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Expression of PPARγ and Its Ligand-dependent Growth Inhibition in Human Brain Tumor Cell Lines

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Peroxisome proliferator-activated receptor γ (PPARγ) belongs to a superfamily of thyroid/steroid hormone receptors and regulates transcription of their target genes in a ligand-dependent manner. Recently, PPARγ was reported to be expressed in several cell lines derived from breast, colon, stomach and lung cancers. Activation of PPARγ by its ligand inhibits the growth of these tumor cells, suggesting that PPARγ ligand is a potential anti-cancer agent in PPARγ-expressing tumors. However, its expression in brain tumors has not been studied. We thus studied the expression in glioma samples with different pathological stages from 20 patients. It was demonstrated that 95% of the glioma tissue expressed PPARγ mRNA. The results prompted us to study whether PPARγ ligand affects the growth of cell lines derived from brain tumors. The receptor expression was studied in 9 cell lines either derived from malignant glioma or neuroblastoma. The expression was detected in a glioma cell line SK-MG-1 and in a neuroblastoma cell line NB-1. Addition of one of the PPARγ ligands, troglitazone, induced growth inhibition in both cell lines. Further analyses revealed that this growth inhibition is caused by a PPARγ-mediated induction of apoptosis. These results suggest that PPARγ ligands could be a potential therapeutic agent for the treatment of the brain tumors expressing this receptor.

Key words: Brain tumor — PPARγ — Troglitazone — Growth inhibition — Apoptosis

The incidence of central nervous system (CNS) neoplasms ranges from 3.8 to 5.1 cases per 100 000 in the population. In the United States, 17 500 patients with brain tumor are diagnosed as new cases among which 12 600 deaths are reported yearly.[1,2] Malignant gliomas constitute 34% of all the brain tumors. The prognosis with the tumor is extremely poor even when it is treated with combined modalities including surgical resection, chemotherapy and irradiation. The patients rarely live beyond 2 years.[3] The effectiveness of a recent trial of a novel treatment, gene therapy, remains to be evaluated.[4] It is thus important to develop other effective measures.

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to a superfamily of thyroid/steroid hormone receptors and regulates transcription of their target genes in a ligand-dependent manner.[5] Activation of PPARγ by its ligands is involved in adipogenesis[6] and anti-inflammatory effect.[5,7] Forced expression of PPARγ in fibroblast and myoblast cell lines results in their adipocyte differentiation.[8] PPARγ and RXRα form a heterodimer, which binds to a regulatory element of the target gene [PPARγ response element (PPRE)] and regulates their expression.[9] The ligands for PPARγ are 15-deoxy-Δ12,14-prostaglandin (PG) J2,[10] which is a PGD2 derivative, and thiazolidinedione drugs (troglitazone, pioglitazone and rosiglitazone).[5] Recently, expression of PPARγ was reported in breast cancer cells,[11] and activation of PPARγ by its ligand inhibited the tumor cell growth, suggesting that the ligand is a potential anti-cancer agent for tumors expressing PPARγ. Additional reports also demonstrated PPARγ expression and growth inhibition by its ligands in cancers of the colon,[12] prostate,[13,14] stomach,[15,16] lung[17] and liposarcoma.[18] Although growth inhibition was observed in a broad range of cancer cell lines, the mechanism of this inhibition seems to vary among the cell lines. In breast, colon, and prostate cancers and liposarcoma, PPARγ ligand induces terminal differentiation,[11,12,14,18] while it induces apoptosis in stomach and lung cancers.[15–17] To our knowledge, expression of PPARγ in CNS tumors has not been studied in detail. We thus studied the expression in glioma samples obtained from 20 patients. Furthermore, we screened the expression of PPARγ in 9 cell lines derived from malignant brain tumors. We found a high incidence of PPARγ expression in malignant glioma samples. Furthermore, a PPARγ ligand, troglitazone, caused apoptosis of two cell lines (glioblastoma-derived SK-MG-1 and neuroblastoma-derived NB-1) that express PPARγ, suggesting that ligands for PPARγ could be potential drugs for the treatment of malignant brain tumor.

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MATERIALS AND METHODS

Tissue samples from surgery Surgical samples from 20 patients were obtained from the tissue bank of our department. Pathological diagnosis of the tumors was astrocytoma (3 cases), anaplastic astrocytoma (5 cases), and glioblastoma (12 cases). The tumor tissues were subjected to total RNA extraction.

