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Effect of activation of PPARγ on cell cycle progression in human gastric carcinoma cells

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Abstract ***Objective:*** To investigate the effect of activation of peroxisome proliferator-activated receptor gamma (PPARγ) on cell cycle arrest of gastric carcinoma cell line MGC803. ***Methods:*** The inhibitory of pioglitazone (PGZ) on proliferation of MGC803 cells was analyzed by MTT assay. Cell cycle was detected by flow cytometry (FCM). The expressions of PPARγ, cyclin D1 and cell cycle protein-dependent kinase CDK4 in MGC803 cells were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). ***Results***: Treatment with 0.1–10 μmol/L PGZ for 96 h significantly inhibited cell proliferation. The proportion of MGC803 cells at G1 phase was significantly increased when treated with 10 μmol/L PGZ for 48, 72 and 96 h, and showed an apparent G1 phase arrest. The expression of PPARγ was at a low level in MGC803 cells and significantly up-regulated when treated with 10 μmol/L PGZ for 48 h ($P < 0.01$). The expression of CDK4 in MGC803 cells was remarkably down-regulated when treated with 10 μmol/L PGZ for 48 h and the expression of cyclin D1 was slightly down-regulated ($P < 0.01$). ***Conclusion:*** Activation of PPARγ significantly induced G1 phase arrest, which was associated with down-regulation of the expressions of CDK4 and cyclin D1.

Key words gastric carcinoma; MGC803 cell; cell cycle; PPARγ

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear hormone receptor that forms heterodimers with the retinoid X receptor and binds to a specific recognition site or peroxisome proliferator response elements (PPRE), in the regulatory region of target genes, e.g. lipoprotein lipase, fat acid synthase, acyl-CoA oxidase. PPARγ has been shown to play a critical role in glucose and lipid metabolism, macrophage function and adipogenesis. There are many known PPARγ agonists such as thiazolidinediones, a synthetic high affinity anti-diabetic drugs, and a number of natural substances including polyunsaturated fatty acids (prostaglandins, leukotrienes, hydroxy-eicosatetraenoic acids) [1]. Recently, the receptor has also been implicated in inflammation and tumorgenesis. Significant evidence from many experimental systems suggests that PPARγ is important in carcinogenesis. PPARγ is up-regulated in malignant tissue, and PPARγ ligands induce terminal differentiation in human breast, colon and lung cancer cells [2–4], and inhibit the growth of human breast, prostate and lung cancer cells [5–7]. These results suggest that PPARγ activation may be implicated in the growth of malignant tumors.

In the present study, we investigated the expression of PPARγ and examined the effects of PPARγ activation on cell cycle in human gastric carcinoma cells.

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Materials and methods

Chemicals

Dimethylsulffoxide (DMSO), ribonuclease (RNase), propidium (PI), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium beomide (MTT), other chemicals and cell culture medium DMEM were purchased from Sigma Chemical Co. (USA). The PPARγ ligand PGZ was purchased from the American Sigma Corporation. All were dissolved in DMSO at 10 μmol/L as a stock solution. The final concentration of DMSO in the culture medium did not exceed 0.1%. All stock solutions were stored at –70 ℃ and were further diluted to appropriate concentrations with medium before use.

The human gastric carcinoma cell line MGC803 was established by Cancer Research Center, Peking University (China). Cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS) 100 units/mL penicillin G 100 μg/mL streptomycin (North China pharmaceutical Group Corporation, China).

Assessment of cell growth

To evaluate the effect of PGZ on cell growth, cell were seeded on a 96-well cell culture plate (Becton Dickinson,

Franklin Lakes, NJ) at a density of 2.0×10^5 cells/well in a volume of 100 μL. Twenty-four hours later, each well was incubated with PGZ at various concentrations (0.01, 0.1, 1.0 and 10 μmol/L). After 72 h, MTT solution was added to each well and cells were incubated for 4 h. The water insoluble formazon was formed during agents incubated in each well. The formazon dye was quantified using a microplate reader (Bio-Rad, Model 550, USA). Cellular proliferation inhibtion rate (CPIR) was calculated using the following formula: CPIR = (1 − Average A value in experimental group / Average A value in control group) × 100%.

Flow cytometry analysis

Cells were seeded in 6-well plates and cultured with 0.1% DMSO or PGZ (10.0 μmol/L) for 48, 72, 96 or 120 h. At the end of incubation, the cells were collected and fixed with 70% ethanol. These samples were treated with RNase (10 μg/mL), stained with propidium iodide (PI) and analyzed with the FACSC alibur flow cytometer (Becton Dickinson, Heidelberg, Germany). All the results were obtained from three independent experiments.

