Retinoid X receptor-γ and peroxisome proliferator-activated receptor-γ expression predicts thyroid carcinoma cell response to retinoid and thiazolidinedione treatment

Joshua P. Klopper,[1] William R. Hays,[1] Vibha Sharma,[1] Margaret A. Baumbusch,[1] Jerome M. Hershman,[2] and Bryan R. Haugen[1]

[1]Division of Endocrinology, Metabolism and Diabetes, Department of Medicine, University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado and [2]Department of Medicine, West Los Angeles VAMC and University of California School of Medicine, Los Angeles, California

Abstract

Poorly differentiated, metastatic thyroid cancer is difficult to treat. These tumors often do not concentrate radioactive iodine and may require chemotherapy, which is suboptimal and toxic. Nuclear hormone receptors peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXR) are variably expressed in thyroid carcinoma cell lines. Expression of these receptors may predict thyroid cancer cell response to treatment with rexinoids and thiazolidinediones. We studied three thyroid carcinoma cell lines: BHP 5-16 (PPARγ−/RXRγ+), BHP 2-7 (PPARγ±/RXRγ−), and DRO-90 (RXRγ+/PPARγ+). BHP 5-16 (RXRγ+) cells treated with rexinoid had decreased proliferation to 69 ± 6% growth compared with vehicle. BHP 2-7 (PPARγ+) cells treated with thiazolidinedione had no decrease in cellular proliferation. DRO-90 (RXRγ+ and PPARγ+) cells had 36 ± 10%, 15 ± 3%, and 13 ± 4% growth when treated with rexinoid, thiazolidinedione, or a combination, respectively. We next investigated the role of apoptosis in the ligand-responsive BHP 5-16 and DRO-90 cells. BHP 5-16 cells underwent no significant apoptosis with rexinoid (1 μmol/L). DRO-90 cells, however, had 3.6 ± 1.3% apoptotic cells with vehicle, 13 ± 3.5% with rexinoid (1 μmol/L), 18 ± 4% with thiazolidinedione (1 μmol/L), and 28 ± 6% with combination treatment (1 μmol/L), suggesting that apoptosis plays a major role in this anaplastic cell line and that the effects of the two ligands are additive. We conclude that receptor expression is necessary for inhibition of thyroid carcinoma growth with ligand treatment but may not be sufficient for response. Additionally, expression of both RXRγ and PPARγ may be necessary for maximal growth inhibition by ligands and may be required for the increased apoptosis. [Mol Cancer Ther 2004;3(8):1011–20]

Received 3/3/04; revised 5/24/04; accepted 6/17/04.

Grant support: NIH grant DK54383, research grant from Takeda Pharmaceuticals, and (in part) by National Cancer Institute grant #R25 CA49981.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Joshua P. Klopper, Division of Endocrinology, Metabolism and Diabetes, Department of Medicine, University of Colorado Cancer Center, University of Colorado Health Sciences Center, Box B151, 4200 East 9th Avenue, Denver, CO 80262. Phone: 303-315-8443; Fax: 303-315-4525. E-mail: joshua.klopper@uchsc.edu

Introduction

Most thyroid carcinomas are well differentiated and respond well to surgery, radioactive iodine therapy, and levothyroxine suppression therapy. However, a significant minority of thyroid cancers are poorly differentiated and often metastatic and do not maintain their capacity to concentrate radioactive iodine (1). These tumors lead to the majority of thyroid carcinoma–related deaths. The tumors may be aggressive papillary or follicular thyroid carcinomas or the almost uniformly fatal anaplastic thyroid carcinoma. Although *in vitro* and *in vivo* studies, including small human trials, have showed modest efficacy in treating anaplastic thyroid carcinoma with agents such as paclitaxel, there is by no means a cure for this deadly disease (2, 3). A novel approach to thyroid cancer therapy, based on features at a molecular as opposed to a histologic level, may help direct novel therapies to patients who would most benefit.

Retinoids are vitamin A derivatives that activate retinoic acid receptors and retinoid X receptors (RXR; ref. 4). Retinoids have been shown to inhibit cellular growth and to induce redifferentiation in some poorly differentiated thyroid cancer cell lines (5–8). Retinoid receptors have six separate isotypes encoded on six separate genes (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ; ref. 9). We have shown that the RXRγ isotype is expressed in a subset of thyroid carcinoma tissue and cell lines but is not expressed in normal thyroid tissue. We have also showed that expression of this receptor predicts growth suppression by treatment with retinoids (10).

Another class of nuclear hormone receptors is the peroxisome proliferator-activated receptors (PPAR). Similar to the RXRs, three isotypes exist (PPARα, PPARβ, and PPARγ; ref. 11). PPARγ is most well known as a regulator of adipocyte differentiation and the target for the thiazolidinedione class of drugs used for the treatment of insulin resistance in type II diabetes mellitus (12). PPARγ acts via formation of a heterodimer with RXR. This heterodimer complex interacts with peroxisome proliferator response elements and regulates the expression of target genes

(13–15). Some studies suggest that the combination of thiazolidinedione and RXR selective retinoids may redifferentiate tumors by a synergistic or additive mechanism (16, 17).

We hypothesize that the nuclear hormone receptor complement of RXRγ and PPARγ in thyroid carcinoma cell lines will predict response to receptor-specific ligand treatment. Furthermore, given the requirement for PPARγ to form a heterodimer with RXR, activation of both receptors should lead to an additive or synergistic response.

Materials and Methods

Chemicals

Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 2% fetal bovine serum (FBS; Hyclone, Logan, UT). LG 346 and LG 1069 were provided by Ligand Pharmaceuticals (San Diego, CA). Pioglitazone was provided by Takeda Pharmaceuticals (Lincolnshire, IL). Rosiglitazone was provided by GlaxoSmithKline Pharmaceuticals (Philadelphia, PA).

Cell Lines

BHP 5-16 and BHP 2-7 are human papillary thyroid carcinoma cell lines (10). DRO-90 human anaplastic thyroid carcinoma cell lines were kindly provided by Dr. G.J. Juillard (University of California-Los Angeles, Los Angeles, CA).

Reverse Transcription-PCR

Qualitative reverse transcription-PCR (RT-PCR) was done by generating sense strand RNA for PPARγ and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Oligonucleotides to the corresponding human ligand binding domain were synthesized by Invitrogen Life Technologies (Carlsbad, CA): for PPARγ, sense 5′-TCTGGCCCACCAACTTTGGG-3′ and antisense 5′-CTTCACAAGCATGAACTCCA-3′; for GAPDH, sense 5′-CTTTGGTATCGTGGAAGGAC-3′ and antisense 5′-GAAATGAGCTTGACAAAGTG-3′. Total RNA was obtained from our experimental cell lines (TriReagent, Sigma Chemical Co., St. Louis, MO), and 5 μg were reverse transcribed using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The reverse transcription procedure involved incubation times and temperatures as follows: 10 minutes at room temperature, 45 minutes at 42°C, and 5 minutes at 90°C. The reverse transcription product was divided into two PCR reactions (PPARγ and GAPDH). The PCR reaction used 98°C for 5-minute denaturation temperature and 1-minute annealing temperature and 72°C for 1-minute elongation temperature. The reaction was carried out over 35 cycles and required 50°C annealing temperature for PPARγ and 60°C annealing temperature for GAPDH.

