Chronic hepatitis B virus (HBV) infection is the most common cause of hepatocellular carcinoma (HCC) worldwide. This study investigated the antineoplastic effects of intrinsic and extrinsic peroxisome proliferator-activated receptor γ (PPARγ) ligands against HBV-associated HCC cells in vitro. Four cell lines that were established from patients with HBV-associated HCC were used. The cells were cultured in various concentrations of the following PPARγ ligands: troglitazone, pioglitazone, rosiglitazone and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2). Cell proliferation, cell cycle and apoptosis were analyzed. PPARγ was expressed in all the cell lines studied. Among the PPARγ ligands, pioglitazone and 15d-PGJ2 clearly inhibited the HBV-associated HCC cell growth and increased the proportion of cells in the sub-G1 phase in the cell-cycle analysis. In apoptosis assays, DNA fragments increased significantly, and the activities of caspase-3 and -9 also increased. A pan-caspase inhibitor and a caspase-3 inhibitor suppressed the PPARγ ligand-induced apoptosis in a dose-dependent manner. These two PPARγ ligands decreased the expression of bcl-2 in most of the cell lines studied. The results suggest that pioglitazone and 15d-PGJ2 have antineoplastic effects on HBV-associated HCC cells. Both of these PPARγ ligands could be candidates for cancer prevention or the chemotherapy of HBV-associated HCC.

Introduction

HCC remains the third highest cause of cancer-related death in Korea, as in the rest of the world, behind lung and stomach cancers, due both to high incidence and poor survival rates (1). In particular, chronic HBV infection is the most frequent etiology of HCC, accounting for more than 75% of all cases in Korea (2). About 50% of patients with chronic hepatitis B (CHB) develop liver cirrhosis within a decade (3-5), and the annual risk of these patients developing HCC ranges from 2 to 4% (6,7).

Although preventing HBV infection through vaccination and adequate antiviral treatment for patients with chronic HBV infection can reduce the risk of HCC (8,9), chemoprevention may be another option for decreasing cancer development in high-risk individuals. The development of HCC in patients with chronic HBV infection is a stepwise process from dysplastic lesions to adenomatous hyperplasia to carcinomas, such as colorectal cancer. Therefore, control of chronic inflammation and reversing cellular dedifferentiation in hepatocarcinogenesis may be potential targets for chemoprevention.

The PPARγ, a member of the nuclear hormone receptor family, plays a role in the regulation of various vital metabolism pathways, such as glucose homeostasis and lipid metabolism (10). Their roles in anti-inflammation, cellular differentiation and induction of apoptosis have also been determined. These receptors heterodimerize with 9-cis-retinoic acid receptors and modulate various target genes after binding to peroxisome proliferator response elements (11).

Treatment with PPARγ ligands has been reported to induce dose-dependent inhibition of cellular growth in some HCC cell lines (12-14) through arrest of the cell cycle in G1 phase and apoptosis, as shown in other human cancer cells (11). However, most of the HCC cell lines examined were not related to chronic HBV infection, and only troglitazone was used as a synthetic ligand in most studies. However, troglitazone has proved to have severe potential hepatotoxicity (15), and thus its clinical application in patients with liver diseases has been limited.
Fortunately, pioglitazone and rosiglitazone have much higher liver toxicity thresholds (16), and administration of these agents may be safe even in patients with some liver diseases (17). To date, however, the studies that examined the antineoplastic effects of these ligands against HCC cells have reported conflicting results (13,18-21).

As a preliminary study to determine the theoretical rationale for using PPARγ ligands for chemoprevention of HCC in patients with HBV-related cirrhosis, this study sought to evaluate the effects of synthetic PPARγ ligands on the proliferation of various HCC cell lines established from patients having HCC with chronic HBV infection (22).

Materials and methods

Cell culture and reagents. Human HCC cell lines, SNU-739, SNU-761, SNU-878 and SNU-886 were supplied by the Korean Cell Line Bank (Seoul, Korea). These cell lines were established from Korean HCC patients chronically infected with HBV (22). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO2 in 95% air. HepG2 was used as a positive control for PPARγ expression. Troglitazone, pioglitazone, rosiglitazone and 15d-PGJ2 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich Inc., St. Louis, MO, USA).

