Peroxisome Proliferator-activated Receptor γ Induces Growth Arrest and Differentiation Markers of Human Colon Cancer Cells

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Peroxisome proliferator-activated receptor γ (PPARγ), one of the nuclear receptors expressed in adipose tissue, plays an important role in adipocyte differentiation. In this study, we investigated the expression of PPARγ and its role in cellular growth and differentiation in six colon cancer cell lines: HT-29, CaCo-2, SW-480, DLD-1, LoVo, and T-84. All six expressed PPARγ mRNA and protein, shown respectively on northern and western blot analyses. Luciferase assay in HT-29 cells, which strongly express PPARγ, showed that troglitazone, a selective ligand for PPARγ, transactivated the transcription of a peroxisome proliferator response element (PPRE)-driven promoter. Furthermore, troglitazone caused a marked decrease in [³H]thymidine incorporation and G1 cell-cycle arrest determined by flow cytometry. Finally, troglitazone induced expression of mRNAs for villin and intestinal alkaline phosphatase, markers for enterocyte differentiation. In conclusion, human colon cancer cells express PPARγ, the ligands of which inhibit cell growth and induce differentiation markers.

Key words: PPARγ — Colon cancer cells — Differentiation

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, has been linked to adipocyte differentiation and the control of cellular lipid uptake.[1, 2] Forced expression and activation of PPARγ in fibroblasts trigger the adipocyte gene expression cascade and lead to the development of the adipose phenotype. PPARγ forms a heterodimeric DNA-binding complex with the 9-*cis*-retinoic acid receptor, recognizes the peroxisomal proliferator response element (PPRE) in the promoter of its target genes, and functions as a transcriptional regulator of genes linked to lipid metabolism. Several polyunsaturated fatty acids, the thiazolidinedione class of antidiabetic drugs such as troglitazone, and the nuclear prostanoid 15-deoxy-$\Delta^{12, 14}$-prostaglandin J_2 (15d-PGJ$_2$) have been identified as ligands for PPARγ.[3–6] Although adipose tissue has been recognized as a principal site of PPARγ expression, this receptor is expressed at low levels in some other tissues such as kidney, liver, small intestine, and large intestine.[7, 8]

Recent reports indicate that PPARγ is expressed at significant levels in human liposarcoma and breast adenocarcinomas and that treatment with PPARγ ligands reduces the growth rate and induces terminal differentiation of these malignant cells.[9, 10] In the large intestine, PPARγ has been shown to be expressed in colonic tumors of azoxymethane-treated rats and in certain human colon cancer cell lines.[11] However, the role of PPARγ in colon cancers has not been elucidated.

In the present study, we investigated the expression of PPARγ and its role in cellular growth and differentiation in several colon cancer cell lines: HT-29, CaCo-2, SW-480, DLD-1, LoVo, and T-84. We found that PPARγ is expressed in these cell lines and that activation of this receptor by its ligands inhibits cell growth and induces cell differentiation.

MATERIALS AND METHODS

Cell cultures All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in 100-mm plastic dishes at 37°C using 5% CO_2 in the following media supplemented with 10% fetal bovine serum (Bop-Whittaker, Walkersville, MD), and 100 units/ml each of penicillin and streptomycin: Dulbecco's modified essential medium (Gibco BRL, Grand Island, NY) for HT-29, CaCo-2, and T-84; Ham's F-12 medium (Gibco BRL) for LoVo; and RPMI-1640 medium (Gibco BRL) for SW-480 and DLD-1. The media were changed every 3 days, and the cells were separated via trypsinization when they reached subconfluence. Experiments were performed on growing cells, and the media were changed 24 h prior to the start of each experiment. The cells were treated with various concentrations of troglitazone (donated by Sankyo Pharmaceuticals, Tokyo), 15dPGJ$_2$ (Cayman Chemical Company, Ann Arbor, MI), and bezafibrate (donated by Kissei Pharmaceuticals, Tokyo).

