RESEARCH ARTICLE

Ubiquitination of p53 is Involved in Troglitazone Induced Apoptosis in Cervical Cancer Cells

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Abstract

Peroxisome proliferator-activated receptor gamma (PPAR-γ), a ligand-dependent nuclear transcription factor, has been found to widely exist in tumor tissues and plays an important role in affecting tumor cell growth. In this study, we investigated the effect of PPAR-γ on aspects of the cervical cancer malignant phenotype, such as cell proliferation and apoptosis. Cell growth assay, Western blotting, Annexin V and flow cytometry analysis consistently showed that treatment with troglitazone (TGZ, a PPAR-γ agonist) led to dose-dependent inhibition of cervical cancer cell growth through apoptosis, whereas T0070907 (another PPAR-γ antagonist) had no effect on Hela cell proliferation and apoptosis. Furthermore, we also detected the protein expression of p53, p21 and Mdm2 to explain the underlying mechanism of PPAR-γ on cellular apoptosis. Our work, finally, demonstrated the existence of the TGZ-PPAR-γ-p53 signaling pathway to be a critical regulator of cell apoptosis. These results suggested that PPAR-γ may be a potential therapeutic target for cervical cancer.

Keywords: Cervix - PPAR-γ - troglitazone - p53 - ubiquitination of p53 - apoptosis

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Introduction

Cervical cancer is the second most common major gynecologic cancer and the leading cause of death from gynecologic malignancies. This cancer type, usually undetected during the early stage, is easily diagnosed in its advanced stage, due to which, the traditional treatment is poor. Obviously, the proposed molecular mechanisms on cervical cancer progression are important for developing potentially novel treatment strategies.

Peroxisome proliferator-activated receptors (PPAR)-γ belongs to the nuclear hormone receptor super-family of ligand-dependent transcription factors (Canbay et al., 2012). PPAR-γ ligands are classified as natural and synthetic (Zhou et al., 2008). The former include long-chain polyunsaturated fatty acid and eicosanoids, and the latter include thiazolidinediones (Han and Roman, 2006). Ligand-activated PPAR-γ, forming a heterodimer complex with the retinoid X receptor (RXR), which then binds to the peroxisome proliferatior response element (PPRE) within the promoter of target genes and modulates their expression. Previous reports have shown that PPAR-γ can play its role in biological processes, including lipid biosynthesis, glucose metabolism, anti-inflammatory response and atherosclerosis (Dong et al., 2009;Ueno et al., 2012). Recently, PPAR-γ has been demonstrated to potentially play an important role in the control of tumor progression, including cell proliferation, apoptosis, angiogenesis and metastasis (Jung et al., 2005). However, to the best of our knowledge, no report is available that addresses the role of PPAR-γ-ubp53 in the apoptosis of cervical cancer. Thus, the present study has been designed to investigate whether there is any association of PPAR-γ and ub-p53 in the advancement of cervical cancer.

Our study used cervical cancer cell lines Hela that endogenously expresses PPAR-γ as an experimental model to study the effects of PPAR-γ ligand Troglitazone (TGZ) and T0070907 on proliferation and apoptosis of cervical cancer cells. It also analyzed the molecular mechanisms of these effects in an attempt to provide experimental clues for the use of PPAR-γ ligands in the treatment of cervical cancer.

Materials and Methods

Cells and reagents

The human Henrietta Lacks cell line (Hela) was obtained from Shanghai Cell Collection (Shanghai, China). Hela cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO BRL, Grand Island, NY) containing 5% fetal bovine serum (FBS, GIBCO-BRL), 2mM L-glutamine, and 100 units/ml penicillin/streptomycin and a 5% CO_2 environment at 37°C.

TGZ and T0070907 were purchased from Tocris.

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Antibodies for PPAR-γ, Bax, Bcl-2, p53, p21, Mdm2, β-actin, ubiquitin and protein A Agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody for pre-caspase3 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences, San Jose, CA, USA. Cck8 cell proliferation Kit and TRI Reagent were purchased from Sigma, St. Louis, MO, USA. MG132 was purchased from Sigma-Aldrich Co (St. Louis, MO). RNeasy Mini Kit was purchased from Qiagen. LightCycler DNA Master SYBR Green I kit, and LightCycler Control Kit DNA were purchased from Roche Diagnostics GmbH, Mannheim, Germany.

