

# 15d-PGJ<sub>2</sub> inhibits cell growth and induces apoptosis of MCG-803 human gastric cancer cell line

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## Abstract

**AIM:** To investigate the influence of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) ligand, 15-deoxy- $\Delta^{12}$ , 14-prostaglandin J<sub>2</sub> (15dPGJ<sub>2</sub>) on the proliferation and apoptosis of MCG-803 human gastric cancer cell lines.

**METHODS:** Cell proliferation was measured by <sup>3</sup>H-TdR assay. Apoptosis was determined by ELISA and TUNEL staining. Protein and mRNA level of bcl-2 family and COXs were measured by Western blotting and Northern blotting respectively. PGE<sub>2</sub> production was examined by RIA.

**RESULTS:** 15dPGJ<sub>2</sub> inhibited cell growth and induced apoptosis of MCG-803 cells. The COX-2 and bcl-2/bax ratios were decreased following 15dPGJ<sub>2</sub> treatment. The PGE<sub>2</sub> production in supernatants was also decreased. These changes were in a dose-dependent manner.

**CONCLUSION:** 15dPGJ<sub>2</sub> may be a useful therapeutic agent for the treatment of gastric cancer.

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## INTRODUCTION

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a nuclear hormone receptor, provided a strong link between lipid metabolism and the regulation of gene transcription<sup>[1,2]</sup>. Recent studies showed that PPAR $\gamma$  was expressed at high levels in human colon cancer cells<sup>[3-5]</sup>. Ligand activation of PPAR $\gamma$  in human colon cancer cells caused a reduction of growth<sup>[3,4]</sup>. In contrast, two independent groups demonstrated that activation of PPAR $\gamma$  promoted the development of colon tumors in mice<sup>[6,7]</sup>. Thus the roles of PPAR $\gamma$  activation in the growth of colon tumors are controversial. In addition to colon cancer, PPAR $\gamma$  activation induced growth arrest in human liposarcoma, prostate cancer and breast cancer<sup>[8-10]</sup>. These results suggest that PPAR $\gamma$  activation may be implicated in the proliferation and apoptosis

of malignant tumor cells.

Gastric cancer is the most common malignant tumor of gastrointestinal tract in the world. The survival rate in gastric cancer is poor. Although PPAR $\gamma$  expression has been studied in various epithelial cancer cell lines such as colon, prostate and breast, no information is available as to whether PPAR $\gamma$  is involved in the regulation of gastric cancer cell survival. In the present study, we investigated the effect of natural PPAR $\gamma$  ligand, 15dPGJ<sub>2</sub> on the growth and apoptosis of gastric cancer cell lines. We have further examined the role of bcl-2 family in the regulation of 15dPGJ<sub>2</sub>-induced apoptosis. In addition, the change of cyclooxygenase (COXs) and the rate-limiting enzyme in the synthesis of prostaglandins (PGs) have been investigated in the process since COXs is recently considered as a promoter of human gastrointestinal cancer.

## MATERIALS AND METHODS

### Cell line and reagents

MCG-803 gastric cancer cell was kindly provided by Cancer Institute, Zhongshan University. Cells were maintained in RPMI-1640 medium and supplemented with 10 % new fetal bovine serum. Antibodies used in this study were obtained from Santa Cruz Biotech. The apoptosis ELISA kit and RIA kit were purchased from Sigma.

### [<sup>3</sup>H] Thymidine incorporation

Cells were planted in 96-well plates and grown for 24 h after they were serum starved for 48 h. They were treated with 15dPGJ<sub>2</sub> for 48 h and pulsed with 5  $\mu$ Ci of [<sup>3</sup>H] thymidine for 4 h. We counted the radioactivity in Beckman LS counter after washing the cells and stopping the reaction with 5 % trichloroacetic acid and solubilizing the cells in 0.5 % of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and repeated at least three times.

