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Functional Activation of PPAR γ in Human Upper Aerodigestive Cancer Cell Lines

Simon K. Wright¹, Beverly R. Wuertz¹, George Harris¹, Raed Abu Ghazallah¹, Wendy A. Miller¹, Patrick M. Gaffney², and Frank G. Ondrey^{1,*}

¹Department of Otolaryngology, University of Minnesota, Minneapolis, Minnesota

²Arthritis and Clinical Immunology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

Abstract

Upper aerodigestive cancer is an aggressive malignancy with relatively stagnant long-term survival rates over 20 yr. Recent studies have demonstrated that exploitation of PPAR γ pathways may be a novel therapy for cancer and its prevention. We tested whether PPAR γ is expressed and inducible in aerodigestive carcinoma cells and whether it is present in human upper aerodigestive tumors. Human oral cancer CA-9-22 and NA cell lines were treated with the PPAR activators eicosatetraynoic acid (ETYA), 15-deoxy- δ -12,14-prostaglandin J2 (PG-J2), and the thiazolidinedione, ciglitazone, and evaluated for their ability to functionally activate PPAR γ luciferase reporter gene constructs. Cellular proliferation and clonogenic potential after PPAR γ ligand treatment were also evaluated. Aerodigestive cancer specimens and normal tissues were evaluated for PPAR γ expression on gene expression profiling and immunoblotting. Functional activation of PPAR γ reporter gene constructs and increases in PPAR γ protein were confirmed in the nuclear compartment after PPAR γ ligand treatment. Significant decreases in cell proliferation and clonogenic potential resulted from treatment. Lipid accumulation was induced by PPAR γ activator treatment. 75% of tumor specimens and 100% of normal control tissues expressed PPAR γ RNA, and PPAR γ protein was confirmed in 66% of tumor specimens analyzed by immunoblotting. We conclude PPAR γ can be functionally activated in upper aerodigestive cancer and that its activation downregulates several features of the neoplastic phenotype. PPAR γ expression in human upper aerodigestive tract tumors and normal cells potentially legitimizes it as a novel intervention target in this disease.

Keywords

oral cancer; thiazolidinedione; transdifferentiation; prostaglandin; genomic

*Correspondence to: Department of Otolaryngology, University of Minnesota, MMC396, 420 Delaware St. SE, Minneapolis, MN 5545.

SUPPORTING INFORMATION

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INTRODUCTION

In spite of advances in molecular biology of squamous cell carcinoma of the head and neck (SCCHN) and multi-modality treatments, the 20–50% survival of stage III and IV disease has not changed appreciably over 20 yr [1]. As a consideration to improve efficacy of conventional treatment or for chemoprevention, nuclear receptor targeting with retinoids continues to be an important focus, however early successes were limited by retinoid toxicity and reversal of differentiation changes upon cessation of treatment [2,3].

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the steroid/thyroid/retinoic acid nuclear receptor superfamily and are promising new targets. One isoform, PPAR γ , heterodimerizes with RXR α to form a functional transcription factor which promotes adipocyte differentiation [4].

Natural and synthetic fatty acids, including the arachidonic acid analog, eicosatetraynoic acid (ETYA), are PPAR γ activators [5]. ETYA and related agents dramatically downregulate cell growth and promote features consistent with differentiation in a variety of cell lines [6–11]. Previous experiments with ETYA demonstrated that structurally distinct lipoxigenase inhibitors have a variety of antineoplastic features not entirely explained by the inhibition of lipoxigenase alone [12–15]. Several anticancer effects of these drugs may be attributable to PPAR γ activation [16,17].

PPAR γ was originally identified in adipocytes [18,19] and strongly directs adipocyte differentiation, even when ectopically expressed in fibroblasts [19,20]. Feasibility of targeting cancer with PPAR γ treatment began with observations of anti-cancer effects after use of these agents in liposarcoma cell lines [21,22] which led to liposarcoma patient studies [23,24]. Clinically, pioglitazone and rosiglitazone, agents of the thiazolidinedione class of drugs which specifically target PPAR γ , are FDA approved treatments for Type II diabetes, and exhibit interesting anticancer properties. PPAR γ -based therapy in breast cancer models demonstrated inhibition of cell cycle progression, induction of apoptosis, extensive lipid accumulation, decreased growth rate and clonogenicity, and promotion of cancer prevention in animal models [25–30]. Activation of PPAR γ in lung cancer cell lines has induced differentiation and apoptosis [20,31–33]. Additional recent studies provide further evidence demonstrating these agents may be useful in colon, neural, ovarian, melanoma, and lung cancer [34–42]; however, mechanisms of downstream action may vary between tissue types. In colon cancer, a principal finding of ciglitazone treatment was alterations in chaperones associated with protein folding [35]. In ovarian cancers, ciglitazone resulted in alterations in glucose transporters. In one recent breast cancer study in MCF-7 cells, ciglitazone and rosiglitazone affected cyclin D1 and augmented doxorubicin activity through cyclin D1 interactions [38]. Another breast study demonstrated modulation of leptin and VEGF as an exploitable treatment with PPAR activators [41]. In non-small cell lung cancer, radiosensitizing effects were observed as well [39]. The potential role for PPAR γ to modulate the early events in carcinogenesis has even spawned considerable interest in this transcription factor as a target for chemoprevention [43]. The fact that these agents affect a transcription factor system and perhaps hundreds of downstream genes, including pathways and cellular processes other than differentiation, may allow for many opportunities for biomarker discovery.

