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Peroxisome Proliferator-activated Receptor Gamma Activation Induces Cell Cycle Arrest via the p53-independent Pathway in Human Anaplastic Thyroid Cancer Cells

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Anaplastic thyroid carcinoma is one of the most aggressive human malignancies. Outcomes of intensive multimodal therapy have been far from satisfactory. Furthermore, *p53* gene dysfunction, often found in this type of cancer, is known to impair the efficacy of the therapeutic agents. Specific ligands for peroxisome proliferator activated receptor gamma (PPAR-γ) induce growth suppression in some tumor cells. In this study, we investigated the role of PPAR-γ in anaplastic thyroid cancer cell lines (OCUT-1, ACT-1). PPAR-γ was expressed and functional in both cell lines. Activation of PPAR-γ with its specific ligands, troglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, inhibited cell growth in a dose-dependent manner through inducing G1 cell cycle arrest. P53 protein expression differed in OCUT-1 and in ACT-1, though the levels stayed constant irrespective of ligand exposure in both cell lines. In contrast, p21 and p27 proteins were induced in a dose-dependent manner in both situations. This study showed that PPAR-γ ligands were able to induce growth suppression in anaplastic thyroid cancer cells via a p53-independent, but p21- and p27-dependent cytostatic pathway. These tumor-suppressive effects of PPAR-γ may provide a novel approach to the treatment of anaplastic thyroid cancer.

Key words: PPAR-γ — Anaplastic thyroid cancer — p53 — p21 — p27

Anaplastic thyroid carcinoma is one of the most aggressive human malignancies. The majority of patients with anaplastic thyroid carcinoma die within a year after receiving an initial diagnosis, and the reported one-year survival rate is only 10 to 20%. Long-term survivors are limited to exceptional cases on whom curative surgery for a small, localized lesion could be performed.[1–3] Usually a combination therapy with drugs and radiation is the only therapeutic strategy in advanced cases, but the outcome of such intensive therapy is far from satisfactory. Furthermore, adverse effects of these intensive combination therapies often occur and can be as severe as the disease itself, worsening the quality of life. Thus, a novel therapeutic approach is highly desirable.

Peroxisome proliferator activating receptor gamma (PPAR-γ) is one of the members of the nuclear receptor superfamily and is reported to be linked to adipocyte differentiation and the control of cellular lipid uptake.[4, 5] Forced expression and activation of PPAR-γ in fibroblasts trigger the adipocyte gene expression cascade and lead to the development of the adipose phenotype. DNA binding requires the formation of a heterodimer of PPAR and RXRα (9-*cis*-retinoic acid receptor) that interacts with a peroxisome proliferator responsive element (PPRE) in the target gene. Several polyunsaturated fatty acids, the thiazolidinedione class of anti-diabetic drugs, such as troglitazone,[6, 7] and the nuclear prostanoid 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ_2)[8, 9] have been identified as ligands for PPAR-γ, and the roles of PPAR-γ-stimulated pathways have been investigated.

The induction of differentiation by the PPAR-γ stimulatory pathway was found not only in adipocytes, but also in tumor cells. The therapeutic approach of inducing differentiation of tumor cells has already been used successfully in the treatment of liposarcoma.[10, 11] Tumor cells in which differentiation had been induced lost their aggressive proliferative character, resulting in dormancy of disease progression. A similar adverse effect of the PPAR-γ stimulatory pathway on cell proliferation has been noted in breast cancer,[12, 13] colon cancer[14, 15] and pancreas cancer cell lines.[16] In those cancer cells, growth arrest was demonstrated following the induction of differentiation. Furthermore, in differentiated thyroid cancer, a similar growth-inhibiting effect of a PPAR-γ ligand was reported.[17] However, the role of PPAR-γ and its stimulatory pathway has not yet been elucidated clearly in anaplastic thyroid cancer, which differs from differentiated thyroid cancer in many respects, such as the manner of disease progression, the therapeutic strategy, and the molecular back-

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ground for its highly aggressive characteristics, including p53 status.

In this study, we demonstrated the expression of PPAR-γ in anaplastic thyroid cancer cell lines and investigated the potential of this receptor as a molecular target for a novel therapeutic strategy.

