Apoptosis

DOI: 10.1007/s10495-006-4003-z

Pioglitazone, a synthetic ligand for PPARγ, induces apoptosis in RB-deficient human colorectal cancer cells

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Published online: 2 March 2006

No published data are available about the expression of peroxisome proliferator-activated receptor γ (PPARγ) and the role of PPARγ in retinoblastoma protein (RB)-deficient human colorectal cancer (CRC) cells (SNU-C4 and SNU-C2A). Our aim was to investigate whether PPARγ is expressed in SNU-C4 and SNU-C2A cells and to elucidate possible molecular mechanisms underlying the effect of pioglitazone, a synthetic ligand for PPARγ, on cell growth in these cell lines. RT-PCR and Western blot analysis showed that both human CRC cell lines expressed PPARγ mRNA and protein. Pioglitazone inhibited the cell growth of both cell lines through G2/M phase block and apoptosis. In addition, pioglitazone caused a down-regulation of the X chromosome-linked inhibitor of apoptosis (XIAP), Bcl-2, and cyclooxygenase-2 (COX-2) under conditions leading to PPARγ down-regulation. These results suggest that pioglitazone may have therapeutic relevance or significance in the treatment of human CRC, and the down-regulation of XIAP, Bcl-2, and COX-2 may contribute to pioglitazone-induced apoptosis in these and other RB-deficient cell lines and tumors.

Keywords: apoptosis; PPARγ; RB-deficient human colorectal cancer; XIAP.

Introduction

Colorectal cancer (CRC) is the third most-common cancer in the world with 1 million new cases diagnosed in 2002, and the number of new cases worldwide has increased rapidly since 1975.[1] Recent advances in understanding the pathogenesis of CRC and the development of new chemotherapeutic agents have improved the prognosis of patients.[2] However, for most patients with advanced CRC, it is still difficult to achieve a complete remission, especially with surgery or chemotherapy. To improve the cure rate of CRC, the identification of potentially new therapeutic agents is needed.

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid.[3] Three distinct PPARs, termed PPARα, PPARβ/δ, and PPARγ, have been identified. They form heterodimers with the retinoid X receptor, thereby acting as ligand-activated transcription factors. PPARs are characterized by distinct patterns of tissue distribution and metabolic functions. PPARα is highly expressed in liver, heart, kidney, and skeletal muscle, where it regulates the expression of proteins involved in fatty acid metabolism. PPARβ/δ is ubiquitously expressed in most tissues. PPARγ is expressed at high levels in adipose tissue and is an important regulator of adipocyte differentiation.[4,5] The synthetic antidiabetic thiazolidinedione (TZD) drugs, such as troglitazone, rosiglitazone, and pioglitazone, are selective ligands for PPARγ.[6] Naturally occurring ligands of PPARγ include 15-deoxy-12,14-prostaglandin J_2 (15d-PGJ_2), the final metabolite of prostaglandin D2 degradation, as well as certain polyunsaturated fatty acids;[7,8] however, it is unknown whether these natural ligands act as regulators of PPARγ function *in vivo*. Recently, in addition to adipose tissue, PPARγ expression was detected in a wide variety of tumor cells.[6,9–19] In tumor cells, PPARγ activation by high-affinity ligands can induce cell cycle arrest,[9,18,19] cell differentiation,[6,10,11,14,16] or apoptosis.[13,15–17] In addition, small-scale clinical trials showed a reduction in tumor progression in patients with breast cancer, prostate cancer, and liposarcoma, who had been treated with TZD.[20] Thus, in addition to lipid metabolism, PPARγ is involved in the proliferation of cancer cells. It has therefore been suggested that PPARγ might be a possible molecular target in treatment of CRC.

Many investigators have focused on the role of PPARγ in CRC because PPARγ is highly expressed in human colon and colorectal cancer.[6,9] The relevance of PPARγ in colon

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cancer cells is still unclear because PPARγ ligands have been reported either to promote the development[21,22] or to reduce the growth rate of colon cancers.[6,9,10] It therefore appears that the action of PPARγ depends on the cell type/genetic profile in general. Although there is increasing evidence that PPARγ activation results in growth inhibition of colon cancer cells, the molecular mechanism of the growth inhibition by PPARγ ligands is not yet clear. The controversial data on the role of PPARγ in colon cancer suggest that PPARγ has more complicated effects on the regulation of cell growth than generally expected. More detailed and broader studies are required before PPARγ ligands can be used as anticancer drugs in CRC patients. Therefore, in this study, our aim was to investigate whether PPARγ is expressed in RB-deficient CRC cells (SNU-C4 and SNU-C2A) in which PPARγ expression has not yet been reported. In addition, we sought to determine whether pioglitazone affects growth of these two cell lines and to clarify the potential molecular mechanisms of pioglitazone-induced apoptosis.

Materials and methods

Cell cultures

Human CRC cell lines SNU-C4 and SNU-C2A were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea), established from poorly differentiated colorectal adenocarcinoma, and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Reagents

Pioglitazone was obtained from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% DMSO in the culture medium. The PPARγ antagonist GW9662 was obtained from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in DMSO at 10^{-2} M as stock solutions. Anti-PPARγ, anti-p21, anti-caspase-3, anti-cytochrome c, anti-bax, and anti-bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against poly (ADP-ribose) polymerase (PARP), X chromosome-linked inhibitor of apoptosis (XIAP), cyclin dependent kinase 1 (cdk1/cdc 2), and cyclin B1 were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-β-actin antibody was from Sigma (St. Louis, MO, USA). The anti-cyclooxygenase 2 (COX-2) antibody was from Cayman Chemical (Ann Arbor, MI, USA). Unless stated otherwise, all chemicals were purchased from Sigma (St. Louis, MO, USA).