Western blotting

After 48h of treatment with TGZ (0, 8, 20 and 40μM) and T0070907 (0, 6.25, 12.5 and 25μM), cells were incubated in lysis buffer containing 20mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 150mmol/L NaCl, 10% glycerol, 1mmol/L Na3VO4, 50mmol/L NaF, 100mmol/L phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture for 20 minutes on ice. After insoluble debris was pelleted by centrifugation at 14, 000g for 15 minutes at 4°C, the supernatants were collected and determined for protein content using the Bradford method. Proteins (80μg) were resolved under denaturing conditions by SDS-PAGE (10%) and transferred onto nitrocellulose membranes. After blocking for 2h in phosphate-buffered saline with 0.1% Tween20 (PBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4°C with the appropriate primary antibody in PBST containing 3% BSA. Membranes were then washed and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG or anti-mouse IgG (1:10000) in PBST for 2h and developed using NBT/BCIP color substrate. The bands on the membrane were scanned for the density and analyzed with the image analyzer (Labworks Software).

Flow cytometry

For apoptosis assay, Hela Cells were seeded onto a six-well plate and allowed to grow to 50% confluence. Cells were collected 48h after treatment with TGZ (0-40 μM), T0070907 (0-25 μM) and subsequently stained with annexin V-FITC apoptosis detection kit as described by the manufacturer. Apoptosis cell fraction was analyzed using FACScan cytometer. Flow cytometry analysis data were performed on FlowJo Software.

Measurement of apoptosis by Annexin V analysis

Hela cell lines were analyzed for apoptosis, using the Annexin V-FITC kit. Briefly, cells (1 ml, 2×10^5/ml per well) were plated in 24 well plates. When cells were grown to 70-80% confluence, cells were treated with TGZ for 48h. Then, cells were washed twice with medium, then incubated with Annexin V-FITC, and then visualized under a fluorescence microscope. Under microscopy, 6 fields were randomly selected from every sample and independent observers performed cell counting in a blind fashion. The apoptotic rate = (number of total apoptotic cells/total number of cells) × 100%.

Cell growth assay

The carcinoma cells (1×10^4 per well) were incubated in triplicate in a 96-well plate and treated with TGZ at different concentrations. Cell survival rate was evaluated by cell count kit-8 assay. Approximately 5,000 cells/well for Hela cell lines was seeded on 96-well plate after incubated for 24, 48 and 72 h. Six parallel experiments in each sample were used to assess the cell viability. Then, 100μl serum-free culture medium and 10 μl CCK-8 solution were added into each well, followed by incubation at 37°C for 2 h. Absorbance at a wavelength of 450nm was recorded using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

Immunoprecipitation

For immunoprecipitation, cytosolic fractions (each containing 400 μg of proteins) were diluted four-fold with HEPES buffer containing 50mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM each of EGTA, EDTA, PMSF and NaVO. The samples were then pre-incubated for 1 h with 20ul protein A Agarose and centrifuged to remove any non-pecifically adhered proteins from the protein A Agarose. The supernatant was then incubated with 2 ug p53 antibody overnight at 4°C. After the addition of Protein A Agarose, the mixture was incubated at 4°C for an additional 2h. Samples were triple washed with HEPES buffer and eluted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer then boiled at 100°C for 5 minutes. Immune complexes were separated by 10% SDS-PAGE and analyzed by Western blotting as described above.

Quantitative Real-Time RT-PCR

Total RNA from the tumor cells was obtained by using the RNeasy Mini Kit. Approximately 0.1 μg of total RNA was used for reverse transcription. Reverse transcription was performed at 22°C for 10 min and then at 42°C for 20min. mRNA copy number of p53 was determined by real-time quantitative RT-PCR using a LightCycler instrument, a LightCycler DNA Master SYBR Green I kit, and LightCycler Control Kit DNA. PCR amplifications were done in glass capillary tubes. Amplification began with a 600s denaturation step at 95°C followed by 40 cycles of denaturation at 95°C for 15s annealing at 62°C for 10s. The sequence of the p53 primers used in these reactions were as follows: p53: 5/-ATA GTG TGG TGG TGC CCT ATG A-3/ (sense), 5/-TGT GAT GAT GGT GAG GAT GG -3/ (antisense). Data were analyzed by using LightCycler

Statistical analysis

Values are expressed as mean ±SD. Statistical analysis of the results was carried out using the Student's t-test or one-way analysis of the variance (ANOVA) followed by the Duncan's new multiple range method or Newman-Keuls test. *P*-values<0.05 were considered significant.

