The Ligands of Peroxisome Proliferator-activated Receptor (PPAR) Gamma Inhibit Growth of Human Esophageal Carcinoma Cells through Induction of Apoptosis and Cell Cycle Arrest

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Abstract. In the present study, we examined the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and the growth-inhibitory effects of Troglitazone and Pioglitazone, selective ligands for PPARγ, using a series of human esophageal carcinoma cell lines (TE-1, -3, -7, -8, -12 and -13). PPARγ expression was detected in all six human esophageal carcinoma cell lines. The esophageal carcinoma cell line TE-13 showed marked growth inhibition in response to Troglitazone and Pioglitazone. Flow cytometry performed on TE-13 cells exposed to Troglitazone showed that the cell cycle was arrested at the G1-phase. This result was confirmed by the finding of reduced cyclin D and cyclin E expression by Western blot analysis. DNA ladder formation was also detected, as was the induction of apoptosis-related proteins. Our results suggested that Troglitazone inhibited the growth of human esophageal carcinoma cell lines via G1 arrest and apoptosis and that PPARγ ligands should be considered as possible target molecules in the treatment of human esophageal carcinomas.

Esophageal cancer is one of the most lethal cancers and remains a significant health problem worldwide (1). In Japan, about 16,500 patients die of this disease each year (2) and the five-year survival rate for esophageal cancer is only about 30% at best. Even so, this figure represents an improvement upon the 5% 5-year survival rate experienced before the advent of combined chemotherapy and radiotherapy treatments that were first trialed about 20 years ago (3). However, despite intensive research into esophageal cancer therapies, at present there is no standard chemotherapeutic regimen for this disease.

PPARγ is a member of the nuclear steroid hormone receptor superfamily and forms a sub-family along with PPARα and PPARδ. PPARγ plays important roles in adipose differentiation and fat metabolism (4-9) and its expression has been detected in multiple tissues including breast, colon, lung, ovary, placenta and activated macrophages (10-16). Thiazolidinedione, used to improve insulin resistance, is known to be a ligand for PPARγ (17,18). Heterodimers of PPARγ and retinoic acid X receptor (RXR) bind to the PPRE/PPAR response DNA elements (19-21), which induce anti-inflammatory reactions (22-24), arteriosclerosis (25,26) and development of adipogenic phenotypes. It has also become widely accepted that PPARγ ligands exert antitumor effects in a variety of tumors, including gastric (27, 28), colon (29-31), sarcoma (32), leukemia (33), hepatocellular carcinoma (34), pancreas (35), breast (36,37) and prostate (38,39) cancers. These results suggest that PPARγ activation may be inhibitory to the growth of malignant tumors. However, the antiproliferative effects of PPARγ activation on esophageal carcinoma have not been precisely elucidated. To confirm the growth-inhibitory effect of PPARγ signaling in esophageal carcinoma, we treated esophageal carcinoma cells with two Thiazolidinediones and examined the subsequent expression of apoptotic genes and cell cycle regulators.

Materials and Methods

Cell culture. Human esophageal carcinoma cell lines TE-1, TE-3, TE-7, TE-8, TE-12 and TE-13 were obtained from Tohoku University (Miyagi, Japan) and cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Because the human gastric cancer cell line MKN45 has already been reported to express PPARγ protein (27), we used this cell line as a
positive control for PPARγ protein expression. MKN45 cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ in air.

Chemicals. Troglitazone, a selective ligand for PPARγ, was kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO) to give a final concentration of 0.05% in culture medium. Pioglitazone, another selective ligand for PPARγ, was kindly provided by Takeda Pharmaceutical Co. (Osaka, Japan) and dissolved in DMSO to give a final concentration of 0.1% in culture medium.

Cell growth assay. Cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described previously (40). To investigate the growth-inhibitory effects of Troglitazone and Pioglitazone on the TE-1, TE-3, TE-7, TE-8, TE-12 and TE-13 cell lines, 2000 cells/well were seeded into 96-well plates and incubated overnight. Cells were then treated with specific concentrations of Troglitazone or Pioglitazone in the presence of FBS for 1, 2, 3, or 4 days, after which 10 ml 12 mM MTT (Molecular Probes Inc, The Netherlands) was added to each well and incubated for 4 h at 37°C. Following the incubation, 150 μl DMSO was added to each well and the cells incubated for 1h at room temperature. Absorbance at 570 nm was obtained for each reaction using an MTT reader (Molecular Device Company).