RNA extraction

Total RNA was extracted from cultured cells with 0.1% DMSO or 50 μM pioglitazone for 3 days using an Ultraspec^{TM}II RNA isolation kit (Biotecx Laboratories, Inc., TX, USA) according to manufacturer's instructions. In brief, cells were harvested and suspended in 1 ml of Ultraspec^{TM} RNA reagent. After incubation for 10 min at 4°C, 0.2 ml chloroform (Sigma) was added, followed by another incubation for 5 min, and then centrifugation at 13,000 rpm for 15 min. After transfer of the supernatant to a new tube, a half volume of cold isopropanol and 0.05 volume of RNA resin were added and then centrifuged. After a wash with 75% ethanol, the pellets were dried, and dissolved with diethyl pyrocarbonate (DEPC)-treated water. The resultant RNA samples were quantified using a spectrophotometer (GeneQuant pro RNA/DNA calculator, Amersham Biosciences) at a wave- length of 260 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

An aliquot of 1 μg of total RNA from each sample was reverse transcribed to cDNA using First-Strand cDNA synthesis Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to manufacturer's instructions with oligo (dT) primer. For detection of the human PPARγ mRNA, a combination of a sense primer, 5′-TCTCTCCGTAATGGAAGACC-3′, and an antisense primer, 5′-GCATTATGAGAGACATCCCCAC-3′, was used as described previously.[15] To identify β-actin mRNA, the following primers were used: sense primer, 5′-TCCATCGTCCACCGCAAATG-3′, and antisense primer, 5′-CAGGGAGACCAAAAGCCTTCATAC-3′. The amplification was carried out in a 100 μl mixture containing 1 μl of the above cDNA product, 0.4 μM each of the sense and antisense primers, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs and 2.5 units of Taq DNA Polymerase (Takara Shuzou Co., Otsu, Shiga, Japan). The reaction conditions were as follows: initial denaturation at 95°C for 2 min and 40 cycles of amplification (95°C for 40 s, 55°C for 50 s, and 72°C for 50 s), followed by a final extension step of 7 min at 72°C. The PCR reaction products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide.

Cell counts

SNU-C4 and SNU-C2A cells were seeded at a density of 2×10^5/well in a six-well plate. After a 24 h incubation period, the cells were treated with 10, 25, or 50 μM of pioglitazone. At days 0, 1, 3, and 5 (at days 0, 2, 4, and 6 days for SNU-C2A cells) after pioglitazone treatment, cells were harvested by trypsinization using trypsin/EDTA and

stained with trypan blue. The vital cells (those not stained with trypan blue) were counted under the microscope. Three independent experiments were carried out.

Cell growth assay

To evaluate the effect of pioglitazone on cell growth in SNU-C4 and SNU-C2A cells, cell viability was determined by [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded on a 96-well plate (Corning Inc., Corning, NY, USA) at a concentration of 1×10^4 cells/well in a volume of 200 μl. Twenty-four hours later, the cells were incubated with pioglitazone at several concentrations for 3 or 5 days. After incubation for the indicated times, 20 μl MTT (Sigma, USA) solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37°C. MTT solution in the medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 100 μl DMSO was added to each well before absorbance at 570 nm was measured.

Cell cycle analysis

After treatment with pioglitazone, floating and adherent cells were harvested, washed in PBS, and fixed in ice-cold 70% (v/v) ethanol for at least 1 h. After washing with PBS, cells were treated with PBS containing 100 mg/ml RNase A (DNase-free) at 37°C for 30 min. After centrifugation, cells were resuspended in PBS containing 50 mg/ml propidium iodide (PI) and stained at room temperature for 30 min. DNA contents were analyzed by FACScan (Becton Dickinson, CA, USA). Cell cycle analysis was performed in triplicate.

Apoptosis assay

The extent of apoptosis was evaluated by annexin V-FITC and flow cytometry. The annexin V assay was used, in which annexin was conjugated with FITC. PI was used as counterstain. Briefly, cells were treated with 50 μM pioglitazone or 0.1% DMSO for the indicated times. After incubation, cells were harvested, washed with PBS (pH 7.4), centrifuged, and stained with annexin V-FITC (Pharmingen) and 2 μg/ml PI in binding buffer (10 mM Hepes, pH 7.4/140 mM NaCl/2.5 mM $CaCl_2$) for 15 min at 37°C in the dark. The samples were analyzed by flow cytometry using a FACScan flow cytometer. Data analysis was performed using CellQuest software (Becton-Dickinson, CA, USA).

Separation of the cytosolic and mitochondrial proteins

Cytosolic and mitochondrial proteins were separated as described by Gao *et al.*[23], with minor modifications. In brief, cells treated with 0.1% DMSO or with 50 μM pioglitazone for 3 days were collected and resuspended in mitochondrial isolation buffer (20 mM Hepes-KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl) and protease inhibitor cocktail (Boehringer Mannheim) supplemented with 10 μM digitonin. Suspensions were incubated at 37°C for 5 min and centrifuged at 12,000 g for 15 min. The supernatant (cytosolic fraction) was collected for Western blotting.

Western blot analysis

Cells treated with 0.1% DMSO or 50 μM pioglitazone were harvested, washed with PBS, and 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, and 10 mg/ml leupeptin. The lysates were centrifuged at 13,000 rpm for 15 min and the concentration of protein in each lysate was determined using Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA, USA) following the manufacturer's suggested procedure. Then, 30 μg of proteins were separated by 8 or 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham Life Science, Inc., Piscataway, NJ, USA), blocked overnight in PBS with 5% skim milk at 4°C, and subsequently probed with primary antibody for 1 h. The blots were also probed with a monoclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA) to be relatively quantified since β-actin was used as a loading control. The membranes were then washed three times with PBS-T and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibody and horseradish peroxidase-conjugated anti-mouse IgG for β-actin, respectively. After being washed three times with PBS-T, immune complexes were visualized using ECL detection reagents (Amersham, Buckinghamshire, UK) following the manufacturer's instructions.