Results

TGZ inhibits cervical cancer cell proliferation

In order to investigate the biological functions of

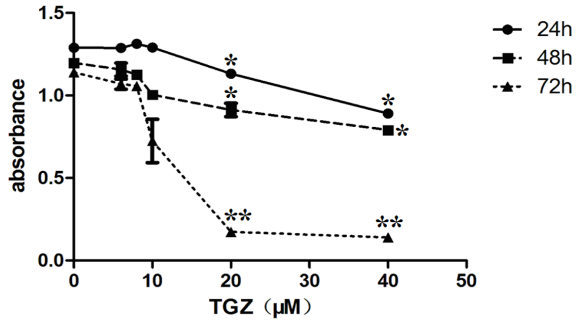

Figure 1. Effects of PPAR-γ Ligand-TGZ on the Viability of Human cervical Cancer Cells. Hela cells were treated with TGZ 0, 6, 8, 10, 20, 40μM for 24, 48, and 72h and cell viability was measured by the Cck8 assay and expressed as % of control culture conditions (0μM TGZ). The data were represented as mean ± SD (n =3). *P<0.05 **P<0.01 versus control groups

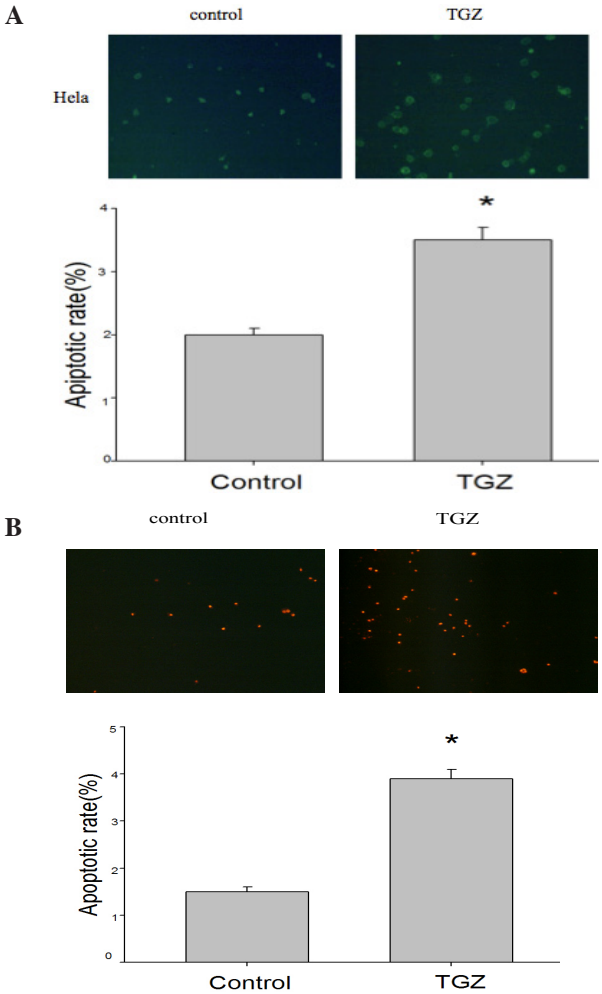

Figure 2. Hela Cells were Treated with TGZ (0, 20μM) for 24 Hours. Cells were analyzed for apoptosis by Annexin V assay. A Prophase apoptotic cells were recognized by binding with FITC on the membrane. B Late apoptotic cells were recognized by binding with FITC on the nuclear chromation and its fragmentation. Data are presented as mean ± SD (n =3). *P < 0.05 versus control groups

PPAR-γ and its role in carcinogenesis, we examined the cell proliferation of cervical cancer cells (Li et al., 2005). In this study, Cell counting assay was used to assess the proliferation ability of Hela cells treated with various