Cell proliferation assay. To evaluate the effects of thiazolidinediones and 15d-PGJ2 on cell viability, HCC cells were treated with troglitazone, pioglitazone, rosiglitazone, and 15d-PGJ2. Cell proliferation was measured with CellTiter 96 Aqueous One solution (Promega, Madison, WI, USA). HCC cells were seeded at 1x10⁴ cells/well in 96-well plates and incubated with 0-100 μM of troglitazone, pioglitazone, rosiglitazone, or 15d-PGJ2 at 37°C for 48 h. Cell viability was determined by a colorimetric assay using a solution containing a tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium] inner salt (MTS), and phenazine methosulfate (PMS) (20 μl/well). Absorbance was determined at 492 nm with background subtraction at 650 nm.

Cell cycle analysis. The effects of various PPARγ ligands on cell cycle distribution were investigated. Cells were plated at a density of 1x10⁵ cells/ml in 6-well plates and incubated with the PPARγ ligands for 48 h at a concentration determined by the MTT assay. After incubation, the cells were harvested by trypsinization and washed with saline (PBS). Cell pellets were resuspended in 0.3 ml PBS, fixed with 0.7 ml cold 100% ethanol, and kept at 4°C. Cells were then washed with PBS and resuspended with 0.5 ml PBS. The cell suspension was incubated with 10 μl RNase A (10 mg/ml) at 37°C for 1 h and stained with 20 μl propidium iodide (1 mg/ml). Flow cytometric analysis of DNA content was performed using a FACScalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and analyzed using Modfit cell cycle analysis software (Verity Software House, Topsham, ME, USA).

Apoptosis assay. For the determination of apoptosis, apoptotic cells were quantified using the cell-death detection ELISA™ (Roche Molecular Biochemicals, Mannheim, Germany). Cells were plated at a density of 1x10⁴ cells/well in 96-well plates. The cells were allowed to attach for 24 h and incubated with the PPARγ ligands for 48 h at various concentrations determined by the MTT assay. After incubation, the cells were lysed with 0.2 ml lysis buffer at room temperature for 30 min and the lysate was centrifuged at 200 x g for 10 min. The lysate was added to streptavidin-coated 96-well plates, and a mixture of biotinylated anti-DNA and peroxidase-coupled anti-DNA antibodies was added. Following a 2-h incubation and subsequent washing, the amount of cytoplasmic nucleosome was quantified by determining the peroxidase staining retained in the immunocomplex, which was determined spectrophotometrically with 2,2'-azino-di[3-ethylbenzothiazoline-sulfonate] as the substrate at an absorbance of 405 nm.

Measurement of caspase protease activity. To elucidate the pathway of apoptosis induced by PPARγ ligands, the enzymatic activities of caspase proteases were measured with a caspase colorimetric assay using caspase colorimetric protease assay sampler kits (BioVision, Mountain View, CA, USA). Cells were plated at a density of 2x10⁵ cells/ml in a 60-mm tissue-culture dish and incubated with 50 μM of pioglitazone and 50 μM of 15d-PGJ2 for 24 h. Cells were harvested by trypsinization, added to 50 μl of lysis buffer, chilled on ice for 10 min and centrifuged at 12,000 x g for 1 min. The supernatants were harvested and the protein concentration was determined by the BCA protein assay method (Pierce, Rockford, IL, USA). The activities of caspase-3, -8, and -9 were measured by proteolytic cleavage of substrates including DEVD-pNA (caspase-3 substrate), IETD-pNA (caspase-8 substrate) and LEHD-pNA (caspase-9 substrate). These colorimetric substrates were solubilized in an assay buffer. After incubation at 37°C for 2 h in the dark, the lysates were measured with a microplate reader (E max microplate reader; Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Caspase-3-, -8-, and -9 activities were determined by direct comparison to the level of caspase activity in the un-induced control.