MATERIALS AND METHODS

Cell lines and cell culture OCUT-1 and ACT-1 are human anaplastic thyroid cancer cell lines. OCUT-1 was recently established and characterized in our laboratory,[18] and ACT-1 was kindly provided by Dr. S. Ohata of Tokushima University. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 to 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37°C with 5% CO_2 in a humidified condition. DNA sequencing for *p53* gene was performed in both OCUT-1[18] and ACT-1 cell lines from exons 5 to 9, and no mutations were confirmed. The cells were treated with various concentrations of troglitazone (provided by Sankyo Co., Ltd., Tokyo) and $15dPGJ_2$ (Cayman Chemical Co., Ann Arbor, MI).

Determination of the PPAR-γ expression We determined the expression of mRNA of PPAR-γ by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was collected from samples using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). One nanogram of total RNA was reverse-transcribed in a 20 μl reaction buffer, containing 1 μl of oligo dT primer, 4 μl of 5× RNA PCR buffer, 1 μl of 10 m*M* dNTPs, 2 μl of 0.1 *M* dithiothreitol (DTT), 0.5 μl of RNA guard (Amersham Pharmacia Biotech, Buckinghamshire, UK) and 1 μl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies). The cDNA samples were amplified in 20 μl of PCR reaction mixture with each primer set and *Taq*-polymerase (Gene Taq, Nippon Gene, Toyama). The primers used for PPAR-γ were 5′-GAGATCACAGAGTATGCCAA-3′ and 5′-CTGTCATCTAATTCCAGTGC-3′.[15] RT-PCR efficiency was confirmed by amplifying human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primers 5′-ACCACAGTCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.[15] PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s. Amplified products were electrophoresed on 2% agarose gel, stained with ethidium bromide.

Protein expression of PPAR-γ was examined by western blotting analysis.[19] Protein was extracted from the cells grown in the exponential phase by lysis buffer (2 m*M* phenylmethanesulfonyl fluoride (PMSF), 0.25 U/ml aprotinin, 5 m*M* DTT, 0.15 *M* NaCl, 1% Triton X-100, 10 m*M* Tris (pH 7.4)). The total protein (20 μg) was electrophoresed on a 10% polyacrylamide gel and transferred to a membrane (Hybond P, Amersham Pharmacia Biotech). The membrane was blocked with 5% skim milk for 2 h at room temperature, and treated with anti-PPAR-γ mouse monoclonal antibody (Clone E-8, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 12 h at 4°C. After three washings with 0.1% Tween 20 in phosphate-buffered saline (PBS) for 5 min each at room temperature, the membrane was incubated for 2 h at room temperature with peroxidase-conjugated secondary antibody (AP181P, Chemicon International, Inc., Temecula, CA), and again washed three times under the same conditions. Peroxidase was detected with an enhanced chemiluminescence detection system (ECL Plus Western Blotting Detection System, Amersham Pharmacia Biotech).

We determined immunoreactivity for PPAR-γ using the streptoavidin-biotin method. Formalin-fixed, paraffin-embedded tissue blocks were obtained from a xenograft of OCUT-1.[18] Immunohistochemical study was performed as described previously.[20] Briefly, sections were dewaxed and then microwave-pretreated. After blocking to reduce nonspecific antibody binding, monoclonal antibody against PPAR-γ (same as above) was reacted for 2 h at room temperature. The section was incubated with peroxidase-conjugated secondary antibody, then reacted with streptoavidin-biotin peroxidase reagent (Histofine Kit, Nichirei Co., Tokyo). Finally, diaminobenzidine with 1% hydrogen peroxidase was applied as a chromogen, and counterstained with hematoxylin.

Luciferase assay for PPRE activation Specific activation of PPRE of the *acyl-coA* gene by troglitazone or $15dPGJ_2$ was demonstrated by luciferase assay as described previously.[21] Briefly, cells were seeded at a concentration of 1×10^6 cells on a 60-mm plastic culture plate and transfected with the plasmids 24 h after having been transferred to fresh culture medium. "SuperFect" Transfection Reagent (Qiagen, Inc., Valencia, CA) was employed according to the manufacturer's instructions for transfection of 2 μg of acyl-CoA oxidase promoter-luciferase plasmid (a gift from Dr. Takashi Osumi[21]) and 0.2 μg of pRL-SV40 (Promega, Madison, WI). The transfected cells were incubated in the complete medium with or without troglitazone for an additional 12 h. The cells were lysed with lysis buffer, and luciferase activity was measured with a Pica Gene reagent kit (Toyo Ink, Inc., Tokyo) with a luminometer. The enzyme activity was normalized for efficiency of transfection, on the basis of sea pansy luciferase activity, and relative values were determined. Transfection experiments were carried out three times independently, and the average values were calculated.