Mitogenic assays HT-29 cells were seeded at a concen-

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tration of 1×10^5 cells/well in 96-well plates and incubated in complete fresh media for 24 h. The cells were subsequently incubated for 20 h with 0, 1, 2.5, 5, 10, 25, 50, or 75 μM 15dPGJ$_2$, troglitazone, or bezafibrate and further incubated with 1 mCi/ml [^{3}H]thymidine (Amersham, Arlington Heights, IL) for 4 h. The cells were washed three times with phosphate-buffered saline (PBS) and separated via trypsinization. Then the [^{3}H]thymidine incorporation was measured by use of the Betaplate System (Pharmacia, Uppsala, Sweden).

Cell counts Cells (5×10^5 cells/ml) were seeded and incubated in complete fresh media for 24 h, then incubated with 0, 25, or 50 μM troglitazone. After 0, 1, 2, and 4 days, the cells were harvested by trypsinization, washed with PBS, resuspended in media, and counted using a hemacytometer.

Northern blot analysis Cells were grown to subconfluence in 100-mm dishes. Total RNA was extracted by the guanidinium thiocyanate method.[12] In some cases, poly(A+)mRNA was isolated by allowing it to bind to oligo(dT) cellulose (Takara, Tokyo). About 10 μg of each RNA was electrophoresed on 1.0% agarose/2.2 M formaldehyde denaturing gel, transferred to Hybond-N+ membranes (Amersham), and UV-cross-linked (1200 mJ). Hybridization was performed using cDNA probes labeled by random priming (Multiprime DNA Labeling System; Amersham) with [α-^{32}P]dCTP (Dupont-NEN, Boston, MA) in Rapid-hyb buffer (Amersham). The human PPARγ probe was constructed by reverse transcriptase polymerase chain reaction (RT-PCR) amplification of a 263-bp PPARγ cDNA fragment from human colon total RNA, using the oligonucleotides 5$'$-GAGATCACAGAG-TATGCCAA-3$'$ and 5$'$-CTGTCATCTAATTCCAGTGC-3$'$. The villin probe was constructed by RT-PCR amplification of a 332-bp villin cDNA fragment from HT-29 cell total RNA, using the oligonucleotides 5$'$-ACCTTCA-CAGGCTGGTTCCT-3$'$ and 5$'$-ATTCCATCGAGGCA-GAGCAG-3$'$. The intestinal alkaline phosphatase (IAP) probe, a 2.5-kb *Eco*RI fragment derived from human IAP cDNA, was purchased from ATCC. The GAPDH probe, a 1.1-kb *Xba*I-*Hin*dIII fragment of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA, was a gift from Dr. T. Nakamura (Osaka University Medical School, Osaka). Quantitated hybridization signals were normalized to the control gene GAPDH and expressed relative to the control values.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis Cells grown to subconfluence in 100-mm dishes were lysed in lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 100 mM NaF, 1 mM PMSF, 0.25 TIU/ml aprotinin, and 10 mg/ml leupeptin. Aliquots containing 50 μg total protein were size-fractionated by SDS-PAGE (5–20% gradi-

ent gels), and the proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). The membranes were blocked with 5% skim milk and were incubated for 1 h at room temperature with goat anti-human-PPARγ polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After three washings with 0.1% Tween 20 in TBS, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-goat IgG (N Pharmaceuticals, Inc., Aurora, OH). The membranes were again washed, and peroxidase was detected with an enhanced chemiluminescence system (ECL, Amersham). The protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Flow cytometry Cell-cycle profiles of HT-29 cells treated with troglitazone were assessed by measuring the DNA content of individual cells by flow cytometry. In preparation for flow cytometry, cells treated with 50 μM troglitazone for 24 h were collected after brief trypsinization, washed with PBS, and fixed with 70% cold ethanol. Then the samples were treated with RNase, stained with 10 mg/ml propidium iodine, and analyzed by a cell sorter (FACScan, Becton Dickinson, Mountain View, CA). Cell-cycle distributions were quantified using Cell-quest software.