Cancer largely represents a lack of differentiation and driving premalignant or cancerous cells into a terminally differentiated state is an attractive therapeutic strategy. Differentiation therapy has been successfully used in acute promyelocytic leukemia, hairy cell leukemia, and childhood neuroblastoma [44,45]. Retinoid therapy has shown mixed results in solid tumor treatment or chemoprevention [2,46,47]. Presently, we examined oral cancer cell lines and tumor specimens for the expression of PPAR γ . We treated cell lines with three chemically distinct ligands of PPAR γ (ETYA, PG-J2, and the prototypic thiazolidinedione, ciglitazone) which caused dose-dependent inhibition of proliferation and cytoplasmic lipid vacuole accumulation and morphologic changes consistent with cellular differentiation with an adipogenic phenotype shift. Electromobility supershift analysis demonstrated specific DR-1 and cyp4A1 consensus sequence binding activity in nuclear extracts which was upregulated with PPAR γ ligand treatment. PPAR γ and RXR α were identified as the predominant binding partners in control and ligand-stimulated nuclear extracts. Functional activation of the PPAR response element (PPRE) demonstrated increased PPRE activation with each ligand and also in the fat specific protein aP2 (fatty acid binding protein, FABP4). Microarray interrogation demonstrated PPAR γ expression was decreased in human head and neck squamous cancer specimens compared to normal oral mucosa. We conclude PPAR γ ligands represent a class of drugs which have value in the treatment of SCCHN and its precursor lesions.

MATERIALS AND METHODS

Cell Culture

Mycoplasma free CA-9-22 and NA EGFR positive oral cavity cell lines [48] were cultured at 37°C, 5% CO₂ as adherent monolayer cultures in RPMI 1640 media supplemented with 2 mM Glutamine, 10% heat-inactivated FBS (Gibco/BRL), 50 u/mL Penicillin, and 50 µg/mL streptomycin. Log-phase cells were routinely subcultured weekly after trypsinization.

Preparation of Nuclear Extracts

Extracts from log-phase squamous cancer cell lines were prepared according to the methods of Dignan, utilizing some of the modifications of Lee as previously published [12]. Protein concentrations of all samples were determined by the Bicinchoninic Acid (BCA) modification of the Biuret Reaction (Pierce Protein Assay kit, Pierce, Rockford, IL) with bovine serum albumin as standards. Plates were read at 560 nm on a Tecan spectrophotometer (Tecan, Durham, NC). All samples were assayed in triplicate and standard curves generated by least squares analysis, to a zero order function utilizing Spectra Software. Correlation coefficients for the functions were greater than 0.97 in all experiments. All protein concentrations were calculated from the central portion of the curve. Standard curves were generated by least squares analysis to a zero order function utilizing Spectra Software. Correlation coefficients (r^2) for the functions were greater than 0.95 in all experiments.

Aerodigestive Cancer Cell Line Western Blotting

Cells were grown in 75 cm² or 150 cm² flasks to 60–80% confluence and treated with respective agents in serum free media. Nuclear extracts were prepared as described under

“Preparation of Nuclear Extracts.” Twenty micrograms of nuclear extract protein were added to equal volume of 2× sample buffer (1.0 mL glycerol, 0.5 mL beta-mercaptoethanol, 3 mL 10% SDS, 1.25 mL 1.0 M Tris-HCl, pH 6.7, 1.5 mg bromophenol blue) and loaded into a 1.5 mm 10% polyacrylamide gel and run at 20 mA for 2 h at 4°C. Gels were removed and incubated in transfer solution (25 mM Tris base, 192 mM glycine, 20% methanol) for 5 min, then transferred to nitrocellulose membrane using a semi-dry transfer unit (Hoeffer). Blots were blocked in 3% milk blocking solution (1× TBS, 3% Milk, 0.05% Tween 20) for 1 h at 4°C. Following TBS rinse (10 mM Tris-HCl, pH 8.0; 150 mM NaCl), blots were incubated in primary antibody at 1:1000 (Santa Cruz Biotech Inc. sc-7273 mouse monoclonal anti-human PPAR γ , Santa Cruz, CA) in blocking solution overnight. Blots were briefly rinsed, then incubated secondary antibody at 1:2000 (Santa Cruz Biotech Inc., goat anti-mouse HRP). Following rinse, the blots were developed using chemiluminescence technique with Santa Cruz reagents per manufacturer protocol, and exposed to film.