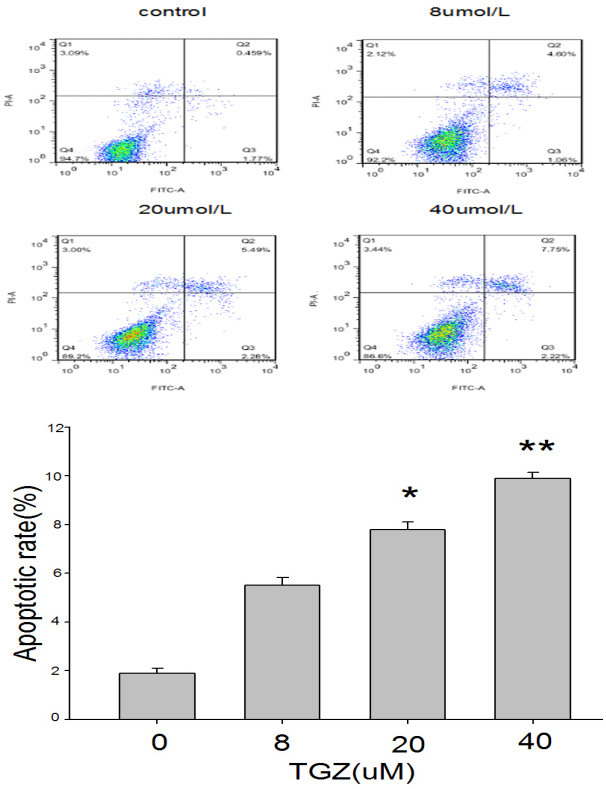

Figure 3. Flow Cytometry Analysis of Cervical Cancer Cells Apoptosis under the Treatment of TGZ. Hela cells were treated with different concentrations of TGZ (0, 8, 20 and 40μM) as indicated for 48 h prior to flow cytometry analysis. Flow cytometry results were plotted from triplicate experiments. Data are presented as mean ± SD (n =3). *P < 0.05; * *P < 0.01 versus control groups

concentrations of TGZ (0, 6μM, 8μM, 10μM, 20μM, 40μM) (Kawa et al., 2002; Nagata et al., 2008). The results showed that TGZ treatment significantly reduced cellular proliferation at 20μM and 40μM as measured by the Cck8 assay after 24h and 48h (P<0.05) (Figure 1). Moreover, the inhibition rate of TGZ was remarkably higher than that of the negative control group at 20μM and 40μM after 72 hours (P<0.01) (Figure 1). Obviously, these data showed that TGZ could inhibit cell proliferation in time- and dose-dependent manner compared with that of the control group (Yoshimura et al., 2003).

TGZ promotes cell apoptosis by Annexin V-FITC binding and flow cytometry assays

In order to investigate the contribution of apoptosis to reduced tumor cell growth mediated by TGZ, we use Annexin V-FITC to detect apoptotic cells by fluorescence microscope (Figure 2). Results were obtained 48h after corresponding medications (Hasan et al., 2011; Pei et al., 2012). It indicated high levels of Annexin V-FITC positive cells in TGZ (20μM) treated groups when compared with control groups in Hela cells, not only during the prophase apoptosis (Figure A), but also the nuclear apoptosis (Figure B). We then used flow cytometric analysis to examine cellular apoptosis. Our results revealed that a dose-dependent increase of the apoptotic cell fraction in Hela cells was identified after cultured with TGZ (Figure 3) (Matsuyama and Yoshimura, 2009). After 2 days of treatment with TGZ, prophase apoptotic cell population

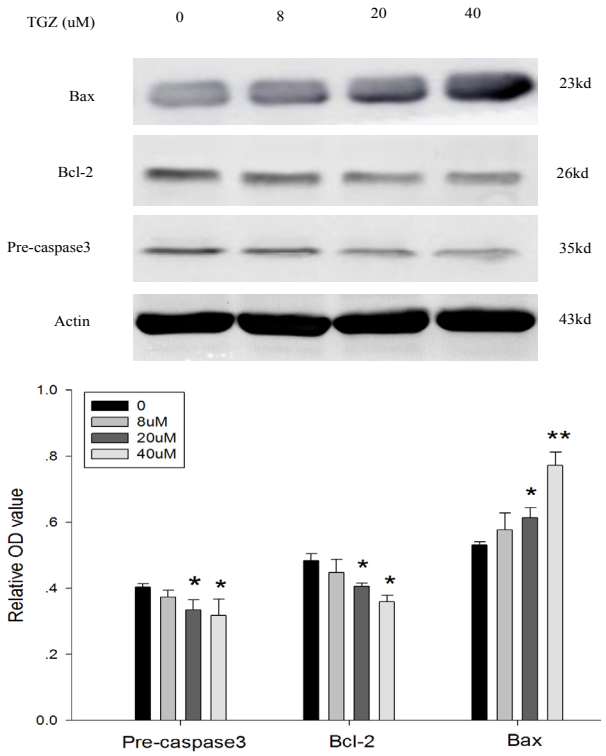

Figure 4. Effect of Troglitazone on the Expression Levels of Bcl-2 Family Members and Pre-caspase3 in Hela Cells. After treatment with troglitazone, cell extracts were prepared and subjected to immunoblotting and probed for various proteins as indicated in the figure. β-actin was used as the loading control. Data are presented as mean ± SD (n =3). *$P < 0.05$; * *$P < 0.01$ versus control groups

