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 cells via a p53-independent, but p21- and p27-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

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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% CO₂ in a humidified condition. DNA sequencing for p53 gene was performed in both OCUT-118) 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 15dPGJ₂ (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 mM dNTPs, 2 µl of 0.1 M dithiothreitol (DTT), 0.5 µl of RNA guard (Amer sham 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′-GAGATCACAGATGTGCCAA-3′ and 5′-CTGTCATCTAATTCAGTGC-3′.15) RT-PCR efficiency was confirmed by amplifying human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primers 5′-ACCACGTGATGCCATCAC-3′ and 5′-TCCACACACCTGTTGCTGTA-3′. 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. Amplification of troglitazone (provided by Sankyo Co., Ltd., Tokyo) and 15dPGJ₂ (Cayman Chemical Co., Ann Arbor, MI).

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PPAR-γ in Anaplastic Thyroid Cancer

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 mM phenylmethanesulfonyl fluoride (PMSF), 0.25 U/ml aprotinin, 5 mM DTT, 0.15 M NaCl, 1% Triton X-100, 10 mM Tris (pH 7.4)). The total protein (20 µg) was electrophoresed on a 10% polyacrylamide gel and transferred to a mem-
adhere to the bottom of the plate. Then, the intended concentrations of troglitazone and 15dPGJ₂ 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 200g 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 15dPGJ₂ 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 concentrations of troglitazone or 15dPGJ₂ 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 15dPGJ₂. In preparation for flow cytometry, cells treated with 50 µM troglitazone or 0.5 µM 15dPGJ₂ 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, Beckman Coulter).
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. 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.

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. PPAR-γ is also 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. 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. Cellular and clinical characteristics of anaplastic thyroid cancer,
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. 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. Dose-dependent activations of PPRE by troglitazone or 15dPGJ2 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 15dPGJ2 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, breast, colon, and pancreatic 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 chro-
nogenic assay, displaying no marked decrease of cell num-
bers after exposure. Ōhta 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 differ-
ences are considered to be due to the nature of the ana-
plastic thyroid cancer cells, i.e., higher proliferative activ-
ity and lower apoptotic ratio compared with differentiated
thyroid cancer.20)

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 tran-
scription factor E2F to progress the cell cycle from the G1
to the S phase. CDK inhibitors, p21cip1 and p27kip1, regulate
this process by inhibiting cyclin/CDK activity and phos-
phorylation 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 differen-
tiation.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 activa-
tion inhibited the growth of cancer cell lines.10) 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
15dPGJ2 treatment, we assessed p21, p27 and p53 protein
expression. Although p53 gene mutation was not demon-
strated in the two cell lines,18) levels of p53 protein expres-
sion 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
15dPGJ2 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 troglita-
zone or 15dPGJ2 treatment. Although p21 is a downstream
effector of p53 to induce cell cycle arrest,29) p53-indepen-
dent 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 phe-
notype seems to be a common feature of various neo-
plasms. 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 car-
cinomas; 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 ther-
apy, 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 path-
way. These tumor-suppressive effects of PPAR-γ may pro-
vide a novel approach to the treatment of anaplastic
thyroid cancer, whether there is a p53 mutation or not.

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