### TUNEL

TUNEL assay was performed using the apoptosis detection system. 15dPGJ<sub>2</sub> (0, 10, 30  $\mu$ M) was added to the culture medium for 48 h. Cells were fixed with 4 % paraformaldehyde in PBS overnight at 4 °C. The samples were washed three times in PBS and permeabilized by 0.2 % Triton X-100 in PBS for 15 min on ice. After washed twice, cells were equilibrated at room temperature for 15-30 min in equilibration buffer (200 mmol/L potassium cacodylate, 0.2 mmol/L dithiothreitol, 0.25 g·L<sup>-1</sup> bovine serum albumin, and 2.5 mmol/L cobalt chloride in 25 mmol/L Tris-HCL, pH 6.6) and incubated in the presence of 5  $\mu$ mol/L fluorescein-12-dUTP, 10  $\mu$ mol/L dATP, 100  $\mu$ mol/L ethylenediaminetetraacetic acid (EDTA), and terminal deoxynucleotidyl transferase at 37 °C for 1.5 h in dark. The tailing reaction was terminated by 2×standard saline citrate (SSC). The samples were washed three times in PBS and analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

### Detection of apoptotic DNA fragmentation

MCG-803 cells were grown in 96-well culture plates. The cells

were incubated with various doses of 15dPGJ<sub>2</sub> for 24 h. Apoptotic DNA fragmentation was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Sigma. This assay was based on a quantitative Sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200  $\mu$ L lysis buffer provided with the kit, the lysates were centrifuged, and 20  $\mu$ L supernatant containing cytoplasmic histone-associated DNA fragments were reacted overnight at 4 °C in streptavidin-coated microtiter wells with 80  $\mu$ L immunoreagent mixture containing biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washED, the immunocomplex-bound peroxidase was probed with 2, 2'-azino-di [3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405 nm.

### RIA for PGE<sub>2</sub>

The amounts of immunoreactive PGE<sub>2</sub> in supernatants were determined by radioimmunoassay (RIA) using a commercially available RIA kit according to the manufacturer's instructions. Briefly, to each polypropylene RIA tube were added 100  $\mu$ L each of anti-PGE<sub>2</sub>, <sup>125</sup>I-PGE<sub>2</sub>, and PGE<sub>2</sub> or the sample. Immune complexes were precipitated 24 h later with 1 ml of 16 % polyethylene glycol solution, and a gamma counter determined the radioactivity in the precipitate. There was no nonspecific interference of the assay by the components of the sample. Determinations were carried out in triplicate and the means and standard deviations were obtained.

### Western blot analysis

The cells were lysed in lysis buffer containing 25 mM Hepes, 1.5 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 g/L eupeptic (pH7.8) at 4 °C with sonication. The lysates were centrifuged at 15 000×g for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (42 mM Tris-HCl, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol and 0.002 % bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated respectively with antibodies against COX-1, COX-2, PPAR $\gamma$ , Bcl-2, bax, bcl-x<sub>i</sub> and  $\beta$ -actin, and then with peroxidase-conjugated secondary antibodies. Detection was performed with enhanced chemiluminescence reagent.

### Northern blot

Total mRNA was isolated and stored at -80 °C until use. The synthetic DNA oligonucleotide probes complemented to COX-1, COX-2, PPAR $\gamma$ , Bcl-2, bax, bcl-x<sub>i</sub> and  $\beta$ -actin mRNA were labeled using terminal deoxynucleotidyltransferase [Boehringer]. Thirty mg of RNA per sample was separated on 1 % agarose-formaldehyde gels and transferred to nylon membranes. The blots were hybridized overnight at 42 °C, washed in 0.2×SSC containing 0.1 % SDS at 50 °C for 10 min, and subjected to autoradiography.

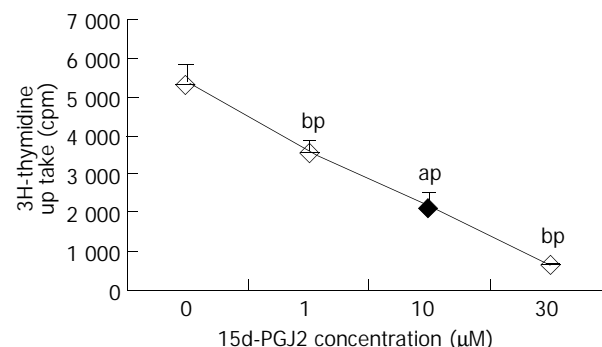
### Statistical analysis

Data were presented as the mean  $\pm$  standard error of the mean, unless otherwise indicated. Multiple comparisons were made for significant differences using analysis of variance, followed by individual comparisons with the Bonferroni post-test. Comparisons between two groups were made with the Student *t* test. A *P* < 0.05 was considered significant.