Quantitative RT-PCR was done for RXRγ as described previously (10). Quantitative RT-PCR was done for PPARγ using RNA extracted from DRO-90 cell lines and followed the same methods as described previously (10). Primers and probes are available on request.

Western Blot Analysis

Protein was isolated from nuclear extracts using a commercial nuclear protein extraction kit (Active Motif, Carlsbad, CA). The protein content of lysates was measured using a commercial DC protein assay kit (Bio-Rad, Hercules, CA). Diluted samples containing equal amounts of protein were mixed with 2× Laemmli sample buffer (Bio-Rad, catalogue no. 161-0737). Proteins were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1× TBST [20 mmol/L Tris-HCl (pH 7.6), 8.5% NaCl, 0.1% Tween 20] containing 5% nonfat dry milk at room temperature for 2 hours and incubated in the appropriate primary antibody in 1× TBST containing 5% nonfat dry milk at 4°C overnight. RXRγ (Y-20) primary antibody and PPARγ (E-8, catalogue no. 7273) monoclonal antibody were obtained (Santa Cruz Biotechnology, Santa Cruz, CA). We used 6.6 μg (1:500 dilution) of RXRγ and 0.13 μg (1:1,500 dilution) of PPARγ primary antibody for overnight incubation. After washing, membranes were incubated for 1 hour at room temperature with antirabbit IgG conjugated to peroxidase at a 1:5,000 dilution for RXRγ and antimouse IgG conjugated to peroxidase at 1:5,000 dilution for PPARγ. The enhanced chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) was used for immunodectection.

Immunoprecipitation

Cells (~12–13 million) were used for each immunoprecipitation experiment. The cells were washed in cold PBS-EDTA, and solubilization buffer (1 mL) was added [buffer contents: 55 mmol/L TEA (pH 7.5), 111 mmol/L NaCl, 2.2 mmol/L EDTA, 0.44% SDS]. The solution was passed through a 20 G syringe five times, frozen at −80°C for 3 hours, and thawed on ice. Triton X-100 (10%, 80 μL) was added followed by protein A/G plus agarose beads (80 μL, sc-2003, Santa Cruz Biotechnology). The mixture was shaken at 4°C for 8 hours. The suspension was spun for 2 minutes at 10,000 rpm to pellet the beads, and the supernatant was removed. PPARγ (5 μL or 200 μg/mL, H-100) rabbit polyclonal antibody (sc-7196, Santa Cruz Biotechnology) was added followed by protein A/G plus agarose beads (80 μL). This was again shaken at 4°C for 8 hours and spun to remove the supernatant. This mixture was washed twice with a wash buffer [50 mmol/L TEA (pH 7.5), 100 mmol/L NaCl, 2 mmol/L EDTA, 0.1% SDS, 0.5% Triton X-100] and washed twice with 10 mmol/L TEA (pH 7.5). The bound antigen was eluted by adding the Western blot 1× sample buffer, heated for 5 minutes at 100°C, and loaded onto a SDS-PAGE gel for Western blot analysis. The methods are the same as above, except for the PPARγ antibody concentration at 1:500.

Cell Growth and Proliferation

Cells were grown to ~80% confluence in 100 mm tissue culture plates. Cells were harvested using trypsin-EDTA (Invitrogen) and counted using a hemocytometer. Cells were transferred to a 96-well plate at a concentration of 500 cells per 200 μL of medium. Each row of eight wells received the same cell type and subsequently the same drug. After cells were allowed to plate down overnight, the medium was aspirated and medium with the appropriate concentration of ligand or equivalent volume of vehicle

was added to each well. Fresh medium with vehicle or ligand was added every 72 hours. At the completion of each time point (3, 6, or 9 days), cell proliferation was assessed following the manufacturer's instructions using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Following a 2-hour incubation at 37°C, each plate was analyzed by a MRX Microplate Reader (Dynatech Laboratories, Chantilly, VA) using Revelation software. Cell proliferation is assessed by determining the absorbance of a 490 nm wavelength of light that is altered by a colorimetric change in solution based on the number of metabolically active and proliferating cells.

Apoptosis

BHP 5-16 and DRO-90 cells were grown to ~80% confluence in 100 mm tissue culture plates. Cells were harvested and counted as described above and transferred to a six-well tissue culture plate at a concentration of 50,000 cells per 3 mL medium for the day 3 plates and 25,000 cells per 3 mL medium for the day 6 plates. After allowing to plate down overnight, the medium was aspirated and fresh medium with the appropriate concentration of ligand or equivalent volume of vehicle was added to the wells. At days 3 and 6, all cells were collected and analyzed by flow cytomery using the Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Eugene, OR) following the manufacturer's instructions.

DR1-Luciferase Transient Transfection

The DRO-90, BHP 2-7, and BHP 5-16 cells were cultured to 80% to 90% confluency (0.8×10^6 to 0.9×10^6 cells) for transfection experiments in a six-well plate. For each well, DR1-TK-luciferase (1 μg), TK-luciferase plasmid (3 μg), and LipofectAMINE 2000 reagent (8 μL; Invitrogen) were used as per the manufacturer's instructions. Each transfection also contained *Renilla* luciferase plasmid (10 ng; Promega) as an internal transfection control. A Rous sarcoma virus promoter luciferase plasmid and an empty TK-luciferase plasmid were transfected in parallel as positive and negative controls, respectively. DNA and the LipofectAMINE reagent were diluted separately in serum-free medium (200 μL) without antibiotics, mixed together, and incubated at room temperature for 30 minutes. The culture plates were washed with PBS and medium was added (1,600 μL). Plasmid LipofectAMINE mixture (400 μL) was added to each well, and the plates were incubated at 37°C with and without pioglitazone. Cells were harvested after 48 hours of incubation at 37°C, subjected to freeze thaw extraction, and assayed for dual firefly and *Renilla* luciferase activity. Luciferase activity was measured in a Monolight 3010 luminometer using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase light units were normalized to *Renilla* luciferase activity.

Results

Expression of PPARγ in Thyroid Carcinoma Cell Lines

Amplification by RT-PCR yielded a DNA fragment of predicted size and revealed PPARγ mRNA in two cell lines: DRO-90 and BHP 2-7 (Fig. 1A). Human GAPDH was used as a positive loading control. Quantitative RT-PCR analysis on two separate samples showed relatively high expression of PPARγ mRNA in DRO-90 and BHP 2-7 (47.8 and 21.0 fg/ng rRNA, respectively). BHP 5-16, however, did not express PPARγ and supported the findings of the qualitative RT-PCR experiment (Fig. 1B). To confirm protein expression of PPARγ in these cell lines, Western blot analysis was done on nuclear protein extracts. Figure 2A shows that PPARγ protein is detectable in all three cell lines despite lack of detectable RNA in BHP 5-16 cells. The RT-PCR analysis additionally confirmed that the well-described PPARγ/PAX8 rearrangement was not present. When corrected for β-actin protein expression, relative levels of PPARγ were higher in DRO-90 cells compared with BHP 2-7 and BHP 5-16 cells (Fig. 2B).

