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Full Paper

Inhibition of Peroxisome Proliferator-Activated Receptor γ Promotes Tumorigenesis Through Activation of the β-Catenin / T Cell Factor (TCF) Pathway in the Mouse Intestine

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Abstract. Although peroxisome proliferator-activated receptor γ (PPARγ) is strongly expressed in the intestinal epithelium, the role of PPARγ in intestinal tumorigenesis has not yet been elucidated. To address this issue, we investigated the effect of PPARγ inhibition and its mechanism on intestinal tumorigenesis using a selective antagonist, T0070907. We treated $Apc^{Min/+}$ mice and carcinogen-induced colon cancer model C57BL/6 mice with T0070907 and counted the number of spontaneous polyps and aberrant crypt foci and observed cell proliferation and β-catenin protein in the colon epithelium. To investigate its mechanism, the changes of β-catenin/TCF (T cell factor) transcriptional activity and location of β-catenin induced by T0070907 were investigated in the colon cancer cell lines. T0070907 promoted polyp formation in the small intestine of $Apc^{Min/+}$ mice and aberrant crypt foci in the colon of C57BL/6 mice. PPARγ inhibition promoted cell proliferation and increased expressions of the c-myc and cyclin D1 genes and the β-catenin protein in the colon epithelium. In vitro, cell proliferation was promoted, but it was inhibited by the transfection of dominant-negative Tcf4. T0070907 increased β-catenin/TCF transcriptional activity and β-catenin protein in the cytsol and nucleus, but relatively decreased it on the cell membrane. PPARγ antagonist promotes tumorigenesis in the small intestine and colon through stimulation of epithelial cell proliferation. β-Catenin contributes to the promotion of tumorigenesis by PPARγ antagonist due to activation of TCF/LEF (lymphoid enhancer factor) transcriptional factor.

Keywords: peroxisome proliferator-activated receptor γ (PPARγ), T0070907, aberrant crypt foci (ACF), β-catenin, intestinal tumor

Introduction

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, is involved in the regulation of growth, differentiation, and metabolism of various cell types via transcriptional regulation of target genes (1). PPARγ has been shown to be abundantly expressed in the intestinal epithelium and in colon cancer cells (2). The role of PPARγ in regulating neoplastic transformation, however, remains controversial. Saez and Lefebvre reported that PPARγ ligands promoted colon polyp formation in $Apc^{Min/+}$ mice (3, 4),

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whereas, in contrast, Sarraf and Tanaka reported that PPARγ ligands inhibited colon carcinogenesis (5, 6). Using $Apc^{Min/+}$ mice, McAlpine showed that PPARγ deficiency enhanced the number of $Apc^{Min/+}$ tumors in both the small intestine and colon (7). In a previous study, we demonstrated the chemopreventive effect of PPARγ ligands against colon cancer development in an azoxymethane (AOM)-induced colon cancer model (8). Niho also demonstrated that PPARγ ligands suppressed tumor formation in $Apc^{Min/+}$ mice (9). These aforementioned findings indicate that activation of PPARγ by its ligands may suppress colon carcinogenesis. However, no studies have investigated the effect of PPARγ inhibition on intestinal tumorigenesis by using a PPARγ-specific antagonist. Therefore, we examined the effects of the PPARγ-specific antagonist T0070907 (10) on colorectal carcinogenesis and its mechanism.

$Apc^{Min/+}$ mice have a mutation of *APC*, which is a major regulator of β-catenin activation, and represent a model of adenomatous polyposis coli (APC) (11). β-Catenin is involved in mediating two major functions in normal cells: a) regulation of cell-cell adhesion as a component of the E-cadherin/catenin adherens complex in the cell membrane and b) mediation of the proliferating signal through the Wingless/Wnt pathway through its expression in the cytoplasm and nucleus (12 – 14). β-Catenin acts as a transcription cofactor with T cell factor/lymphoid enhancer factor (TCF/LEF) in the Wnt signaling pathway. Free pools of β-catenin are tightly regulated by the tumor suppressor proteins, APC and GSK3β, and destabilized by phosphorylation at Ser33, Ser37, and Thr41 (15). Mutations of any of APC or β-catenin itself can lead to inhibition of β-catenin degradation, resulting in an increase in the cytoplasmic pools (16 – 20) and activation of TCF/LEF-mediated transcription.

On the other hand, the relationship between PPARγ and β-catenin was reported in some studies, but it is still unclear for colon cancer. In the maintenance of preadipocytes, activation of β-catenin by overexpression of Wnt or a GSK3β phosphorylation-defective mutant of β-catenin blocks adipogenesis via inhibition of PPARγ-associated gene expressions (21, 22). Conversely, activation of PPARγ by its ligands stimulates the degradation of β-catenin (23). Jansson reported a direct interaction between PPARγ and β-catenin in colon cancer cells (24). From the above, it is evident that β-catenin and PPARγ mutually inhibit each other's activity, and it is thought that a balance between β-catenin and PPARγ signaling is important for the maintenance of normal cell differentiation and proliferation (25).

In this study, we investigated the effect of PPARγ inhibition using a selective antagonist, T0070907, on the development of intestinal polyps in $Apc^{Min/+}$ mice and colonic aberrant crypt foci (ACF) in a carcinogen-induced colon cancer mouse model. Furthermore, we aimed to elucidate the role of β-catenin in the promotion of intestinal tumorigenesis by PPARγ antagonists.