Cell culture Human cell lines derived from malignant gliomas (T98G, U251-SP, SK-MG-1, U-251MG, AO2, U178 and Jones) were obtained from Memorial Sloan Kettering Cancer Institute (New York, NY). U-251*nu/nu* was cloned by transplanting U-251MG cells into nude mice. Human neuroblastoma cell line (NB-1) was obtained from Hirosaki University School of Medicine (Hirosaki, Aomori). The cells were cultured in Eagle's medium (Nissui, Tokyo) supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 m*M* L-glutamine at 37°C in a humidified atmosphere (95% air and 5% CO_2). HepG2 cells derived from human hepatoblastoma (American Type Culture Collection (ATCC), Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 m*M* L-glutamine.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis and cDNA probe preparation A cDNA probe for human PPARγ was prepared by RT-PCR amplification using total RNA from HepG2 cells. The sequences of oligonucleotides used were as follows: PPARγ; sense (296–322) 5′-TTCTCCAGCATTTCTACTCCACATTAC-3′, antisense (888–914) 5′-ATGGTGATTTGTCTGTTGTCTTTCCTG-3′.[19] For RT-PCR, 1 μg of total RNA and oligo(dT) primer at a final concentration of 1 μ*M* in 5.5 μl was heated to 70°C for 3 min, followed by cooling on ice for 1 min. cDNA synthesis was initiated by incubating with 200 units of recombinant reverse transcriptase (Superscript II, Life Technologies, Gaithersburg, MD) under the conditions recommended by the supplier. One-hour reaction at 37°C was terminated by heating at 100°C for 5 min. After precipitation in ethanol, the cDNA was dissolved into 50 μl of a buffer (10 m*M* Tris-HCl pH 8.0, 10 m*M* EDTA). A 2-μl aliquot of the cDNA, 2 μl of the oligonucleotide primers (10 μ*M* each), 0.5 μl of dNTP mixture (100 m*M*) and *Taq* DNA polymerase (AmpliTaq, Applied Biosystems, Foster City, CA) were used for subsequent PCR. The conditions for the PCR were: denaturation at 94°C for 30 s, annealing at 54°C for 1 min and extension at 74°C for 1 min. The cycle number ranged from 20 to 30. The amplified cDNA fragments (619 bp) were subcloned into pGEM-T Easy vector (Promega, Madison, WI). Authenticity of the cDNA was verified by sequencing and the cDNA was used as the probe for northern blot analysis. The same primers were used to detect the expression of PPARγ in the tumor and the cell lines. The previously described cDNA probe for a house-keeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also used for the northern blot analysis.[20]

Northern blot analysis Cells were cultured in 100-mm dishes (Falcon 3003, Becton Dickinson Labware, Franklin Lakes, NJ) to subconfluence. Total RNA was extracted by the acid guanidine phenol/chloroform method.[21] Ten micrograms of total RNA from each cell line was electrophoresed on 0.8% agarose gel, blotted on a nitrocellulose membrane (Gene Screen Plus, NEN, Boston, MA) and hybridized with the ^{32}P-labeled cDNA probe for PPARγ. The conditions for hybridization and for washing were as described previously.[20] After detecting PPARγ mRNA expression, the same membrane was rehybridized with the radiolabeled cDNA probe for GAPDH.[21] To determine the amount of each mRNA, the hybridized membrane was exposed to the imaging plate, and the radioactivity of a specific band was measured by using a Fujix Bioimage Analyzer (BAS 2000, Fuji Photo Film, Tokyo). The radioactivity of the PPARγ mRNA band was corrected based on that of GAPDH.

Chemicals Troglitazone was dissolved in dimethyl sulfoxide (DMSO).

Cellular proliferation assay The effect of troglitazone on the proliferation of each cell line was studied as follows. Approximately 1×10^3 cells were seeded in 96-well plates (Becton Dickinson Labware). After overnight culture, troglitazone (0–20 μ*M*) was added (day 0), and the viable cells were estimated with a WST assay kit (Dojindo, Kumamoto) on days 0, 1, 3, and 4.