Quantitative reverse-transcription-polymerase chainreaction (real-time RT-PCR)

RT-PCR was done by generating sense strand RNA for PPARγ, CDK4, cyclin D1, β-actin and human glyceraldehydes-3-phosphate dehydrogenase (GADPH). Oligonucle-otides to the corresponding human ligand binding domain were synthesized by TaKaRa Biotechnology (Dalian, China): for PPARγ, sense 5'-TCTGGCCCAC-CAACTTTGGG-3', and antisense 5'-CTTCACAAGCAT-GAACTCCA-3'; for CDK4, sense 5'-CATGTAGACCAGG ACCTAAGG-3', and antisense 5'-AACTGGCGCATCA-GATCCTAG-3'; for cyclin D1, sense 5'-CTGTCGCTG-GAGCCCGTGAAAAAG3', and antisense 5'-GAAGTT-GTTGGGGCTCCTCAGGTT-3'; for β-actin sense 5'-GTGGGGCGCCCAGGCACCAC-3', and antisense 5'-CTCCTTAATGTCACGCACGATTT-3'; for GADPH, sense 5'-CTTTGGTATCGTGGAAGGAC-3', and antisense 5'-GAAATGAGCTTGACAAAGTG-3'. Total RNA was obtained from our experimental cell lines (Trizol, Invitrogen Life Techenologys), and 1 μg and reverse transcribe using random hexamers and avian myelobblastosis virus reverse transcriptase (Promega, Madison, WI). The reverse transcription procedure involved incubation times and temperatures as follows: 10 min at room temperature, 45 min at 42 ℃, and 5 min at 90 ℃. The reverse transcription product was divided into 5 PCR reactions (PPARγ, CDK4, cyclin D1, β-actin and GADPH). The PCR reaction used 94 ℃ for 5-min denaturation temperature and 60 ℃ for 1-min annealing temperature and 72 ℃ for 1-min elongation temperature. The reaction was carried out over 35 cycles. The inductions of PCR was separated on a 1.5% agarose gel and then analyzed by Image Master VDS (Pharmacia Biotech) using Total Lab software.

Statistical analysis

The results were expressed as the mean ± SD and statistically compared with the control group or compared between different drugs by using the one-way AVOVA and multiple comparison of SAS Base 6.12 sorftware (SAS Inc, USA). $P < 0.05$ was statistically significant difference.

Results

PGZ induced growth inhibition

To study the effect of PGZ on human cancer cells, the cells were treated with various concentrations of PGZ for 72 h and cell proliferation was determined by MTT reduction methods. Results demonstrated that treatment with PGZ resulted in a dose-dependent inhibition of proliferation of MGC803 cells (Table 1).

PGZ induced cell cycle arrest at G1 in human cancer cells

Treatment of MGC803 cells with PGZ resulted in a dose-dependent inhibition of cell growth as compared with their untreated controls at all the time points observed. This may involve an arrest of cells at specific check points in the cell cycle. After treatment with PGZ

Table 1 The analysis of cell proliferation in all groups by MTT (%, $\bar{\chi}$ ± s, $n = 5$)

Group (μmol/L)	CPIR (72 h)
0	–
0.01	7.12*
0.1	36.3*
1.0	49.8*
10.0	65.1**

* $P < 0.05$ vs control; ** $P < 0.01$ vs 0.01, 0.1 and 1.0 μmol/L groups

Table 2 The effect of 10 μmol/L PGZ on cell cycle alteration t of MGC803 cells (%, $\bar{\chi}$ ± s, $n = 5$)

Group	G1	S	G2/M
48 h			
Control	67.7 ± 0.3	18.8 ± 1.2	13.5 ± 0.2
PGZ	69.1 ± 0.4	18.1 ± 1.2	12.8 ± 0.2
72 h			
Control	64.0 ± 1.0	22.1 ± 0.9	14.0 ± 0.3
PGZ	69.8 ± 1.4*	18.8 ± 0.3*	11.4 ± 0.2
96 h			
Control	60.3 ± 1.0	24.7 ± 1.9	15.0 ± 1.2
PGZ	76.1 ± 1.4*,**	17.6 ± 1.1*	6.3 ± 0.2
120 h			
Control	63.1 ± 1.3	24.5 ± 1.2	12.4 ± 0.6
PGZ	68.9 ± 1.4	25.6 ± 1.1	5.7 ± 0.3

* $P < 0.05$ vs control; ** $P < 0.01$ vs 48 and 72 h groups

(10 μmol/L) for 48, 72, 96 and 120 h, the results showed an appreciable arrest of cells in G1/S (Table 2 and Fig. 1).