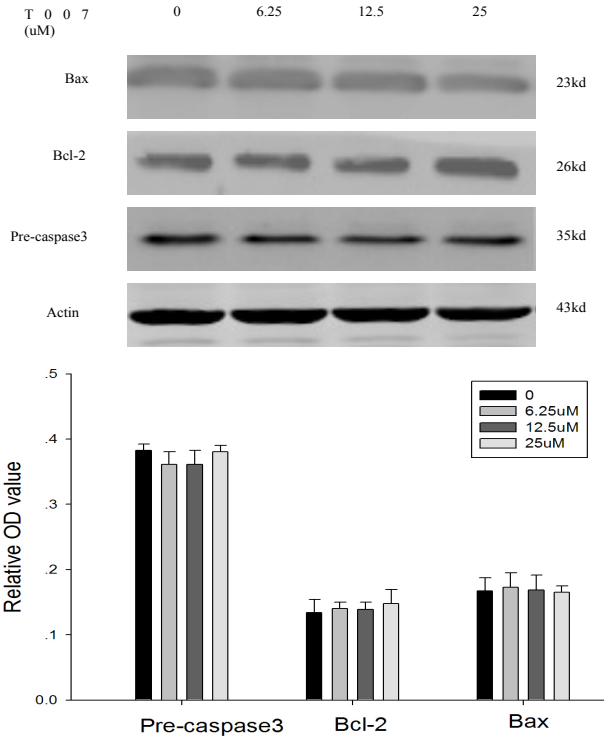

Figure 5. Effect of T0070907 on the Expression Levels of Bcl-2 Family Members and Pre-caspase3 in Hela Cells. After treatment with T0070907, cell extracts were prepared and subjected to immunoblotting and probed for various proteins as indicated in the figure. β-actin was used as the loading control

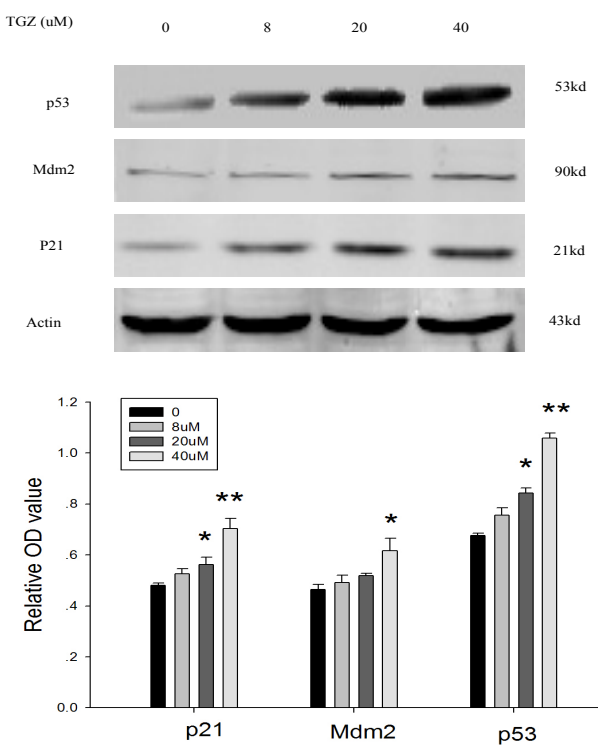

Figure 6. The Relative Amounts of p53-related Apoptosis Proteins Changes after Troglitazone Treatment in Hela Cells. After treatment with troglitazone, cell extracts were prepared and subjected to immunoblotting and probed for various proteins as indicated in the figure. β-actin was used as the loading control. Data are presented as mean ± SD (n =3). *$P < 0.05$; **$P < 0.01$ versus control groups

characterized by PI (-) and Annexin-V (+) was 0.459% in control, 4.6% in 8μM, 5.49% in 20μM, and 7.75% in 40μM of TGZ, respectively. There was also a dose-dependent increase in nuclear apoptosis. Cell population characterized by PI (+) and Annexin-V (+) was 1.77% in control, 1.06% in 8μM, 2.28% in 20μM, and 2.22% in 40μM of TGZ (Park et al., 2005). Taken together, these data demonstrated that TGZ could promote cell apoptosis in Hela cells.

