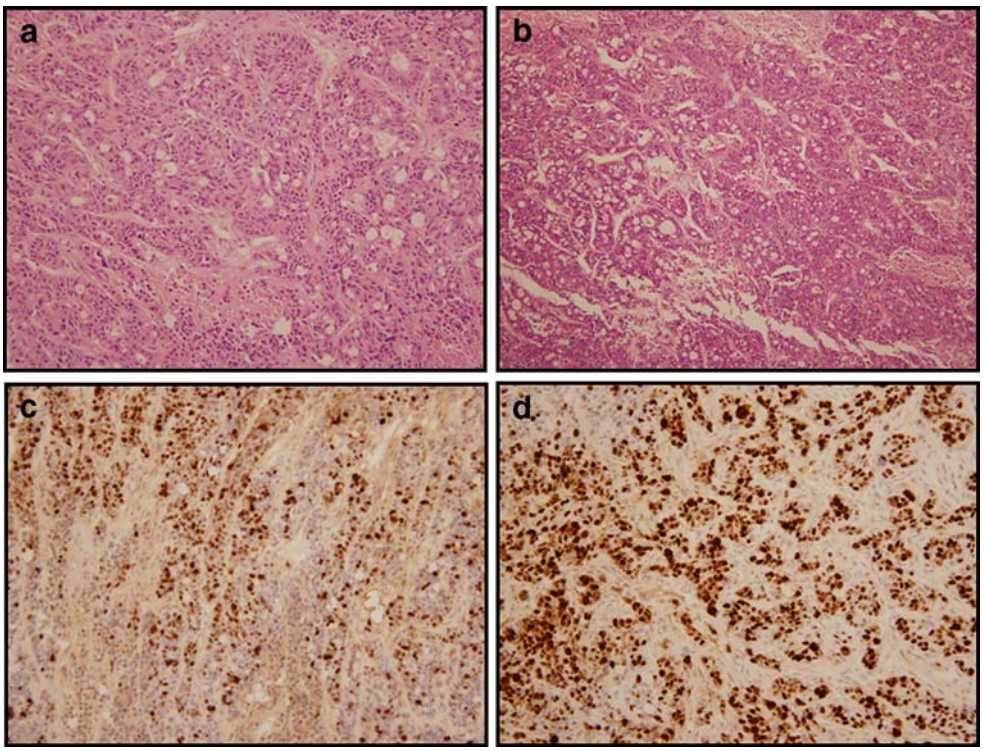

Fig. 5 Immunohistochemical stain of xenografted tumor tissue. **a** Irregular glandular proliferation and tumor necrosis were observed in xenografted tumors in the control group. (H&E stain ×200). **b** Irregular glandular proliferation and tumor necrosis were observed in the xenografted tumors of the control group. (H&E stain ×200). **c** The Ki-67 labeling index of the control group was 50–60% (Ki-67 stain ×200). **d** The Ki-67 labeling index of the treatment group was 80–90% (Ki-67 stain ×200)

oral administration of a 10 mg/kg dose of pioglitazone in an anti-diabetic treatment of female and male rats. In a previous bioavailability study, pioglitazone was shown to have an absolute bioavailability of >80% with a small volume of distribution (0.2–0.3 L/kg) [18]. Usually, tissue concentrations of drugs are lower than plasma levels when the drug has a small distribution. This difference means that real extracellular concentrations of pioglitazone were likely lower than 0.4–1.2 μM/L in that study. Previous in vitro studies have found a suppression in the growth of cells treated with thiazolidinediones, such as troglitazone, pioglitazone, rosiglitazone and with 15-d Δ PGJ2 at 1, 10, and 100 μM/L concentration [5–7]. These studies did not describe whether the subject cancer cell lines had the APC gene mutation or not. In our in vitro experiment, cell growth was promoted by treatment with 0.01, 0.1, and 1 μM/L concentrations of 15-d Δ PGJ2 and pioglitazone. Although only 0.1 μM/L 15-d Δ PGJ2 and pioglitazone produced statistically significant cell growth differences (P=0.003, P=0.001), 0.01 μM/L 15-d Δ PGJ2 and pioglitazone also showed a trend of tumor promotion.

Nude mouse tumor xenograft is a widely accepted model for the evaluation of the anti-tumor efficacy and associated toxicities of a test agent. We administered a 15 mg/kg dose of pioglitazone to mice xenografted with human HT-29 cells to observe tumor growth effects with a low pioglitazone concentration. As in our in vitro study, tumor growth of a xenografted mass was promoted by treatment with pioglitazone. Lefebvre et al. [8] and Saez et al. [9] have also presented evidence that APC^{min} mice have an increased number of polyps when subjected to oral thiazolidinediones.

High doses of 15-d Δ PGJ2 and pioglitazone, in contrast, can suppress tumor growth. This finding is consistent with results from previous studies demonstrating suppression of colon cancer by treatment with thiazolidinediones [5, 6]. When all results are considered together, our study indicates that 15-d Δ PGJ2 and pioglitazone induce tumor growth at relatively lower concentrations (0.01–0.1 μM/L) and suppresses cancer growth at higher concentrations (10–100 μM/L) in APC-mutated HT-29 cells in vitro and in vivo.

The mechanism of tumor growth at low concentrations of 15-d Δ PGJ2 and pioglitazone is suspected to involve the Wnt/β-catenin pathway, which plays a critical role in the development of colon cancer [12]. Wnt signaling controls the proliferation and differentiation of epithelial cells [19]. The APC gene mutation is the most common cause of Wnt-signaling dysfunction in colon cancer. The mutation prevents the down-regulation of β-catenin, resulting in stabilization and accumulation of large quantities of the protein in the cytoplasm [11]. This enhanced β-catenin expression promotes constitutive transcription of Wnt target genes [20]. Several target genes of the Wnt/β-catenin pathway have been identified, including c-Myc, and cyclin D1 [21, 22]. Because the HT-29 cell line has an APC gene mutation, we hypothesized that tumorigenesis of HT-29 cells in the presence of low concentrations of 15-d Δ PGJ2 and pioglitazone may be associated with APC gene mutation. We used western blot analyses of β-catenin and c-Myc in our in vitro and in vivo studies to examine the relationship between tumor growth and the Wnt-signaling transduction pathway. As with tumor growth, levels of β-catenin and c-Myc increased at lower doses of 15-d Δ PGJ2 and pioglitazone. Jansson et al. [23] recently reported that PPAR-γ is found in the same protein complex as β-catenin and TCf-4 (T cell factor-4), and concluded that PPAR-γ seems to associate with both β-catenin and TCf-4. Our study also suggests that the mechanism of tumorigenesis may be linked to the Wnt-signaling transduction pathway.

Recently, the incidence of old people with diabetes markedly increased and most of these cases have taken PPAR-γ ligands to reduce insulin resistance. For any of these cases that have APC-mutated colon polyps or early colon cancer, treatment with PPAR-γ ligands might actually be harmful. So, we think that well-controlled clinical trials should be conducted to confirm our results and to verify the clinical application.

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