## RESULTS

### 15dPGJ<sub>2</sub> inhibited growth of MCG-803 cells

To evaluate the effect of 15dPGJ<sub>2</sub> on the growth of MCG-803 cells, 15dPGJ<sub>2</sub> (1, 10, 30  $\mu$ M) was added to the culture medium for 48 h. Cell growth was determined by <sup>3</sup>H-dRT assay (Figure 1). 15dPGJ<sub>2</sub> inhibited MCG-803 cell growth in a dose-dependent manner.



**Figure 1** Effect of 15d-PGJ<sub>2</sub> on cell growth by H<sup>3</sup>-TdR assay (48 h). MCG-803 cells were incubated with various concentrations of 15d-PGJ<sub>2</sub> for 48 h. The value is represented as mean  $\pm$  SEM (*n*=3). <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 versus control group.

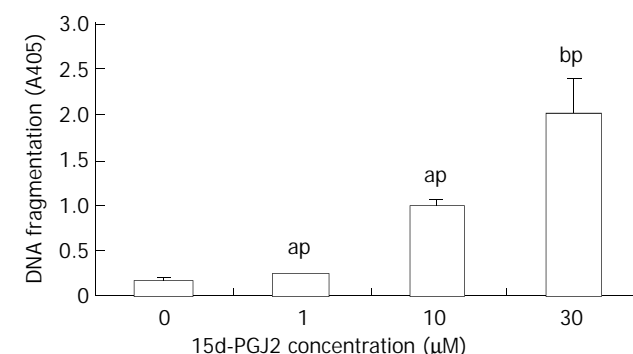
### 15dPGJ<sub>2</sub> induced apoptosis in MCG-803 cells

There are several methods to evaluate apoptosis. In this study, we used ELISA and TUNEL to check for apoptosis of MCG-803 cells after 24 h treatment with 15dPGJ<sub>2</sub>. The percentage of TUNEL-positive cells increased from 13.7  $\pm$  1.5 % to 48.3  $\pm$  2.9 % (Table 1). The same results were obtained by ELISA. The effect of 15dPGJ<sub>2</sub> at concentrations from 1  $\mu$ mol/L to 30  $\mu$ mol/L on DNA fragmentation in MCG-803 cells is shown in Figure 2. 15dPGJ<sub>2</sub> was found to significantly induce DNA fragmentation after the onset of incubation and this effect was in a dose-dependent manner.

**Table 1** TUNEL assay performed using apoptosis detection system

15d-PGJ <sub>2</sub> (μM)	Control	10	30
Percentage of TUNEL-positive cells	13.7±1.5 %	34.9±2.7 % <sup>bp</sup>	48.3±2.9 % <sup>bp</sup>

15dPGJ<sub>2</sub> (0, 10, 30  $\mu$ M) was added to the culture medium for 48 h. The percentage of TUNEL-positive cells was determined. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 compared with respective controls. The value is represented as mean  $\pm$  SEM (*n*=3). <sup>b</sup>*P* < 0.01 versus corresponding control groups.

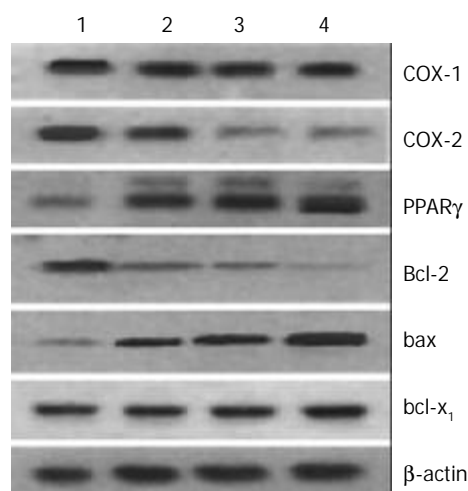


**Figure 2** Effect of 15d-PGJ<sub>2</sub> on DNA fragmentation in MCG-803 cells. Cytoplasmic histone-associated DNA fragments were determined using a commercial ELISA kit. Culture of MCG-803 cells for 24 h in the presence of 15d-PGJ<sub>2</sub> resulted in dose

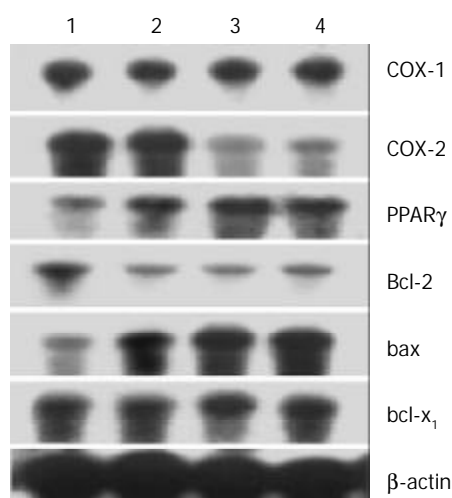
dependent DNA fragmentation. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  compared with the controls. The value is represented as mean  $\pm$  SEM ( $n = 3$ ).