Cell cycle assay by flow cytometry. TE-13 cells were treated with 50 mM Troglitazone in DMSO to a final concentration of 0.05% in culture medium. After 24-or 48-h incubation, the cells were collected by centrifugation and permeabilized with ice-cold 70% ethanol for at least 0.5 h. After washing with PBS, the cells were treated with 0.5 mg/ml RNase A (DNase-free) and incubated at 37°C for 20 min. After centrifugation, the cells were resuspended in PBS containing 50 μg/ml propidium iodide and stained at 4°C for 10 min. DNA content was analyzed by CYTORON ABSOLUTE (Johnson and Johnson Company).

Analysis of DNA fragmentation. Soluble DNA was extracted after 12-h treatment with 50 mM Troglitazone (in DMSO to a final concentration of 0.05%). Cells floating in medium were collected by centrifugation, while cells that had remained attached to the dish were detached by scraping. The cells were pelleted by centrifugation and resuspended in Tris-EDTA buffer (pH 8.0). Cell plasma membranes were analyzed using the Quick Apoptotic DNA Ladder Detection Kit (Bio Vision Research Product, USA) and residual material electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed under UV illumination.

Apoptosis assay by FITC-annexin V/propidium iodide assay. Apoptosis was also determined using FITC-annexin V and propidium iodide (PI). For FITC-annexin V staining, TE-13 cells were treated with 50 μM Troglitazone in DMSO to a final concentration of 0.05% in culture medium. After 12 h, the cells were collected by centrifugation and analyzed for FITC-annexinV and PI staining using the Vibrant Apoptosis assay Kit (Molecular Probes, Inc.) followed by the CYTORON ABSOLUTE kit (Johnson and Johnson Company).

Western blot analysis. TE-13 cells were treated with several doses of Troglitazone and total proteins extracted at several time-points. Protein concentrations were measured using the Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) according to the manufacturer’s protocol. Aliquots of 20 μg protein were separated by 5-10% SDS-PAGE (Ready Gels J; Bio-Rad) and transferred to polyvinyl difluoride membranes (ATTO, Tokyo, Japan). After blocking overnight in TBS with 5% skim milk at 4°C, blots were reacted with primary polyclonal antibodies against human p16, p27, cyclin A, cyclin D1, cyclin E, PPARγ, PARP, caspase 3, caspase 8, cytochrome C, Bax, Bid, Bel-XL, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and washed. After reaction with horseradish peroxidase-conjugated anti-rabbit IgG, anti-goat IgG or anti-mouse IgG, immune complexes were visualized using ECL detection reagents (Amersham International) according to the manufacturer’s protocols.

Results

PPARγ expression and growth inhibition by Troglitazone. To examine whether the esophageal cancer cell lines expressed PPARγ protein, Western blot analysis was performed as demonstrated in Figure 1. PPARγ protein expression was clearly detected in all six cell lines examined as a 51 kD band, as previously reported (27). Different expression levels were observed across the cell lines with the highest level detected in the TE8 cell line. The sensitivity of esophageal carcinoma cell lines to growth inhibition by Troglitazone is shown in Figure 2. Each cell line was treated with 0.1, 1, 10, or 100 μM of Troglitazone and the effect
Figure 2. Cell growth assay. TE-1, TE-3, TE-7, TE-8, TE-12 and TE-13 were grown for 1, 2, 3, or 4 days with (0.1, 1, 10, or 100μM) and without Troglitazone. The multiplication curve in time progress was displayed for every concentration. Growth inhibition was analyzed using the MTT assay as described in "Materials and Methods." Clear growth inhibition was observed in a time- and dose-dependent manner in 4 cancer cell lines (TE-7, 8, 12 and 13).

Figure 3. Cell cycle assay by flow cytometry. G1 peak and sub-G1 peak can be seen in a time-dependent manner after treatment. The cells were incubated without or with 50μM Troglitazone for 12 or 24 h.
observed for 24, 48, 72 and 96 h after treatment. The MTT assay was used to measure the inhibitory effect at each time-point compared to non-treated control cells. Interestingly, Troglitazone significantly inhibited the growth of TE-7, -8, -12 and -13 cells in time-and dose-dependent manners. A time- and dose-dependent anti-tumor effect was also observed for TE-1 and -13 cells after treatment with Pioglitazone, also known to be a PPARγ ligand (data not shown). The growth inhibitory effects of Troglitazone and Pioglitazone were not related to PPARγ expression level.

The inhibitory mechanism of Troglitazone on TE-13 cells. To determine whether the cells were arrested or became apoptotic in response to Troglitazone, cell cycle analysis was performed by flow cytometry (Figure 3). The proportions of TE-13 cells at each cell cycle stage with and without Troglitazone treatment were compared. At 24 h after treatment, increased numbers of G1-phase and decreased numbers of G2/M-phase cells were observed and this trend was enhanced 48 h after treatment. This suggested that the proportion of cells arrested at G1-phase was increased after Troglitazone treatment. Moreover, compared with control cells, the sub-G1 cell population was markedly increased after 48-h treatment, which strongly suggested the presence of apoptosis.