Materials and Methods

Reagents and antibodies

The PPARγ-specific antagonist T0070907 was purchased from Cayman Chemical (Ann Arbor, MI, USA). PPARγ siRNA and polyclonal antibody against PPARγ (H-100) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Azoxymethane (AOM) and MTT (methylthiazolyldiphenyl-tetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). Monoclonal antibody against β-catenin and polyclonal antibody against Phospho-β-Catenin (Ser33/37/Thr41) were purchased from BD Transduction Laboratories (San Diego, CA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively.

Cell lines

The human colon cancer cell lines Lovo and HT-29 were obtained from Health Science Research Resources Bank (Osaka) and American Type Culture Collection (Manassas, VA, USA), respectively.

Animals

The mice were treated humanely according to the National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All animal experiments were approved by the institutional Animal Care and Use Committee of Yokohama City University School of Medicine. Five-week-old male C57BL/6 mice were purchased from CLEA Japan (Tokyo) and male C57BL/6-*Apc*$^{Min/+}$ mice ($Apc^{Min/+}$ mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Three to five mice were housed per metallic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room air-conditioned at 24 ± 2°C and 55% humidity, under a 12-h light/dark cycle.

Spontaneous intestinal polyp formation model

To investigate the effect of PPARγ inhibition on intestinal polyp formation, six-week-old mice were divided into groups of 8 or 9 male $Apc^{Min/+}$ mice per group, and each group was given 0 (control), 25, 50, or 100 ppm of the PPARγ antagonist T0070907, mixed into the diet, for 7 weeks. The daily intake of T0070907 in the 100-ppm group was estimated to be approximately 10 mg/kg body weight based on the diet consumption.

Food and water were provided ad libitum to the animals. The animals were then observed for clinical signs and mortality. The body weights and food consumption were measured weekly. The intestines were divided into three sections: the colon and two segments of the small intestine: proximal (half of oral portion in small intestine) and distal (half of the anal portion in small intestine). These segments were opened longitudinally and fixed in 10% neutral buffered formalin. The number of the polyps was determined by examination under a stereoscopic microscope. Polyps that were bigger than 1.0 mm in the maximum axis were counted.

Induction of ACF in a mouse model of AOM-induced colon cancer

Six-week-old C57BL/6 mice were divided into 2 groups composed of mice treated with 500 ppm T0070907 or not treated. The mice of both groups were given two weekly intraperitoneal injections of 10 mg/kg of AOM. In six weeks after the treatment, the mice were sacrificed and samples were collected. The numbers of ACF and aberrant crypts (ACs) were counted as described previously (8).

Immunohistochemistry of normal colon epithelium

Paraffin-embedded sections were deparaffinized and subjected to immunohistochemical staining for β-catenin with an anti-mouse β-catenin monoclonal antibody using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and for BrdU using the staining kits (BD Biosciences, San Jose CA, USA) in accordance with the manufacturer's instructions. The primary β-catenin antibody was diluted 1:800 and nuclear counterstaining was performed with hematoxylin.

BrdU labeling index

The Bromodeoxyuridine (BrdU) labeling index was expressed as the ratio of the number of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. The criteria for selecting the crypts in which to conduct the measurements were as follows: a clearly visualized and continuous cell column on each side of the crypt, a completely visible crypt lumen, and opening of the crypt in the middle area of each colon. Twenty crypts were counted in each mouse, and all animals were evaluated.

Cell proliferation and apoptosis assay

Cell proliferation was measured by the MTT assay. Cells were plated in 96-well plates at a concentration of 5×10^3 cells each well. A 0.4-μg sample of dominant-negative Tcf4 plasmid (26) (kindly provided by Dr. Tetsuji Yamada, Biochemistry Division, National Cancer Center Research Institute) or 0.4 μg of pTRE2-pur as a mock control was transfected using Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the procedure recommended by the manufacturer. After the transfection, cells were treated with 10 μM T0070907 for 24 h, and then 0.5% MTT solution was added to each well. The absorbance at 595 nm was determined using a microplate reader (Model 550; Bio-Rad, Richmond, CA, USA). The experiments were performed in quadruplicate and repeated three times.

To evaluate the apoptotic activity, annexin V staining was performed using the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences) in accordance with the manufacturer's instructions. Cells were subsequently analyzed by FACScan flow cytometry.

Inhibition of PPARγ function using siRNA

Lovo cells at 70% confluence were transfected with PPARγ siRNA by Lipofectamin 2000 in accordance with the procedure recommended by the manufacturer. The cells were treated with 10 nM PPARγ siRNA for 24 h. We used Stealth RNAi Negative Control Medium GC (Invitrogen) for the control specimens. Inhibition of PPARγ expression was confirmed by real-time RT-PCR and PPARγ expression was suppressed by over 80% as compared with that in the control (data not shown).

Reporter gene assay

Untreated cells or cells treated with T0070907 or PPARγ siRNA for 24 h were transfected with either 0.4 μg TOPflash (containing TCF/LEF-binding sites, the basic thymidine kinase promoter, and the firefly luciferase reporter gene) (Upstate, Lake Placid, NY, USA) or 0.4 μg FOPflash (containing mutated TCF /LEF-binding sites) (Upstate) by Lipofectamin 2000. All samples were normalized by transfecting 0.1 μg phRL-tk (Promega, Madison, WI, USA). At 24 h after the transfection, luciferase activities were measured with the Dual Luciferase Reporter Assay System (Promega). This experiment was performed in triplicate and repeated three times.

Western blot analysis

Colon epithelial protein was extracted with T-PER (PIERCE, Rockford, IL, USA). Cytsolic and nuclear protein was collected using ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosciences, Darmstadt, Germany). Protein concentrations were determined by using Protein Assay Reagent (Bio-Rad). Protein were separated by SDS/PAGE and transferred to a polyvinylidene difluoride membrane. After the transfer, the membranes were blocked with Blocking One-P (Nacalai Tesque, Kyoto) and probed with each primary antibody.