### PPAR mRNA and protein expression in MCG-803 cells increased by 15dPGJ<sub>2</sub>

Since 15dPGJ<sub>2</sub> is a potent PPAR agonist in the events leading to cell apoptosis, we have investigated its effects on the expression of PPAR $\gamma$  mRNA and protein. MCG-803 cells were treated with 15dPGJ<sub>2</sub> in the range of 1–30  $\mu$ M for 6 h. Figure 4 shows that PPAR $\gamma$  mRNA expression was significantly increased in a dose-dependent manner. A Western blot analysis further showed that the mRNA levels encoding PPAR $\gamma$  upon treatment with 15dPGJ<sub>2</sub> could be related to the variation of the corresponding protein (Figure 3).



**Figure 3** COX-1, COX-2, PPAR $\gamma$ , bcl-2, bax, and bcl-XL protein levels in MCG-803 cells treated with 15d-PGJ<sub>2</sub>. Cell lysates were collected and processed at 6 h. The whole cellular protein was electrophoresed in SDS-PAGE gel. Western blot was performed using antibodies against COX-1, COX-2, PPAR $\gamma$ , bcl-2, bax, bcl-XL.  $\beta$ -actin was used as a lane-loading control. (1) control, (2) 1  $\mu$ M, (3) 10  $\mu$ M, (4) 30  $\mu$ M.



**Figure 4** COX-1, COX-2, PPAR- $\gamma$ , bcl-2, bax, bcl-XL mRNA levels in MCG-803 cells treated with 15d-PGJ<sub>2</sub> for 6 h by Northern blot. (1) control, (2) 1  $\mu$ M, (3) 10  $\mu$ M, (4) 30  $\mu$ M.

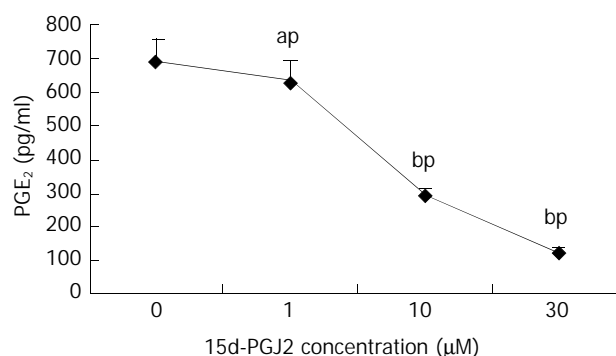
### Effect of 15dPGJ<sub>2</sub> on bcl-2, bcl-XL and bax expression in MCG-803 cells

To further elucidate the mechanism of 15dPGJ<sub>2</sub> induced apoptosis in MCG-803 cells, we evaluated the involvement of bcl-2 family in the apoptosis process by Western blot and

Northern blot analysis. The protein and mRNA of bax were increased and bcl-2 decreased in a dose-dependent manner 6 h after 15dPGJ<sub>2</sub> treatment (Figures 3, 4). No change was observed in protein and mRNA expression of bcl-XL (Figures 3, 4).

### Effect of 15dPGJ<sub>2</sub> on COXs expression and PGE<sub>2</sub> production in MCG-803 cells

To assess the role of COXs in 15dPGJ<sub>2</sub> induced apoptosis, we first examined the changes of COX-1 and COX-2 in MCG-803 cells treated with 15dPGJ<sub>2</sub>. As shown in Figures 3 and 4, the COX-2 protein increased 6 h after 15dPGJ<sub>2</sub> treatment and no change was observed in protein expression of COX-1. In Northern blot analysis, similar results were obtained. The expression of COX-1 mRNA was not changed and COX-2 mRNA was not regulated by 15dPGJ<sub>2</sub> in dose dependent manners. We used specific RIA to measure the production of PGE<sub>2</sub> as COXs catalyzed the rate-limiting step in the biosynthesis of prostaglandins. Parallel to the inhibition of COX-2, decrease of PGE<sub>2</sub> was found in culture medium of HCG-803 cells stimulated by 15dPGJ<sub>2</sub> (Figure 5). These results further confirmed that COX-2, not COX-1 activity was changed by 15dPGJ<sub>2</sub>.



