Ligands of the peroxisome proliferator-activated receptor γ inhibit hepatoce llular carcinoma cell proliferation

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Abstract. This study was designed to investigate the regulatory role of the peroxisome proliferator-activated receptor γ (PPARγ) in the growth of hepatocellular carcinoma cells under the hypothesis that the levels of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mRNA and the phosphorylated Akt (pAkt) protein would be affected by the presence of different receptor ligand concentrations. SMMC-7721 hepatocellular carcinoma cells were cultured in the presence of different concentrations of either 15-deoxy-prostaglandin J2 (15-d-PGJ2) or pioglitazone and experiments were conducted in order to determine cell growth changes and measure levels of PTEN mRNA and pAkt protein. Our results after treatment with MTT showed the addition of ligands to the cultured cells inhibited their proliferation in a time- and dose-dependent manner. Also, flow cytometry after PI treatment showed the presence of ligands in the growth media could increase the proportion of G0/G1 phase cells, and decrease the proportion of S phase cells. Finally, the same cells exhibited increased levels of the PTEN mRNA by RT-PCR and pAkt protein by western blot analysis. Taken together, our results support the notion that PPARγ ligands can inhibit the proliferation of hepatocellular carcinoma cells in a time- and dose-dependent manner, and that this is at least in part due to the resulting upregulation of PTEN expression.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a class of ligand-dependent sequence-specific nuclear transcription factors that can be divided into three subtypes: PPARα, PPARβ (PPARδ) and PPARγ. PPARγ has complex biological functions and is involved in fat and glucose metabolism, monocyte activation and the inflammatory response; moreover, recent studies have found that it can also regulate tumor cell differentiation and inhibit tumor cell proliferation, features that can play roles in the development of gastrointestinal tumors (1-5). The human PPARγ gene is located in the P25 region of chromosome 3 and spans more than 100 kb. Each species of PPARγ protein is highly conserved, and the amino acid sequences of PPARγ protein in human and mice share a 95% homology (3). The post-transcriptional regulatory effect of PPARγ depends on its binding to ligands, but the exact range of endogenous ligands remain unidentified (4). There are two classes of PPARγ ligands known to exist currently, natural and synthetic ligands. 15-Deoxyprostaglandin J2 (15-d-PGJ2) and pioglitazone are the representatives of the two classes, respectively (5). After ligand binding in the nuclear membrane, heterodimers are formed with retinoic acid receptors, and those dimers bind to the peroxisome proliferator or responsive element in target gene promoter regions to activate their transcription (6). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a recently discovered tumor suppressor gene whose cognate protein can act as both lipid and protein phosphatase (7). A large number of studies have shown that PPARγ ligands can induce cell apoptosis, thereby inhibiting the growth of cancer cells (1-3). The activation of PPARγ can inhibit the proliferation of pancreatic, breast and colorectal cancers, by upregulating the expression of PTEN (8-10). We hypothesized that a PTEN ligand able to stimulate PPARγ could inhibit proliferation in hepatocellular carcinoma cells. In this study, the effect of PPARγ ligands on the growth of hepatocellular carcinoma cells and the changes in PTEN expression during the process were observed *in vitro*, in order to help elucidate a possible mechanism of PPARγ activation regulating the liver cancer growth.

Materials and methods

Cell culture. SMMC-7721 hepatocellular carcinoma cells (Shanghai Cell Institute of Chinese Academy of Sciences) were grown adhering to the walls of flasks, using RPMI-1640 culture solution (Gibco, Carlsbad, CA, USA) containing 10% newborn calf serum (Lanzhou Minhai Biology, Gansu, China), 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator containing 95% O_2 and 5% CO_2 at 37°C.

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Determination of cell viability via MTT assay. MTT was prepared by dissolving 50 mg MTT powder (Amresco LLC, Solon, OH, USA) in 10 ml phosphate-buffered saline (PBS) and stirring with a magnet for 30 min, followed with filtration with a 0.22-μm microporous membrane for sterilization. The sterile solution was stored at 4°C. SMMC-7721 cells in logarithmic growth phase were inoculated into 96-well plates at $5x10^3$ cells/well and incubated for 24 h. Four groups were set up for 15-d-PGJ2 and pioglitazone treatments, a control group with 0 μmol/l of added drug, one with 10 μmol/l, one with 25 μmol/l, and another with 50 μmol/l. 15-d-PGJ2 and pioglitazone were both purchased from Cayman Chemical, Inc. (Ann Arbor, MI, USA) and prepared with dimethyl sulfoxide (DMSO) into a storage concentration of 50 mmol/l. The final concentration of DMSO in cell culture medium was not more than 0.1%. The drugs were added to the wells after cells grew adhering to the wall. A blank control group was set up for zero setting. The cells were incubated in a CO_2 incubator at 37°C for 24, 48 and 72 h after being treated with either drug, then 100 μg MTT were added to each well before and cells were incubated for another 4 h. Then the media were discarded, and the cells were washed with PBS. Finally, 150 μl DMSO were added. The cells were placed on a vibrating platform for 10 min, and the A_{490} values were measured with a Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell viability was calculated from the equation: Cell viability = (A_{490} value in experimental group/A_{490} value in control group) x 100%.