Transfections and luciferase assays HT-29 cells were seeded at a concentration of 1×10^6 cells/60-mm dish and transfected with the plasmids 24 h after having been transferred to fresh media. Transfection was done by using LipofectAMINE reagent (Gibco BRL) mixed with 2 μg of acyl CoA oxidase promoter-luciferase plasmid (kindly donated by Dr. Osumi)[13] and 0.2 μg of pRL-SV40 (Promega, Madison, WI) for 3 h. The transfection mix was replaced by complete media with or without 50 μM troglitazone and further incubated for 12 h. The cells were lysed with 1$\times$ luciferase lysis buffer (Toyo Ink, Inc., Tokyo). Luciferase activity was measured using the PicaGene reagent kit (Toyo Ink) in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany). The enzyme activity was normalized for efficiency of transfection, on the basis of sea pansy luciferase activity, and relative values were determined. Transfection experiments were carried out three times independently, and the average values were calculated.

Statistical analysis Data are expressed as mean$\pm$SE. Statistical analyses were carried out using Student's unpaired t test. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

PPARγ expression in human colon cancer cell lines We determined the expression of PPARγ mRNA and protein in six colon cancer cell lines: HT-29, CaCo-2, SW-480, DLD-1, LoVo, and T-84. As shown in Fig. 1A,
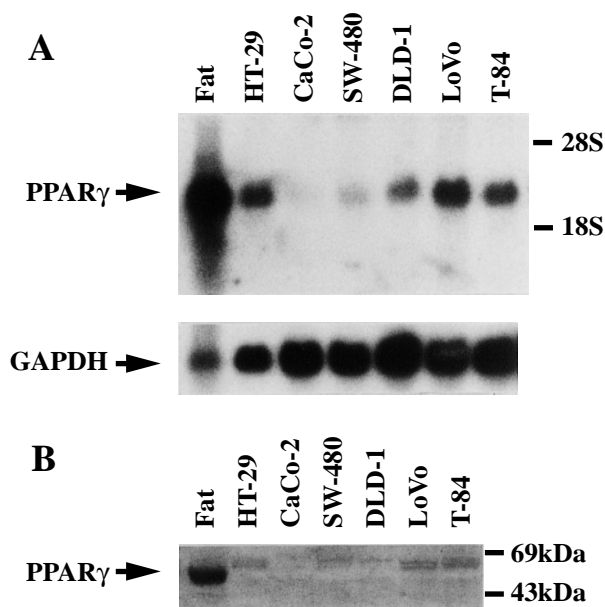
Fig. 1. A: PPARγ mRNA expression in human colon cancer cell lines. Approximately 10 μg of each RNA obtained from the colon cancer cell lines was electrophoresed on 1.0% agarose/2.2 *M* formaldehyde denaturing gel and transferred to nitrocellulose membranes. Hybridization was performed using a human PPARγ cDNA probe labeled with [α-^{32}P]dCTP. The GAPDH control is shown in the bottom panel. B: PPARγ protein expression in human colon cancer cell lines. Approximately 50 μg of each protein obtained from the colon cancer cell lines was separated on SDS-PAGE, probed with an anti-PPARγ antibody, and visualized with enhanced chemiluminescence.
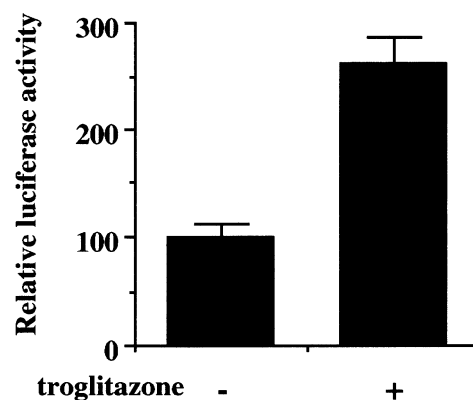

Fig. 2. PPRE transactivation in HT-29 cells. HT-29 cells were transiently transfected with an acyl CoA promoter luciferase construct and pRL-SV40. After treatment with 50 μM troglitazone for 12 h, cells were harvested, and the Dual luciferase assay was performed as described in "Materials and Methods." Data are expressed as mean±SE (*n*=3).