Data were expressed as mean±SD (n=8). Statistical analyses were carried out using Student's unpaired t test. $P<0.05$ was considered statistically significant.

Analysis of DNA fragmentation due to apoptosis The cells were cultured with troglitazone (0 or 20 μ*M*) for 3 days. They were lysed in 100 μl of cell lysis buffer (10 m*M* Tris-HCl pH 7.4, 10 m*M* EDTA, and 0.5% Triton X-100) for 10 min on ice and centrifuged at 12 000 rpm for 20 min. The resulting supernatant containing the fragmented DNA was treated with RNase A (0.4 mg/ml) for 1 h at 37°C followed by proteinase K (0.4 mg/ml) for 1 h at 37°C. After precipitation with 5 *M* NaCl and isopropanol, DNA was collected and resuspended in 20 μl of TE buffer (10 m*M* Tris-HCl; 1 m*M* EDTA, pH 8.0). ApoAlert LM-PCR Ladder Assay Kit (CLONTECH, Palo Alto, CA) was used to detect DNA fragmentation according to the manufacturer's protocol.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling) method Approximately 1×10^4 cells were cultured in Lab-Tek Chamber Slide (Nalgen Nunc International, Naperville, IL). The cells were treated with 20 μ*M* troglitazone for 4 days. DNA fragmentation was analyzed by the TUNEL method[22] using

ApoAlert DNA Fragmentation Assay Kit (CLONTECH).

Flow cytometry Flow cytometry was performed to detect annexin V binding and to analyze cell cycle distribution. Annexin V binding was studied by using the ApoAlert Annexin V-FITC Kit (CLONTECH) according to the manufacturer's protocol. After 72, 96 and 144 h of troglitazone (0 or 20 μM) treatment, the cells were harvested, resuspended in 200 μl of binding buffer, and then 5 μl of FITC-conjugated annexin V and 10 μl of propidium iodide were added. For cell cycle analysis, the cells were treated with troglitazone (0 or 20 μM) for 24 h. The cells were fixed in 70% ethanol for 1 h at 4°C, centrifuged at 6000 rpm for 3 min, washed twice with phosphate-buffered saline (PBS), and resuspended in 0.2 ml of PBS with 50 units/ml RNase A. After incubation for 30 min at 37°C, they were stained with 50 μg/ml propidium iodide for 30 min at 4°C. A flow cytometer (Coulter EPICS XL, Coulter Corp., Miami, FL) was used for both analyses, after passing the cells through a nylon-mesh filter. The percentages of cells in G0/G1, S, and G2/M of the cell cycle were analyzed and quantitated with the Multicycle software (Coulter Electronics, Coulter Corp.).

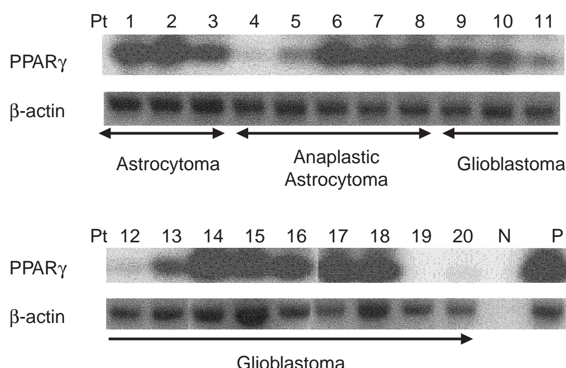

Fig. 1. PPARγ mRNA expression in surgically resected glioma samples. Expression of PPARγ mRNA in glioma samples was determined by RT-PCR analysis. See "Materials and Methods" for the conditions of RT-PCR. PPARγ mRNA was detected in 19 out of 20 samples (95%). In 5 out of 19 samples, however, PPARγ mRNA expression was relatively low. N, negative control (no RNA-RT product); P, positive control (RT-PCR product from HepG2 total RNA).

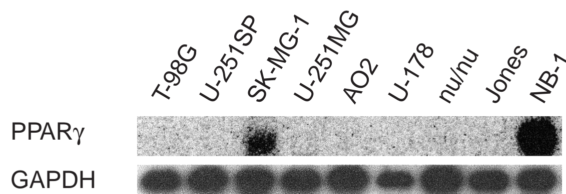

Fig. 2. PPARγ mRNA expression in brain tumor cell lines. Expression of PPARγ mRNA in brain tumor cell lines was determined by northern blot analysis. Its expression was detected in a neuroblastoma cell line NB-1 and glioma-derived SK-MG-1. HepG2 (hepatoblastoma cell line) was used as a positive control.