Expression of RXRγ in Thyroid Carcinoma Cell Lines

Quantitative RT-PCR was done using sense strand RNA corresponding to the ligand binding domain of the RXRγ

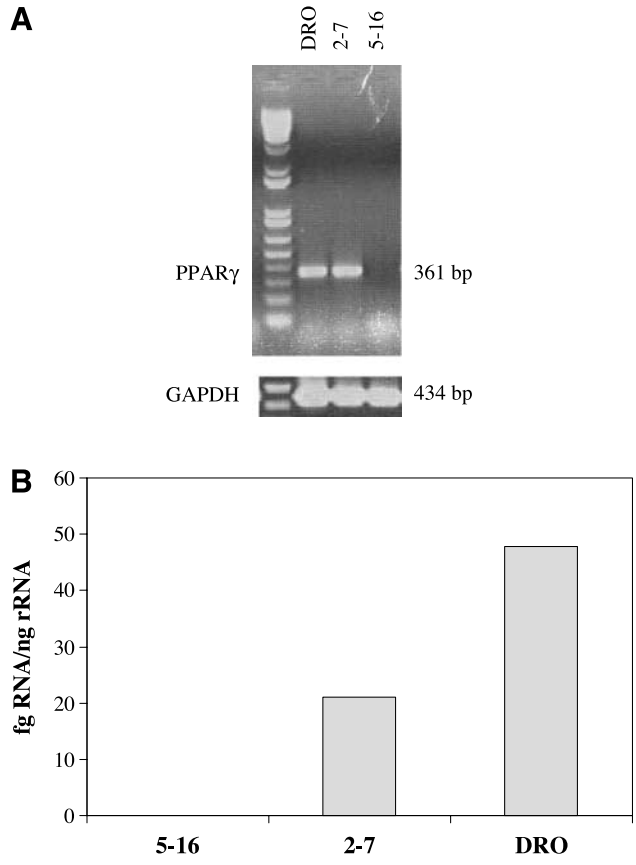

Figure 1. RT-PCR and quantitative RT-PCR analysis of PPARγ isotypes in thyroid carcinoma cell lines. **A,** RT-PCR. Total RNA (5 μg) was used from each of the three thyroid carcinoma cell lines (DRO-90, BHP 5-16, and BHP 2-7), reverse transcribed using random primers, and amplified by PCR using specific primers for PPARγ and GAPDH. The PCR products were resolved by electrophoresis and stained with ethidium bromide. **B,** quantitative RT-PCR. Total RNA (1 μg) was used for the PPARγ RT-PCR analysis (ABI PRISM 7700, Perkin-Elmer, Boston, MA), and absolute values were derived from a standard curve using a known amount of sense strand RNA (femtograms of sense strand RNA).

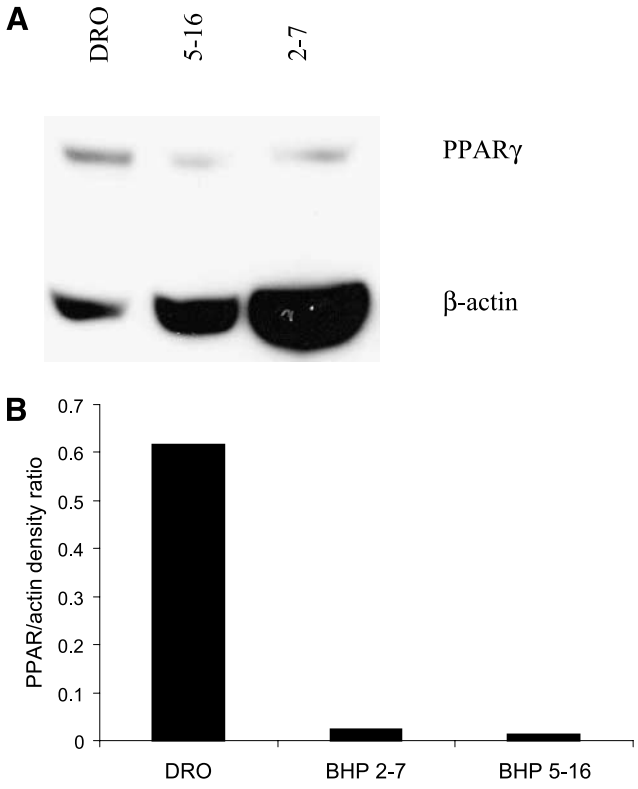

Figure 2. Western blot analysis of PPARγ in thyroid cancer cell lines using a monoclonal antibody. **A,** Western blot. Nuclear protein extract (60 μg) from three thyroid cancer cell lines was size separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. The blot was blocked with 10% nonfat milk and incubated with 1:1,500 dilution of a mouse monoclonal antibody against human PPARγ (COOH terminus, Santa Cruz Biotechnology). A 1:5,000 dilution of secondary antibody was used. β-actin was measured as a loading control. **B,** densitometry analysis. Densitometry was done on the Western blot in **A,** density levels of PPARγ are corrected for β-actin loading control.

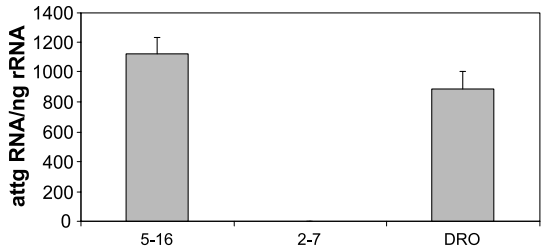

Figure 3. Quantitative RT-PCR analysis of RXRγ isotype in thyroid carcinoma cell lines. Total RNA (1 μg) was used for the RXRγ RT-PCR analysis (ABI PRISM 7700, Perkin-Elmer), and absolute values were derived from a standard curve using a known amount of sense strand RNA [attograms (*attg*) of sense strand RNA]. Isoform RNA was normalized to total input RNA (18S rRNA measured from 1 ng total RNA).

receptor as a standard (10). Total RNA (1 μg) from each cell line was analyzed for RXRγ mRNA expression. BHP 5-16 and DRO-90 cell lines had high expression of RXRγ mRNA (1,120 ± 112 and 890 ± 117 ag/ng rRNA, respectively). BHP 2-7 had no detectable levels of RXRγ mRNA (Fig. 3).

To confirm the expression of RXRγ protein in our three thyroid cancer cell lines, Western blot analysis was done on nuclear extract protein. BHP 5-16 and DRO-90 cell lines showed clear expression of RXRγ protein, whereas the BHP 2-7 cell line lacked detectable RXRγ protein (Fig. 4).

Cell Line Proliferation in Response to Ligand Treatment

Cells were grown in the presence of vehicle or ligand for 9 days. BHP 5-16 (PPARγ±/RXRγ+) cellular proliferation was decreased in the presence of LG 346 (1 μmol/L) and combination of LG 346-pioglitazone (1 μmol/L; Fig. 5A). There was no suppression of growth in the presence of pioglitazone (1 μmol/L) alone. Proliferation in the presence of LG 346 (1 μmol/L) was 69 ± 6% of growth compared with cells grown in vehicle. This was similar with the combination of LG 346-pioglitazone with proliferation of 78 ± 9% compared with cells grown in vehicle.

BHP 2-7 (PPARγ±/RXRγ) proliferation did not seem affected by any ligand treatment, including PPARγ-specific treatment with thiazolidinedione (Fig. 5B).