Determination of cell cycle via flow cytometer. SMMC-7721 cells were seeded in 10-cm culture dishes and the media was changed after culture for 24 h. Fresh medium containing 50 μmol/l 15-d-PGJ2 or pioglitazone was added to each dish; cells were incubated for a further 72 h and then washed using PBS. Propidium iodide (PI) was used for staining the DNA inside the cells. The cell cycle analysis, giving the proportion of cells on each phase, was determined by flow cytometry (Bio-Rad Laboratories, Inc.).

Detection of levels of PTEN mRNA expression in cells via reverse transcription-polymerase chain reaction (RT-PCR). RNA extraction. After SMMC-7721 treatment of cells in culture with 0, 25 and 50 μmol/l of either 15-d-PGJ2 or pioglitazone for 72 h, the total RNA was extracted by Tri-Blue reagent (Shenneng Bocai, Shanghai, China) using the one-step method. Briefly, the old culture solution was discarded, and cells were washed twice with pre-cooled PBS. Cell pellets in 1.5 ml Eppendorf tubes were mixed with 1 ml Tri-Blue. Next, 200 μl chloroform was added to each tube, and each tube mixed well in a vortex for 30 sec. After the centrifugation, the supernatant was retained and isopycnic isopropanol was added, followed by centrifugal removal of the supernatant. Cells were finally washed twice using alcohol, followed by drying at room temperature and addition of DEPC water.

RT. Total RNA was used for reverse transcription using MMLV reverse transcriptase. Briefly, 1 μl total RNA, 1 μl Oligo(dT) 18-Primer (0.5 μg/μl) and 10 μl DEPC-treated H_2O were all mixed together uniformly. A low speed centrifugation step was carried out before incubation at 70°C for 5 min to denature secondary structures. Then the samples were placed on ice, and the reactions were prepared by adding 4 μl 5X reaction buffer and 1 μl R1 bolock ribonuclease inhibitor; then 1 μl 10 nM dNTP mix. The tubes were briefly centrifuged, and then incubated at 37°C for 5 min. Next, 1 μl RevertAid MuLV Reverse Transcriptase was added, mixed and incubated for further 60 min. The reactions were stopped at 70°C; and the tubes were placed on ice. The equivalent cDNA obtained was amplified by PCR to detect PTEN mRNA levels with amplified β-actin as the internal reference product. All steps were repeated at least 3 times for each experimental group.

PCR. PTEN primers were synthesized by Shenneng Bocai. PTEN: Upstream primer, 5'-AGACCATAACCCACC ACAGC-3' and downstream primer, 5'-ACCAGTTCGTCCC TTTCCAG-3'; amplified fragment length, 123 bp. β-actin: Upstream primer, 5'-AGCGGGAAATCGTGCGTG-3' and downstream primer, 5'-CAGGGTACATGGTGGTGCC-3'; amplified fragment length, 287 bp. According to the instructions, PTEN primers were dissolved into 10 μmol/l working solution using DEPC-H_2O to prepare 50 μl PCR reactions. The reactions were placed in a thermocycler instrument (Bio-Rad Laboratories, Inc.). The protocol for PTEN amplification was set up with an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 20 sec, and extension at 72°C for 40 sec; and a final extension at 72°C for 10 min. For β-actin amplification the protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were stored at 4°C. Finally, agarose gel electrophoresis was performed to analyze the results.