PGZ affected PPARγ expression

MGC803 cells used in this study expressed PPARγ. After exposure to 10 μmol/L PGZ for 48, 72 and 96 h, PPARγ mRNA expression was up-regulated remarkably in MGC803 cells (Fig. 2).

Regulation of CDK4 and cyclin D1 mRNA expression by PGZ

Since cyclin D1 and the CDK4 played key roles in cell cycle progression from the G1 to S phase, we investigated the regulatory effects of PGZ on the expression of these molecules. Cyclin D1 was only slightly down-regulated (Fig. 3), and CDK4 was remarkably down-regulated by PGZ in MGC803 cells for 48 h (Fig. 4).

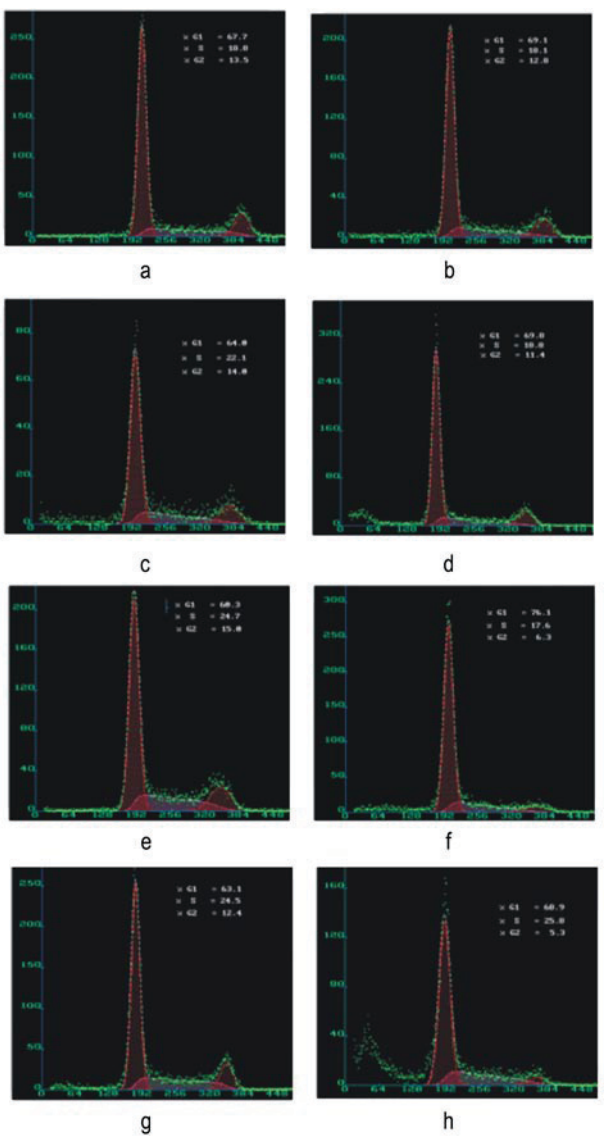

Fig. 1 The cell cycle of MGC803 cells detected by FCM. (a) control for 48 h; (b) MGC803 cells treated with PGZ for 48 h; (c) control for 72 h; (d) MGC803 cells treated with PGZ for 72 h; (e) control for 96 h; (f) MGC803 cells treated with PGZ for 96 h; (g) control for 120 h; (h) MGC803 cells treated with PGZ for 120 h

Discussion

Increasing evidence suggests that PPARγ may be implicated in cell cycle progress. So far, PPARγ expression and effects of PPARγ agonists on cell cycle have been investigated in pancreas cancer, breast cancer and liver cancer [8–12]. However, whether or not gastric cancer cells express PPARγ is not known. In addition, little is known

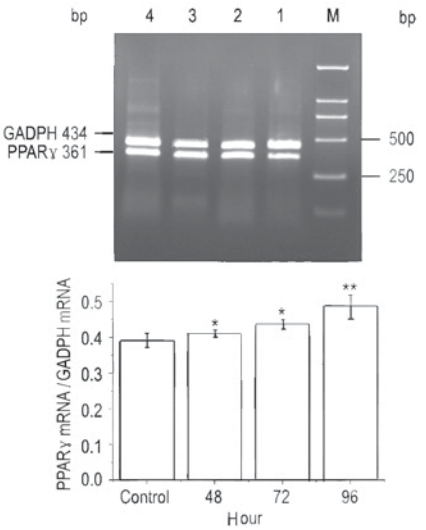

Fig. 2 Effect of 10.0 μmol/L PGZ on mRNA expression of PPARγ in MGC803 cells. Lane 1: control; Lane 2: 48 h after treatment; Lane 3: 72 h after treatment; Lane 4: 96 h after treatment; M: DNA marker. * $P < 0.05$, vs control; ** $P < 0.01$, vs control