Electromobility Shift Assays

Probe preparation—Double stranded DNA oligonucleotide probes for OCT-1 were obtained commercially (Promega, Madison, WI). The OCT-1 transcription factor consensus sequence contained 5′-TGTCGAATG-CAAATCACTAGAA-3′. The DR-1 probe and mutant was obtained commercially and contained the sequence: 5′-AGC TTC AGG TCA GAG GTC AGA GAG CT-3′ (Santa Cruz Biotech Inc, sc-2547), 5′-AGC TTC AGC ACA GAG CAC AGA GAG CT-3′ (Santa Cruz Biotech, Inc, sc-2548), respectively. The published PPAR γ recognition sequence for the Cyp4A1 probe was additionally utilized. The Cyp4A1 probe was manufactured by GibcoBRL as a sense and anti-sense oligonucleotide as well as a mutated sequence. The Cyp4A1 probe contained the published Cyp4A1 sequence, 5′-TGA AAC TAG GGT AAA GTT CA-3′. The mutated sequence for Cyp4A1 was 5′-TGA AAC TAG CAT AAA GCA CA-3′ with four base pair substitutions in the direct repeat element of the Cyp4A1 gene. The Cyp4A1 DNA strands were annealed by heating in annealing buffer (0.1 mM Tris HCL, 1.0 M NaCl, 1× React2 (BRL), 300 pM oligonucleotide) to 60° C, then cooling to room temperature. Consensus oligonucleotide probes were labeled with T4 polynucleotide kinase (Promega) and gamma-32-ATP (6000 Ci/mM, Amersham, Arlington Heights, IL).

EMSA Binding Reactions and Shift Assays—EMSA binding reactions consisted of 5 μ g of nuclear extract protein prepared as under “Preparation of Nuclear Extracts” above and identically as previously published. The gels were dried and imaged by phosphor imaging with a Packard Cyclone Phosphor-imager utilizing MP imaging screens and analyzed by Optiquant software (Packard Technologies, Downers Grove, IL). All EMSA reactions were carried out on at least three separate nuclear extract preparations. For supershift experiments, 1 μ g of antibody for RXR alpha (sc553 $\times$) or PPAR γ (sc7273) (Santa Cruz Biotech Inc.) was added 12 h prior to the binding reactions with the labeled probes, and the EMSAs were carried out at 4°C. Densitometry analysis was performed using Optiquant software.

Transient Transfections/Luciferase Assays—All cell lines were transiently transfected with a PPRE reporter plasmid (TK-PPREx3-LUC [PPRE 3(5′-TCGA-CAGGGGACCAGGACAAAGGTCACGTTTCGGGAGTC-GAC), three copies] kindly gifted

by Dr. Ron Evans, Salk Institute, La Jolla, CA). An additional luciferase construct for the promoter of the PPAR γ responsive lipid metabolism gene aP2 was utilized for a second series of experiments (kindly gifted by Dr. David Bernlohr, University of Minnesota, Minneapolis, MN). The p21 promoter reporter gene plasmid (obtained from Dr. Bert Vogelstein, Addgene plasmid 16451) expresses the firefly luciferase gene under the control of a 2400 base pair segment of the p21 promoter. Briefly, cells at 60–80% confluence were exposed to 2 μ g/mL TK-PPREx3-LUC reporter (or aP2, or p21) construct plasmid and co-transfected with 0.4 μ g/mL β -gal containing DNA reporter. Lipofectamine, at 10 μ g/mL in Opti-MEM (Life Technologies, Gaithersburg, MD), was utilized for transfection. After 4 h, the media was washed free from the cells, and complete media was used for 24 h further incubation unless otherwise indicated. Following specified treatments, relative luciferase activity was assayed with a Dual-Light reporter gene assay system (Tropix, Bedford, MA) as per kit instructions. A Tropix model TR717 dual injection plate luminometer with Winglow software was utilized for the analyses. Luciferase was scaled to β -gal as an internal standard. For PPAR γ antagonist experiments, cells were pre-incubated for 1 h with T0070907 prior to incubation with activators. Three assays were performed in triplicate wells for a total of nine replicates per data point.

Cell Proliferation Assays—Log-phase NA and CA-9-22 cells were plated in tissue culture treated 96-well, flat-bottom plates (Falcon, Becton Dickinson Labware, Franklin, NJ) at 5000 cells/well in complete media overnight. The following morning, media was replaced with serum-free media containing PPAR γ ligands or the vehicle alone (ETOH or DMSO) for 72 h. Proliferation was determined by using the MTT reaction assay (Boehringer Mannheim, Indianapolis, IN) as per manufacturer instructions reading the plates on a Tecan plate spectrophotometer at 560 nm. For PPAR γ antagonist experiments, cells were pre-incubated for 1 h with T0070907 prior to incubation with activators. Six replicates were used for each data point and experiments repeated twice.

Clonogenic Assays—CA-9-22 and NA cells were trypsinized during log phase growth and 400 cells per well were plated in 5% media, and allowed to attach overnight. The following morning, treatment agents or appropriate vehicle controls were added. After 10 d, cell colonies representing cell numbers of 25 or more were counted after staining with crystal violet. The treatments were conducted in triplicate, experiments were repeated with similar results twice, and results were expressed as fractional number of colonies over control.

Oil Red O Studies—Cell lines were grown to 50–60% confluence in 12-well flat bottom plates; respective ligands were added in 2% media and incubated for 72 h. Cells were then harvested by trypsinization, resuspended, and fixed in CytoRich (Tripath Imaging, Inc, Burlington, NC). Oil Red O solution (0.2% in isopropyl alcohol) was then applied for 15 min; cells were then rinsed with serial concentrations of isopropyl alcohol and digital photographs were taken. Pixel counting experiments were performed using Adobe Photoshop CS5 and stained areas of fat containing vacuoles were expressed on a per cell basis.