Statistical analysis

Data are expressed as mean values ± standard deviations (SD). Analyses were carried out using Student's *t*-tests to determine whether there were significant differences in cell viability between pioglitazone-treated and control cells. A $p< 0.05$ was considered significant.

Results

Expression of PPARγ in SNU-C4 and SNU-C2A cells

PPARγ is constitutively expressed in a variety of cell types.[24,25] No published data are available about the expression of PPARγ and the role of PPARγ in RB-deficient human colorectal cancer (CRC) cells (SNU-C4 and SNU-C2A), which were established from two Korean patients. To determine whether PPARγ is expressed in SNU-C4 and SNU-C2A cells, we examined its expression by RT-PCR and Western blot analysis in these two cell lines. As shown in Figures 1 and 2, PPARγ mRNA and protein were readily detectable in SNU-C4 and SNU-C2A cells. To investigate whether pioglitazone affects the expression of PPARγ, we checked the expression of PPARγ by RT-PCR and Western blot analysis. It showed that pioglitazone treatment resulted in a decrease or even disappearance of PPARγ at both the mRNA and protein levels, as shown in Figures 1 and 2.

Effect of pioglitazone on the cell growth of SNU-C4 and SNU-C2A cells

To evaluate the effect of the specific PPARγ ligand pioglitazone on the cell growth of human CRC cells, SNU-C4 and SNU-C2A cells were treated with pioglitazone (0, 10, 25, or 50 μM) for 1, 3, and 5 days or 2, 4, and 6 days, respectively. Cell viability was determined either by directly counting viable cells based upon trypan blue staining or by MTT assay. Pioglitazone significantly inhibited the cell growth in a dose- and time-dependent manner in both cell lines, as shown in Figure 3.

Figure 1. Expression of PPARγ mRNA in SNU-C4 (A) and SNU-C2A (B) cells treated with 0.1% DMSO (C) or 50 μM pioglitazone (P) for 3 days. Total RNA was extracted and RT-PCR was performed as described in the "Materials and Methods." The sizes of RT-PCR products of PPARγ and β-actin were 474 bp and 487 bp, respectively.

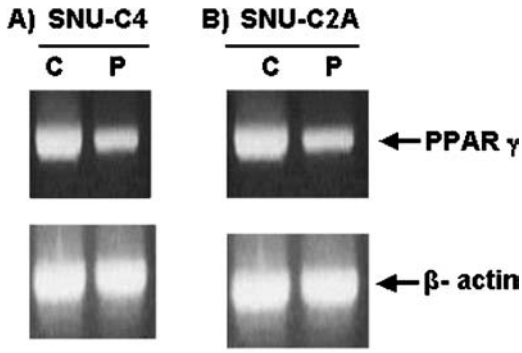
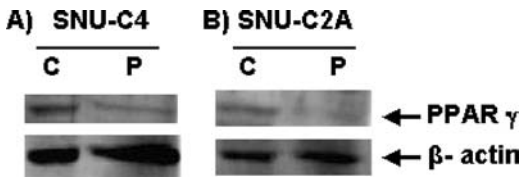


Figure 2. Expression of PPARγ protein in SNU-C4 (A) and SNU-C2A (B) cells treated with 0.1% DMSO (C) or 50 μM pioglitazone (P) for 3 days. Proteins were obtained from both cell lines, separated on SDS-PAGE, probed with an anti-PPARγ antibody or anti-β-actin antibody, and visualized with enhanced chemiluminescence.



Growth inhibitory effect of pioglitazone was partly independent of PPARγ

To test whether these growth-inhibitory effects of pioglitazone were mediated by PPARγ activation, PPARγ antagonist GW9662 was added to the culture medium before treatment with pioglitazone and cell viability was determined by MTT assay. As shown in Figure 4, the inhibitory effect of pioglitazone was partially reversed by the addition of GW9662. A similar result was obtained in the SNU-C2A other cell line (data not shown).

Cell cycle analysis

To investigate the underlying mechanism of growth inhibition by the ligands for PPARγ, we used FACS analysis to reveal the effect of pioglitazone on the cell-cycle profile. The flow cytometry analysis showed a decrease in the relative proportions of G1-cells and an increase in the relative proportions of G2/M-cells in SNU-C4 and SNU-C2A cells after treatment with pioglitazone (Table 1). These results indicate that pioglitazone inhibited growth of SNU-C4 and SNU-C2A cells by inducing a block in the G2/M phase of the cell cycle.