Detection of the expression of PTEN and pAkt protein via western blot analysis. SMMC-7721 cells were inoculated into 6-well plates and cultured for 24 h under normal conditions. Once the cells adhered to the walls, the medium was changed. The cells were next incubated with 0, 10, 25 and 50 μmol/l of either 15-d-PGJ2 or pioglitazone for 72 h. After washing cells twice with PBS, 150 μl cell lysis buffer [20 mmol/l Tris (pH 7.5), 4 mmol/l EDTA (pH 8.0), 2% SDS] were added to each well to crack open the cell membranes via a cell sonicator (JY96-II; Ningbo Xinzhi Biologic Science and Technology Co., Ltd., Ningbo, China). After a 10,500 x g centrifugation step for 10 min, the supernatants containing the soluble protein fractions were transferred into pre-cooled EP tubes. Protein concentrations were determined by the BCA method. Next, 20 μg total protein were added to loading buffer at a ratio of 4:1, and boiled for 5 min; after the protein denaturation, the samples were loaded onto a gel for electrophoresis and then transferred onto a PVDF membrane (Roche Diagnostics, Basel, Switzerland). Standard western blot protocols were followed (protein blotting system; Bio-Rad Laboratories, Inc.). Mouse monoclonal PTEN antibody (dilution, 1:1,000, catalog no. ab79156) or mouse monoclonal pAkt (dilution, 1:1,000, catalog no. ab105731), purchased from Abcam (Cambridge, MA, USA) were incubated with the membranes overnight at 4°C. TBP was used for membrane washing. Then, HRP-labeled goat anti-mouse secondary antibody (dilution, 1:5,000; catalog no. ab6785; Abcam, Cambridge, MA, USA) was added and incubated at room temperature

Table I. Effects of 15-d-PGJ2 and pioglitazone on the proliferation of SMMC-7721 cells (mean ± SD).

Groups	Drug concentration (μmol/l)	24 h	48 h	72 h
15-d-PGJ2	10	87.61±1.73	81.98±1.85	77.86±2.21
	25	65.70±1.81	61.83±1.72	54.89±1.90
	50	48.52±1.82	47.93±1.94	30.79±2.51
Pioglitazone	10	93.56±2.25	84.73±2.07	82.04±2.19
	25	70.63±2.22	63.67±1.55	58.26±2.34
	50	54.43±1.84	40.96±2.29	33.79±2.93

15-d-PGJ2, 15-deoxyprostaglandin J2.

for 1 h. TBST was used for washing the membrane three times for 5 min. Finally, the ECL chemiluminescence method was used for visualizing the results. Additionally, an α-tubulin antibody (dilution, 1:5,000; catalog no. 5335) was used as the internal reference. Mouse anti-human PTEN monoclonal antibody (dilution, 1:1,000; catalog no. 9556) and mouse anti-human pAkt monoclonal antibody (dilution, 1:1,000; catalog no. 12694) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Peroxidase (HRP)-labeled goat anti-mouse secondary antibody (dilution, 1:5,000; catalog no. 4410) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Statistical analysis. The SPSS 12.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analyses. All variables were presented as mean ± standard deviation (mean ± SD); analysis of variance (ANOVA) and t-test were used for inter-group differences. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of 15-d-PGJ2 and pioglitazone on proliferation of SMMC-7721 cells. SMMC-7721 cells were treated with different concentrations of 15-d-PGJ2 or pioglitazone. It was found that both of the PPARγ ligands had a significant inhibitory effect on the proliferation of SMMC-7721 cells. The inhibitory effects were more pronounced with increasing drug concentrations (p<0.05). Table I obtain details of time- and dose-dependence on cellular proliferation.

Cell cycle effects of 15-d-PGJ2 and pioglitazone on SMMC-7722 cell cycle. 15-d-PGJ2 and pioglitazone can inhibit the proliferation of SMMC-7721 cells, therefore we used PI and flow cytometry to further investigate the ligand effects on the cell cycle. The proportion of G0/G1 phase SMMC-7721 cells after treatment with 50 μmol/l 15-d-PGJ2 or pioglitazone for 72 h was significantly higher than the same proportion in cells without treatment (p<0.05). However, the proportion of S phase cells was significantly decreased (p<0.05), and the proportion of G2/M phase cells was not increased significantly (p<0.05) in the high-dose treatment groups. This apparent inhibition at the level of replication can be seen in Table II.

Table II. Effects of 15-d-PGJ2 and pioglitazone on SMMC-7722 cell cycle (mean ± SD).