PPARγ mRNA was expressed in all six. PPARγ mRNA was expressed at high levels in HT-29, LoVo, and T-84, at an intermediate level in DLD-1, and at low levels in SW-480 and CaCo-2.

Fig. 1B shows the expression of PPARγ protein in these cell lines. The protein showed a molecular mass of approximately 55 kDa in all human colon cancer cell lines, which is consistent with the reported value for human PPARγ protein.[14] The expression levels of PPARγ protein were in good accordance with those of mRNA.

PPRE transactivation in HT-29 cells To determine if the PPARγ expressed in the cell lines was functional, we transfected HT-29 cells with an acyl-CoA oxidase promoter-luciferase reporter plasmid containing a PPRE.[13] Over 12 h, luciferase activity in the HT-29 cells treated with troglitazone, a specific ligand for PPARγ,[5] for 12 h was approximately three-fold that in untreated cells (Fig. 2).

PPARγ ligands decrease cell growth and induce G1 arrest in HT-29 cells Since PPARγ expressed in HT-29 cells was functional, we next examined whether ligand activation of PPARγ influences the cell growth and the cell cycle of colon cancer cells. Treatment with 15dPGJ$_2$, a natural ligand for PPARγ, as well as with troglitazone, reduced the [^{3}H]thymidine incorporation of HT-29 cells in a dose-dependent manner. However, treatment with bezafibrate, a specific ligand for PPARβ,[15] did not affect [^{3}H]thymidine incorporation (Fig. 3A). Furthermore, treatment with troglitazone resulted in a dose-dependent inhibition of cell proliferation (Fig. 3B).

The effect of troglitazone on the cell cycle profile was analyzed by flow cytometry. Representative cell cycle profiles of cells stained with propidium iodide are shown in Fig. 4. The cells exhibited a decreased fraction of S and G2/M-phase cells resulting from an increased accumulation of cells at G0/G1.

The above results indicate that ligand activation of PPARγ inhibits cellular growth and induces cell cycle arrest at G1 in colon cancers. Previous studies have shown that ligand activation of PPARγ induces growth inhibition and cell cycle withdrawal in liposarcoma and breast cancer cells.[9, 10] Our results are compatible with those reported in such malignant cells.

PPARγ activation induces the expression of villin and IAP mRNAs in HT-29 cells PPARγ activation is known to induce terminal differentiation with cell growth inhibition in adipogenic cell lines.[16] We hypothesized that growth inhibition by PPARγ activation would be accompanied by differentiation of the colon cancer cell lines studied here. To clarify the effect of PPARγ activation upon differentiation of colon cancer cells, we examined the expression of villin and IAP transcripts, which have been used as markers for enterocyte differentiation.[17, 18] Fig. 5 illustrates the increase in both villin and IAP
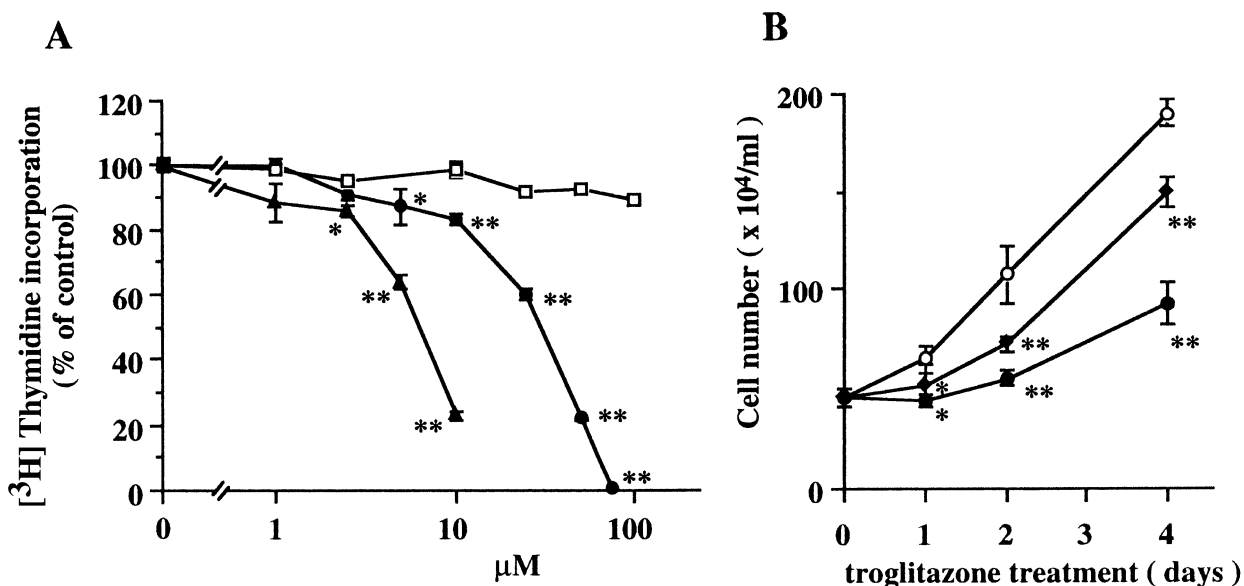
A