**Figure 5** Effect of 15d-PGJ<sub>2</sub> on PGE<sub>2</sub> production by RIA (24 h). MCG-803 cells were incubated at various concentrations of 15d-PGJ<sub>2</sub> for 24 h. The value is represented as mean  $\pm$  SEM ( $n = 3$ ). <sup>a</sup> $P < 0.05$  and, <sup>b</sup> $P < 0.01$  versus corresponding control groups.

## DISCUSSION

Peroxisome proliferator-activated receptor (PPAR)  $\gamma$ , a member of the steroid nuclear hormone receptor superfamily<sup>[11]</sup>, has been known to trigger adipocyte differentiation and lipid storage by regulating the expression of genes critical for adipogenesis<sup>[12]</sup>. PPAR $\gamma$  functioned as a ligand-dependent transcription factor<sup>[13]</sup>, which upon heterodimerization with the retinoid X receptor (RXR)<sup>[14]</sup>, bound to specific response elements termed peroxisome proliferator response elements (PPRE). This in turn regulated the expression of target genes<sup>[15,16]</sup>. Activation of this receptor has been implicated in tumor promotion, cellular differentiation and apoptosis<sup>[17]</sup>. There are a number of naturally occurring agents that activate PPAR $\gamma$ . Some J-series prostaglandins have been found to bind to PPAR $\gamma$  in low micromolar range<sup>[18,19]</sup>. The PGD<sub>2</sub> derivative, 15-deoxy-12, 14-PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>) was a high affinity ligand ( $K_d = 300$  nM) that demonstrated anti-inflammatory<sup>[20,21]</sup> and anti-neoplastic activity<sup>[22]</sup>.

The anti-neoplastic activity of PPAR $\gamma$  ligand prompted us to determine whether 15dPGJ<sub>2</sub> would trigger MCG-803 cells to undergo apoptosis. The results from Western blotting and Northern blotting have demonstrated that the PPAR $\gamma$  gene and protein expression were clearly observed in MCG-803 cells. 15dPGJ<sub>2</sub> stimulated the expression of PPAR $\gamma$  mRNA and its product. Coincubation of MCG-803 cells with 15dPGJ<sub>2</sub> potentially inhibited cell growth and induced apoptosis in a dose-

related fashion. The dose-dependent suppression of cell growth in cancer cells was also reported after a number of synthetic PPAR $\gamma$  ligands such as thiazolidinediones (TZDs) and non-steroidal anti-inflammatory drugs (NSAIDs) treatment<sup>[9]</sup>. These results suggest that activation of the PPAR $\gamma$  pathway by 15dPGJ<sub>2</sub> induces MCG-803 cells to undergo apoptosis.

In this study, the well-known important mediator of apoptosis, bcl-2 family was examined in MCG-803 cells treated with 15dPGJ<sub>2</sub>. In bcl-2 family, bcl-2 and bcl-X<sub>L</sub> were anti-apoptotic, whereas bax, bad and bak were pro-apoptotic<sup>[23]</sup>. The ratio of anti-apoptotic factor to pro-apoptotic factor in the cells was assumed to be a critical mechanism in maintaining normal homeostasis. A higher concentration of bax compared with bcl-2 enhanced susceptibility to apoptosis. Nevertheless, the cell continued to survive if bcl-2 predominated over bax. One of the most significant problems in the treatment of cancer was the resistance of cancer cells to chemotherapy-induced apoptosis. Of gastric cancers, 72 % bcl-2 overexpression and 33-40 % bax frameshift mutation were observed<sup>[24-26]</sup>. A higher ratio of bcl/bax strongly blocked gastric cancer cell apoptosis by inhibiting cytochrome C release from mitochondria and Caspase-3 activation<sup>[27-31]</sup>. Our data indicated that 15dPGJ<sub>2</sub> led to a dramatic decrease in bcl-2 and increase in bax. The bcl-X<sub>L</sub> remained unchanged after 15dPGJ<sub>2</sub>. In addition, the decreased ratio of bcl/bax was detected prior to commencement of cell apoptosis after 6 h treatment. These results suggested a direct effect of PPAR pathway on bcl/bax expression. Therefore we concluded that the change of bcl-2 family might play a key role in 15dPGJ<sub>2</sub>-induced apoptosis in MCG-803 cells.