Genomics Analysis—Patient characteristics and biopsy samples from patients and volunteer control subjects were obtained in accord with guidelines set forth by the Institutional Review Board of the Human Subjects Protection Committee at the University of Minnesota. The University of Minnesota Cancer Center Tissue Procurement Facility obtained tumor samples from surgical resection specimens from patients undergoing surgery for SCCHN using standardized procedures. All samples were immediately placed on ice and, within 30 m of devascularization, frozen in liquid nitrogen following removal of portions needed for pathological diagnosis. Histological analyses were performed to ensure that each specimen contained greater than 50% tumor tissue and less than 10% necrotic debris, and those samples not meeting these criteria were rejected. Healthy control subjects without a history of oral cancer, pre-malignant lesions or periodontal disease were recruited through the University of Minnesota School of Dentistry. Following administration of a local anesthetic, a 6 mm punch biopsy of buccal mucosa was obtained and flash frozen in liquid nitrogen until mRNA extraction (40% mucosa/60% submucosa in specimens).

Extraction of Total RNA and Probe Preparation—Fifty to 100 mg of tissue was submerged in 1 mL of Trizol reagent (Life Technologies) and immediately homogenized using a rotor-stator homogenizer (Powergen 700, Fischer Scientific) under RNase free conditions. Total RNA was extracted from the samples using the Trizol extraction protocol following a 1-min spin at $12,000 \times g$ to pellet particulate matter. Total RNA was precipitated by incubating with 0.5 mL of isopropyl alcohol for 10 m followed by centrifugation at $12,000g$ for 10 m at 4°C . The pellet was washed twice with 75% ethanol, dissolved in RNase free water and stored at -80°C until further use. Agarose gel electrophoresis was performed on each sample prior to further analysis to confirm the presence of nondegraded RNA.

Five to 10 μg of total RNA was used to prepare biotinylated cRNAs for hybridization using the standard Affymetrix protocol (Expression Analysis Technical Manual, Affymetrix, Inc., 2000). Briefly, RNA was converted to first strand cDNA using a T7-linked oligo(dT) primer (Genset, La Jolla, CA), followed by second strand synthesis (Life Technologies). The double stranded cDNA was then used as a template for labeled in vitro transcription reactions using biotinylated ribonucleotides (Enzo, Farmingdale, NY). Fifteen micrograms of each labeled cRNA was hybridized to Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA) using standard conditions in an Affymetrix fluidics station.

Analysis of Microarray Data

Pre-processing of microarray data—Scanned Affymetrix array data were uploaded into the GeneData Cobi 4.0 (www.genedata.com) database maintained by the Supercomputing Institute's Computational Genomics Laboratory at the University of Minnesota. Pre-processing of the Affymetrix arrays was carried out using GeneData Refiner 3.0 (www.genedata.com) software to correct for variations in hybridization intensity due to gradient effects, dust specks, or scratches. Gene expression intensity for each array was scaled to an arbitrary value of 1500 intensity units to allow comparisons across all arrays. Expression intensity values for each gene were derived using Refiner by applying the Microarray Suite 5.0 algorithm.

Statistical analysis—Genes differentially expressed between the 41 SCCHN and 13 normal oral mucosal biopsies were identified using a Satterthwaite *t*-test [49] to robustly estimate significance despite unequal variance among groups.

Tumor Specimen Western Blotting—The protein layer from Trizol extracted tumors was used for the tumor immunoblotting experiments identically as per manufacturer instructions with 40 µg tumor protein loaded per lane. Specimens were obtained from central portions of the tumor and contained tumor cells and surrounding stroma and immediately frozen in dry ice. This was performed through an IRB approved tissue procurement protocol allowing for an extra biopsy from the tumor but not normal tissue or margin tissue from the tumor specimen, all at the time of surgery. Specimens were supplied without patient identifying data. The tumors for the Western blot experiment were from a different cohort of patients than the microarray experiments.

Biochemicals—Electrophoresis grade acrylamide and bis-acrylamide, PMSF, DTT, NaCl (Sigma Chemical, St. Louis, MO), Nonidet P40 (U.S. Biochemical Corp., Cleveland, OH), MgCl₂, KCl (Quality Biological Corp., Gaithersburg, MD), EDTA (Research Genetics, Huntsville, AL), 1M HEPES, Glycerol (Gibco/BRL), Ammonium Persulfate, TEMED, 10× TBE (Bio-Rad Laboratories, Richmond, CA), 15-deoxy-delta-12-14-PG-J2, PPAR γ antagonist T0070907 (Cayman Chemical Company, Ann Arbor, MI), Ciglitazone, Pioglitazone, ETYA (Biomol, Plymouth Meeting, PA).

Statistical Analysis

All analyses were conducted utilizing 2-sided Student *T*-tests with 95% confidence intervals calculated with a statistical analysis program (Statmate, Graph Pad Software, San Diego, CA). The overall analysis of the Affymetrix chip data have been previously published [50]. For the analysis of the PPAR gene expression data, a 2-sided *t*-test with a correction for unequal variances was employed (using a Welch Correction).