Caspase inhibitor assay. To determine whether apoptosis by PPAR ligands was caspase-dependent, we investigated the effects of caspase inhibitors on apoptosis of HBV-associated HCC cells. To assess cytosolic histone-bound DNA fragments, the cell-death detection ELISA™ method was used. Briefly, cells were incubated in the presence of 50 μM of pioglitazone, 50 μM of 15d-PGJ2 with or without a pan-caspase inhibitor, Z-VAL-FMK, or a caspase-3-specific inhibitor, Z-DEVD-FMK (R&D Systems, Minneapolis, MN, USA) for 48 h. Cell death was assayed according to the manufacturer’s protocol.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) procedures to determine PPARγ, bcl-2 and bax expression. The mechanism of apoptosis was investigated by examining mRNA expression of an anti-apoptotic protein, Bcl-2, and a pro-apoptotic protein, Bax, with RT-PCR. Total RNA was extracted from cultured cells using the RNA-Bee
solution kit following the manufacturer's protocol (Tel-Test, Friendswood, TX, USA). First-strand cDNA was synthesized by reverse transcription in a total volume of 20 μl reaction mixture containing 1 μg of RNA, 1X reaction buffer, 1 mM dNTP, 5 μM random primers, 20 units RNase inhibitor, and 20 units AMV reverse transcriptase (Promega). The reaction mixture was incubated at 42 °C for 1 h and terminated by heating at 95 °C for 5 min. PCR was performed with 2 μl of cDNA in a 50-μl reaction mixture of 1X PCR buffer, 0.2 mM of each dNTP, 20 pmol primer, and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Primer sequences are shown in Table I. The conditions for amplification were as follows: denaturation at 95°C for 14 min, denaturation at 95°C for 1 min, annealing at 55-58°C for 1 min, extension at 72°C for 1 min (25-30 cycles), and a final extension at 72°C for 7 min. PCR products (10 μl) were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized by image analysis.

Statistical analysis. Values are expressed as the mean ± SD. Student's t-test was used to evaluate differences between the control samples and PPARγ ligand-treated samples. Inhibition of apoptosis was estimated by the differences between the PPARγ ligand-treated sample and caspase inhibitor-treated sample with PPARγ ligands. p<0.05 was considered statistically significant.

Results

The expression of PPARγ mRNA in HBV-associated HCC cell lines. In a similar manner to the results obtained from HepG2, all of the HBV-associated HCC cell lines studied, SNU-739, SNU-761, SNU-878 and SNU-886, demonstrated expression of PPARγ mRNA using RT-PCR (Fig. 1).

The effects of various PPARγ ligands on the proliferation of HBV-associated HCC cell lines. All of the PPARγ ligands used in the study suppressed cellular growth in a dose- and time-dependent manner (Fig. 2). Particularly, the inhibitory effects of pioglitazone and 15d-PGJ2 were evident in all the HBV-associated HCC cell lines studied. HepG2 growth was also inhibited by 15d-PGJ2 and troglitazone, but not markedly.

The effects of PPARγ ligands on cell cycle in HBV-associated HCC cell lines. Pioglitazone and 15d-PGJ2 increased the percentage of apoptotic cells that were in subG1 phase compared...
to the controls in all of the HBV-associated HCC cell lines studied (Fig. 3). The cells in G0/G1 phase were markedly
decreased by 15d-PGJ2 in all the HCC cell lines including HepG2. Troglitazone increased apoptotic cells only in
SNU-761. In contrast, rosiglitazone showed no prominent cell cycle changes in the HCC cell lines studied.

**The effects of PPARγ ligands on apoptosis in HBV-associated HCC cell lines.** Dose-dependent increments in apoptosis were seen in all of the HCC cell lines studied after treatment with pioglitazone and 15d-PGJ2 in a cell-death detection ELISA assay (Fig. 4). The apoptotic effect of troglitazone was evident only in SNU-761 at a concentration of 100 μM. Cells were cultured with 0-100 μM of a ligand for 48 h, and apoptosis was measured using a cell-death detection ELISA kit. Values are expressed as a percentage relative to the control. The data are presented as the mean ± SD of triplicate samples.

Figure 4. Apoptosis induced by various PPARγ ligands in HBV-associated HCC cell lines. Dose-dependent increments in apoptosis were seen in the studied cell lines treated with pioglitazone and 15d-PGJ2. The apoptotic effect of troglitazone was evident only in SNU-761 at a concentration of 100 μM. Cells were cultured with 0-100 μM of a ligand for 48 h, and apoptosis was measured using a cell-death detection ELISA kit. Values are expressed as a percentage relative to the control. The data are presented as the mean ± SD of triplicate samples.

**The effects of PPARγ ligands on the expression of bcl-2 and bax mRNA.** The expression of bcl-2 was suppressed by pioglitazone and 15d-PGJ2 in a time-dependent manner. Pioglitazone decreased the expression of bcl-2 in SNU-739, -761 and -878 cells, while bax expression did not change. 15d-PGJ2 decreased bcl-2 expression in the HCC cell lines studied.