TGZ induces apoptosis via p53-related apoptotic pathway

In order to identify the apoptotic mechanism induced by TGZ on cervical cancer cell, the anti-apoptotic protein precaspases-3 and Bcl-2, pro-apoptotic protein Bax expression levels, the key players in the apoptotic cascade, were examined after 48h of exposure (Wu et al., 2009). The results showed that the expression levels of pre-caspase3 and Bcl-2 was down-regulated, and Bax was up-regulated with increasing doses of TGZ, especially at 20μM and 40μM (Figure 4). To test whether PPAR-γ selective antagonist T0070907 affected cell apoptosis in Hela cells, we also examined Bcl-2, Bax and pre-caspase3 protein expression levels with various concentrations of T0070907 (0-25 μM) (Zaytseva et al., 2011). The results showed that the tested concentrations of T007 had no significant effect on apoptosis (Figure 5). Moreover, to identify whether PPAR-γ ligand TGZ could induce cell apoptosis via a p53-related mechanism, we examined protein expression levels of p53 and p53-downstream genes (p21, Mdm2) (Cheon et al., 2009;Kim et al., 2011).

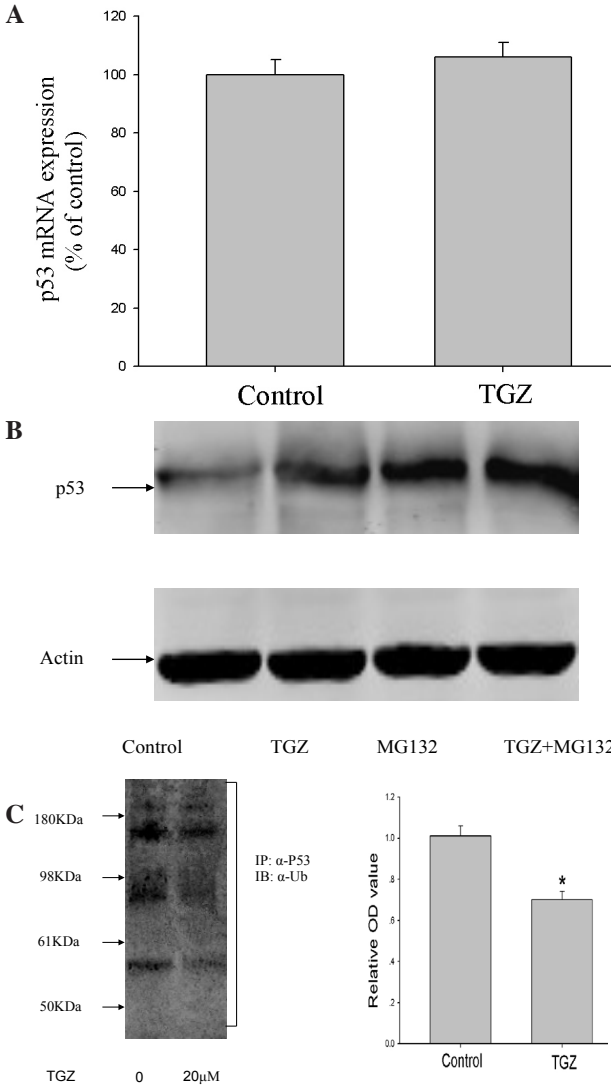

Figure 7. Expression of p53 mRNA, Protein and Ubiquitination in Cervical Cancer Cells. A Total RNA from cervical cancer cell was extracted, expression of p53 mRNA has been evaluated by quantitative RT-PCR. Hela cells were treated with TGZ (20 μM) after 24h of incubation. Data are mean±standard deviation and are expressed as % control. B Hela cells were pretreated with TGZ for 24h and then treated with proteasomal inhibitor MG132 (10 μM) for 6h. p53 expression was analyzed by Western blotting using anti-p53 antibody. The arrowhead indicates the position of p53. C The expression of p53 and ubp53 were demonstrated by immunoprecipitation with p53 antibody. P53 and ubp53 expression levels were examined by western blot. The arrowhead indicates the position of p53 and ubp53. Data are presented as mean ± SD (n =3). *$P < 0.05$ versus control groups

The results showed that p53, Mdm2 and p21 were all up-regulated with increasing doses of TGZ (Figure 6). Taken together, these results suggested that p53-dependent apoptosis may be involved in the TGZ induced apoptosis of cervical cancer cells.