RESULTS

Expression of PPARγ in glioma patients' specimens To examine whether PPARγ is expressed in glioma samples surgically excised from patients, we performed RT-PCR. As shown in Fig. 1, PPARγ mRNA expression was detected in 19 out of 20 patients (95%). In 5 out of 19 samples, PPARγ mRNA expression was relatively low. There was no correlation between its expression and the pathological gradings of the gliomas.

Expression of PPARγ in brain tumor cell lines Expression of PPARγ mRNA was studied in nine human brain tumor cell lines by northern blot analysis. As shown in Fig. 2, the highest expression was observed in a neuroblastoma cell line NB-1, and moderate expression levels were seen in glioma-derived SK-MG-1 and hepatoblastoma-derived HepG2. The absence of PPARγ mRNA was ascertained by RT-PCR in the negative cell lines (data not shown).

Since PPARγ itself is one of the PPARγ-responsive genes, we next examined whether the level of PPARγ mRNA was increased by troglitazone in the positive cell lines, SK-MG-1 and NB-1. In SK-MG-1, it was increased at 12 h after troglitazone, and in NB-1 from 12 to 48 h (Fig. 3).

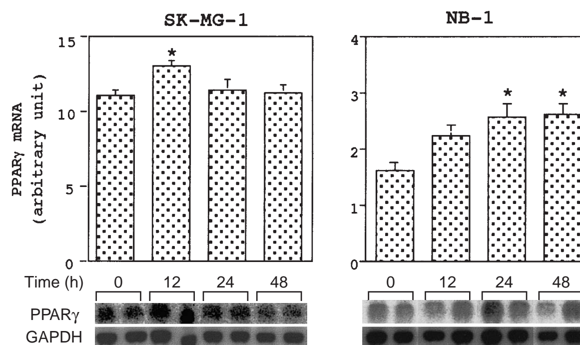

Fig. 3. Effect of troglitazone on PPARγ-responsive gene. Lower panels depict representative autoradiographs after hybridization with the cDNAs for PPARγ and GAPDH using duplicate samples for each time point. The experiment was repeated three times and the levels of PPARγ mRNA corrected based on that of GAPDH mRNA are presented as mean±SD. Expression of mRNA of PPARγ, whose gene is PPARγ-responsive, was induced by troglitazone treatment, indicating that the receptor expressed in these cell lines is functionally active. * $P<0.05$.

Inhibition of cell growth by PPARγ ligand To study the effect of PPARγ ligand on the growth of the tumor cell lines, WST assays were performed. In the six glioma cell lines expressing no PPARγ mRNA, troglitazone treatment did not affect their cell growth (Fig. 4, A–F). Note that troglitazone seems to affect the growth of AO2 cells that do not express PPARγ. However, no significant growth inhibition due to treatment with troglitazone was observed at any time point. In the SK-MG-1 cells expressing PPARγ, cell viability was similar in the troglitazone-treated and untreated groups until day 3 (Fig. 4G). However, on day 4, troglitazone treatment resulted in a significant decrease in the cell viability compared with the non-treated cells. Similarly in another PPARγ-expressing cell line, NB-1, troglitazone significantly decreased the cell viability on days 3 and 4 (Fig. 4H).