B

Fig. 3. PPARγ ligand effects upon cell growth. A: HT-29 cells were treated with 0, 1, 2.5, 5, 10, 25, 50, or 75 μM PPAR ligand for 24 h, and [³H]thymidine incorporation was measured. Data represent the mean±SE from six identical experiments and are expressed as a percentage of the values measured in untreated control cells. B: The same numbers of HT-29 cells were cultured with 0, 25, or 50 μM troglitazone. Cell numbers were determined at the indicated time points. Data are expressed as mean±SE (n=4). ∗P<0.05 or ∗∗P<0.01, significantly different from the untreated control cells. A: ● troglitazone, ▲ 15dPGJ₂, □ bezafibrate. B: ○ 0 μM, ◆ 25 μM, ● 50 μM.
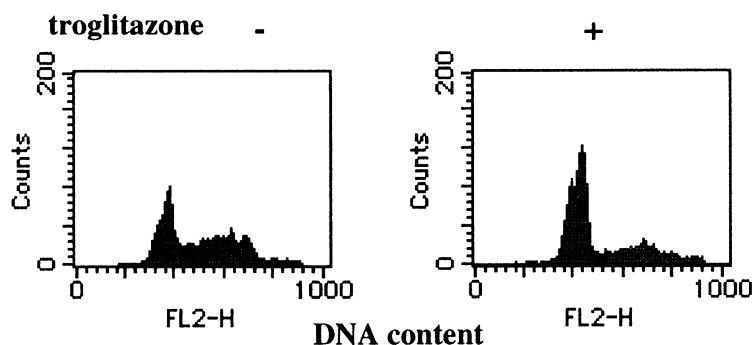

Fig. 4. PPARγ ligand effects upon the cell-cycle profile. After treatment with 50 μM troglitazone for 24 h, HT-29 cells were collected, and cell-cycle analyses with propidium iodide were performed using flow cytometry. Cell-cycle distributions quantified by Cell-quest software were as follows. Untreated cells: G1, 42.6%; S, 29.8% and G2/M, 23.4%. Troglitazone-treated cells: G1, 67.2%; S, 13.3% and G2/M, 15.9%.

mRNA levels in response to PPARγ activation in HT-29 cells. The villin mRNA level showed an approximately 4-fold increase following 48 h of 25 μM troglitazone treatment (Fig. 5A). The IAP mRNA level showed an approximately 2.5-fold increase after 48 h of 25 μM troglitazone treatment (Fig. 5B).