Cyclo-oxygenase (COX) was the rate-limiting enzyme that catalyzed the initial step in biosynthesis of prostaglandins (PG) from arachidonic<sup>[32,33]</sup>. COX is encoded by two separate genes, COX-1 and COX-2, both of which participate in formation of a variety of eicosanoids. COX-1 is expressed constitutionally in most tissues and has been proposed to be a housekeeping gene involved in cytoprotection of gastric mucosa, vasodilation in the kidney, and control of platelet aggregation. In contrast, COX-2 is an inducible immediate early gene that is upregulated by various stimuli including mitogens, cytokines, growth factors, and tumor promoters. Accumulating evidence has shown that increased PGE<sub>2</sub> levels via overexpression of the inducible COX-2 isoform were important in the development of human cancer<sup>[34]</sup>. Previous studies have demonstrated that COX-2 expression was aberrantly increased in various human epithelial cancers, including those of colorectal<sup>[35,36]</sup>, esophageal<sup>[37]</sup>, gastric<sup>[38]</sup>, lung<sup>[39]</sup> and bladder origin<sup>[40]</sup>. Data in this study also showed apparent COX-2 expression in MCG-803 cells. These findings suggest that cellular upregulation of COX-2 and PGE<sub>2</sub> may be a common mechanism in epithelial carcinogenesis.

In gastrointestinal system, it has been reported that COX-2 overexpression led to the inhibition of apoptosis or altered cell cycle kinetics in epithelial cells<sup>[41,42]</sup>. Numerous epidemiological studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) decreased incidence of gastrointestinal cancers and COX-2 was recognized as a major target of NSAIDs. Inhibition of COX-2 by NSAIDs or COX-2-specific inhibitors caused cell death in cancer cells<sup>[43-49]</sup>, indicating that COX-2 was an important molecular target for prevention and treatment in gastrointestinal cancers. In this study, a crucial issue yet to be resolved was whether COX-2 inhibition played a role in the induction of apoptosis by 15dPGJ<sub>2</sub> in human gastric carcinoma. We found decreased expression of COX-2 mRNA and protein as well as PGE<sub>2</sub> production in MCG-803 after 15dPGJ<sub>2</sub> treatment. Our current study demonstrated that the decrease in COX-2 expression occurred prior to apoptosis, suggesting that down-regulation of COX-2 might be an upstream event of 15dPGJ<sub>2</sub>-induced apoptosis. These changes may imply that

15dPGJ<sub>2</sub> interferes with the expression of COX-2 and PGE<sub>2</sub> production, thereby contributing to induced apoptosis in MCG-803 cells.

The premise is that COX-2 inhibitor has antitumor effect is based on the assumption that prostaglandins and other COX-2 generated downstream mediators promote tumor cell proliferation, survival, and angiogenesis in an autocrine and/or paracrine manner<sup>[50-53]</sup>. It was also reported that selective cyclo-oxygenase-2 inhibitor induced apoptosis and down-regulated bcl-2 expression in cancer cells<sup>[54]</sup>. Sheng H also found modulation of apoptosis and Bcl-2 expression by PGE<sub>2</sub> in human colon cancer cells<sup>[55]</sup>. However, Hsu AL reported that the cyclo-oxygenase-2 inhibitor celecoxib induced apoptosis by blocking Akt activation in human prostate cancer cells independent of Bcl-2<sup>[56]</sup>. In this study no data proved that Bcl-2 was one of the downstream mediators when COX-2 was inhibited by 15dPGJ<sub>2</sub>. The precise mechanisms on the molecular interaction of COX and bcl-2 expression in 15dPGJ<sub>2</sub> induced gastric apoptosis should be established by more profound analysis.

In summary, our data demonstrate that 15dPGJ<sub>2</sub> inhibited the growth of human gastric cancer cells (MCG803). It is proposed that the decrease of bcl-2/bax ratio and COX-2 be responsible for the apoptotic process in MCG803 cells. Furthermore, PPAR $\gamma$  ligand is a new strategic approach to provide new anticancer therapies.

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