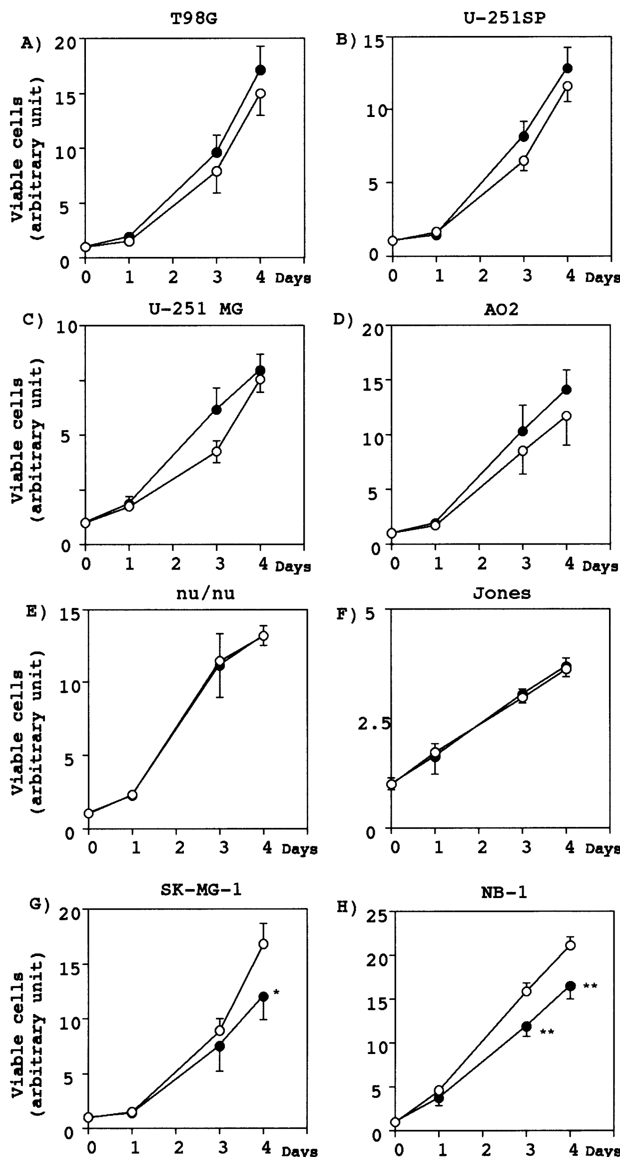

Fig. 4. Effect of troglitazone on cell growth in PPARγ-positive and negative cell lines. The numbers of viable cells were determined by WST assay on days 0 (addition of troglitazone), 1, 3 and 4. A–F, PPARγ-negative cell lines; G, H, cell lines expressing PPARγ. The data are presented as mean±SD (n=8). Note that significant growth inhibition was observed in only two cell lines, SK-MG-1 (G) and NB-1 cells (H). ○ troglitazone (−), ● troglitazone (20 μM). * P<0.01 vs. troglitazone (−).

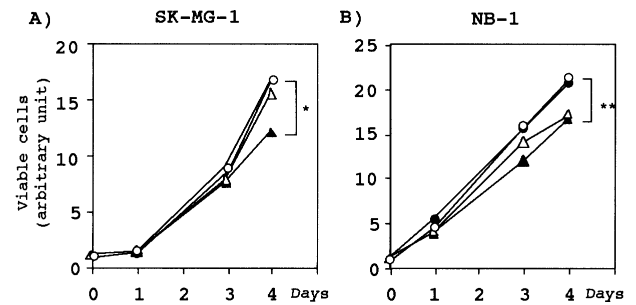

Fig. 5. Dose of troglitazone required for growth inhibition. The cell viability was analyzed by WST assay in the presence of 10, 15 and 20 μM. Only 20 μM troglitazone decreased the cell viability in SK-MG-1 cells. Growth of NB-1 cells was inhibited by 15 μM and 20 μM troglitazone. The data are presented as mean±SD (n=8). ○ troglitazone (−), ● troglitazone (10 μM), △ troglitazone (15 μM), ▲ troglitazone (20 μM). * P<0.01, ** P<0.005 vs. troglitazone (−).

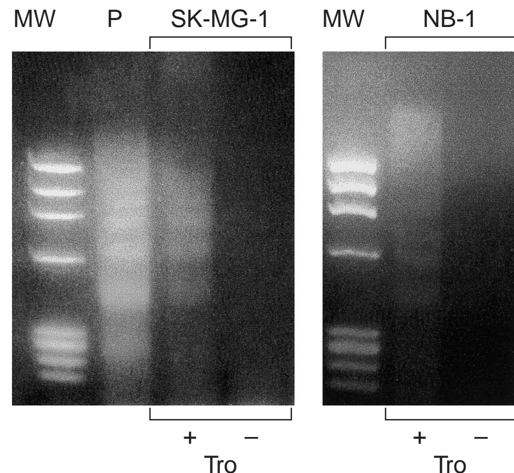

Fig. 6. DNA ladder formation with troglitazone treatment. DNA fragmentation was analyzed by the LM-PCR method. In both SK-MG-1 and NB-1 cells, a DNA ladder pattern was seen after treatment with troglitazone for 3 days. MW, molecular weight marker; P, positive control (fetal thymus DNA).