In the large intestine, cellular proliferation is confined to the basal portions of the crypts, and the cells migrate upward from the crypt to the villous surface in about 2 to 6 days. During this migration, the cells undergo differentiation with the transcriptional activation of a number of cell type-specific genes, including those for enzymes, transporters, and structural proteins that reside within the apical microvilli. IAP is one such enzyme expressed exclusively in the brush-border of villus-associated enterocytes.[17] The microvillar structural protein villin is also selectively expressed in the villous cells.[18] Therefore, both IAP and villin are considered to be markers for
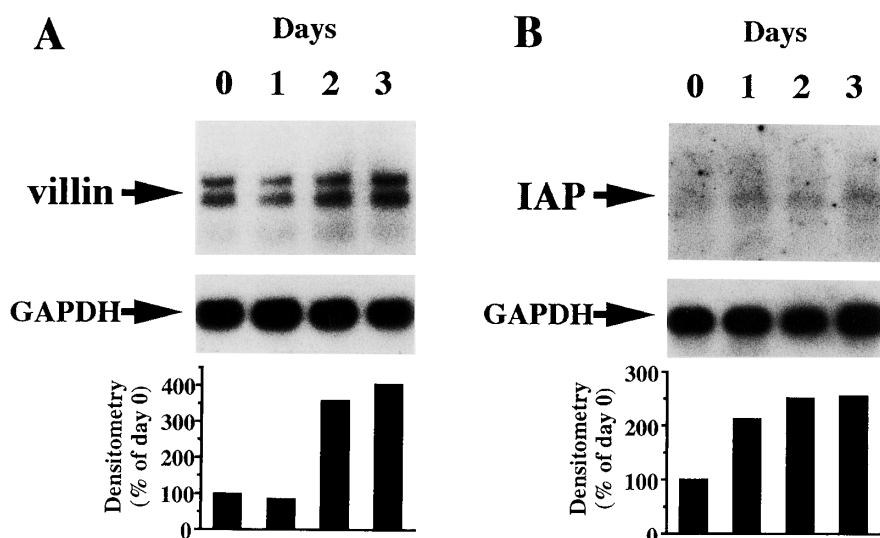
Fig. 5. PPARγ ligand effects upon the expression of villin and IAP transcripts. HT-29 cells were treated with 25 μM troglitazone for the indicated times. Then, total RNA (A) or poly(A+)mRNA (B) isolated from HT-29 cells was electrophoresed on 1.0% agarose/2.2 M formaldehyde denaturing gel, transferred to nitrocellulose membranes and hybridized with a villin or IAP cDNA probe, respectively. Blots were hybridized with a GAPDH cDNA probe. Normalized hybridization signals of villin and IAP mRNA are expressed relative to the value on day 0 (lower panel).

enterocyte differentiation along the crypt-villus axis. Overall, our results suggest that ligand activation of PPARγ can induce growth arrest and differentiation of HT-29 cells. Interestingly, immunohistochemical studies have revealed that a high expression of PPARγ is observed in the more differentiated murine colonic epithelial cells facing the intestinal lumen compared with cells in the lower parts of the crypts.[19] PPARγ may play a physiological role in the differentiation of normal colonic epithelial cells.

In summary, this study shows that PPARγ is expressed in human colon cancer cells and that PPARγ ligands inhibit cell growth and induce differentiation markers.

These tumor-suppressive effects of PPARγ may provide an approach to the treatment of colorectal cancer, which is one of the leading causes of cancer deaths in Japan. Chemotherapy for colorectal cancers is far from optimal because it is associated with serious toxicity and its tumor-suppressive effect is unsatisfactory. Since induction of differentiation is a non-toxic therapeutic approach, PPARγ ligands such as troglitazone, a widely used antidiabetic drug, may be candidates for a novel, non-toxic approach to the treatment of colorectal cancers.

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Note Added in Proof: While this manuscript was under review, differentiation and reversal of malignant changes in colon cancer through PPARγ was reported by others (Sarraf, P. *et al., Nat. Med.*, **4**, 1046–1052 (1998)).