Horseradish-peroxidase–conjugated secondary antibodies and the ECL detection kit (Amersham, London, UK) were used for the detection of specific proteins. All images were taken by LAS3000 (Fuji Film, Tokyo). The results were normalized to the expression level of GAPDH for the total cell, tubulin for the cytosol, and histone for the nucleus.

Gene expression analysis

Total RNA was extracted from the mouse colon epithelium and colon cancer cell lines, Lovo and HT29, using the RNeasy Mini Kit (QIAGEN). Total RNA was reverse-transcribed into cDNA and amplified by real-time RT-PCR using the ABI PRISM 7700 System (Applied Biosystems, Foster City, CA, USA). The probes and primer pairs specific for cyclin D1, c-myc, PPARγ, and β-actin were purchased from Applied Biosystems. The concentrations of the target genes were determined using the competitive CT method and the values were normalized to an internal control.

Immunofluorescence analysis of colon cancer cells

Colon cancer cells were plated on collagen-1–coated glass coverslips followed by exposure to 10 μM T0070907 for 12 h. The coverslips were paraformaldehyde-fixed and permeabilized with 100% ethanol at −20°C. Fixed cells were incubated with primary antibodies and stained with Alexa Fluoro-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). Confocal laser scanning microscopic images were then generated (Carl Zeiss, ObErkochen, Germany). The primary β-catenin antibody and PPARγ antibody were diluted 1:800 and 1:500, respectively.

Statistical analyses

All results are expressed as mean ± S.D. values. Statistical analysis for the multiplicity of the colon tumors was conducted using ANOVA. Other statistical analyses were performed by Student's *t*-test. The results were considered to be statistically significant when P values were <0.05.

Results

Enhancement of spontaneous polyp formation in the $Apc^{Min/+}$ mice by the selective PPARγ antagonist

Fig. 1A shows the number and distribution of intestinal polyps in the $Apc^{Min/+}$ mice treated with the PPARγ antagonist T0070907 or vehicle. Most polyps were observed in the small intestine, with only a few apparent in the colons, both in the T0070907- and vehicle-treated groups. A significant increase in the number of polyps in the distal, but not proximal, portion of the small intestine was observed. Similarly, a significant increase in the total polyp number was observed in the mice treated with the PPARγ antagonist as compared with the control. In contrast, basal spontaneous polyp formation was markedly less pronounced in the colon than in the small intestine in this model. There were no significant differences in the body weights or food intake of the mice among the groups. To examine the effect of T0070907 on adipogenesis, we measured the serum levels of triglyceride, free fatty acid, and total cholesterol in each mouse before it was killed. There was no significant difference among the groups (data not shown). None of the mice died during the observation period.

Inhibition of PPARγ promotes colon epithelial cell proliferation and ACF formation in the mouse model of AOM-induced colon tumorigenesis

Significant increases in the number of ACF and ACs were observed following treatment with the PPARγ antagonist in the colon of the mouse model of AOM-induced tumorigenesis (Fig. 1B).

The expression of PPARγ in the mouse intestine was examined by western blot analysis. PPARγ expression was lowest in the proximal intestine and in the more distal portion showed higher expression (Fig. 1C).

We performed BrdU immunohistochemical staining of the colon epithelium of the mouse model of AOM-induced colon tumorigenesis to investigate the effects of T0070907 treatment on the cell proliferative activity. A significant increase of the BrdU labeling index was observed in the colon of the mice treated with T0070907 as compared with the control mice (Fig. 2A).

Inhibition of PPARγ increases the gene expression level of cyclin D1 and c-myc and protein level of β-catenin in the colon epithelium

Analysis of the gene expressions of c-myc and cyclin D1 in the colon epithelium was performed by real-time RT-PCR. Expressions of both the c-myc and cyclin D1 genes were significantly increased following treatment with T0070907 (500 ppm) (Fig. 2B). Next, we investigated the protein level of β-catenin. Western blot analysis showed β-catenin increased by the treatment of T0070907 (Fig. 2C). These results indicate that the inhibition of PPARγ increases colon epithelial cell proliferation by promoting the transcription of these genes due to β-catenin increase, thereby accelerating the formation of ACF.

Immunohistochemistry of β-catenin in normal colonic epithelium following treatment with T0070907