Cell viability analysis Cell viability after exposure to the troglitazone or $15dPGJ_2$ was measured by MTT (methylthiazoletetrazolium bromide) assay.[22] Cells (1×10^3 cells/well) were seeded in each well of a 96-well plastic culture plate and left overnight under the same conditions to

adhere to the bottom of the plate. Then, the intended concentrations of troglitazone and 15dPGJ$_2$ were added to each well, and the cells were incubated for 7 days. After the incubation period, MTT was added to the final concentration of 0.5 mg/ml, and the cells were incubated again for 2 h under the same conditions. The culture plate was centrifuged at 200*g* for 5 min, and supernatant was removed. Dimethyl sulfoxide was added for reaction, and the absorbancy was measured with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) and calculated using the supplied software. The experiments were carried out three times independently, in triplicate each time, and the average values of the three independent experiments were calculated.

Cell growth inhibition analysis The adverse effect of troglitazone or 15dPGJ$_2$ on cell growth was also determined by chronogenic assay. Cells were seeded in 10-cm plastic culture plates and allowed to sit overnight to adhere to the bottom of the plates. Next, the intended concentration of troglitazone or 15dPGJ$_2$ was added, and the cells were incubated under the same conditions. We observed the cell morphology under a microscope before collecting the cells with brief trypsinization and counting them with a cell counting machine (Coulter Counter, Beckman Coulter, Tokyo) at 3, 5 and 7 days after exposure. The experiments were carried out in triplicate each time and repeated more than three times independently, and the average values were calculated.

Flow cytometry We used flow cytometry to measure the DNA content of individual cells, which allowed us to assess the cell-cycle profiles of the cells treated with troglitazone or 15dPGJ$_2$. In preparation for flow cytometry, cells treated with 50 μM troglitazone or 0.5 μM 15dPGJ$_2$ for 6 days were collected after brief trypsinization, washed with PBS and fixed with 70% cold ethanol. The samples were then treated with ribonuclease (R6513: Sigma-Aldrich Corp., Saint Louis, MO), stained with 10 mg/ml propidium iodide and analyzed by a cell sorter (FACScan,

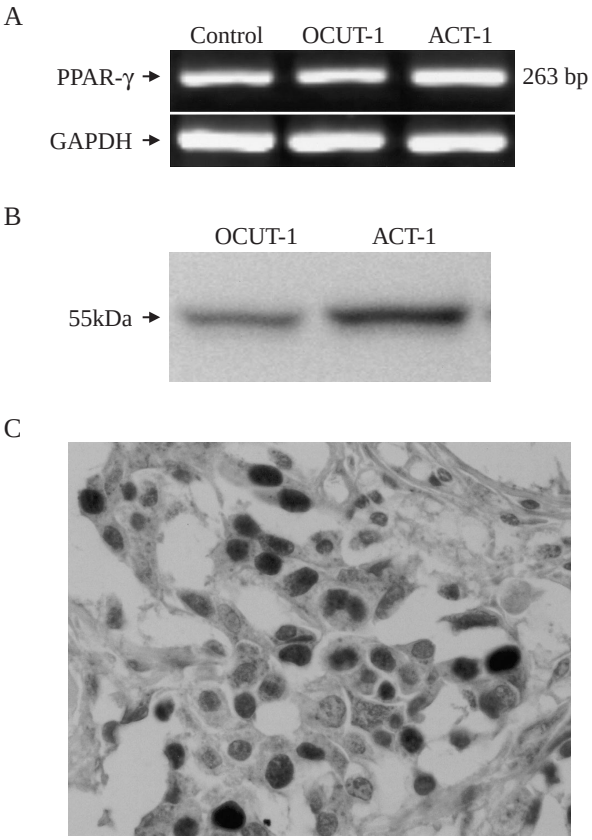

Fig. 1. Expression of PPAR-γ in thyroid cancer cell lines. In both cell lines, mRNA was clearly detected by RT-PCR analysis. Adipose tissue was used as a control (A). A single band of 55-kDa protein was detected by western blotting (B). PPAR-γ immunoreactivity was found in the nucleus of cancer cells (C).