To examine the role of apoptosis in this phenomenon, treated and non-treated TE-13 cells were checked for DNA fragmentation. DNA was extracted at 0, 6, or 12 h after treatment with Troglitazone and the results showed clear DNA ladder formation from 12 h after treatment (Figure 4). The presence of apoptosis was confirmed by using an FITC-annexin V/PI assay, as shown in Figure 5. Results indicated that 24 h after Troglitazone treatment, 33.8% of TE-13 cells were apoptotic. These results indicated that the growth inhibitory effects of Troglitazone were mediated through G1 arrest and apoptosis.

Effect of Troglitazone on the expression of cell cycle regulators. The cell cycle is positively regulated by several regulators, including G1 cyclins that bind to cell cycle-dependent kinases (cdk), while it is also negatively-regulated by inhibitors. It has been previously reported that the p27 protein, a strong inhibitor of cyclin D, cdk2 and cdk4, is up-regulated by Troglitazone change along with G1 arrest. Therefore, in the present study we examined the expression of positive and negative cell cycle regulators in TE-13 cells following Troglitazone treatment. TE-13 cells were treated with Troglitazone for 6, 12, 24, or 48 h and Western blot analysis performed on extracted proteins to
examine the expression of cyclin A, cyclin D1, cyclin E, p53, p27, p21 and p16. Cyclin D1 protein expression was clearly detected as a 34 KD band and showed a markedly decreased expression level in a time-dependent manner (Figure 6A). A marked decrease in cyclin E expression was also observed at 24 and 48 h after treatment. As for the negative regulators, p27 protein was detected as a 27 KD band and showed 2-fold higher expression at 48 h compared to the control level, while a 4-fold increase in p16 protein expression was observed at 12 h after treatment. These results were consistent with previous studies (35) and supported the finding of cell cycle arrest at the G1-stage. p53 and p21 gene expression were not detected in TE-13 cells (data not shown).

Effect of Troglitazone on the expression of apoptosis-related genes.
We then examined possible apoptotic signal transduction pathways following PPARγ-receptor-mediated treatment of gastric cancer cells. The process of apoptosis is complicated. Drugs that damage DNA can induce apoptosis via the perturbation of inner mitochondrial membrane permeability. The sudden increase in mitochondrial membrane permeability is a central coordinating event in the apoptotic process with the immediate release of cytochrome C from mitochondria (41). In the cytosol, cytochrome C combines with Apaf-1 in the presence of ATP to activate caspase 9, which in turn activates effector caspases such as caspase 3. Thus the activation of caspase 3 and subsequent degradative events appear to be triggered by the release of cytochrome C from the intermembrane mitochondrial space. Other known apoptotic mechanisms include signaling via the death receptor-ligand system and through the phospho-inositol-triphosphate (PI3)-Akt-Apaf-1 system. Death receptors (42,43), members of the TNF receptor family, activate caspase 8 which then transduces signals to mitochondria via Bid protein (44) and at the same time activate caspase 3, resulting in the induction of apoptosis. Caspase 3 is the effector molecule that causes DNA fragmentation. As for the kinase system in apoptosis regulation, Akt phosphorylates Bad protein (45), a member of the Bcl-2 family that promotes apoptosis. Phosphorylation of the Bad protein leads to its inactivation, with the failure to form heterodimer complexes with Bcl-2 or Bcl-XL. Therefore, activated Akt inhibits apoptosis.

In the present study, changes in cytochrome C, Bid, Bax, MDM 2, caspase 8, Bcl-XL, caspase 3 and PARP protein expression were analyzed by Western blot analysis (Figure 6B). Interestingly, expression levels of the 32 KD caspase 3 protein were increased 3-fold compared to control levels at 6 h after Troglitazone treatment. Thus, it is possible that this increase in caspase 3 expression may underlie the induction of PARP gene expression 24 h after Troglitazone treatment and suggests the involvement of programmed cell death in TE-13 cells. The possible induction of apoptosis was further supported by the finding of increased cytochrome C induction also present at 6 h after Troglitazone treatment, with approximately 1.2-fold induction. This also suggested that the induction of the apoptosis pathway was initiated via PPARγ. This hypothesis was further confirmed by the finding of increased Bid protein expression and a marked decrease in Bcl-XL protein expression. Caspase 8 levels were increased at 12 h after Troglitazone treatment, which suggested the involvement of the death receptor pathway.