To investigate changes of quantity and localization

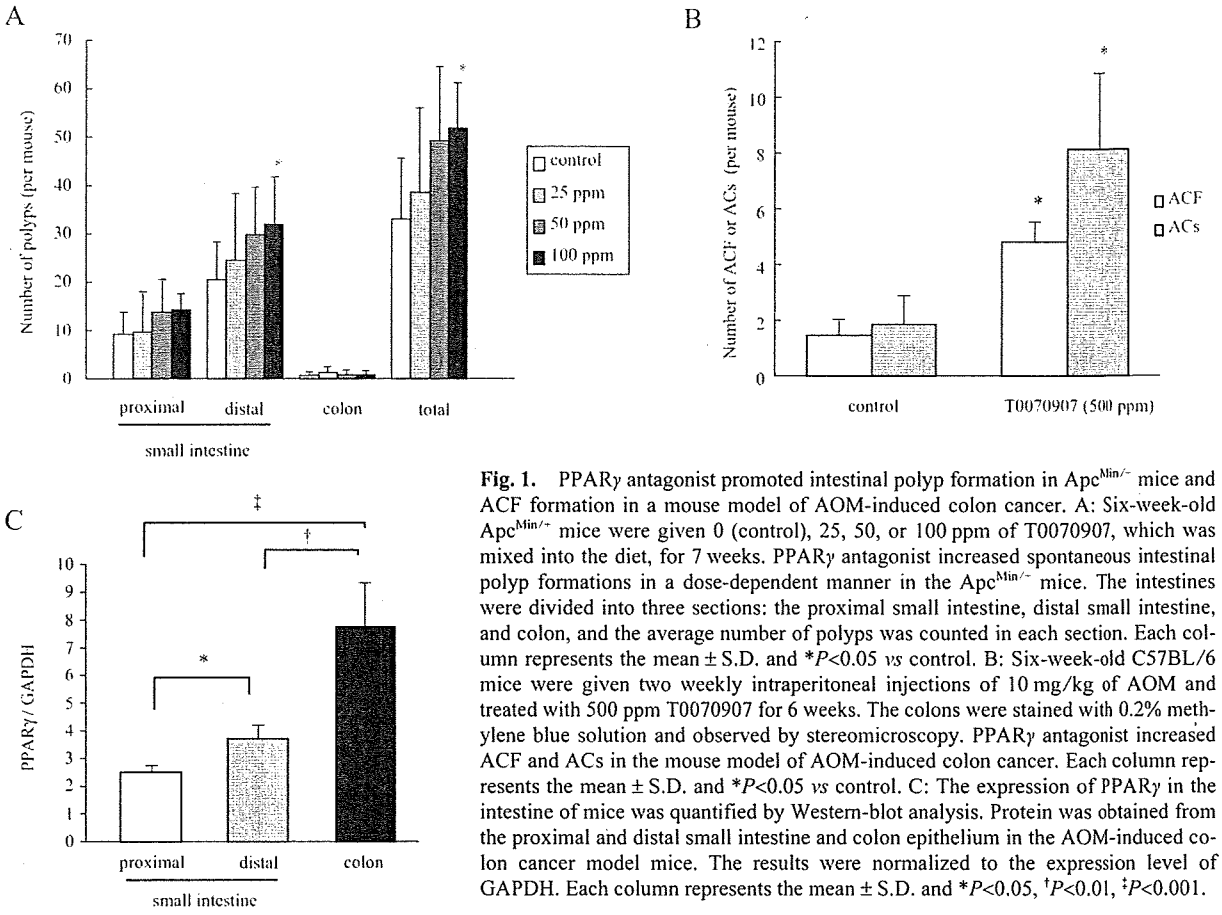

Fig. 1. PPARγ antagonist promoted intestinal polyp formation in $Apc^{Min/-}$ mice and ACF formation in a mouse model of AOM-induced colon cancer. A: Six-week-old $Apc^{Min/+}$ mice were given 0 (control), 25, 50, or 100 ppm of T0070907, which was mixed into the diet, for 7 weeks. PPARγ antagonist increased spontaneous intestinal polyp formations in a dose-dependent manner in the $Apc^{Min/-}$ mice. The intestines were divided into three sections: the proximal small intestine, distal small intestine, and colon, and the average number of polyps was counted in each section. Each column represents the mean ± S.D. and $*P<0.05$ *vs* control. B: Six-week-old C57BL/6 mice were given two weekly intraperitoneal injections of 10 mg/kg of AOM and treated with 500 ppm T0070907 for 6 weeks. The colons were stained with 0.2% methylene blue solution and observed by stereomicroscopy. PPARγ antagonist increased ACF and ACs in the mouse model of AOM-induced colon cancer. Each column represents the mean ± S.D. and $*P<0.05$ *vs* control. C: The expression of PPARγ in the intestine of mice was quantified by Western-blot analysis. Protein was obtained from the proximal and distal small intestine and colon epithelium in the AOM-induced colon cancer model mice. The results were normalized to the expression level of GAPDH. Each column represents the mean ± S.D. and $*P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$.

of β-catenin in the colon epithelium, we performed immunohistochemistry for β-catenin (Fig. 2D). Imuunohistochemistry revealed no clear difference of the location and the amount of β-catenin between control and T0070907-treated mice.

PPARγ antagonist does not affect the apoptotic activity but increases the cell proliferative activity via TCF/LEF transcriptional factor in colon cancer cells

To elucidate the mechanism underlying the effect of PPARγ inhibition on tumor formation, we investigated the effect of the PPARγ antagonist T0070907 on the cell proliferative and apoptotic activity using the cultured colon cancer cell lines Lovo and HT-29. MTT assay revealed that T0070907 increased the cell proliferative activity of the colon cancer cells (Fig. 3A). Furthermore, knockdown of TCF/LEF transcriptional factor by induction of dominant-negative Tcf4 plasmid inhibited the increase of cell proliferation by T0070907 (Fig. 3: B and C). On the other hand, the assay using annexin V showed no difference in the apoptotic activity between the control and T0070907-treated cells (data not shown).

Inhibition of PPARγ increases the expressions of c-myc and cyclin D1 via transcriptional activation of β-catenin

Real-time RT-PCR revealed that T0070907 significantly increased the gene expressions of both c-myc and cyclinD1 in the colon cancer cell lines Lovo and HT29 (Fig. 4A). We then performed the β-catenin/TCF reporter assay (TOPflash/FOPflash system) to investigate the transcriptional activity of β-catenin. PPARγ antagonist increased β-catenin/TCF reporter activity in Lovo and HT29 cells (Fig. 4B). We checked this effect using PPARγ siRNA, which also increased β-catenin /TCF reporter activity in Lovo cells (Fig. 4C).