RESULTS

PPAR γ Protein Expression and Induction in Squamous Aerodigestive Cancer

NA and CA-9-22 oral cancer cell lines were treated with PPAR γ activators and western blot and electromobility shift assays performed. We used three structurally diverse PPAR γ activators including eicosatetraynoic acid (ETYA) (a synthetic analog of arachidonic acid known to have anticancer properties), ciglitazone (a thiazolidinedione), and prostaglandin 15-deoxy-delta-12-14-PG-J2 (PG-J2). At baseline, small amounts of immunoreactive PPAR γ protein were present at the anticipated molecular weight in nuclear extracts from both cell lines, and this was increased after 40 h of ETYA, ciglitazone, and PG-J2 treatment (Figure 1A). The 40-h time point was chosen for these initial experiments to be consistent with original experiments on PPAR activation [19,51]. These experiments were repeated three times with similar results. This data demonstrates that PPAR γ protein level increases in the nucleus with PPAR γ activator treatment in two oral cancer cell lines.

Next, to identify a potential nuclear binding partner for PPAR γ from the western blots, we incubated CA-9-22 cells with 10 μ M ETYA for 40 h and performed EMSAs utilizing the published PPAR γ recognition sequence from the cyp4A1 gene promoter [21]. Nuclear extracts from untreated CA-9-22 cells contained small amounts of protein as demonstrated in lane 1 of Figure 1B. This protein supershifted with antibody to PPAR γ and RXR α (lane 3 and 4), demonstrating that both binding partners for an active heterodimer exist in the nuclear extracts of CA-9-22 squamous carcinoma cells. Ten μ M ETYA treatment caused increased binding of proteins in nuclear extracts to the cyp4A1 PPAR γ recognition sequence (lane 5). Supershift analysis with PPAR γ and RXR α antibodies demonstrated 3.31 fold increases in PPAR γ binding (lane 7) compared to untreated CA-9-22 cells (lane 3). Unlabeled competition experiments with 100-fold excess of the cyp4A1 oligonucleotide are shown in lane 2 and 6. The experiments were repeated and Oct-1 was used as a housekeeping control for the experiments (not shown). These data demonstrate oral cavity cancer cells have the capacity to upregulate nuclear PPAR γ nuclear protein in response to three classes of PPAR γ activators (Figure 1A), and that the upregulated nuclear protein has the capacity to bind to a PPAR γ response element containing PPAR γ /RXR α dimers after ETYA treatment (Figure 1B).

To analyze functional activity of the PPAR gene, PPAR γ luciferase reporter gene experiments were conducted. Figure 2 demonstrates significant activation of the PPAR luciferase reporter after 24 h treatment in CA-9-22 and NA cells with ETYA (Figure 2A and B * P < 0.0001). Next, PG-J2 treatment of both cell lines caused increases in the PPAR luciferase reporters (* P < 0.0001). The maximal activation of the reporter genes occurred at a 5 μ M concentration of PG-J2 in both cell lines (Figure 2C and D). Above 5 μ M, under the reporter gene conditions, apoptosis/cytotoxicity was often evident by inverted phase light microscopy. Similarly, ciglitazone treatment of both cell lines caused increases in the PPAR reporters in both cell lines (Figure 2E and F, * P < 0.0001). The maximal activation of the reporter genes occurred at 5–10 μ M concentration in both cell lines. These data demonstrate functional activation of PPAR γ in oral cancer cell lines. This fortifies the western blot and EMSA data identifying PPAR γ in nuclear protein extracts on western blots and EMSAs (Figure 1). Interestingly, at 20 μ M ciglitazone, cells developed an apoptotic appearance by light microscopy with floating cells present in significant numbers. Consequently, we found inconsistencies in the activations at concentrations of PG-J2 and ciglitazone above 5 μ M. This was accompanied by 50% or greater decreases in the amount of β -gal activation (data not shown), probably attributable to apoptosis/cytotoxicity.

PPAR γ Ligands Induce Growth Arrest and Decrease Clonogenicity of Squamous Aerodigestive Cancer Cells

To examine anti-cancer properties of PPAR γ activation in oral cancer, cell proliferation and colony formation were analyzed using ligand treatments with and without inhibitors. ETYA treatment of both cell lines resulted in decreased proliferation in a dose-dependent fashion at concentrations of 5–40 μ M ETYA (Figure 3A and B). The IC₅₀ concentrations for the effects observed with ETYA are between 5 and 10 μ M for CA-9-22 cells and between 10 and 20 μ M for NA cells. Both cell lines also demonstrated significant dose-dependent decreases in cell proliferation with PG-J2 at 2.5 μ M (the reported IC₅₀ concentration) and

above (Figure 3C and D) [16]. Ciglitazone caused similar dose-dependent effects with an IC₅₀ of approximately 5 μ M for both cell lines. In sum, three structurally diverse PPAR γ activators decreased cell proliferation at similar concentrations to PPAR γ nuclear elevations and functional activation in Figures 1 and 2. This demonstrates an associative link between PPAR γ activation and decreased proliferation in oral cancer cell lines.