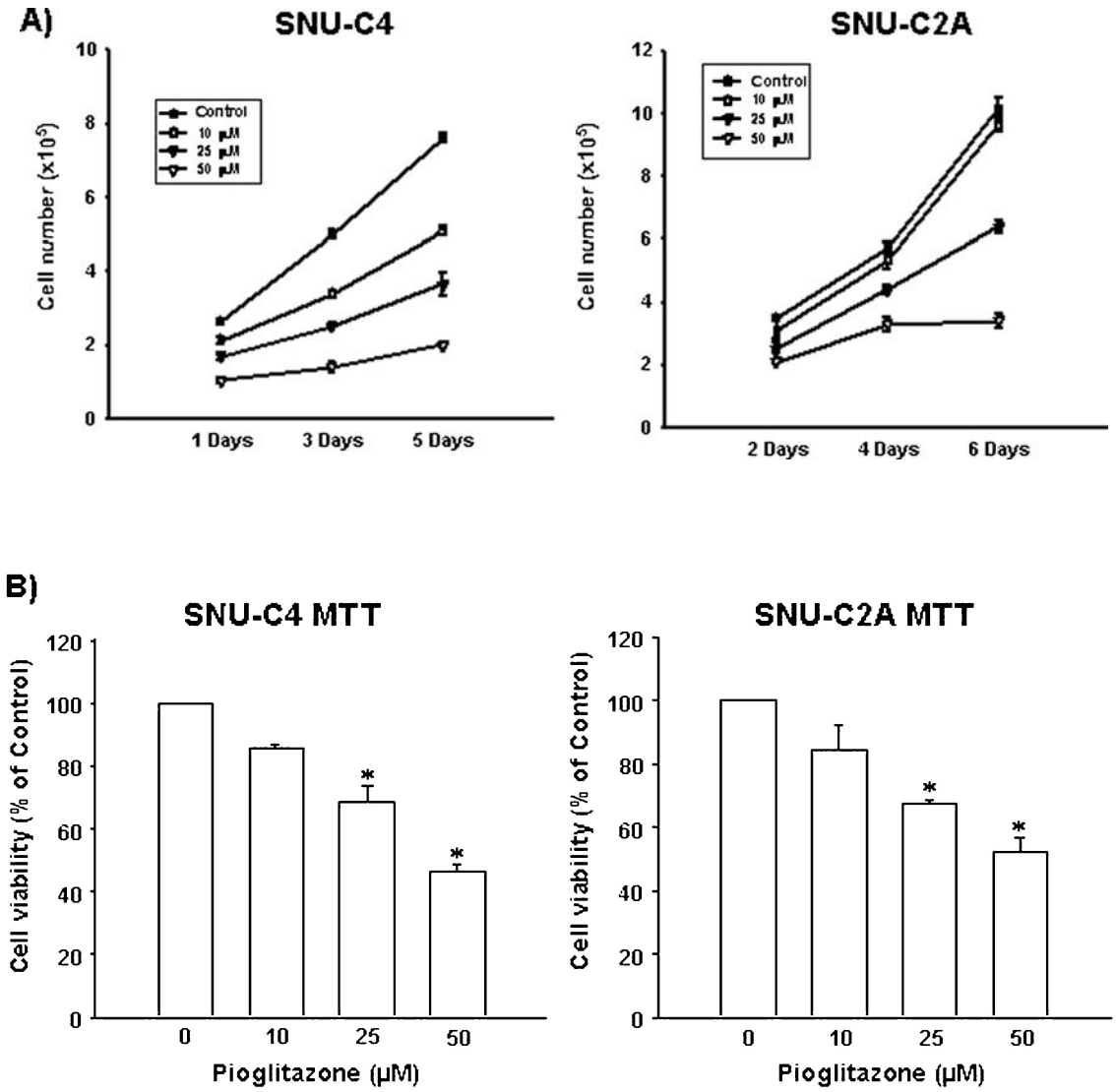
Effect of pioglitazone on the expression of cell cycle-related proteins

Western blot analysis was used to detect changes in expression of cell cycle-regulated proteins by pioglitazone treatment. Pioglitazone strongly decreased or even fully inhibited the expression of the G2/M phase-regulating protein cyclin B1 and cdk1/cdc2, and increased the expression of p21 in both cell lines (Figure 5). These changes may indicate the mechanism by which the G2/M phase is blocked by pioglitazone treatment.

Induction of apoptosis

To delineate whether the inhibition of cell growth by pioglitazone was the result of increased induction of apoptosis, SNU-C4 and SNU-C2A cells were treated with pioglitazone for various time intervals (50 μM for 3 and 5 days) and stained with annexin V conjugated with FITC and counterstained with PI. Pioglitazone treatment resulted in an

Figure 3. Effects of pioglitazone on the growth of SNU-C4 and SNU-C2A cells. (A) Cell counts; SNU-C4 cells and SNU-C2A cells were seeded one day before the treatment and treated with pioglitazone (0, 10, 25, or 50 μM) for 1, 3, and 5 days or 2, 4, and 6 days, respectively. After treatment for indicated times, cell viability was determined by direct viable cell counting; viable cells were determined by trypan blue staining in which unstained cells were considered viable. These data represent the mean ± SD of three independent experiments. (B) MTT assay; cell viability was determined by MTT assay and expressed as a percentage of viability under control culture conditions. These data represent the mean ± SD of three independent experiments. $^*p<0.05$, vs. control cells.



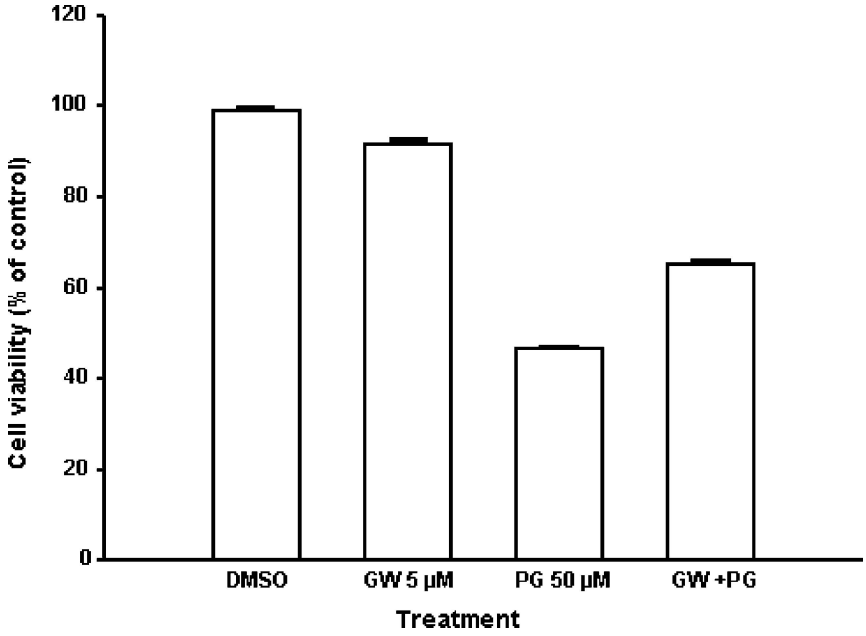
increase of apoptotic cell proportions in a time-dependent manner (Figure 6).