DRO-90 (PPARγ+/RXRγ+) proliferation was significantly decreased in the presence of LG 346 (1 μmol/L) and pioglitazone (1 μmol/L). Additionally, final concentration combination of LG 346-pioglitazone (1 μmol/L) provided the greatest suppression of proliferation at 13 ± 4% compared with cells grown in vehicle (Fig. 5C). Morphologically, the DRO-90 cells began to change after 72 hours of treatment (Fig. 6).

Given the marked suppression of DRO-90 cell proliferation, we did a dose titration of ligands alone and in combination to assess the lowest concentration of drug that would suppress growth. Additionally, we used a different rexinoid, LG 1069, to confirm that our response to RXR

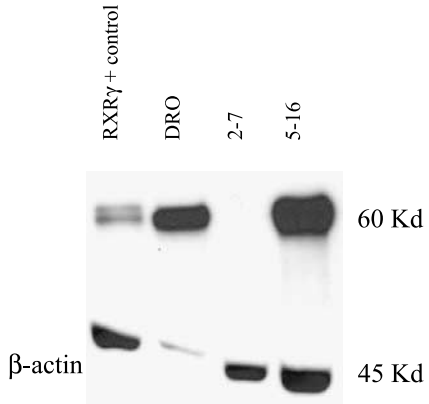

Figure 4. Western blot analysis of RXRγ isotype in thyroid carcinoma cell lines. Nuclear extract protein (100 μg) was size separated on a 10% SDS-PAGE gel. RXRγ+ control is RXRγ cDNA in a PCGN2 plasmid construct transfected into CV-1 cells. The nitrocellulose membrane was incubated with RXRγ primary antibody (Santa Cruz Biotechnology) or β-actin (loading control) and developed using a horseradish peroxidase secondary antibody system.

ligands was a class effect of the drug. LG 1069 (50 nmol/L) caused a 77 ± 4% decreased cellular proliferation at day 9. Pioglitazone (50 nmol/L) caused an 87 ± 4% decreased cellular proliferation at day 9. Even combination doses (25 nmol/L) of each ligand (50 nmol/L total) provided 94 ± 2% suppression of cellular proliferation (Fig. 7).

The above experiments were all repeated replacing pioglitazone with rosiglitazone, and the results were similar including the dose titration experiment (data not shown).

Functional Analysis of PPARγ in Thyroid Cancer Cell Lines

Expression of RXRγ mRNA and protein correlated well with functional suppression of growth in the thyroid cancer cell lines treated with rexinoids. PPARγ mRNA expression did not directly correlate with protein expression or functional growth inhibition by thiazolidinedione. To further explore this apparent discrepancy, whole cell protein extracts were subjected to immunoprecipitation (polyclonal PPARγ antibody) for the three thyroid cancer cell lines. Figure 8 shows that only the DRO-90 cells have detectable PPARγ protein using this technique. These data correlate with the suppression of growth by thiazolidinedione in Fig. 5. To determine if these cell lines contain functional PPARγ receptors, we did transient transfection using the DR1-TK-luciferase peroxisome proliferator response element reporter (gift from B. Forman, Department of Molecular Medicine, The City of Hope National Medical Center, Duarte, CA). Figure 9 shows that DR1-mediated luciferase activity was stimulated with thiazolidinedione only in DRO-90 cells (Fig. 9A), suggesting that only these cells have functional PPARγ. Figure 9D shows that this effect in DRO-90 cells is dose dependent.

Measurement of Apoptosis in BHP 5-16 and DRO-90 Cells

To investigate the mechanisms of decreased cell proliferation, we measured rates of apoptosis in BHP 5-16 and DRO-90 cells treated with rexinoid. BHP 5-16 cells did not have a significant percentage of cells (only 1.7 ± 0.4%) that undergo apoptosis with LG 346 (1 μmol/L) at 6 days despite 23 ± 2% suppression of cellular proliferation at day 6 with the same treatment conditions. In contrast, DRO-90 cells had 13 ± 3.5% of cells that undergo apoptosis with LG 346 (1 μmol/L) at day 6 (Fig. 10).

Given the strong suppression of cellular proliferation in DRO-90 cells with all ligands used, we studied the apoptotic effects of RXR and PPARγ ligands alone and in combination (Fig. 11). As shown, the percentage of cells undergoing apoptosis was significantly higher after 6 days of treatment with either ligand alone (13 ± 3.5% for LG 346 and 18 ± 3.8% for pioglitazone) as compared with treatment with vehicle (3.6 ± 1.3%). Additionally, 0.05 μmol/L