Discussion
In this study, we demonstrated the dose-dependent growth inhibition of Troglitazone on several esophageal carcinoma cell lines, which was consistent with results obtained for
other cancer cell lines. Pioglitazone, another PPARγ ligand, also had growth-inhibitory effects on the esophageal carcinoma cell lines (data not shown). While there have been increasing numbers of reports of PPARγ ligand-mediated growth inhibition on various carcinomas over the past ten years, clinical studies have not yet established consistent cell growth-inhibitory effects and the mechanism of the growth inhibition of tumor cell requires investigation. We first postulated that the growth-inhibitory effect was related to PPARγ protein expression level. However, we found no correlation between the amount of PPARγ protein present in a particular cell line and the level of cell growth inhibition. This lack of relationship between PPARγ expression and cell growth inhibition has been reported for other cancer cell lines (46) and indicates that receptor level cannot be used to predict the effectiveness of Troglitazone prior to treatment.

We then examined whether the cell growth inhibitory effect of Troglitazone was related to G1-phase cell cycle arrest. Using the TE-13 cell line, G1-stage arrest was observed by flow cytometric cell cycle analysis. We also demonstrated that expression levels of the CDK inhibitors p16 and p27 were significantly increased, while levels of the CDKs cyclin D1 and cyclin E1 were decreased. Moreover, reduction in expression levels of MDM2 protein, which inhibits upstream of p16 and p27, was also observed.

The induction of apoptosis by PPARγ ligands has been demonstrated in a variety of cell lines. Takahashi et al. (47), demonstrated that Troglitazone induced morphological changes in TE-7 cells. In the present study, we examined the induction of apoptosis during Troglitazone-mediated cell growth inhibition in the esophageal cancer cell line TE-13. DNA fragmentation was observed 12 h after Troglitazone treatment, while induction of apoptosis was demonstrated after 24 h with the appearance of annexin in the cell membranes. Along with the time-dependent increase in the proportion of sub-G1 cells observed by flow cytometry, we found that the level of apoptosis also increased in a time-dependent manner. Based on these findings, we then investigated the precise signal transduction pathways involved in the apoptotic process. The expression levels of a variety of apoptosis-related proteins were examined. As caspase 8 protein expression increased soon after Troglitazone treatment (6 and 12 h), it is possible that the caspase 8-dependent pathway underlies the Troglitazone effects. A receptor for this pathway is TNF-related apoptosis-inducing ligand (TRAIL) that shows homology to Fas ligand. Our results indicated that the expression of Bid, Bax and cytochrome C proteins was also time-dependently increased after Troglitazone treatment. Caspases 8 and 2 have been implicated upstream of Bak/Bax activation in several other systems (48). In the case of caspase 8, this effect is mediated through the cleavage of Bid into active Bid that in turn activates Bak and Bax, while the mechanism of caspase 2-mediated apoptosis is less clear (49). Bax acts as a proapoptotic protein and is inhibited by survival factors such as Akt. Phosphorylation by activated Akt can inhibit the proapoptotic protein Bad, leading to increased free (antiapoptotic) Bcl-XL (45). Our results suggested that activation of the mitochondrial apoptotic pathway might be a relatively late phase (24 to 48 h). However, Lucarelli et al. (50) reported that Troglitazone-mediated survival correlated with Akt activation. In our study, activation of Akt occurred 6 h after Troglitazone treatment using an antibody that recognized the activated form of this kinase, with Akt activation decreased after 12 h (data not shown). A study examining the induction of apoptosis by a PPARγ ligand observed early and late phases of apoptosis (51). Akt activation can be induced by mechanisms other than PPARγ activation and this may help explain the apparent biphasic nature of apoptosis-related protein expression. Caspase 3 and PARP may represent the early phase of apoptotic signal transduction as they were activated very soon after Troglitazone treatment. The natural ligand of PPARγ is 15d-prostaglandin J2 (PGJ2), which is dependent on the production of proliferative prostaglandins, such as PGE2 (52), that modulate the activity of the cyclooxygenase-2 (COX-2) enzyme. While Li et al. (53), reported that Troglitazone was associated with the down-regulation of COX-2 mRNA and protein expression, further research is necessary to define the relationship between PPARγ and COX2.

In conclusion, our results indicated that PPARγ is expressed in human esophageal carcinoma cell lines and that its ligands induce growth arrest and changes associated with differentiation, as well as induction of apoptosis and cell cycle arrest in human esophageal carcinoma. Thus, PPARγ ligands represent a new class of molecular targeting agents that may be useful in the treatment of human esophageal carcinoma.

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