We next examined the dose of troglitazone required for the growth inhibition. In SK-MG-1 cells, 10 *μM* and 15 *μM* troglitazone did not affect the cell growth. Only 20 *μM* troglitazone decreased the cell viability (Fig. 5A). Growth of NB-1 cells was inhibited by 15 *μM* and 20 *μM* troglitazone (Fig. 5B). These results demonstrate that troglitazone at 20 *μM* inhibits the growth of both cell lines.

Effect of troglitazone on the differentiation of SK-MG-1 and NB-1 cells Treatment of SK-MG-1 or NB-1 cells with 20 *μM* troglitazone for 4 days did not cause any morphological changes such as elongation of dendritic processes, suggesting that the ligand for PPARγ does not affect the differentiation of the two cell lines (data not shown). We thus speculated that troglitazone inhibited the growth of the two cells by inducing apoptosis. The possibility was tested as follows.

Detection of apoptosis in brain tumor cell lines Apoptosis is characterized by a series of typical morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis.[22] The biochemical hallmark of apoptosis is degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180–200 base pairs.[22] To study whether troglitazone treatment of SK-MG-1 or NB-1 induces apoptosis, we analyzed the presence or absence of fragmented DNA by the LM-PCR method in cells treated with the drug. As shown in Fig. 6, in SK-MG-1 and NB-1, no DNA fragmentation could be observed in the untreated groups. However, on day 3, a DNA ladder pattern was demonstrated in both cell lines treated with troglitazone. DNA

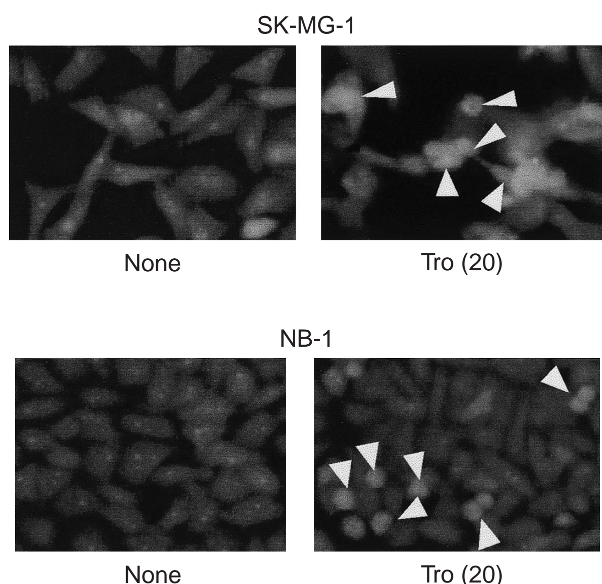

Fig. 7. TUNEL staining of SK-MG-1 and NB-1 cells in the presence of troglitazone. TUNEL-positive cells (arrow head) were detected in both SK-MG-1 and NB-1 cells treated with 20 *μM* troglitazone for 4 days.

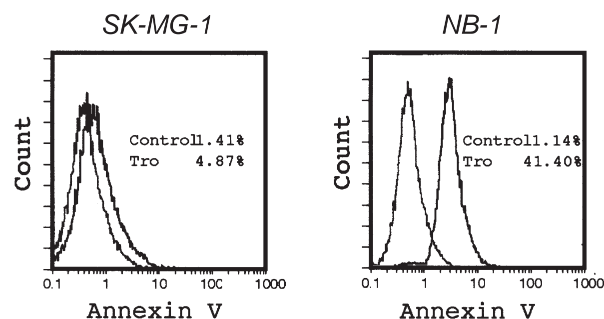

Fig. 8. Study of annexin V staining in SK-MG-1 and NB-1 cells. After annexin V staining, the fraction corresponding to annexin V-positive cells was determined by flow cytometry and indicated as percentage of the total cell population. Annexin V-positive cell fraction was increased by troglitazone treatment.