Release of cytochrome c and activation of the caspase cascade by pioglitazone

To gain insight into the mechanism by which the apoptotic process might occur via pioglitazone, we tested whether caspase activation was involved. Caspase 3 expression was detected in both SNU-C4 and SNU-C2A cells with 0.1% DMSO, and incubation with 50 μM pioglitazone resulted in reduced caspase-3 protein expression in both cell lines (Figure 7A). Because the anti-caspase-3 antibody does not recognize cleaved caspase-3, the observed reduction indicates a cleavage and activation of caspase-3 after pioglitazone treatment. PARP is a substrate of activated caspase-3, and its cleavage can be observed in cells expressing caspase-3. We examined the presence of cleaved PARP when the cells undergo apoptosis by pioglitazone. As shown in Figure 7B, PARP was cleaved in the cells that were treated with pioglitazone.

To confirm the involvement of caspase in this pathway, we investigated the effect of pretreatment with the pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp

Figure 4. Growth-inhibitory effect of pioglitazone was partly independent of PPARγ. Cells (1×10^5 cells/ well) were incubated in the presence of pioglitazone (PG, 50 μM), GW9662 (GW, 5 μM), or their combinations (GW+PG) for 3 days. In the case of combinations, GW9662 was incubated for 1 h before treatment with pioglitazone. After incubation, cell viability was determined by MTT assay and expressed as a percentage of viability under control culture conditions. These data represent the mean ± SD of three independent experiments.



(OMe) fluoromethyl ketone (Z-VAD-fmk), on pioglitazone-induced apoptosis. We observed that 50 μM Z-VAD-fmk only partially blocked the ability of pioglitazone to induce apoptosis in SNU-C4 (Figure 7C) and SNU-C2A cells (data not shown). These data suggest that a caspase-independent mechanism may also contribute to the pioglitazone-induced apoptosis in these cell lines. Because cytochrome c is known to be released from mitochondria to cytosol in response to the death signal and to activate the caspase cascade, we examined the release of cytochrome c from mitochondria by Western blot analysis. As shown in Figure 7D, pioglitazone treatment causes a release of cytochrome c into the cytosol in both cell lines.

Changes in expression levels of various apoptosis-related proteins by pioglitazone

As shown above, apoptosis induction by pioglitazone was partially associated with activation of caspase-3, suggesting that PPARγ activation might modulate other apoptosis-related proteins. To further elucidate the

Table 1. The relative proportion of cells (%) in the G1-, S-, and G2/M-phases are shown in control cells and cells treated with pioglitazone*

	% Cell cycle distribution		
Treatment	**G1**	**S**	**G2/M**
SNU-C4 cells			
Control	61.4 ± 1.4	6.2 ± 0.1	32.4 ± 1.2
50 μM PG	48.9 ± 0.5	6.2 ± 0.1	44.9 ± 0.6
SNU-C2A cells			
Control	63.6 ± 1.1	15.8 ± 0.6	20.7 ± 1.1
50 μM PG	49.3 ± 1.0	13.6 ± 0.8	37.0 ± 1.7

*Cell cycle distribution was determined after 3 days of treatment. The percentages were an average calculated on the results of three separate experiments. The value was represented as mean ± SD (n = 3). PG denotes cells treated with pioglitazone.

mechanism of pioglitazone-induced apoptosis in these cell lines, we used Western blot analysis to evaluate the involvement of the Bcl-2 family in the apoptosis process. As shown in Figure 8A and 8B, Bax expression was increased and Bcl-2 expression was decreased in pioglitazone-treated cells. However, no change was observed in Bcl-xL (data not shown). Expression of XIAP was also analyzed by Western blotting. XIAP expression was down-regulated by treatment with pioglitazone (Figure 8C).

Figure 6. The effect of pioglitazone on induction of apoptosis of SNU-C4 (A) and SNU-C2A cells (B). Cells were treated with 0.1% DMSO or 50 μM pioglitazone for 3 or 5 days. The percentage of apoptotic cells was determined by annexin V-FITC/PI staining as described in the "Materials and Methods." Data presented are mean $\pm$ SD from three independent experiments. C denotes control cells; P denotes cells treated with pioglitazone.