PPARγ antagonist increases β-catenin protein by suppressing its degradation

We quantified the protein expression levels of β-catenin in Lovo and HT-29 cells that were left untreated or treated with the PPARγ antagonist. T0070907 increased β-catenin protein level in both cell lines (Fig. 5A). To elucidate the mechanism underlying the increase of β-catenin protein by T0070907, we performed real-time RT-PCR to examine the production of β-catenin and western blot analysis of β-catenin and

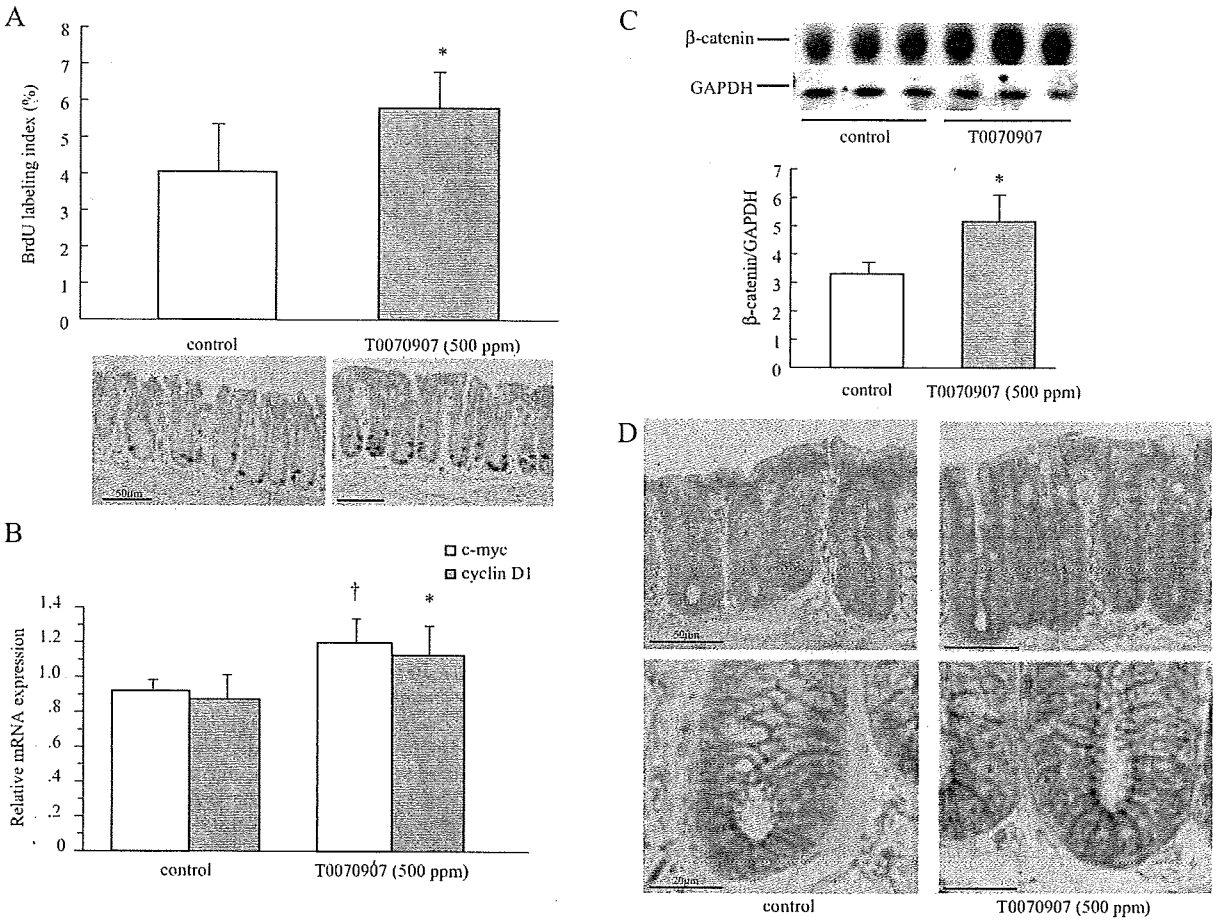

Fig. 2. PPARγ antagonist increased cell proliferation in colon epithelium via increase of β-catenin, c-myc, and cyclin D1 in AOM-induced colon cancer model mouse. A: BrdU was administered intraperitoneally at a dose of 50 mg/kg, 1 h prior to the sacrifice of the mice. BrdU-positive cells were detected by immunohistochemistry. The BrdU labeling index was expressed as the ratio of the number of positively-stained nuclei to the total number of nuclei counted in the crypts of the colon. Lower photographs show representative immunohistochemical staining for BrdU in each group. Each column represents the mean ± S.D. and $^{*}P<0.05$. B: Real-time RT-PCR analysis for cyclin D1 and c-myc expressions in the colon epithelium. Samples were obtained from normal colon epithelium in the AOM-induced colon cancer model mouse. Each column represents the mean ± S.E.M and $^{*}P<0.05$, $^{\dagger}P<0.01$. C: Western blot analysis for β-catenin was performed. Total protein was obtained from normal colon epithelium. Upper photographs showed representative western blot signals for β-catenin and GAPDH. Each column represents the mean ± S.D. and $^{*}P<0.05$. D: Immunohistochemistry of β-catenin in normal colon epithelium obtained from the AOM-induced colon cancer model mouse. There was no clear difference of the location and the amount of β-catenin between control and T0070907-treated mice. Nuclear counterstaining was performed with hematoxylin.

phospho-β-catenin to examine the degradation of β-catenin. Real-time RT-PCR revealed no difference in the β-catenin mRNA expression level between the control and T0070907-treated cells (data not shown). On the other hand, western-blot analysis revealed an increase in the amount of β-catenin and a decrease in the amount of phospho-β-catenin following treatment with T0070907 (Fig. 5A). These results indicated that T0070907 increased β-catenin protein not due to an increase of β-catenin production, but a decrease of β-catenin degradation.