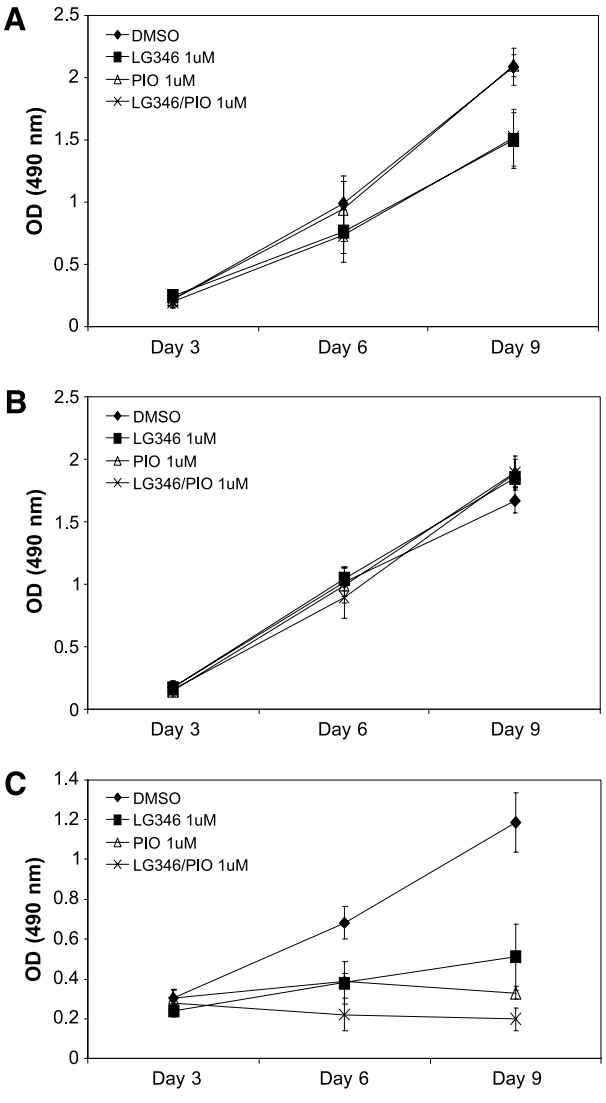

Figure 5. Cell proliferation response to vehicle versus ligand. **A,** treatment of BHP 5-16 (RXRγ+, PPARγ±) cells with vehicle versus ligand. Cells were grown in 2% FBS-RPMI in the presence of 1 μmol/L LG 346, pioglitazone (*PIO*), or LG 346-pioglitazone combination (500 nmol/L of each) or vehicle (DMSO) for 3, 6, and 9 days. At each time point, cells were harvested and analyzed using a nonradioactive cell proliferation assay. Cell proliferation is represented by absorbance at 490 nm. *Points,* average of seven experiments for LG 346 with eight data replicants in each experiment and three experiments for pioglitazone and LG 346-pioglitazone combination with eight data replicants in each experiment; *bars,* SE. **B,** treatment of BHP 2-7 (RXRγ−, PPARγ±) cells with vehicle versus ligand. Cells were grown in 2% FBS-RPMI in the presence of 1 μmol/L LG 346, pioglitazone (*PIO*), or LG 346-pioglitazone combination (500 nmol/L of each) or vehicle (DMSO) for 3, 6, and 9 days. At each time point, cells were harvested and analyzed using a nonradioactive cell proliferation assay. Cell proliferation is represented by absorbance at 490 nm. *Points,* average of six experiments for LG 346 with eight data replicants in each experiment and three experiments for pioglitazone and LG 346-pioglitazone combination with eight data replicants in each experiment; *bars,* SE. **C,** treatment of DRO-90 (RXRγ, PPARγ+) cells with vehicle versus ligand. Cells were grown in 2% FBS-RPMI in the presence of 1 μmol/L LG 346, pioglitazone (*PIO*), or LG 346-pioglitazone combination (500 nmol/L of each) or vehicle (DMSO) for 3, 6, and 9 days. At each time point, cells were harvested and analyzed using a nonradioactive cell proliferation assay. Cell proliferation is represented by absorbance at 490 nm. *Points,* average of eight experiments for LG 346 with eight data replicants in each experiment and four experiments for pioglitazone and LG 346-pioglitazone combination with eight data replicants in each experiment; *bars,* SE.

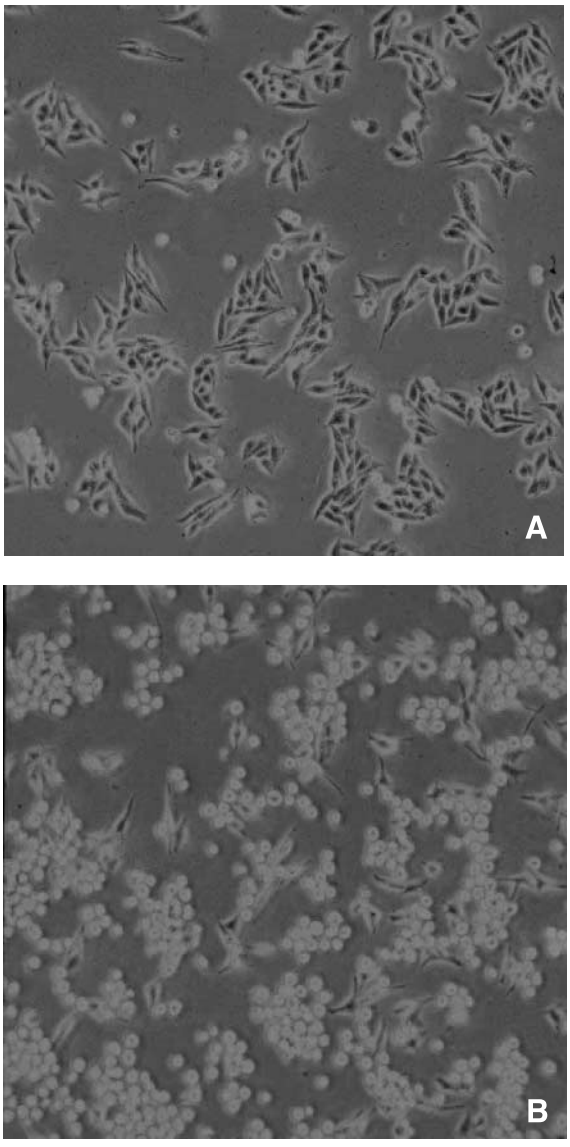

Figure 6. DRO-90 cells in culture. Cells were grown in 100 mm tissue culture plates in 2% FBS-RPMI for 72 hours in the presence of vehicle (DMSO; **A**) or LG 346-pioglitazone combination treatment (1 μmol/L; **B**). **A,** typical polygonal, flat cell morphology. **B,** cells become ''balled up'' with drug treatment. This morphology is seen prior to the cells lifting from the plate surface.

of each ligand together (0.1 μmol/L combination) generated more apoptotic cells (24 ± 5.4%) than 1 μmol/L of either ligand alone, suggesting a synergistic effect of these two ligands on apoptosis. We confirmed apoptosis in DRO-90 cells using poly(ADP-ribose) polymerase (PARP) cleavage. Although rexinoid alone did not show the appropriate PARP cleavage band on Western blot analysis, treatment with thiazolidinedione and combination thiazolidinedione-rexinoid yielded the expected PARP cleavage product (data not shown).

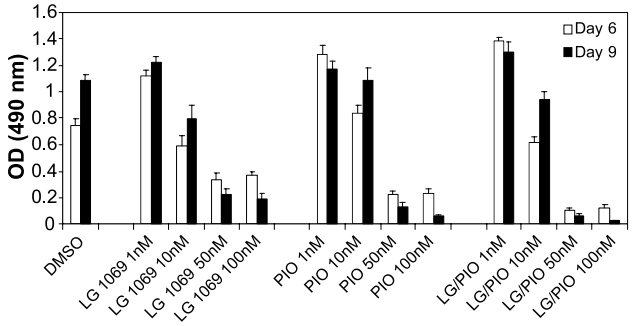

Figure 7. Dose titration of DRO-90 (RXRγ+, PPARγ+) cells with vehicle versus ligand. Cells were grown in 2% FBS-RPMI in the presence of stepwise increases of LG 1069, pioglitazone (*PIO*), and LG-pioglitazone combination treatment from 1 to 100 nmol/L for 6 and 9 days. At each time point, cells were harvested and analyzed using a nonradioactive cell proliferation assay. Cell proliferation is represented by absorbance at 490 nm. *Columns,* average of two experiments with eight data replicants in each experiment; *bars,* SE.

Discussion

In this report, we have showed that thyroid carcinoma cell line expression of RXRγ and/or PPARγ seems to be necessary for response to receptor-specific ligands. While necessary, however, expression of PPARγ alone does not seem to be sufficient to provide decreased cellular proliferation in response to a thiazolidinedione. Furthermore, apoptosis seems to play a limited role in the mechanism of decreased cellular growth in response to a rexinoid ligand; however, the level of apoptosis is synergistically enhanced when a cell line expressing both RXRγ and PPARγ receptors is treated with a combination of ligands.

The anticancer activity of vitamin A and its derivatives has been studied since the 1960s. The initial work was conducted on rat models of lung carcinoma (5). The prototype

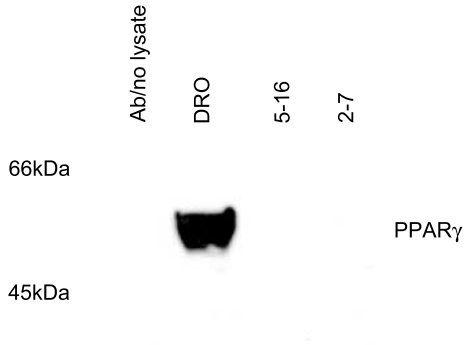

Figure 8. PPARγ immunoprecipitation. Immunoprecipitation used ~12–13 million cells each of DRO-90, BHP 2-7, and BHP 5-16. Whole cell extract was incubated with a polyclonal antibody against PPARγ, and the resultant protein was size separated on a 10% SDS-PAGE gel. The nitrocellulose membrane was incubated with a mouse monoclonal PPARγ primary antibody (Santa Cruz Biotechnology) and developed using a horseradish peroxidase secondary antibody system. *Ab/no lysate,* negative control.