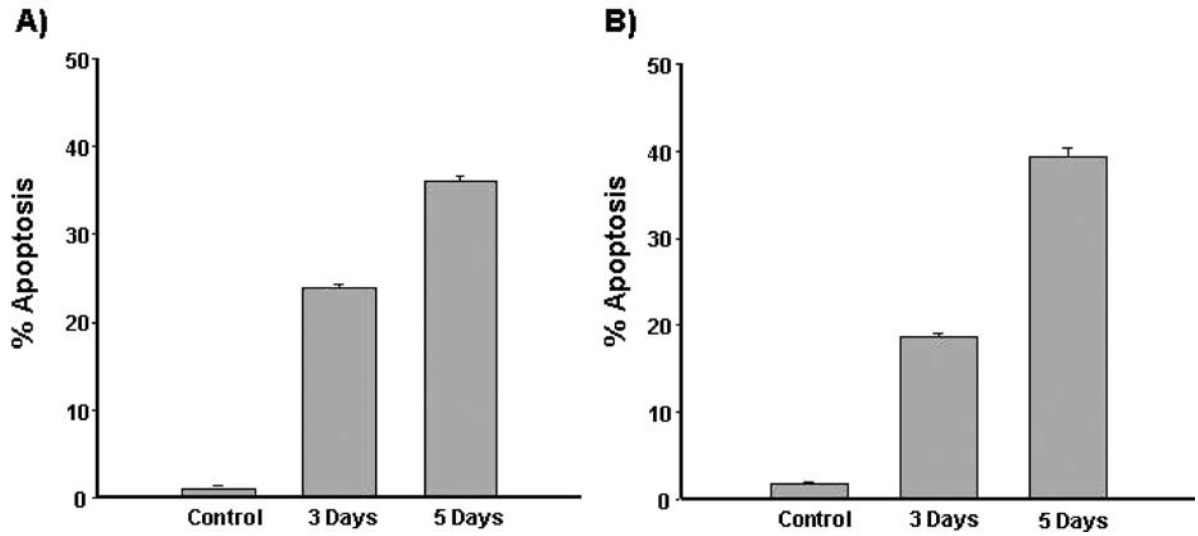


Figure 7. Expression of caspase-3, PARP, and cytochrome c in SNU-C4 and SNU-C2A cells treated with 50 μM pioglitazone for 3 days. Approximately 30 μg of each protein obtained from the cells were separated by SDS-PAGE, probed with anti-caspase-3 (A), anti-PARP (B), and anti-cytochrome c (D) antibodies, and visualized with enhanced chemiluminescence. (C) Partial inhibition of pioglitazone-induced apoptosis by pretreatment with benzyloxycarbonyl-Val-Ala-Asp (OMe) fluotomethyl ketone (Z-VAD-fmk). C denotes control cells (0.1% DMSO); P denotes pioglitazone-treated cells. Values are mean $\pm$ SD of three independent experiments.

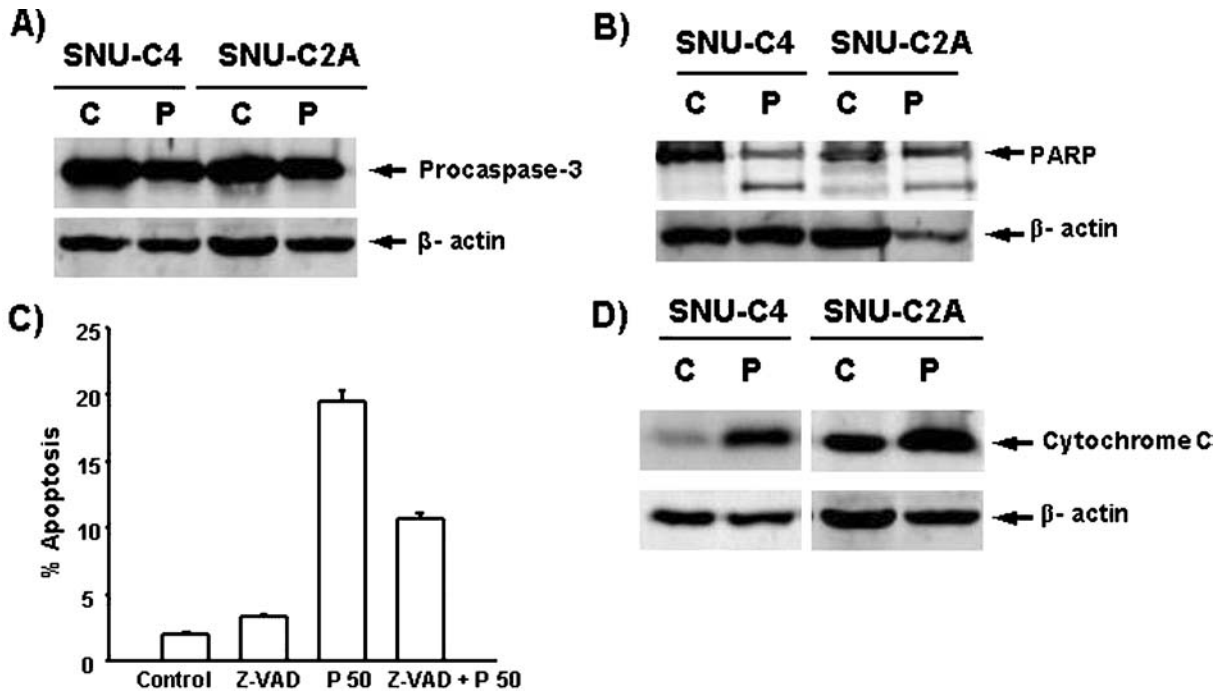


Figure 5. Effect of pioglitazone on the expression of cyclin B1, cdc2, and p21 in SNU-C4 and SNU-C2A cells. SNU-C4 and SNU-C2A cells were treated with 50 μM pioglitazone for 3 days. Approximately 30 μg of each protein obtained from the cells were separated by SDS-PAGE, probed with anti-cyclin B1 (A), anti-cdc2 (B), and anti-p21 antibodies (C), and visualized with enhanced chemiluminescence. C denotes control cells; P denotes cells treated with pioglitazone.