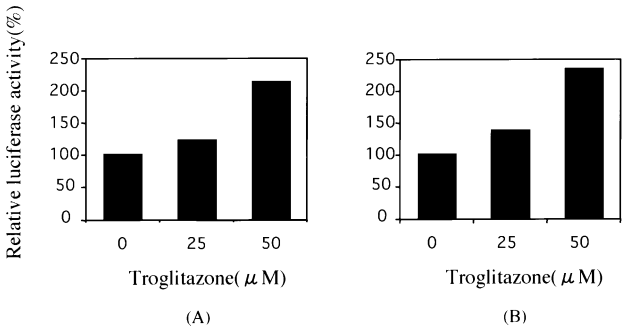

Fig. 2. Specific activation of PPRE of the *acyl-coA* gene by troglitazone was demonstrated by luciferase assay. Dose-dependent activities were found in OCUT-1 (A) and ACT-1 (B) cells. Values are the means of three independent experiments.

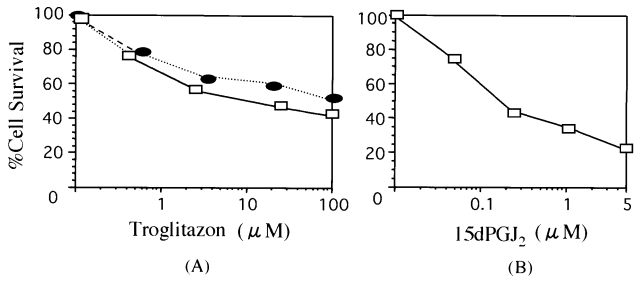

Fig. 3. Results of cell viability assay (MTT method). Cell viability was reduced in every cancer cell line examined. Troglitazone (A) and 15dPGJ$_2$ (B) inhibited cell viability in a dose-dependent manner. Values are the means of three independent experiments. □ OCUT-1, ● ACT-1.

Becton Dickinson, Mountain View, CA). Cell cycle distributions were quantified using Cell-quest software.

Cell cycle-related gene expression Approximately 1×10^6 cells were incubated with 50 μM troglitazone or 0.5 μM 15dPGJ$_2$ for 48 h, then the protein was extracted as described above. Changes in expression of p53, p21 and p27 were determined by western blotting, which was performed as described above. The intensity of the specific band was quantified with a densitometer (Atto, Tokyo). Specific antibodies against p53 (Clone DO-7, DAKO A/S, Copenhagen, Denmark), p21 (Clone F-5, Santa Cruz Biotechnology, Inc.) and p27 (Clone K-25020, Transduction Laboratories, Lexington, KY) were used.

Statistical analysis Data are expressed as mean values. Statistical analyses were carried out using Student's unpaired *t* test. $P<0.05$ was considered statistically significant.

RESULTS

Expressions of PPAR-γ were demonstrated in both of the thyroid cancer cell lines examined. In both cell lines, expression of PPAR-γ mRNA was clearly detected by RT-PCR analysis (Fig. 1A). Also, a 55-kDa protein was detected as a simple specific band by western blot analysis (Fig. 1B). PPAR-γ immunoreactivity was found in the nucleus of cancer cells in OCUT-1 (Fig. 1C).

Specific activation of PPRE of the *acyl-coA* gene by troglitazone was demonstrated by luciferase assay. Dose-dependent activities were found in OCUT-1 (Fig. 2A) and ACT-1 (Fig. 2B). A similar result was demonstrated with 15dPGJ$_2$ (data not shown).

Results from the cell viability (MTT) assay are shown in Fig. 3. Troglitazone and 15dPGJ$_2$ reduced the viability of both types of cells in a dose-dependent manner. The results of chronogenic assay are shown in Fig. 4. A growth-inhibitory effect of troglitazone was found after 4 days of exposure in OCUT-1 cells, and similar results were obtained in the ACT-1 cell line (data not shown).