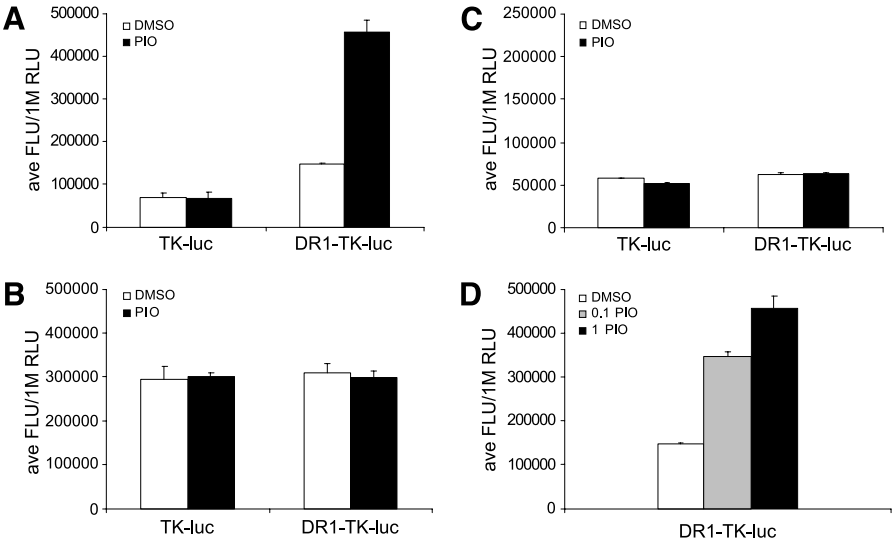

Figure 9. DR1-luciferase transient transfection. DRO-90 (**A**), BHP 5-16 (**B**), and BHP 2-7 (**C**) cells were cultured to 80–90% confluency (0.8×10^6 to 0.9×10^6 cells) for transfection experiments to assess the functional activity of PPARγ in response to pioglitazone (*PIO*). Luciferase activity was measured in a Monolight 3010 luminometer using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase light units were normalized to *Renilla* luciferase activity. **D,** dose response to pioglitazone (0.1 and 1 μmol/L) was assessed in the responsive DRO-90 cell line.

for retinoid-based therapies comes from the experience with using all-*trans* retinoic acid in combination with chemotherapy for the treatment of acute promyelocytic leukemia. This treatment combination has revolutionized the treatment of acute promyelocytic leukemia and is one of the few success stories in cancer therapy, wherein this treatment effectively eradicates acute promyelocytic leukemia (4). Retinoids have also been shown to induce growth arrest of MCF-7 breast cancer cell lines *in vitro*, and the RXR selective ligand LG 1069 has prevented the development of estrogen receptor–negative mammary tumors in an *in vivo* transgenic mouse model (6, 18). Additionally, LG 1069 is Food and Drug Administration approved (bexarotene, Targretin) for the treatment of T-cell lymphoma.

The effects of retinoids have also been studied with thyroid carcinoma cell lines. Van Herle et al. (19) first noted a significant and dose-dependent reduction in follicular carcinoma cell number and [^{3}H]thymidine uptake in the presence of 13-*cis*-retinoic acid (10 μmol/L). Additionally, Schmutzler et al. (7) investigated the ability of all-*trans* retinoic acid to induce redifferentiation in human thyroid carcinoma cell lines (including follicular and anaplastic carcinoma cell lines) by increasing type I 5′ deiodinase activity. Follicular thyroid carcinoma cell lines showed an increased activity, whereas anaplastic cell lines did not. The first report of retinoid therapy in a patient with advanced thyroid cancer was the use of isotretinoin (13-*cis*-retinoic acid, Accutane) in a patient with follicular thyroid cancer and lung metastases (20). The therapy caused symptomatic improvement in the patient's shortness of breath, decreased serum thyroglobulin level, and decreased tumor metabolism (^{18}FDG positron emission tomography). In a small clinical trial conducted by Simon et al. (8), 20 patients with advanced thyroid carcinoma (excluding anaplastic thyroid carcinoma) were treated with 13-*cis*-retinoic acid for at least 5 weeks. The investigators studied markers of redifferentiation including thyroglobulin level, radioactive iodine uptake, and tumor size by standard imaging. A response in one marker was seen in ~65% of patients, whereas two or more markers indicated redifferentiation in ~40% of patients. Boerner et al. (21) studied 23 patients with advanced, progressive, metastatic follicular cell–derived thyroid cancer in what has been the longest clinical trial to date. Patients were treated with 13-*cis*-retinoic acid (0.3–1 mg/kg/d) for up to 9 months. Tumor glucose uptake decreased while on therapy and went back up at the cessation of therapy. Twenty-eight percent of patients had new or increased I-131 uptake in tumors. Unfortunately, there was no control group in this study and only two patients had a clinically significant response (of 21 evaluated and with I-131 therapy used in conjunction to isotretinoin). Finally, an initial clinical trial of LGD1550 (a synthetic retinoic acid receptor agonist) included seven patients with advanced thyroid cancer, in whom four had stable disease for at least 20 weeks (and up to 56 weeks) and one who had a transient decrease in serum thyroglobulin level (22).

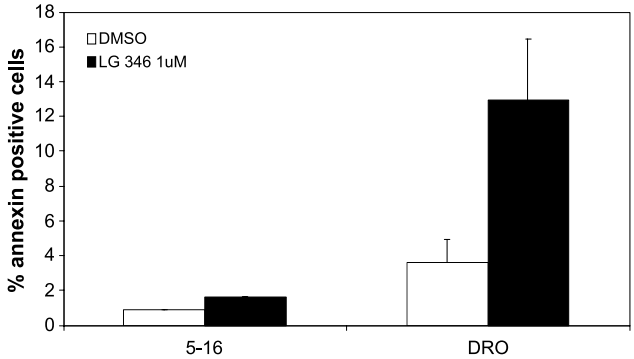

Figure 10. Measurement of BHP 5-16 (RXRγ+, PPARγ±) versus DRO-90 (RXRγ+, PPARγ+) cell apoptosis. Cells were grown in 2% FBS-RPMI in the presence of LG 346 (1 μmol/L) or DMSO for 6 days. Apoptosis was analyzed by flow cytometry using the Vybrant Apoptosis Assay. Apoptosis is indicated by % Annexin-positive cells.