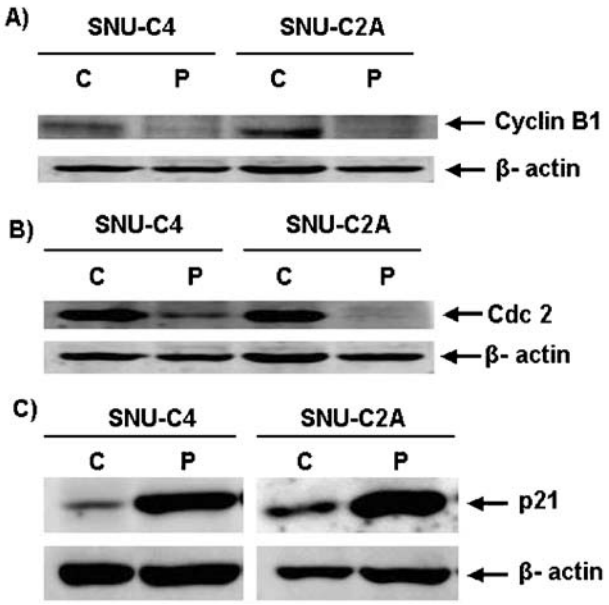
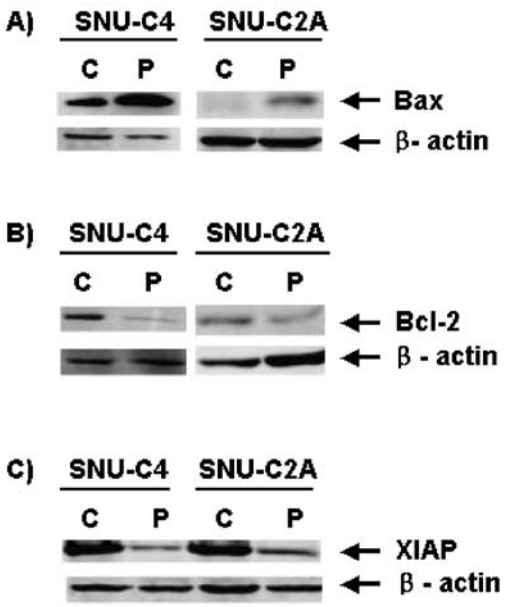


Figure 8. Effect of pioglitazone treatment on expression of apoptosis-related proteins. SNU-C4 and SNU-C2A cells were incubated in the presence or absence of 50 μM pioglitazone for 3 days at 37°C. Proteins were extracted and Bax (A), Bcl-2 (B), and X chromosome-linked inhibitor of apoptosis (XIAP) (C) were detected by Western blot analysis. Each lane was loaded with 30 μg protein. C denotes control cells; P denotes cells treated with pioglitazone.



Effect of pioglitazone on COX-2 expression in SNU-C4 and SNU-C2A cells

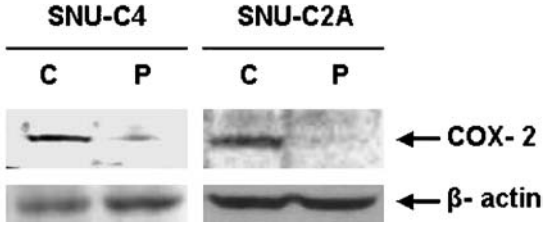
To assess the role of COX-2 in pioglitazone-induced apoptosis, we examined the change in COX-2 expression in SNU-C4 and SNU-C2A cells treated with pioglitazone. As shown in Figure 9, after treatment with 50 μM pioglitazone for 3 days in both cell lines, the expression of COX-2 was decreased.

Discussion

In the present study, we have demonstrated that PPARγ was expressed in SNU-C4 and SNU-C2A cells and that pioglitazone treatment caused a down-regulation of PPARγ in both cell lines (Figures 1 and 2). We evaluated the effects of PPARγ ligand on the cell growth of human CRC cell lines (SNU-C4 and SNU-C2A) using pioglitazone. Pioglitazone is a TZD derivative, representing a novel class of oral drugs for the treatment of diabetes. Troglitazone, also a TZD derivative, causes severe hepatic dysfunction leading to hepatic failure,[26–28] but the mechanism of hepatotoxicity is not known.[28] Earlier investigators have demonstrated the different effects of troglitazone and pioglitazone on cell behavior,[29,30] but previous results have largely been obtained from experiments with troglitazone. Further studies are needed to clarify whether other TZD derivatives have the same effects as troglitazone.

In this study, we used pioglitazone as a PPARγ ligand, instead of troglitazone, because troglitazone has severe hepatic toxicity.[26–28] The treatment of SNU-C4 and SNU-C2A cells with pioglitazone inhibited cell growth in a concentration- and time-dependent manner (Figure 3). This finding is in agreement with the results of Kitamura *et al.*,[10] who showed troglitazone inhibited the growth of colon cancer cells, even though we used a different cell line and different ligand from those in that study. A number of studies have demonstrated that activation of PPARγ by a PPARγ agonist inhibits cell growth and induces cell cycle arrest.[31] On the other hand, PPARγ

Figure 9. Expression of cyclooxygenase-2 (COX-2) by pioglitazone treatment. SNU-C4 and SNU-C2A cells were incubated in the presence or absence of 50 μM pioglitazone for 3 days at 37°C. Proteins were extracted, and COX-2 protein was detected by Western blot analysis. Each lane was loaded with 30 μg protein. C denotes control cells; P denotes cells treated with pioglitazone.



is not always required for some PPARγ ligands to induce cell growth arrest. For example, TZD inhibits cell growth equally efficiently in PPARγ +/+ and PPARγ −/− mouse embryonic stem cells.[32] These studies clearly suggest that TZD may inhibit cell growth through PPARγ-dependent and/or PPARγ-independent mechanisms in a given cell context. Because pioglitazone is an agonist for PPARγ, its growth-inhibitory effect may be through the activation of PPARγ. However, a PPARγ-specific antagonist GW9662 could partially block the growth-inhibitory effect of pioglitazone (Figure 4). These data also suggest that the growth-inhibitory effect of pioglitazone involves a PPARγ-independent pathway, as well as a PPARγ-dependent pathway.

