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

Mihoko Kato, Takashi Nagaya, Miyuki Fujieda, Kiyoshi Saito, Jun Yoshida and Hisao Seo

1Department of Endocrinology and Metabolism, Division of Molecular and Cellular Adaptation, Research Institute of Environmental Medicine, Nagoya University, Chikusa-ku, Furo-cho, Nagoya 464-8601 and 2Graduate School of Medicine, Department of Neurosurgery, Nagoya University, Tsu ranai-cho, Showa-ku, Nagoya 466-8550

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. 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. The effectiveness of a recent trial of a novel treatment, gene therapy, remains to be evaluated. 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. 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

To whom correspondence should be addressed.
E-mail: hseo@riem.nagoya-u.ac.jp

660
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, A02, 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 Hiroaki University School of Medicine (Hiroaki, Aomori). The cells were cultured in Eagle’s medium (Nissui, Tokyo) supplemented with 10% FBS, 50 µg/ml penicillin, 50 µg/ml streptomycin, and 2 mM l-glutamine at 37°C in a humidified atmosphere (95% air and 5% CO2). 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 µg/ml penicillin, 50 µg/ml streptomycin, and 2 mM 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 are as follows: PPARγ sense (296–322): 5′-TTCTCCAGCATTCTAC-TCCACATTAC-3′, antisense (888–914): 5′-ATGTTGAT-TTGCTGGTGTTCCTGTCG-3′. For RT-PCR, 1 µg of total RNA and oligo(dT) primer at a final concentration of 1 µM 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 using 4°C and then ethanol was added to precipitate the DNA. The DNA was collected and resuspended in 20 µl of water. The cDNA was then amplified using the following primers: forward 5′-ATCGCGCTTTGAGTCG-3′, reverse 5′-ATGGTGAT-TTGCTGGTGTTCCTGTCG-3′. Approximately 1×10^4 cells were cultured in Lab-Tek Chamber Slide (Nalge Nunc International, Naperville, IL). The cells were treated with troglitazone (0 or 20 µM) for 3 days. They were lysed in 100 µl of cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA). A 2-µl aliquot of the cDNA, 2 µl of the oligonucleotide primers (10 µM each), 0.5 µl of dNTP mixture (100 mM) 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 housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also used for the northern blot analysis.

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. Ten micrograms of total RNA from each cell line was electrophoresed on 0.8% agarose gel, blotted to a nitrocellulose membrane (Gene Screen Plus, NEN, Boston, MA) and hybridized with the 32P-labeled cDNA probe for PPARγ. The conditions for hybridization and for washing were as described previously. After detecting PPARγ mRNA expression, the same membrane was rehybridized with the radiolabeled cDNA probe for GAPDH. 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 µM) 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 µM) for 3 days. They were lysed in 100 µl of cell lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM 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 20 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 using 4°C and then ethanol was added to precipitate the DNA. The DNA was collected and resuspended in 20 µl of TE buffer (10 mM Tris-HCl; 1 mM 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 1×10^3 cells were cultured in Lab-Tek Chamber Slide (Nalgen Nunc International, Naperville, IL). The cells were treated with 20 µM troglitazone for 4 days. DNA fragmentation was analyzed by the TUNEL method using Apoptosis of Brain Tumor by PPARγ Ligand
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.).

**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. 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.

**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 tro-
glitazone 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 mor-
phological changes such as elongation of dendritic pro-
cesses, 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 possi-

![SK-MG-1](image1)

None Tro (20)

![NB-1](image2)

None Tro (20)

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.

![SK-MG-1](image3)

Annexin V

Control 41.4% Tro 4.67%

![NB-1](image4)

Annexin V

Control 14.1% Tro 41.4%

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-
postive cell fraction was increased by troglitazone treatment.

![SK-MG-1](image5)

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 \( \mu M \) 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. This translocation can be detected by annexin V, which binds preferentially to PS in the presence of \( \text{Ca}^{2+} \). 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. 4.14% 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 PPAR\( \gamma \) in most (95%) of the glioma samples surgically excised from 20 patients (Fig. 1). In contrast to the high incidence of PPAR\( \gamma \) 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 PPAR\( \gamma \)-negative cells.

The present study for the first time demonstrated that a PPAR\( \gamma \) 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 PPAR\( \gamma \)-negative cells supports the notion that the growth-inhibitory effect of the ligand is mediated by its receptor. 

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

This in vitro study together with the finding that PPAR\( \gamma \) is frequently expressed in glioma tissues raises the possibility that treatment with a PPAR\( \gamma \) 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 PPAR\( \gamma \) ligand specifically to brain tumors.

**ACKNOWLEDGMENTS**

We are indebted to Sankyo Pharmaceutical Co. (Tokyo) for the provision of troglitazone. This work was supported in part by a grant from the Ministry of Health, Labour and Welfare.