**The effects of caspase inhibitors on PPARγ ligand-induced apoptosis.** With doses ranging from 25 to 100 μM of the pan-caspase inhibitor, Z-VAD-FMK and the caspase-3 inhibitor, Z-DEVD-FMK, the apoptosis induced by pioglitazone and 15d-PGJ2 was reversed in a dose-dependent manner in the HCC cell lines studied (Fig. 6).

Figure 5. Caspase activation by pioglitazone (top) and 15d-PGJ2 (bottom). Caspase-3 and caspase-9 were increased significantly in the HBV-associated HCC cell lines studied, as compared to the control, while caspase-8 was not affected. The HCC cells were incubated with 50 μM pioglitazone and 50 μM 15d-PGJ2 for 24 h. The enzymatic activities of the caspase proteases were measured using a caspase colorimetric assay. Values are expressed as percentages relative to the control. The data are presented as the mean ± SD of three independent experiments. *p<0.05 and **p<0.01, compared to the controls.

**The effects of PPARγ ligands on the expression of bcl-2 and bax mRNA.** The expression of bcl-2 was suppressed by pioglitazone and 15d-PGJ2 in a time-dependent manner. Pioglitazone decreased the expression of bcl-2 in SNU-739, -761 and -878 cells, while bax expression did not change. 15d-PGJ2 decreased bcl-2 expression in the HCC cell lines studied.
studied and increased \( bax \) expression concurrently in SNU-761 cells (Fig. 7).

**Discussion**

PPAR\( \gamma \) ligands have significant antineoplastic effects against various cancer cells, including HCC cells, but whether they can inhibit HBV-associated HCC, which might involve unique hepatocarcinogenesis, is not clear. In this study, we demonstrated that HBV-associated HCC cells, like other HCC cells, expressed PPAR\( \gamma \) and were sensitive to various PPAR\( \gamma \) ligands. The PPAR\( \gamma \) ligands inhibited cell growth in the HCC cells examined in a dose-dependent manner.

Among the PPAR\( \gamma \) ligands used in this study, pioglitazone and 15d-PGJ\(_2\) inhibited cell growth most markedly, although previous studies found that pioglitazone and rosiglitazone inhibited the growth of various HCC cell lines less than troglitazone. Pioglitazone inhibited the cellular growth of HepG2 (13,19,21), HLF (19), Hep3B and PLC/PRF/5 (21) at high concentrations (100 \( \mu \)M) only. Rosiglitazone showed very weak or no effect on HepG2, Hep3B and PLC/PRF/5 (18,21). These findings were confirmed in our recent study.

![Figure 6](image_url)
time-dependent manner, and mRNA levels were measured using RT-PCR. Pioglitazone decreased expression of \( \text{bcl-2} \) and \( \text{bax} \) expression in HBV-associated HCC cell lines. Pioglitazone decreased the expression of \( \text{bcl-2} \) in SNU-739, -761, and -878 cells, while \( \text{bax} \) expression did not change. 15d-PGJ\(_2\) decreased \( \text{bcl-2} \) expression in all the HCC cell lines studied and increased \( \text{bax} \) expression concurrently in SNU-761 cells. The cells were cultured with 50 \( \mu \text{M} \) pioglitazone and 50 \( \mu \text{M} \) 15d-PGJ\(_2\) in a time-dependent manner, and mRNA levels were measured using RT-PCR.

Figure 7. The effects of pioglitazone and 15d-PGJ\(_2\) on \( \text{bcl-2} \) and \( \text{bax} \) mRNA expression in HBV-associated HCC cell lines. Pioglitazone decreased the expression of \( \text{bcl-2} \) in SNU-739, -761, and -878 cells, while \( \text{bax} \) expression did not change. 15d-PGJ\(_2\) decreased \( \text{bcl-2} \) expression in all the HCC cell lines studied and increased \( \text{bax} \) expression concurrently in SNU-761 cells. The cells were cultured with 50 \( \mu \text{M} \) pioglitazone and 50 \( \mu \text{M} \) 15d-PGJ\(_2\) in a time-dependent manner, and mRNA levels were measured using RT-PCR.