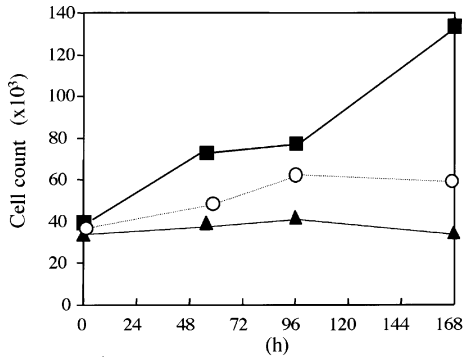

Fig. 4. Results of chronogenic assay. A growth-inhibitory effect of troglitazone in OCUT-1 cells was found after 4 days of exposure. Similar results were seen in the ACT-1 cell line (data not shown). Values are the means of three independent experiments. ■ 0 μM, ○ 10 μM, ▲ 25 μM.

Fig. 5 demonstrates the morphology of the cultured cells after 7 days of exposure to troglitazone. No remarkable change in cellular morphology was visible after exposure to troglitazone.

The results of flow-cytometric assay are shown in Fig. 6. ACT-1 cells treated with troglitazone exhibited decreased fractions of S (48.3% to 18.6%) and G2/M (15.3% to 5.6%)-phase cells resulting from an increased accumulation of cells at G0/G1 (36.5% to 75.8%). A similar increased accumulation of cells at the G0/G1 phase (63.4% to 86.2%) was also demonstrated in the OCUT-1 cell line.

Changes in expression of p53, p21 and p27 after exposure to troglitazone or 15dPGJ$_2$ were measured by western blotting. A single specific band with the expected molecular weight was observed in every experiment (Fig. 7). Only faint expression of p53 protein was found in OCUT-1 cells, and the level was not influenced by exposure to troglitazone or 15dPGJ$_2$. Although a much higher level of p53 protein expression was found in ACT-1 than OCUT-1 cells (150-fold), the level stayed constant irrespective of troglitazone or 15dPGJ$_2$ exposure. In contrast, p21 and p27 proteins were induced significantly in OCUT-1 and ACT-1 cells after troglitazone or 15dPGJ$_2$ treatment. In OCUT-1 and ACT-1 cells, p21 was induced 2-fold after 50 μM troglitazone treatment. P27 was also induced in both cell lines, by 3- and 4-fold, after 50 μM troglitazone treatment, respectively. Not only troglitazone, but also 15dPGJ$_2$ could induce p21 expression by 3.5-fold after 1.0 μM 15dPGJ$_2$ treatment in OCUT-1 and ACT-1. P27 induction was also in the same range (at 5- and 4-fold after 1.0 μM 15dPGJ$_2$ treatment in OCUT-1 and ACT-1 cells, respectively).

DISCUSSION

PPAR-γ is a member of the nuclear receptor superfamily, which includes receptors for steroid, retinoid and thyroid hormones and vitamin D. When activated, these receptors directly bind to DNA and activate various transcription factors. As consequences of ligand-induced activation of these receptors, various alterations in cellular function can be induced, such as differentiation, growth suppression, apoptosis and metamorphosis.[23] PPAR-γ is known to be expressed in almost every organ and cell type. Moreover, recent reports indicate that PPAR-γ is also expressed in many types of malignant cells.[24] Expression of PPAR-γ and an adverse effect on cell growth in follicular or papillary cancer cells of the thyroid, that is, in differentiated thyroid cancers, have been reported.[17] Cellular and clinical characteristics of anaplastic thyroid cancer,

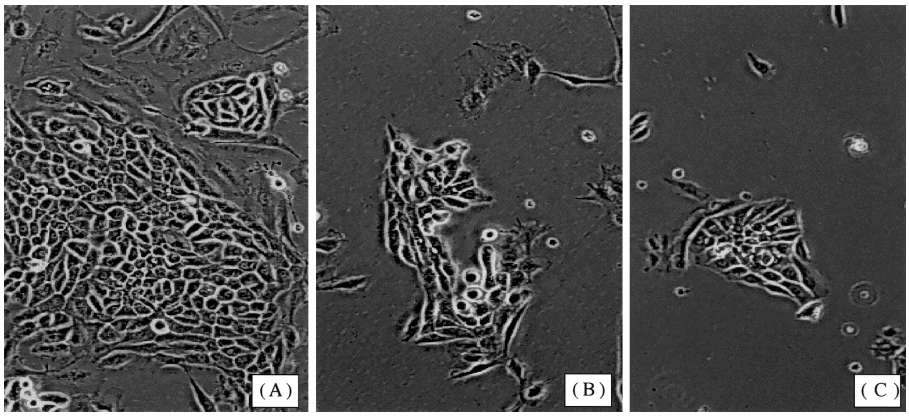

Fig. 5. No remarkable change in cellular morphology was seen after 7 days of exposure to troglitazone. A, control; B, troglitazone 25 μM; C, troglitazone 50 μM.