(Received January 9, 2002/Revised March 22, 2002/Accepted March 28, 2002)

**REFERENCES**

1) Boring, C. C., Squires, T. S. and Tong, T. Cancer statistics, 1993. *CA Cancer J. Clin.*, 43, 7–26 (1993).
2) Boring, C. C., Squires, T. S., Tong, T. and Montgomery, S. Cancer statistics, 1994. *CA Cancer J. Clin.*, 44, 7–26 (1994).
3) Lesser, G. J. and Grossman, S. The chemotherapy of high-grade astrocytomas. *Semin. Oncol.*, 21, 220–235 (1994).
4) Wakabayashi, T., Mizuno, M. and Yoshida, J. Gene therapy of central nervous system tumors. *Neurul Med. Chir. (Tokyo)*, 38, 763–771 (1998).
5) Desvergne, B. and Wahli, W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.*, 20, 649–688 (1999).
6) Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. and Evans, R. M. 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte differentiation factor PPAR-gamma. *Cell*, 83, 803–812 (1995).
7) Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J. C., Najib, J., Maclouf, J. and Tedgui, A. Activation of human aortic smooth-muscle cells is inhibited by PPAR-alpha but not by PPAR-gamma activators. *Nature*, 393, 790–793 (1998).
8) Tontonoz, P., Hu, E. and Spiegelman, B. M. Stimulation of adipogenesis in fibroblasts by PPAR-gamma 2, a lipid-activated transcription factor. *Cell*, 79, 1147–1156 (1994).
9) Spiegelman, B. M. PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes*, 47, 507–514 (1998).
10) Kliwer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C. and Lehmann, J. M. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell*, 83, 813–819 (1995).
11) Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S. and Spiegelman, B. M. Terminal differentiation of human breast cancer through PPAR-gamma. *Mol. Cell*, 1, 465–470 (1998).
12) Kitamura, S., Miyazaki, Y., Shinomura, Y., Kondo, S., Kanayama, S. and Matsuzawa, Y. Peroxisome proliferator-
activated receptor γ induces growth arrest and differentiation markers of human colon cancer cells. *Jpn. J. Cancer Res.*, 90, 75–80 (1999).

13) Kubota, T., Koshizuka, K., Williamson, E. A., Asou, H., Said, J. W., Holden, S., Miyoshi, I. and Koefler, H. P. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res.*, 58, 3344–3352 (1998).

14) Mueller, E., Smith, M., Sarraf, P., Kroll, T., Aiyer, A., Kaufman, D. S., Oh, W., Demetri, G., Figg, W. D., Zhou, X. P., Eng, C., Spiegelman, B. M. and Kantoff, P. W. Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. *Proc. Natl. Acad. Sci. USA*, 97, 10990–10995 (2000).

15) Takahashi, N., Okumura, T., Motomura, W., Fujimoto, Y., Kawabata, I. and Kohgo, Y. Activation of PPAR-gamma inhibits cell growth and induces apoptosis in human gastric cancer cells. *FEBS Lett.*, 455, 135–139 (1999).

16) Sato, H., Ishihara, S., Kawashima, K., Moriyama, N., Suetsugu, H., Kazumori, H., Okuyama, T., Rumi, M. A., Fukuda, R., Nagasue, N. and Kinoshita, Y. Expression of peroxisome proliferator-activated receptor (PPAR) gamma in gastric cancer and inhibitory effects of PPAR-gamma agonists. *Br. J. Cancer*, 83, 1394–1400 (2000).

17) Chang, T. H. and Szabo, E. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. *Cancer Res.*, 60, 1129–1138 (2000).

18) Tontonoz, P., Singer, S., Forman, B. M., Sarraf, P., Fletcher, J. A., Fletcher, C. D., Brun, R. P., Mueller, E., Aliotk, S., Oppenheim, H., Evans, R. M. and Spiegelman, B. M. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. *Proc. Natl. Acad. Sci. USA*, 94, 237–241 (1997).

19) Greene, M. E., Blumberg, B., McBride, O. W., Yi, H. F., Kronquist, K., Kwan, K., Hsieh, L., Greene, G. and Nimer, S. D. Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. *Gene Expr.*, 4, 281–299 (1995).

20) Nagaya, T., Murata, Y., Yamaguchi, S., Nomura, Y., Ohmori, S., Fujieda, M., Katunuma, N., Yen, P. M., Chin, W. W. and Seo, H. Intracellular proteolytic cleavage of 9-cis-retinoic acid receptor alpha by cathepsin L-type protease is a potential mechanism for modulating thyroid hormone action. *J. Biol. Chem.*, 273, 33166–33173 (1998).

21) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162, 156–159 (1987).

22) Saraste, A. and Pulkki, K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc. Res.*, 45, 528–537 (2000).

23) Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, 184, 39–51 (1995).