Next, diminished clonogenic growth, a second anticancer property, was assessed in CA-9-22 cells after PPAR γ ligand treatment. ETYA at 5, 10, 20, and 40 μ M was effective in significantly reducing clonogenic growth of both cell lines when assessed at 10 d (Figure 4A and B). The IC₅₀ for the effect was approximately 20 μ M in both cell lines (IC₅₀ for PPAR activation by ETYA is 10 μ M) [5]. PG-J2 treatment significantly decreased clonogenic growth with all concentrations of the agent tested (1, 2, 5, and 10 μ M). The IC₅₀ for clonogenic growth was between 1 and 2 μ M (reported IC₅₀ for PPAR γ activation with PG-J2 is 2 μ M). At 10 μ M, PG-J2 clonogenic growth was nearly abolished with greater than 95% inhibition of colony formation. With ciglitazone, the effects were less pronounced. Ciglitazone at 4.5 μ M had little effect on clonogenic growth, but concentrations of 9 and 18 μ M had significant dose dependent effects, reducing growth by up to 40% after treatment. Each experiment was repeated with similar results. Taken together, these results demonstrate that three structurally diverse PPAR γ activators decrease two hallmark features of the neoplastic phenotype, proliferation and clonogenic growth.

To confirm the specificity of PPAR γ effects, antagonist studies were performed. After screening a variety of PPAR γ agonists, we utilized T0070907, a PPAR γ antagonist which covalently binds to Cys313 of PPAR γ , inducing conformational changes blocking the recruitment of transcriptional cofactors to PPAR γ /RXR heterodimer. We pre-incubated CA-9-22 cells for 1 h and then performed PPAR γ luciferase reporter gene assays as well as MTT assays. Reporter gene assays revealed significant reversal of PPAR γ reporter gene activation when T0070907 was co-incubated with all three PPAR γ activators (Figure 5A–C). Additionally, we wanted to investigate whether these results would translate to other observations we made on MTT assays. With ETYA and ciglitazone, significant reversal of inhibition of cell proliferation occurred with 20% increases towards controls with ETYA and 15–20% with ciglitazone. With PG-J2 and T0070907, however, this effect was absent (Figure 5D). This likely reflects PPAR γ -independent anti-proliferative effects on CA-9-22 cells. We next repeated the clonogenic assay experiments with T0070907 and CA-9-22 cells. We observed the antagonist itself had no effect on clonogenic potential. However, in the case of both ETYA and ciglitazone (Supplemental Figure S1), T0070907 antagonized the effects of both agents on clonogenic potential. Only minimal, insignificant changes in clonogenic growth were observed after 5 μ M PG-J2 and T0070907 combination treatments, however, there was a significant change in clonogenic growth in the cells treated with 2.5 μ M PG-J2 (35% of control colonies) versus PG-J2 and T0070907 (47% of control independent effects on cell growth were more likely to occur with PG-J2). These data strengthen support for PPAR γ mediated antiproliferative effects on cell growth and decreased clonogenicity, since a PPAR γ antagonist helps abrogate the effects observed. These data are enhanced for ETYA and ciglitazone, compared to PG-J2.

To preliminarily examine a mechanism for the observation of decreased clonogenicity and proliferation, we next tested the CA-9-22 cells for activation of the cell cycle checkpoint protein, p21. We utilized the commercially available FDA approved drug pioglitazone, a thiazolidinedione related to ciglitazone, in CA-9-22 cells. We found the p21 promoter was significantly activated 3.6–5.6 fold at 24 and 48 h after pioglitazone treatment (Table 1). Several experiments were performed at various timepoints from 0–48 h and the observable increases occurred at 24 and 48 h (one-way ANOVA, $P < 0.0001$). We additionally examined Beas 2B and MSK Leuk1 cells and discovered a 10 μ M ciglitazone induction of p21 promoter-reporter gene activity of 25–75% over controls at 6 h. Finally, we examined our genomics data for associations between p21 and PPAR γ and did not find coordinate regulation between these two genes ($R^2 = 0.0236$; $P = 0.4999$) for the oral cancer specimens and the tumor cohort ($R^2 = 0.0098$; $P = 0.5570$). Together these data suggest that p21 may be a mechanistic target for thiazolidinedione action in squamous malignancy and premalignancy, but other effects must be occurring as well through other PPAR γ dependent genes.

Ligands of PPAR γ Induce Lipid Accumulation and Induce Differentiation Markers

The effect of PPAR γ ligands on cell morphology was assessed by light microscopy using oil-red-o staining (Supplemental Figure S2). Cell lines treated with PPAR γ ligands demonstrated a morphologic change characterized by the acquisition of cytoplasmic lipid droplets. Cell pseudopod formation was observed in many cells in the plates, which is one feature of squamous epithelial differentiation [52]. At higher doses, cell rounding and decreased cell density was typically observed, consistent with cell death and inhibition of proliferation, respectively. Even in these cells, lipid droplets are observed. Decreased cell density was a consistent finding across cell lines and all PPAR γ ligands. Vehicle-only control cells grew to high densities. These experiments were repeated several times with similar results.