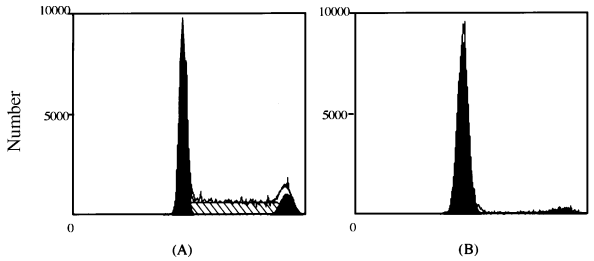

Fig. 6. Results of flow-cytometric assay. ACT-1 cells treated with troglitazone for 6 days exhibited a decreased fraction of S- and G2/M-phase cells resulting from an increased accumulation of cells at G0/G1. Similar results were seen with the OCUT-1 cell line (data not shown). A, control; B, troglitazone.

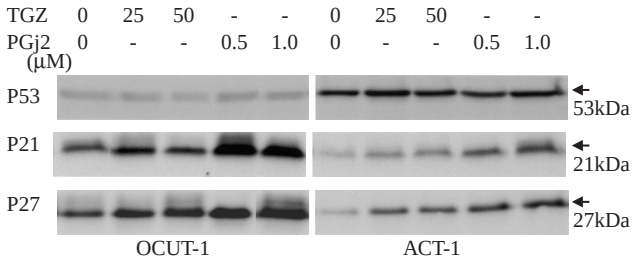

Fig. 7. Changes in expression of cell cycle-related genes are shown. A single band of 53-kDa protein was detected at a low level in OCUT-1 (A) cells and at a high level in ACT-1 cells (B). P53 protein was expressed at the same level in cells treated with troglitazone or $15dPGJ_2$ as in untreated cells (upper panel). A single band of 21-kDa protein (middle panel) or 27-kDa protein (lower panel) was detected in OCUT-1 (A) or ACT-1 (B) cells, respectively, by western blotting. Both p21 and p27 proteins were expressed at a higher level in cells treated with troglitazone or $15dPGJ_2$ than in untreated cells.

one of the most aggressive human malignancies, are very different from those of differentiated thyroid cancers. Moreover, there is not yet a definitive therapeutic strategy for treatment of anaplastic thyroid cancer. Thus, in this study, we investigated the expression of PPAR-γ and its role in anaplastic thyroid cancer in an attempt to assess its possible therapeutic implications. We found that PPAR-γ was expressed in anaplastic thyroid cancer cell lines, where PPAR-γ transcription was clearly demonstrated. PPAR-γ protein was stably expressed in thyroid cancer cells and was located in the nucleus. These results showed that the normal pattern of PPAR-γ expression was conserved in these anaplastic thyroid cancer cell lines.

PPAR-γ forms a heterodimeric DNA-binding complex with RXRα, which recognizes PPRE in the promoter of its target genes, and functions as a transcriptional regulator of gene-linked lipid metabolism.[25] To determine whether the PPAR-γ expressed in these cell lines was functional, we examined the specific activation of PPRE of the *acyl-coA* gene by luciferase assay.[21] Dose-dependent activations of PPRE by troglitazone or $15dPGJ_2$ were found in these cell lines, suggesting that PPAR-γ could be activated selectively by its specific ligands in anaplastic thyroid cancer cell lines.