The mechanisms for pioglitazone-induced inhibition of cell growth in SNU-C4 and SNU-C2A cell lines are unknown. The present study demonstrated that pioglitazone treatment resulted in G2/M phase block via the down-regulation of cyclin B1 and cdc2 and up-regulation of p21 in both cell lines (Table 1, Figure 5).

Fajas *et al*.[33] suggested that PPARγ activation in the presence of RB results in the arrest of cells at the G1 phase of the cell cycle, whereas in the absence of RB, cells accumulate in G2/M, endoduplicate, and undergo apoptosis. Both cell lines used in this study are RB-deficient; thus, the effect of the PPARγ ligand on the cell cycle was a G2/M phase block. This finding agrees with the observations of Fajas *et al*.,[33] who demonstrated that rosiglitazone inhibited the cell growth of SaOS cells by means of a G2/M phase block.

We also demonstrated that pioglitazone induced apoptosis in these two cell lines (Figure 6). Consistent with the induction of apoptosis, we observed activation of caspase-3 and the cleavage of PARP, the substrate for active caspase-3 (Figure 7). Pretreatment with Z-VAD-fmk, a pan-caspase inhibitor, partially blocked the pioglitazone-induced apoptosis (Figure 7). This finding suggests that the apoptosis signaling by pioglitazone in SNU-C4 and SNU-C2A cells is mediated by both caspase-dependent and -independent pathways. Some reports have identified caspase-3-independent apoptotic pathways, such as apoptosis induced by exogenous nitric oxide, transforming growth factor-β, cell-permeable peptide SN50, arsenic trioxide (As_2O_3), NF-κB inhibition, or a low-molecular weight fraction of human seminal plasma.[34–40] It also has been reported that a caspase-independent mechanism is partly involved in the 15d-PGJ_2-induced apoptosis of malignant cells,[41,42] which is similar to our findings, even though the ligand for PPARγ used in this study differed from those used in previous studies.

Several studies have shown that there are mitochondrial potential changes and changes in expression of anti-apoptotic proteins during caspase-3-independent apoptosis.[37,40] Bcl-2 and Bcl-xL negatively regulate the sensitivity to apoptosis by inhibiting the mitochondrial pathway, whereas Bcl-xS and Bax are positive regulators or inducers of this pathway.[43] Our data indicated that pioglitazone leads to a dramatic decrease in Bcl-2 and an increase in Bax (Figure 8). The Bcl-xL remained unchanged after pioglitazone treatment (data not shown). We also observed down-regulation of XIAP by pioglitazone in SNU-C4 and SNU-C2A cells (Figure 8). XIAP is the most potent anti-apoptotic inhibitor;[44] thus, modulation of the expression of pro-apoptotic and anti-apoptotic molecules contributes to the decision of the cell about whether to proliferate or undergo apoptosis. These results indicate that the down-regulation of Bcl-2 and XIAP and up-regulation of Bax protein may contribute to pioglitazone-induced apoptosis in SNU-C4 and SNU-C2A cells. Interestingly, our finding that XIAP is down-regulated by pioglitazone in these cell lines is consistent with a previous report on hepatocellular carcinoma cells in which XIAP was also shown to be down-regulated by 15d-PGJ_2.[42] However, the mechanism by which PPARγ ligand down-regulates XIAP expression remains unknown. Further studies are needed to elucidate whether PPARγ ligand selectively down-regulates XIAP via transcriptional, translational, or posttranslational control.

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the biosynthesis of prostaglandins from arachidonic acid.[45,46] COX is encoded by two separate genes, COX-1 and COX-2, both of which participate in the formation of a variety of eicosanoids. COX-1 is constitutively expressed in most tissues; however, COX-2 is an inducible gene that is up-regulated by various stimuli, including mitogens, cytokines, growth factors, and tumor promoters. Previous studies have demonstrated that COX-2 expression is aberrantly increased in various human epithelial cancers in the colorectum, stomach, and bladder.[47–49] Yang *et al*. showed that activation of PPARγ pathway by ciglitazone treatment induced apoptosis and inhibited COX-2 expression in HT-29 human colon cancer cells,[50] but the result was not confirmed in an observation by Lefebvre *et al*.[21] Our data showed that pioglitazone inhibited the expression of COX-2 in SNU-C4 and SNU-C2A cells. Inhibition of COX-2 expression may at least in part explain the mechanism by which pioglitazone induces apoptosis in these cell lines. Further research is necessary in order to elucidate the relationship between PPARγ and COX-2.

Conclusion

Our study clearly shows that PPARγ is expressed in SNU-C4 and SNU-C2A cells and that treatment of these cells with pioglitazone inhibits cell growth through a G2/M phase block and apoptosis. Furthermore, down-regulation of cyclin B1 and cdc2 and up-regulation of p21 may be involved in the G2/M phase block by pioglitazone. Pioglitazone induces apoptosis via caspase-dependent and caspase-independent pathways, accompanied by the decreased expression of Bcl-2, XIAP, and COX-2 and increased expression of Bax. These results suggest that pioglitazone may be of therapeutic importance in the treatment of human CRC. However, further

studies of the effects of pioglitazone on tumor growth *in vivo* and investigations of the molecular mechanism involved in pioglitazone-induced apoptosis are necessary.

Acknowledgment

Supported by the Korea Science and Engineering Foundation through the Medical Research Center for Cancer Molecular Therapy at Dong-A University.

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