PPARγ antagonist increases cell proliferation via increase of the β-catenin expression in the nucleus

To confirm the increase of β-catenin expression in the nucleus, we fractionated the cell protein into cytosol and nucleus, and then measured the β-catenin expression in each of the compartments by Western-blot analysis. T0070907 increased β-catenin expression in the cytosol and nucleus (Fig. 5B). Consecutively, we performed immunofluorescent staining for β-catenin and PPARγ to investigate the intracellular localization of β-catenin by confocal microscopy. β-Catenin was strongly expressed on the cell membrane and weakly expressed in the

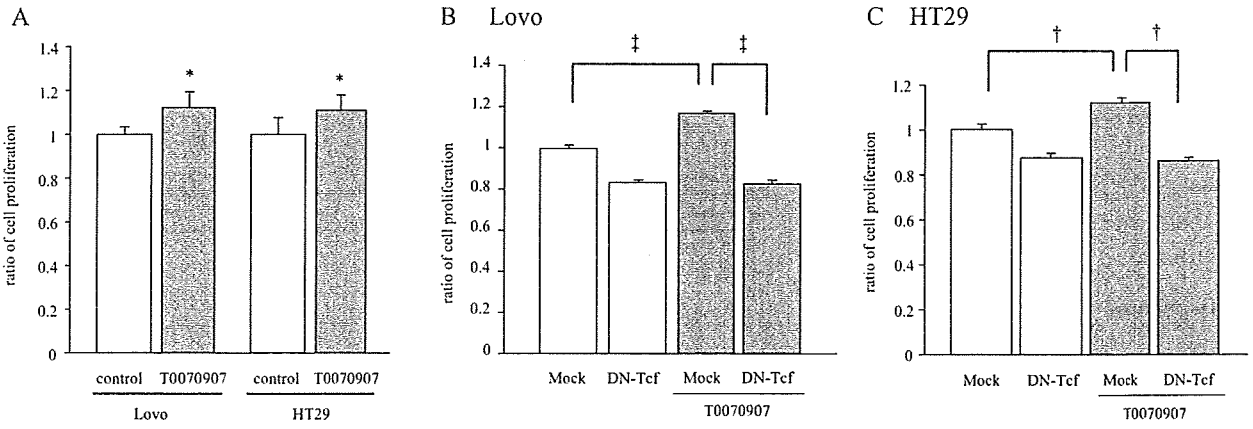

Fig. 3. PPARγ antagonist increased cell proliferation via Tcf4 in the colon cancer cells. A: MTT assay was performed in colon cancer cell lines to examine the effect of PPARγ antagonist for cell proliferation in vitro. Lovo and HT29 cells were treated and untreated with 10 μM T0070907 for 24 h. The PPARγ antagonist significantly increased the cell proliferations in both cell lines. The ratio in the control was defined as 1.0. Each column represents the mean ± S.D. *$P<0.05$ *vs* control. B and C: Cell proliferation assay in Lovo cells (B) and HT29 cells (C) transfected with dominant–negative Tcf4 (DN-Tcf) or mock vector. At 24 h after transfection, Lovo and HT29 cells were either left untreated or untreated with T0070907 for 24 h. Each column represents the mean ± S.D. †$P<0.01$, ‡$P<0.001$ *vs* control.