Since PPAR-γ expressed in these cells was functional, we next examined the influence of ligand-induced activation of PPAR-γ on cell viability. As described in the results section, treatment with troglitazone or $15dPGJ_2$ resulted in a dose-dependent inhibition of cell viability and induced G1-phase cell-cycle arrest in these anaplastic thyroid cancer cell lines. Previous studies have shown that ligand activation of PPAR-γ induces a similar reduction of viability and cell cycle withdrawal in liposarcoma,[10] breast,[12] colon,[15] and pancreatic[16] cancer cells. Our results are compatible with those reports. Moreover, we found no marked change in cell morphology. Thus, the effect of the PPAR-γ stimulatory pathway might not be cytotoxic but

rather cytostatic. This was supported by the result of chronogenic assay, displaying no marked decrease of cell numbers after exposure. Ohta *et al.*[17] reported the induction of apoptosis after treatment of troglitazone in differentiated thyroid cancer cell lines. However, we could not find any apoptotic cell death in our anaplastic thyroid cancer cells by morphologic or flow cytometric analysis. These differences are considered to be due to the nature of the anaplastic thyroid cancer cells, i.e., higher proliferative activity and lower apoptotic ratio compared with differentiated thyroid cancer.[26]

Activation of the G1 phase cyclin and cyclin-dependent kinase (CDK) complexes results in the phosphorylation of retinoblastoma gene products (Rb) and the release of transcription factor E2F to progress the cell cycle from the G1 to the S phase. CDK inhibitors, $p21^{cip1}$ and $p27^{kip1}$, regulate this process by inhibiting cyclin/CDK activity and phosphorylation of Rb, resulting in G1 arrest. Recently, some CDK inhibitors, including p21 and p27, have been shown to be induced through PPAR-γ activation, playing crucial roles in post-mitotic growth arrest and adipocyte differentiation.[27] Furthermore, Wakino *et al.*[28] also reported that a ligand of PPAR-γ attenuated both the mitogen-induced degradation of p27 and the mitogenic induction of p21 to inhibit proliferation in vascular smooth muscle cells. It has been reported that induction of p27 with PPAR-γ activation inhibited the growth of cancer cell lines.[16] However, the downstream effector(s) of PPAR-γ activation has not been identified in relation to growth arrest. To characterize the cell cycle-related gene induction with troglitazone or $15dPGJ_2$ treatment, we assessed p21, p27 and p53 protein expression. Although *p53* gene mutation was not demonstrated in the two cell lines,[18] levels of p53 protein expression varied in OCUT-1 and ACT-1 cells, suggesting alterations in *p53* gene expression. Nonetheless, the level of p53 protein was not altered following troglitazone or $15dPGJ_2$ exposure. These results indicate that the *p53* tumor-suppressor gene plays only a small role in this cell-cycle arrest pathway. In contrast, p21 and p27 proteins were induced in OCUT-1 and ACT-1 cells after troglitazone or $15dPGJ_2$ treatment. Although p21 is a downstream effector of p53 to induce cell cycle arrest,[29] p53-independent induction of p21 has also been demonstrated.[30, 31] These results strongly suggested that cell cycle arrest at G1 in this study was independent of the p53 pathway and instead occurred via the p21 and p27 pathways.

P53 has been implicated in the control of the cell cycle, DNA repair and synthesis, cell differentiation, genomic plasticity and programmed cell death. p53 is mutated in about half of almost all types of cancer arising from a wide spectrum of tissues. A relationship between *p53* gene mutation and tumor progression to a more aggressive phenotype seems to be a common feature of various neoplasms. As far as thyroid neoplasms are concerned, several reports indicate that p53 mutations are more frequent (25–85%) in undifferentiated carcinomas of the thyroid gland, since they have rarely been detected in differentiated carcinomas; it was also reported that such mutations are a step toward the development of a tumoral undifferentiated phenotype.[32–34] Conventional therapies to treat anaplastic thyroid cancer, such as chemotherapy and irradiation therapy, have largely depended on the *p53* gene status[35–37] Although we could not find *p53* gene mutation in OCUT-1 and ACT-1, the level of p53 protein expression differed markedly between the cell lines, indicating an alteration of p53 expression in at least one of these cell lines. Thus, our result might provide a novel therapeutic approach to treating anaplastic thyroid cancer with altered *p53* gene expression.

In summary, this study showed that PPAR-γ ligands induced cell cycle arrest in anaplastic thyroid cancer cells via a p21- and p27-dependent, and p53-independent pathway. These tumor-suppressive effects of PPAR-γ may provide a novel approach to the treatment of anaplastic thyroid cancer, whether there is a p53 mutation or not.

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