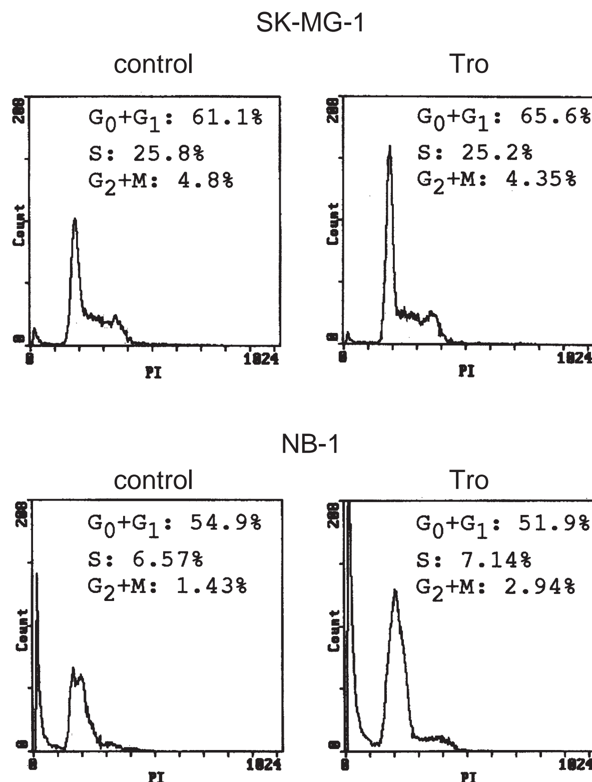

Fig. 9. Cell cycle analysis under treatment with troglitazone. Cell cycle analysis was done after troglitazone treatment for 24 h, using a flow cytometer. The cell cycles of both SK-MG-1 and NB-1 cells were not affected by troglitazone treatment.

fragmentation was further analyzed by the TUNEL method. As shown in Fig. 7, TUNEL-positive cells were detected in SK-MG-1 and NB-1 cells treated with 20 μM troglitazone for 4 days.

An additional characteristic feature in the early stage of apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane.[23] This translocation can be detected by annexin V, which binds preferentially to PS in the presence of Ca^{2+}. Annexin V-positive cells were slightly increased in SK-MG-1 after 72, 96, and 114 h of troglitazone treatment. As shown in Fig. 8, annexin V staining at 96 h demonstrated that annexin V-positive cells increased under troglitazone treatment (1.41% in the untreated vs. 4.87% in the treated). In the case of NB-1 cells, annexin V-positive cells were slightly increased at 72 h and 96 h after troglitazone treatment (data not shown). Furthermore, at 144 h, there was a significant increase of annexin V-positive cells (1.14% in the untreated vs. 41.4% in the treated) (Fig. 8). As shown in Fig. 9, the cell cycle in SK-MG-1 and NB-1 cell lines was not affected by the treatment with troglitazone.

DISCUSSION

RT-PCR demonstrated expression of PPARγ in most (95%) of the glioma samples surgically excised from 20 patients (Fig. 1). In contrast to the high incidence of PPARγ expression in glioma specimens, expression was demonstrated in only one glioma-derived cell line, SK-MG-1 and one neuroblastoma-derived cell line, NB-1. It is speculated that clonal selection of the brain tumor cells might favor the growth of PPARγ-negative cells.

The present study for the first time demonstrated that a PPARγ ligand can inhibit the growth of cells derived from malignant glioma and neuroblastoma when they express the receptor. The finding that troglitazone did not inhibit the growth of PPARγ-negative cells supports the notion that the growth-inhibitory effect of the ligand is mediated by its receptor.[12, 13]

Our present study also demonstrated that a PPARγ ligand induces apoptosis of both SK-MG-1 and NB-1 cells without affecting the cell cycle. Although the pathway for the PPARγ ligand-induced apoptosis in these cell lines is not defined, the findings are compatible with those reported by Takahashi *et al.*[15] and Sato *et al.*[16]

This *in vitro* study together with the finding that PPARγ is frequently expressed in glioma tissues raises the possibility that treatment with a PPARγ ligand could be a new therapeutic approach for malignant brain tumors. However, the pro-apoptotic effect of troglitazone is rather marginal, so a more potent analogue will be required. Further study is also necessary to determine how to deliver the PPARγ ligand specifically to brain tumors.

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