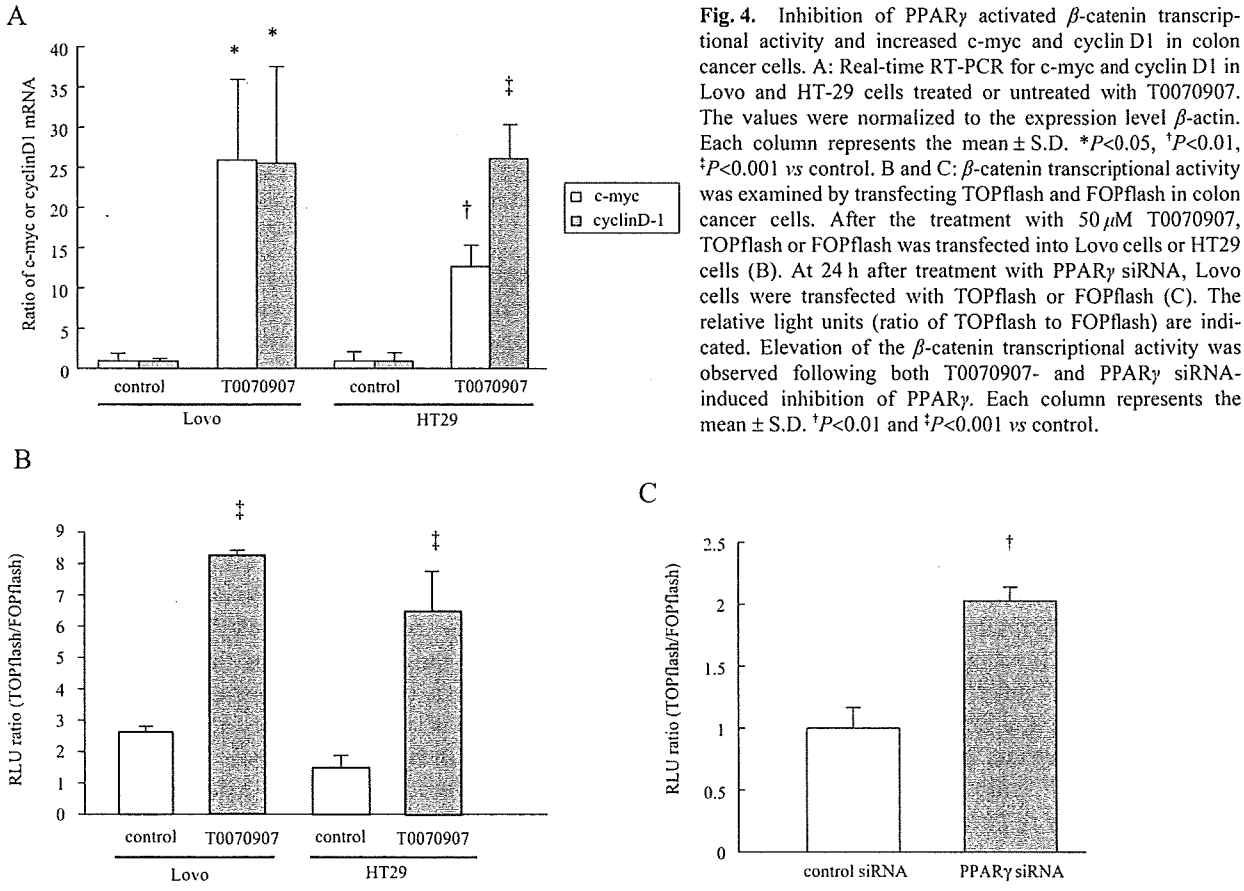

Fig. 4. Inhibition of PPARγ activated β-catenin transcriptional activity and increased c-myc and cyclin D1 in colon cancer cells. A: Real-time RT-PCR for c-myc and cyclin D1 in Lovo and HT-29 cells treated or untreated with T0070907. The values were normalized to the expression level β-actin. Each column represents the mean ± S.D. *$P<0.05$, †$P<0.01$, ‡$P<0.001$ *vs* control. B and C: β-catenin transcriptional activity was examined by transfecting TOPflash and FOPflash in colon cancer cells. After the treatment with 50 μM T0070907, TOPflash or FOPflash was transfected into Lovo cells or HT29 cells (B). At 24 h after treatment with PPARγ siRNA, Lovo cells were transfected with TOPflash or FOPflash (C). The relative light units (ratio of TOPflash to FOPflash) are indicated. Elevation of the β-catenin transcriptional activity was observed following both T0070907- and PPARγ siRNA-induced inhibition of PPARγ. Each column represents the mean ± S.D. †$P<0.01$ and ‡$P<0.001$ *vs* control.