TGZ inhibits ubiquitination of p53 in cervical cancer cell

In order to further investigate the mechanism on the up-regulation of p53, we examined the expression of p53 mRNA in human cervical cancer cell lines using quantitative RT-PCR (Ohtani et al., 2004;Li et al., 2013). In this assay, the mRNA levels of p53 were not affected by TGZ in a quantitative RT-PCR analysis (fig 7a), suggesting that TGZ up-regulated p53 at the post-transcriptional level. To evaluate whether this effect was mediated via the ubiquitin-proteasome system, the proteasome inhibitor MG132 was used to block the proteasomal degradation in Hela cells (Okamoto et al., 2009; Choi et al., 2011; Tian et al., 2012). Our results showed that TGZ-mediated upregulation of p53 was prevented by the proteasome inhibitor MG132 (Figure 7b). Then, we examined the ubiquitination of p53. In our study, ubiquitination of p53 was reduced in TGZ treatment cells versus control group (Figure 7c). Taken together, these findings demonstrated that TGZ increased p53 expression through inhibition of ubiquitin-mediated p53 proteasomal degradation.

Discussion

The effect of PPAR-γ ligands on cell growth inhibition and apoptosis has been investigated in various cancer cells, including cervical cancer cell (Nagamine et al., 2003). Although these studies demonstrated that several PPAR-γ ligands can induce cell growth inhibition, little is known about the mechanism mediating these effects in cancer cells (Yu et al., 2006). The goal of this study was to provide further mechanistic insight into PPAR-γ ligands through the TGZ-PPAR-γ-p53 pathways.

In recent years, PPAR-γ agonists including TGZ have been reported to suppress the growth of cancer cells of diverse tissue origin. In our study, the CcK8 assay showed TGZ inhibited growth of cervical cancer cells in a time- and dose-dependent manner. The inhibitory effect of TGZ on the proliferation of cervical cancer cells was associated with induction of apoptosis, flow cytometry and Annexin V were conducted.

In addition, we respectively investigated effects of PPAR-γ ligands, TGZ and T0070907, on cell apoptosis in human cervical cancer cell line in vitro. Our studies found that PPAR-γ ligand TGZ could promote cell apoptosis but T0070907 did not work, and it was in agreement with other study on colorectal carcinoma. Then, we detected the apoptosis-related gene protein such as pro-apoptotic gene bax, p21, p53, and anti-apoptotic gene bcl-2 pre-caspase3. In our experiment, down-regulation of Bcl-2, pre-caspase3 coupled with up-regulation of Bax and p21 was observed with increasing doses of TGZ (Nam et al., 2007). And, the change of p53 was also observed after the treatment in Hela cells. Therefore, our findings that PPAR-γ ligand, TGZ, decreasesd both Bcl-2 and pre-caspase3 but increased p53, p21 and bax support the notion that the activation of PPAR-γ subsequently leaded to cell apoptosis via activation of p53 pathway (Hassan et al., 2013).

The p53 tumor suppressor is a sequence-specific DNA-binding transcriptional factor that plays a central role in regulating growth arrest and apoptosis during the stress response. post-translational modification plays an important role in p53 regulation and ubiquitination of p53 has emerged as a fundamental mechanism of p53 control. In our study we tested the expression of p53 and ubiquitination of p53. The results showed that down-regulation of p53 ubiquitination and up-regulation of p53

with the increasing concentration of TGZ. We further demonstrated the suppressive effect of TGZ was mainly due to its induction of apoptosis, probably resulting from the TGZ-PPAR-γ-p53 pathways.

In summary, our study demonstrated that PPAR-γ played an important role in human cervical cancer carcinogenesis, especially in tumor proliferation and apoptosis (Liu et al., 2005). Further experiment remains to be done to evaluate the tumor-promoting properties of PPAR-γ using an in vivo tumor mouse model, hoping that we can provide a useful therapeutic strategy for cancer intervention in cervical cancer.

Acknowledgements

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