To test the hypothesis that PPAR γ activators may cause some level of transdifferentiation into an adipose phenotype, cells were tested for their ability to activate an adipose differentiation-specific gene aP2 (aka FABP4). In both CA-9-22 cells and NA cells, ETYA at 10 and 20 μ M activated the aP2 luciferase reporter gene, with significant activation occurring with as little as 5 μ M ETYA (Figure 6A and D). Ciglitazone treatment demonstrated significant dose-dependent increases in aP2 reporter gene activation at 4.5, 9, and 18 μ M concentrations in the CA-9-22 cells (Figure 6C and F). Similar levels of activation were observed with PG-J2 treatment (Figure 6B and E). Next, RT-PCR was conducted with PG-J2 and these experiments demonstrated increased FABP4 and adipsin mRNA levels after PG-J2 treatment, without increases in PPAR γ mRNA levels (Figure 6G). In aggregate, these data demonstrate increased activation and expression of adipose differentiation-specific genes after PPAR γ ligand treatment.

PPAR γ Expression in Head and Neck Tumors

In order to identify whether PPAR γ was an identifiable target in actual human tissues, specimens from our head and neck cancer tumor bank/data base were interrogated for their level of PPAR γ expression using microarray technology. Thirty four of 44 tumor specimens

expressed PPAR γ at detectable levels (63%) whereas 100% (13/13) normal oral cavity specimens expressed PPAR γ (Figure 7A and B). As a group, the normal oral cavity specimens had a higher level of expression of PPAR γ (342.6 ± 87.22) than the tumors (180.7 ± 45.26) ($P = 0.0023$). More specifically, oral cavity and oropharynx cancers had lower expression of PPAR γ ($P = 0.0006$) compared to normals than the overall cohort. In addition, PPAR α and δ were also interrogated from the array. There were no significant differences in PPAR α ($P = 0.5875$) expression in the tumors versus the control specimens (Figure 7B and E), however, PPAR δ expression was significantly diminished in the tumors (833.1 ± 38.11) compared to the controls (1163 ± 89.9) ($P = 0.0038$) (Figure 7C and F). There were no differences in PPAR expression across the differentiation spectrum of the malignancies (well, moderately, poor/moderate, and poorly differentiated) for PPAR γ ($P = 0.9613$). Similarly, there was no statistically significant difference between patients experiencing recurrent disease for any of the PPAR proteins ($P > 0.1112$). Demographics for this cohort are published on page 56, Table 1 of Ginos et al. [50].

We also compared baseline PPAR γ expression between oral cancer CA-9-22 cells and three keratinocyte cell line models for differences (bronchial epithelial cells: Beas 2B and transformed oral cavity epithelial cells: HOK 16B and MSK Leuk1). On qPCR we found similar expression in the oral cancer and bronchial cells, which was somewhat higher than the transformed oral cavity cell lines (HOK 16B and MSK Leuk1 cells). These data indicate that unstimulated oral cavity cell lines and cancer cells have baseline PPAR γ expression, and that this expression may be influenced by immortalization (data not shown).

Western analysis also revealed PPAR γ positive immunoreactivity in six of nine head and neck cancer specimens at the expected molecular weight of PPAR γ (Figure 7G). Three of the specimens exhibited two separate bands at molecular weights reported for PPAR γ 1 and 2 isoforms suggesting both isotypes are present in head and neck tumors [53]. The percent of tumors which expressed PPAR γ upon gene bank interrogation and western analysis was similar (66% vs. 63%). Since PPAR γ protein is expressed in human tumor specimens, this provides further evidence that it could be putative target for intervention clinically.

DISCUSSION

There is a vital and broad need to investigate new molecular targets for therapeutic interventions for oral cancer and its prevention. A variety of PPAR γ activators have anticancer properties in solid tumor carcinogenesis, however, studies in oral cancer are not common. One such study with troglitazone provides evidence that thiazolidinediones can prevent cancer in 4 NQO tongue carcinogenesis in rats [45]. In an effort to improve understanding of this underserved niche, we identified PPAR γ receptors in human squamous aerodigestive cancer cell lines and tumors. We then discovered several classes of in vitro PPAR γ activators decreased cell proliferation, clonogenicity and promoted a differentiated adipocyte-like phenotype in our oral cancer cell lines. This preclinical evidence provides rationale for further investigation of PPAR γ activation treatment strategy (and some putative biomarkers) for oral malignancy or premalignancy.

PPAR γ has not been investigated sufficiently in squamous cancer differentiation or transdifferentiation. Experiments examining ETYA effect on lymphoma, glioma, prostate, and head and neck cancer cell lines (under the premise that ETYA was an eicosanoid synthesis inhibitor) revealed the unexplained finding of lipid uptake in several cell lines, as well as growth inhibition [19,20,51]. This was felt to be a cell death phenomenon at the time. ETYA has the capacity to activate PPAR α and PPAR γ [20], and ETYA treatment of 3T3-L1 pre-adipocytes stimulates “terminal differentiation”, presumably through its PPAR γ mediated properties [5]. In our experiments, ciglitazone and PG-J2 treatment support the ETYA observations. PPAR γ specificity for the events is strengthened by antagonist experiments with T0070907. We also observed the formation of cytoplasmic lipid droplets after PPAR ligand treatment. Further, PPAR γ activators promoted upregulation of FABP4 and possibly a partial adipogenic phenotype in these oral cancer cells. Together, the accumulation of cellular lipid subsequent to FABP4 activation is consistent with at least rudimentary adipocyte differentiation. In another study, PPAR γ ligands induced upregulation of general markers of differentiation, including gelsolin, Mad, and p21 in non-small cell lung cancer. However, no lipid specific markers or oil red O staining were observed under the experimental conditions in that study [32].