Group	Cell cycle distribution (%)		
	G0/G1 phase	S phase	G2/M phase
Control	63.45±1.49	32.19±1.83	4.36±0.64
15-d-PGJ2 (50 μmol/l)	76.87±1.09	18.28±1.46	4.85±0.58
Pioglitazone (50 μmol/l)	78.31±1.28	16.90±1.08	4.79±0.73

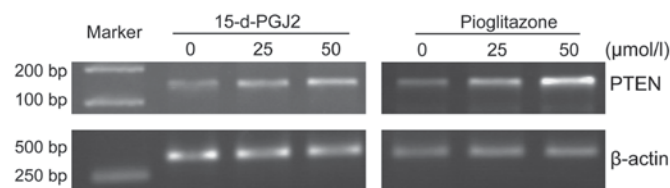15-d-PGJ2, 15-deoxyprostaglandin J2.

Figure 1. Agarose gel electrophoresis after RT-PCR showing the effects of 15-d-PGJ2 and pioglitazone on the expression of the PTEN mRNA in SMMC-7721 cells. Lane 1, 15-d-PGJ2 (0 μmol/l); lane 2, 15-d-PGJZ (25 μmol/l); lane 3, 15-d-PGJ2 (50 μmol/l); lane 4, pioglitazone (0 μmol/l); lane 5, pioglitazone (25 μmol/l); and lane 6, pioglitazone (50 μmol/l). 15-d-PGJ2, 15-deoxyprostaglandin J2; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

Effects of 15-d-PGJ2 and pioglitazone on the expression levels of PTEN mRNA in SMMC-7721 cells. After treating SMMC-7721 cells with 15-d-PGJ2 or pioglitazone for 72 h, it was found that the expression levels of PTEN mRNA were significantly increased in a drug concentration manner for both ligand treatments (Fig. 1).

Effects of 15-d-PGJ2 and pioglitazone on the expression levels of PTEN and pAkt protein in SMMC-7721 cells. After either 15-d-pGJ2 or pioglitazone treatment for 72 h, the expression of PTEN protein in SMMC-7721 cells was increased in a drug concentration manner (p<0.05). At the same time, the
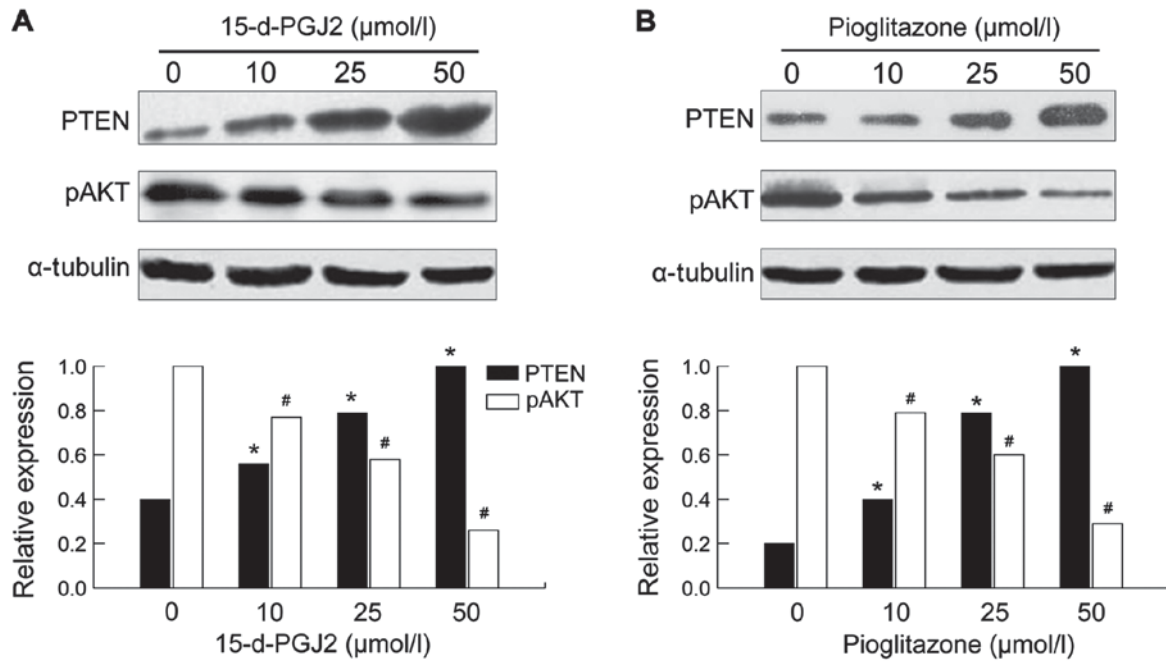
Figure 2. Effect of 15-d-PGJ2 and pioglitazone on the expressions of PTEN and pAkt protein in SMMC-7721 cells. (A) Western blot analysis showed the expression level of PTEN was increased successively after being treated with 10, 25 and 50 μmol 15-d-PGJ2, compared with that after being treated with 0 μmol/l 15-d-PGJ2 (*p<0.05); the expression level of pAkt was decreased successively ($^\#$p<0.01). (B) The expression level of PTEN was increased successively after being treated with 10, 25 and 50 μmol pioglitazone, compared with that after being treated with 0 μmol/l pioglitazone (*p<0.05); the expression level of pAkt was decreased successively ($^\#$p<0.01). 15-d-PGJ2, 15-deoxyprostaglandin J2; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