In addition, thiazolidinediones are selective receptor modulators (31). Each PPAR\( \gamma \) ligand binds with various cofactors in the nucleus and may act as an agonist or antagonist in different cells. This also occurs with selective estrogen receptor modulators (SERMs) such as tamoxifen. Originally, tamoxifen was described as an estrogen receptor antagonist in the breast, but was later discovered to be an agonist in the uterus. Since the coactivator and corepressor ratios differ in each cell type and the conformational change of PPAR\( \gamma \) receptors after binding to specific ligands also differs, antitumor effects depend on specific ligands and cancer cells (32). This could also explain why the PPAR\( \gamma \) antagonist GW9662 and thiazolidinedione analogs have shown contradictory antineoplastic effects. These ligands might act as an agonist in specific cancer cells. In a microassay test, gene expression profiles differed between troglitazone and pioglitazone in HepG2 cells (33), suggesting that chemical structure determines biological endpoints.

Pioglitazone, a main member of the thiazolidinediones, has played a role in advancement of the treatment of type 2 diabetes. Although thiazolidinediones share similar glucose-lowering effects through a probable PPAR\( \gamma \) pathway, each agent is unique in terms of its anti-proliferative effects, as shown in this study. Hepatotoxicity is more common with troglitazone than with any other thiazolidinediones including pioglitazone. Moreover, pioglitazone has shown anti-inflammatory and cancer-preventive effects in human and mouse liver-disease models, such as acute ischemic liver disease (34), non-alcoholic steatohepatitis (17), and premalignant liver disease (35). If pioglitazone has more specific effects on liver cells, it could be a potential liver-specific antineoplastic agent.

In addition, 15d-PGJ\(_2\) is a cyclopentenone-type prostanoid (PG) derived by the dehydration of prostaglandin \( \Delta_2 \). Unlike other PGs, 15d-PGJ\(_2\) is recognized as a potent endogenous ligand for PPAR\( \gamma \). The inhibitory effects of 15d-PGJ\(_2\) have been noted in various cancer cells (36). HepG2 was prominently inhibited by 15d-PGJ\(_2\), compared to troglitazone (13). The mechanism of its anti-proliferative effects has not been elucidated fully. Not only is 15d-PGJ\(_2\), the most potent endogenous ligand for PPAR\( \gamma \), it also inhibits cell growth via PPAR\( \gamma \)-independent mechanisms, such as the NF\( \kappa \)B pathway (37), the apoptosis pathway including death receptor 5 (DR5), and a specific receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (38). In our...
study, the anti-proliferative and apoptotic effects of 15d-PGJ2 in HCC cell lines were consistent with previous studies, suggesting that 15d-PGJ2 is also a potential antineoplastic agent.

The increased activities of caspase-9 and -3 with no change in caspase-8 activity that we observed also suggest that the apoptosis induced by pioglitazone and 15d-PGJ2 is related to an intrinsic pathway. Similar results with troglitazone in SNU-761 suggest that PPARγ ligands have a common pathway in the induction of apoptosis. Specific caspase-inhibitor assays confirmed that apoptosis was essentially dependent on caspases. As the concentrations of the inhibitors increased, the cellular growth inhibitory effect of PPARγ ligands was reversed.

Although the main mechanisms of apoptosis remain poorly understood, some publications have reported that the intrinsic apoptotic pathway is related to the expression of pro- and anti-apoptotic proteins such as Bax and Bcl-2. For example, bax was upregulated in C6 glioma cells (39) and in renal cancer cells, while bcl-2 was downregulated (40) after treatment with PPARγ ligands. In our study, expression of bcl-2 and bax changed depending on the PPARγ ligands administered and the HCC cell line examined. Pioglitazone and 15d-PGJ2 reduced bcl-2 expression in most cells, while bax mRNA expression did not change, except in the SNU-761 cell line. Among the HBV-associated HCC cells, SNU-761 upregulated bax expression, but not bcl-2, in the presence of 15d-PGJ2. Rosiglitazone had no clear effects on apoptotic gene expression, as shown in other assays. These results suggest that PPARγ ligands, especially pioglitazone and 15d-PGJ2, induce apoptosis at the transcriptional level by diminishing the ratio of bcl-2/bax.

In conclusion, pioglitazone and 15d-PGJ2 induced prominent apoptosis, possibly through the caspase-dependent intrinsic pathway in all the HBV-associated cell lines. The effects of troglitazone were evident in one HCC cell line, SNU-761, but not as prominent in the other cell lines as with pioglitazone and 15d-PGJ2. Rosiglitazone had minimal effects on the cell lines studied. Pioglitazone and 15d-PGJ2 may be candidates for chemoprevention or chemotherapy of HBV-associated HCC. Further investigation is needed to confirm their antineoplastic effects in vivo and to elucidate their potential effects as chemopreventive agents for patients with B viral cirrhosis.

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