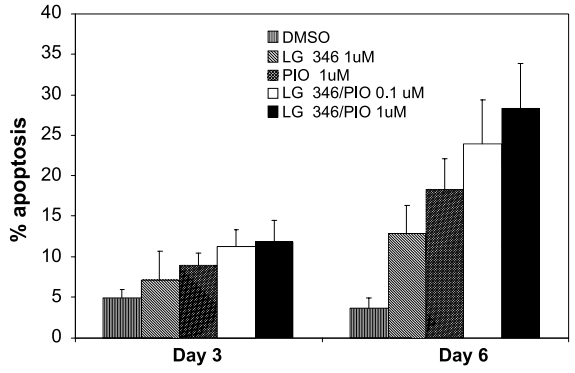

Figure 11. Measurement of DRO-90 (RXRγ+, PPARγ+) cell apoptosis. Cells were grown in 2% FBS-RPMI in the presence of 1 μmol/L LG 346, 1 μmol/L pioglitazone, 0.1 μmol/L LG 346-pioglitazone combination (50 nmol/L of each), 1 μmol/L LG 346-pioglitazone combination (500 nmol/L of each), or DMSO for 3 and 6 days. Apoptosis was analyzed by flow cytometry using the Vybrant Apoptosis Assay. Apoptosis is indicated by % Annexin-positive cells.

In the current study, we specifically investigated the RXRγ receptor subtype of the retinoid/rexinoid superfamily. We observed an ~30% decrease in cellular proliferation with RXRγ selective ligands used alone in RXRγ+ cell lines. Based on the proliferation curves, cellular growth was slowed but not stopped entirely.

The PPARs are members of the nuclear hormone receptor superfamily. The three isotypes of PPAR (α, β and γ) show distinct tissue distribution, with PPARγ being highly expressed in adipose tissue and macrophages (11). Like other nuclear hormone receptors, PPARγ contains a DNA binding domain and COOH-terminal ligand binding domain that mediates dimerization and transactivation functions (23). There are many examples of PPARγ activation promoting differentiation, most notably as the ''master regulator'' of adipocyte differentiation (13). The cell differentiation extends to numerous cell lines, however, including hepatocytes, fibroblasts, breast cells, and myocytes (14). The ligands for PPARγ include naturally occurring prostaglandins and most notably the thiazolidinedione class of antidiabetic drugs (12). Although PPARγ activation clearly leads to adipocyte differentiation, numerous *in vitro* studies have showed thiazolidinedione-induced differentiation in nonadipocyte cell lines (15).

Ligand activation of PPARγ has been studied in human prostate cancer cell lines via *in vitro* and *in vivo* studies. Segawa et al. showed that the PPARγ receptor mRNA was specifically seen in the prostate cancer cells, prostatic intraepithelial neoplasia, and was faintly seen in cells showing evidence of benign prostatic hypertrophy. Interestingly, PPARα and PPARβ mRNA was detected in all cell lines including normal prostate tissue (24). The authors concluded that PPARγ expression is induced in prostate cancer, and its presence may be a potential target for redifferentiation therapy. A trial of 41 men with histologically confirmed prostate cancer and no symptomatic metastases were treated p.o. with troglitazone (a thiazolidinedione and PPARγ activator), and a significant proportion of subjects had stabilization of their prostate-specific antigen (25). Additionally, thiazolidinedione ligands have been shown to induce apoptosis in hepatocellular cell lines and colon cancer cell lines *in vitro* (26, 27).

The effects of liganded PPARγ receptors on thyroid carcinoma cell lines have been studied as well. Martelli et al. (28) studied numerous human thyroid carcinoma cell lines with regard to PPARγ expression. In this study, RNA extracted from a normal thyroid cell line did have low-level expression of PPARγ by RT-PCR analysis. Five of six carcinoma cell lines expressed PPARγ and had decreased cellular growth at 48 hours, increased G_1 cell cycle arrest, and increased apoptosis when treated with ciglitazone (a thiazolidinedione). Interestingly, when PPARγ was overexpressed in the PPARγ-negative cell line, its growth kinetics slowed and apoptosis increased even without liganded treatment. Ciglitazone caused marked decrease in tumor cell colony growth. Ohta et al. (29) used troglitazone to study its effects on papillary carcinoma cells lines that differentially expressed PPARγ receptors. Troglitazone (10 μmol/L) caused a significant decrease in cell number in culture in PPARγ-positive papillary carcinomas compared with those that were PPARγ negative at 72 hours. This work was carried to a nude mouse model, wherein 500 mg/kg/d of troglitazone given over 5 days/wk for 7 weeks visibly inhibited the growth of implanted tumor cells to a significant degree. These investigators also showed that the BHP 2-7 cells, which express PPARγ mRNA, showed significant growth inhibition with 10 μmol/L pioglitazone. We did not observe this effect in these cells using 1 or 10 μmol/L pioglitazone. While we confirmed that the BHP 2-7 cells express PPARγ mRNA, the level of protein expression was relatively low (Fig. 2B) and these cells did not have functional PPARγ (Fig. 9C). Additionally, BHP 2-7 is RXRγ negative; therefore, heterodimerization of RXRγ and PPARγ cannot occur in this cell line.

RXRs and PPARγ preferentially form heterodimers. The exploitation of this PPAR/RXR heterodimer has occurred in breast cancer research. Rubin et al. showed that a combination of LG10135 (a RXR ligand) and troglitazone inhibited breast aromatase activity (which activates estrogen biosynthesis in the breast and contributes to breast carcinogenesis). The combination showed a greater effect than either ligand alone (both of which decreased aromatase activity; ref. 30). Elstner et al. (31) showed that a thiazolidinedione-rexinoid combination therapy for inhibition of breast cancer cell growth was seen only with those cells that were bcl-2 protein positive. This study shows that a nuclear hormone receptor complement alone does not completely predict a cellular response to liganded treatment and that a variety of other factors, coactivators and/or corepressors, may influence cellular response.

We have identified a thyroid carcinoma cell line, DRO-90, that expresses RXRγ and PPARγ mRNA and protein. Interestingly, this cell line showed a more substantial decrease in cellular proliferation with either RXRγ or PPARγ

ligands than the other cell lines that only expressed one nuclear hormone receptor type. We believe this shows an accentuated response due to the heterodimerization of receptors even with only one receptor-specific ligand. More impressively, however, was the effect of treatment with both thiazolidinediones and rexinoids. The decrease in cellular proliferation was at least additive as a combination of each ligand at 50% of the dose of either ligand alone had a greater reduction in DRO-90 cellular proliferation. Furthermore, expression of both receptors was associated with increased apoptosis in response to each ligand. We used PARP cleavage to verify apoptosis and did not find the expected band on Western blot analysis for cells treated with rexinoid alone. This may represent that the amount of apoptosis initiated by the rexinoid ligand did reach the threshold for detection required to be seen using the PARP antibody. Another possibility is that PARP cleavage detects apoptosis associated with the caspase enzyme system (primarily caspase-3) and that rexinoid treatment induces apoptosis through different caspase enzymes or different mechanisms. DRO-90 cells had a much greater percentage of cells in apoptosis after treatment with either ligand alone when compared with the BHP 5-16 (RXRγ+) cells that had a minimal response when treated with a rexinoid. Combination therapy seemed to have a synergistic effect as a 10-fold smaller concentration of ligands together pushed more cells into apoptosis at 6 days than either ligand alone.

To our knowledge, this is the first published set of experiments on human thyroid carcinoma cell lines using a molecularly based rationale with combination treatment of two different nuclear hormone receptor ligands. The lack of cellular response to ligands without its appropriate receptor lends support to the fact that this is a receptor driven, ligand-activated effect and not a drug effect through a secondary pathway. However, it is important to note that other factors such as coactivator/corepressor activity, DNA methylation, and histone acetylation all probably play a role in liganded effect (32, 33). These effects and pathways will still need to be investigated further.