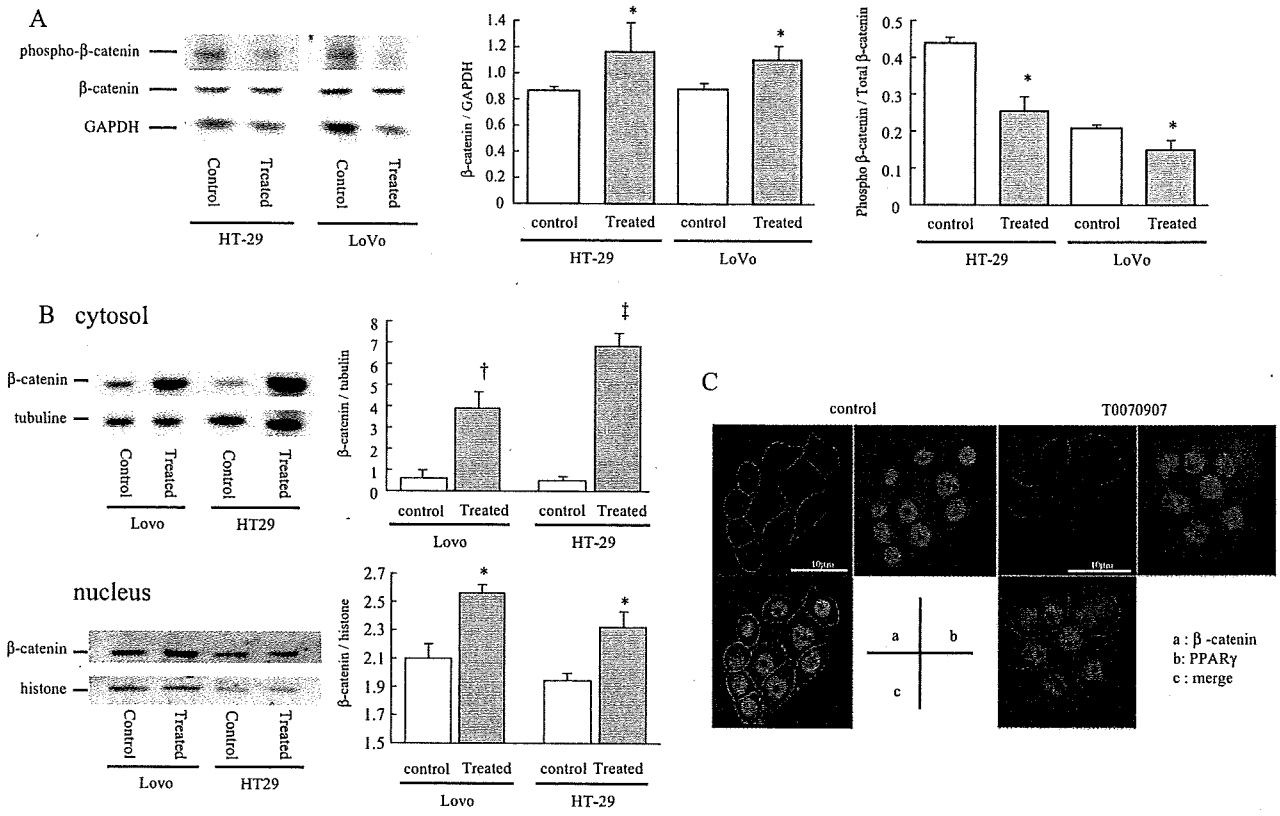

Fig. 5. PPARγ antagonist increased β-catenin protein in the whole cell, cytosol and nucleus by suppressing its phosphorylation. A: Western-blot analysis for β-catenin and phospho-β-catenin (Ser 33/37/Thr 41) was performed in the Lovo cells and HT29 cells. T0070907 increased β-catenin and decreased phospho-β-catenin in the both types of cells. Photographs show representative Western-blot signals for β-catenin, phospho-β-catenin, and GAPDH. Each column represents the mean ± S.D. and $*P<0.05$ *vs* each control. B: Western-blot analysis for β-catenin with the protein extract from the cytosol and nucleus. β-Catenin was particularly increased in the cytosol. Each column represents the mean ± S.D. and $*P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ *vs* each control. C: PPARγ (Green) and β-catenin (Red) immunofluorescence in HT-29 cells treated or not treated with T0070907. Treatment with the PPARγ antagonist increased β-catenin protein in the cytosol and relatively decreased it on the cell membrane.

nucleus in the control cells. On the other hand, in the T0070907-treated cells, relative translocation of β-catenin from the membrane to the cytosol was observed (Fig. 5C). PPARγ was localized mainly in the nucleus and did not merge with β-catenin in either the control or T0070907-treated cells.

Discussion

We clearly demonstrated that inhibition of PPARγ using the selective antagonist T0070907 promoted spontaneous polyp formation, even in the absence of any treatment with chemical carcinogens, in the small intestine of $Apc^{Min/+}$ mice. These results strongly indicate that inhibition of the PPARγ pathway alone may be sufficient to accelerate polyp formation in the small intestine of $Apc^{Min/+}$ mice. McAlpine et al. reported that PPARγ deficiency in gene knockout mice enhanced the number of $Apc^{Min/+}$ tumors (7). The present study supports their report. However, the pharmaceutical effect of PPARγ inhibitor on intestinal tumorigenesis was described for the first time in the present study. A significant increase in the number of polyps in the distal, but not proximal, portion of the small intestine was observed. This result may be explained by the higher level of PPARγ expression in the distal portion compared to the proximal portion (Fig. 1C). Moreover, colon polyps in $Apc^{Min/+}$ mice were not increased by treatment with 100 ppm T0070907, but the number of ACF in the AOM-induced colon cancer model mice was increased by 500 ppm T0070907. We speculate two reasons for the discrepancy of these results: 1) Colon carcinogenesis could not be properly evaluated because the number of colon polyps in $Apc^{Min/+}$ mice was very small (less than 1.0 per mouse). 2) The expression of PPARγ in the colon is higher than in the small intestine (Fig. 1C). Therefore, more T0070907 is needed to suppress the effect of PPARγ in the colon than in the

small intestine. However, once the effect of PPARγ was sufficiently inhibited, the difference should be large.

Cell proliferation and β-catenin protein were increased and cyclin D1 and c-myc expressions were up-regulated in the colon epithelium of PPARγ antagonist–treated mice, but the location of β-catenin protein was not changed. These results suggest that inhibition of PPARγ increases colonic epithelial cell proliferation by increasing the β-catenin protein that is promoting transcription of cyclin D1 and c-myc.

To investigate the mechanism, we investigated the effect of PPARγ inhibition on the cell proliferation and apoptotic activity in colon cancer cell lines. PPARγ inhibition increased the cell proliferation but not the apoptotic activity. Cell proliferation increased by the PPARγ antagonist was inhibited by dominant-negative Tcf4 induction. This result supports that the PPARγ antagonist plays its role via TCF/LEF transcriptional factor.

Increase in the β-catenin/TCF transcriptional activity was observed in association with inhibition of PPARγ in cultured cells by reporter assay, with target gene activation, that is, of cyclin D1 and c-myc. As cyclin D1 and c-myc act as factors accelerating the G1/S phase and as proto-oncogenes (27, 28), it appears that PPARγ inhibition promotes cell proliferation and tumorigenesis by accelerating the cell cycle via enhancing β-catenin /TCF transcriptional activation. Similar increase in gene transcriptional activity was also observed following knockdown by PPARγ siRNA.

Because the PPARγ antagonist did not increase the mRNA expression level of β-catenin (data not shown) but decreased the phosphorylation level of β-catenin protein, the compound may increase the amount of β-catenin via suppressing the degradation of β-catenin. Furthermore, in the immunohistchemistry and western blot analysis of colon cancer cells, PPARγ antagonist does not only increase total β-catenin but also transfers the main location of β-catenin protein from the cell membrane into the cytosol and nucleus. These results may indicate that the PPARγ antagonist stabilizes β-catenin protein mainly in the cytosol and nucleus, and it causes a relative decrease of the β-catenin on the cell membrane.

In conclusion, we clearly demonstrated that inhibition of PPARγ using a selective antagonist promoted both spontaneous polyp formation in the small intestine of $Apc^{Min/+}$ mice and carcinogen-induced ACF formation in the colon. Inhibition of PPARγ increased β-catenin expression especially in the nucleus, resulting in enhanced expression of cyclin D1 and c-myc via TCF /LEF transcriptional factor, which in turn, promoted epithelial cell proliferation. Our results imply that PPARγ plays an important role of suppressing tumorigenesis in the intestine by attenuating epithelial cell proliferative activity.

Acknowledgments

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