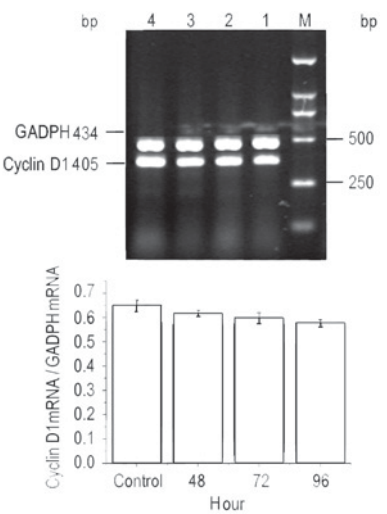

Fig. 3 Effect of 10.0 μmol/L PGZ on mRNA expression of cyclin D1 in MGC803 cells. Lane 1: control; Lane 2: 48 h after treatment; Lane 3: 72 h after treatment; Lane 4: 96 h after treatment; M: DNA marker

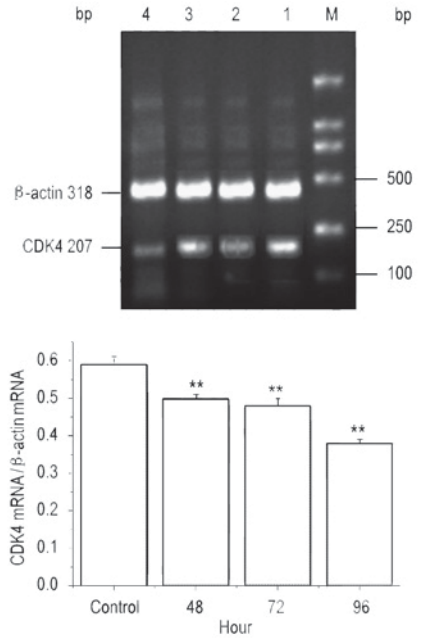

Fig. 4 Effect of 10.0 μmol/L PGZ on mRNA expression of CDK4 in MGC803 cells. Lane 1: control; Lane 2: 48 h after treatment; Lane 3: 72 h after treatment; Lane 4: 96 h after treatment; M: DNA marker. ** $P < 0.01$ vs control

about the action of PPARγ activation on cell behavior in gastric cancer cells. In the present study, we tried to clarify the above two questions in a human gastric cancer cell line, MGC803.

In the present study, we found that PGZ displayed a significantly inhibitory effect on the proliferation of MGC803 cells. Furthermore, the MGC803 cells treated with PLAB (10 μmol/L) for 48 h exhibited a dramatic accumulation of cells in G1 phase of the cell cycle associated with down-regulation of expression of CDK4 and cyclin D1, two key molecules that control the G1 to S progression.

Although the exact mechanism of growth inhibition of tumor cells by PGZ is not well understood, increasing evidence suggests that it is associated with the alteration of the cell cycle. Targeting the key regulators of the G1/S transition such as cyclins, CDKs and CDK inhibitors may be one of the mechanisms by which PGZ induce cell cycle arrest in a variety of tumor cells. In terms of regulation of the cell cycle, cyclin-dependent kinases play a most critical role. In the G1 and S phase, CDK4 and CDK2 kinases are activated by binding to cyclin D and cyclin E/A, respectively. The CDK4/cyclin D1 complex is one of the major regulatory elements governing the G1 to S progression [13]. To further analyze the molecular mechanism by which PGZ causes cell cycle arrest, we evaluated CDK4 and cyclin D1 mRNA levels. In our study, cyclin D1 was only slightly down-regulated by PGZ, and CDK4 was strongly down-regulated by PGZ, suggesting that cell cycle arrest is mediated by the limitation of the supply of CDK4 to cyclin D1 / CDK4 complex formation, which is an essential step in regulating passage into mitosis.

Based on the outcome of this study and available literature knowledge, we suggest PPARγ pathways by which PGZ results in cell cycle arrest. It is tempting to suggest that PGZ has strong potential for development as a chemopreventive and possibly as a therapeutic agent against gastric carcinoma.

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