In summary, expression of RXRγ and/or PPARγ seems to predict response to ligand treatment. However, it seems that, for PPARγ activation and effect to occur, it must happen in the setting of a heterodimer coupling with RXRγ. Finally, coactivation of a RXRγ-PPARγ heterodimer complement lends an additive effect to cause decreased cellular proliferation *in vitro*, and a large part of this effect is the result of cellular apoptosis.

Acknowledgments

We gratefully acknowledge the use of the Gene Expression Core Facility and Flow Cytometry Core Facility of the University of Colorado Cancer Center.

References

1. Haugen BR. Management of the patient with progressive radioiodine non-responsive disease. Semin Surg Oncol 1999;16:34 – 41.

2. Ain KB, Tofiq S, Taylor KD. Antineoplastic activity of Taxol against human anaplastic thyroid carcinoma cell lines *in vitro* and *in vivo*. J Clin Endrocrinol Metab 1996;81:3650 – 3.

3. Ain KB, Egorin MJ, DeSimone PA. Treatment of anaplastic thyroid carcinoma with paclitaxel: phase 2 trial using ninety-six-hour infusion. Thyroid 2000;10:587 – 94.

4. Altucci L, Gronmeyer H. The promise of retinoids to fight against cancer. Nat Rev 2001;1:181 – 93.

5. Schmutzler C, Köhrle J. Retinoic acid redifferentiation therapy for thyroid cancer. Thyroid 2000;10:393 – 406.

6. Wu K, Zhang Y, Xu X, et al. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. Cancer Res 2002;62: 6376 – 80.

7. Schmutzler C, Brtko J, Bienert K, Köhrle J. Effects of retinoids and role of retinoic acid receptors in human thyroid carcinomas and cell lines derived therefrom. Exp Clin Endocrinol Diabetes 1996;104:16 – 9.

8. Simon D, Koehrle J, Reiners C, et al. Redifferentiation therapy with retinoids: therapeutic option for advanced follicular and papillary thyroid carcinoma. World J Surg 1998;22:569 – 74.

9. Rochaix Ph, Moteil-Onteniente S, Rochette-Egly C, Caratero C, Voigt JJ, Jozan S. Reduced expression of retinoic acid receptor β protein (RAβ) in human papillary thyroid carcinoma: immunohistochemical and Western blot study. Histopathology 1998;33:337 – 43.

10. Haugen BR, Larson LL, Pugazhenthi U, et al. Retinoic acid and retinoid X receptors are differentially expressed in thyroid cancer and thyroid carcinoma cell lines and predict response to treatment with retinoids. J Clin Endrocrinol Metab 2004;89:272 – 80.

11. Elbrecht A, Chen Y, Cullinan CA, et al. Molecular cloning, expression and characterization of human peroxisome proliferator activated receptors γ1 and γ2. Biochem Biophys Res Commun 1996;224:431 – 7.

12. Kersten S, Desvergne B, Wahil W. Roles of PPARs in health and disease. Nature 2000;405:421 – 4.

13. Gelman L, Fruchart JC, Auwerx J. An update on the mechanisms of action of the peroxisome proliferator-activated receptors (PPARs) and their roles in inflammation and cancer. Cell Mol Life Sci 1999;55: 932 – 43.

14. Vamecq J, Latruffe N. Medical significance of peroxisome proliferator-activated receptors. Lancet 1999;354:141 – 8.

15. Murphy GJ, Holder JC. PPAR-γ agonists: therapeutic role in diabetes, inflammation and cancer. Trends Pharmacol Sci 2000;21:469 – 74.

16. Mehta RG, Williamson E, Patel MK, Koeffler HP. A ligand of peroxisome proliferator-activated receptor γ, retinoids, and prevention of preneoplastic mammary lesions. J Natl Cancer Inst 2000;92: 418 – 23.

17. Totntonoz P, Singer S, Forman BM, et al. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor γ and the retinoid X receptor. Proc Natl Acad Sci USA 1997;94:237 – 41.

18. Dokmanovic M, Chang B, Fang J, Roninson IB. Retinoid-induced growth arrest of breast carcinoma cells involves co-activation of multiple growth-inhibitory genes. Cancer Biol Ther 2002;1:24 – 30.

19. Van Herle AJ, Agatep ML, Padua DN III, et al. Effects of 13 *cis*-retinoic acid on growth and differentiation of human follicular carcinoma cells (UCLA R0 82 W-1) *in vitro*. J Clin Endrocrinol Metab 1990;71: 755 – 63.

20. Borner AR, Simon D, Muller-Gertner HW. Isotretinoin in metastatic thyroid cancer. Ann Intern Med 1997;127:246.

21. Boerner AR, Petrich T, Weckesser E, et al. Monitoring isotretinoin therapy in thyroid cancer using ^{18}F-FDG PET. Eur J Nucl Med Mol Imaging 2002;29:231 – 6.

22. Soignet SL, Miller VA, Pfister DG, et al. Initial clinical trial of a high-affinity retinoic acid receptor ligand (LGD1550). Clin Cancer Res 2000;6: 1731 – 5.

23. Nolte RT, Wisely GB, Westin S, et al. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. Nature 1998; 395:137 – 43.

24. Segawa Y, Yoshimura R, Hase T, et al. Expression of peroxisome proliferator-activated receptor (PPAR) in human prostate cancer. Prostate 2002;51:108 – 16.

25. Mueller E, Smith M, Sarraf P, et al. Effects of ligand activation of peroxisome proliferator-activated receptor γ in human prostate cancer. Proc Natl Acad Sci 2000;97:10990 – 5.

26. Yoshizawa K, Cioca DP, Kawa S, Tanaka E, Kiyosawa K. Peroxisome proliferator-activated receptor γ ligand troglitazone induces cell cycle arrest and apoptosis of hepatocellular carcinoma cell lines. Cancer 2002;95:2243–51.

27. Yang W, Furcht H. Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. Carcinogenesis 2001;22:1379–83.

28. Martelli ML, Iuliano R, Pera IL, et al. Inhibitory effects of peroxisome proliferator-activated receptor γ on thyroid carcinoma cell growth. J Clin Endrocrinol Metab 2002;87:4728–35.

29. Ohta K, Endo T, Haraguchi K, Hershman JM, Onaya T. Ligands for peroxisome proliferator-activated receptor γ inhibit growth and induce apoptosis of human papillary thyroid carcinoma cells. J Clin Endrocrinol Metab 2001;86:2170–7.

30. Rubin GL, Duong JH, Clyne CD, et al. Ligands for the peroxisomal proliferator-activated receptor γ and the retinoid X receptor inhibit aromatase cytochrome *P*450 (*CYP19*) expression mediated by promoter II in human breast adipose. Endocrinology 2002;143:2863–71.

31. Elstner E, Williamson EA, Zang C, et al. Novel therapeutic approach: ligands for PPARγ and retinoid receptors induce apoptosis in bcl-2-positive human breast cancer cells. Breast Cancer Res Treat 2002;74:155–65.

32. Berger J, Daxenbichler G. DNA methylation of nuclear receptor genes—possible role in malignancy. J Steroid Biochem Mol Biol 2001;80:1–11.

33. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 2000;92:1210–6.