expression of the protein of the downstream pAkt gene was decreased and the difference was more significant with higher amounts of drug concentration used (p<0.05) (Fig. 2).

Discussion

PPARγ is a class of ligand-dependent sequence-specific nuclear transcription factors, and 15-d-PGJ2 and pioglitazone are its natural and synthetic ligands, respectively (11). The effects of 15-d-PGJ2 and pioglitazone on the growth of SMMC-7721 hepatocellular carcinoma cells under different concentrations and different treatment lengths were observed by MTT assay. The results showed that both drugs had a significant time- and dose-dependent inhibitory effect on the cell growth. Furthermore, an analysis of the relation between the inhibitory effect and the cell cycle performed by flow cytometry, found that 15-d-PGJZ and pioglitazone can both significantly increase the proportion of G0/Gl phase cells and decrease the proportion of S phase cells. This suggests that the inhibitory effect is at least partially related to the inhibition of cell DNA synthesis and mitosis, which blocks the transition of cells from G1 to S phase.

The tumor suppressor function of PTEN has been attributed to its lipid phosphatase activity (12). PTEN can catalyze the dephosphorylation of phosphorylated phosphatidylphthalide 4,5-diphosphoinositol (PIP2) and phosphatidic acid 3,4,5-triphosphoinositol (PIP3) (13). Previous studies have shown that PTEN can specifically inhibit the activation of phosphatidylinositol-3-kinase-dependent protein kinase B (PKB or Akt), and PI3K catalyzes the phosphorylation of phosphoinositide on cell membranes to produce PIP2 and PIP3, the latter of which further activates the downstream

Akt (14). Therefore, PTEN can antagonize the activity of PIP3 and PIP2, and reduce their concentration in cells, thereby inhibiting the phosphorylation of Akt (15,16). Inactivation of PTEN may promote cell proliferation and tumor angiogenesis, by inhibiting cell apoptosis (17-19).

It was found in this study that, after treatment with 15-d-pGJ2 or pioglitazone, SMMC-7721 cells increased the expression levels of their PTEN mRNA in a ligand concentration manner. Furthermore, western blot analysis found that the expression of the PTEN protein was also increased; and correspondingly, the expression of the downstream pAkt gene product (belonging to the PI3K pathway) was decreased more significantly with increasing ligand concentrations. Finally, the results of the MTT assay showed that the proliferation of SMMC-7721 cells was inhibited by the PPARγ ligands in a dose-dependent manner. Taken together, the above results suggest that the inhibitory effect of the PPARγ ligands, 15-d-pGJ2 or pioglitazone, on the proliferation of the hepatocellular carcinoma cells may be achieved by upregulating the expression of PTEN (a cancer suppressor gene), thus affecting the PI3K pathway and inhibiting the phosphorylation of Akt.

At the same time, there is evidence that PTEN can also act as a cell cycle regulator by stimulating P27 expression (8). P27 is an important cyclin kinase inhibitor that can inhibit the activity of G1 phase cyclin-dependent kinase (CDK) and induce the G1 phase cell arrest (9). The results of flow cytometry in our study showed that the proportion of G0/G1 phase cells was increased and that of S phase cells was decreased significantly after addition of PPARγ ligands. This suggests that PPARγ ligands inhibit the proliferation of SMMC-7721 by blocking the transition to S phase, and this could be due to

upregulation of the expression of PTEN, leading to an increase in the p27 protein level, which would induce a G1 phase cell arrest.

In this study, two ligands of PPARγ, 15-d-PGJ2 and pioglitazone, were used to confirm their inhibitory effect on the proliferation of SMMC-7721 hepatocellular carcinoma cells, and this inhibitory effect was related to the expression of PTEN, a tumor suppressor gene. In conclusion, we believe that PPARγ ligands may act as tumor suppressors, which categorizes them as potential clinical treatments against liver cancer.

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