Generally, differentiation is accompanied by decreased proliferation. The decrease in proliferation and clonogenicity we observed, in the context of lipid accumulation after FABP4 upregulation, is consistent with a possible transdifferentiation phenomenon occurring in both squamous cancer cell lines tested. In pre-adipocyte cell culture models, the first step of adipocyte differentiation involves cell cycle and cell proliferation arrest. More importantly, from a cancer treatment or cancer prevention perspective, proliferation and clonogenicity arrest are important prerequisites for effective therapeutic compounds. Additionally, the evidence that differentiation phenomena are occurring in the cell lines adds an interesting dimension to the potential clinical translation of these findings. In particular, early phase clinical trials might include an intermediate endpoint biomarker panel related to squamous or adipocyte lineage differentiation, in addition to more typical surrogate endpoints like proliferation markers or targeted endpoints (e.g., PPAR γ).

We also found PPAR expression in the normal human tissue samples and human head and neck tumors. The diminished expression of PPAR γ nuclear receptors in tumor tissues is intriguing from an experimental therapeutic standpoint of retinoid cancer therapies. Much effort has been dedicated to analyze RAR β expression in aerodigestive cancers. A principal finding is that diminished expression is associated with poorer survival in early stage lung cancer [54]. From this, a concept developed that restoring the expression of the RAR portion of nuclear receptor heterodimers would improve survival and this could be accomplished with either genetic overexpression or induction with retinoids through feed-forward growth loops which increase RAR β expression [55–58]. Similarly, the promotion of increased PPAR γ is perhaps exploitable as both a treatment goal and biomarker in future studies.

In conclusion, PPAR γ activation in these oral cancer cells includes (1) upregulation of two PPAR γ reporter genes, (2) nuclear PPAR γ protein upregulation upon western blot analysis, and 3) upregulation of nuclear PPAR γ /RXR α heterodimers on EMSA using the PPAR γ recognition sequence CYP4A1. The hypothesis of using PPAR γ treatments for oral cancer

has been recently tested clinically in oral leukoplakia patients with the FDA approved thiazolidinedione pioglitazone (NCI contract N-01-15000; clinicaltrials.gov NCT 00099021).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PPAR	peroxisome proliferator-activated receptors
ETYA	eicosatetraenoic acid
PG-J2	15-deoxy- δ -12,14-prostaglandin J2
SCCHN	squamous cell carcinoma of the head and neck
VEGF	vascular endothelial growth factor
RXR	retinoid X receptor
EGFR	epithelial growth factor receptor
PPRE	PPAR response element
FABP4 (aP2)	fatty acid binding protein

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Figure 1.

(A) Western blot analysis of nuclear extracts from cells treated for 40 h with ETYA, ciglitazone, and PG-J2. Concentrations are given in μM . Molecular weight markers (MW given in kD at the expected weight of PPAR γ). (B) EMSA analysis of CA-9-22 oral cancer cells treated for 4 h with untreated control (lanes 1–4) or 10 μM ETYA (lanes 5–8). PPAR γ heterodimers are supershifted in the SS bands with both PPAR γ and RXR α antibodies. WT = wild type competition of the bands. Densitometry analysis shows 3.31 fold increase in PPAR γ supershift over untreated control (lane 7 vs. lane 3).

Figure 2. PPRE reporter gene analysis of oral cancer cells treated with ETYA, PG-J2, and ciglitazone for 40 h. Concentrations are given in μM . RLU are relative light units over the control for the treated cells. High concentrations of PG-J2 (10 μM) and ciglitazone (20 μM) were associated with cytotoxicity/apoptosis. * $P < 0.0001$. Experiments repeated three times. Nine replicates per data point are depicted.

Figure 3.

Growth inhibition in oral cancer cell lines with PPAR γ activators. Upon MTT assay of cells treated with varying concentrations of PPAR γ ligands for 72 h, dose-dependent inhibition of cell proliferation was seen in all agents. * $P < 0.05$, ** $P < 0.0001$. Six replicates per group, experiments repeated three times.

Figure 4. Clonogenic assays of oral cancer cell lines with PPAR γ ligand treatment. Inhibition in colony number was noted in all three agents. * $P < 0.05$, ** $P < 0.01$. The cells were plated, treated and clonogenic growth was assessed at 10 d.

Figure 5.

Treatment of CA-9-22 cells with PPAR γ antagonist, T0070907, abrogates effect of PPAR γ ligand treatment. CA-9-22 cells were transiently co-transfected with PPREx3-luciferase reporter gene and β -galactosidase reporter gene to account for transfection efficiency. After 24 h treatment with PPAR γ ligands and 10 μ M T0070907, lysates were assayed for luciferase activity. (A) 5 μ M ETYA, (B) 5 μ M ciglitazone, (C) 1 μ M PG-J2. Reporter activity is expressed as fold over control. Panels are representative of two similar experiments. Nine replicates per data point are utilized. * P < 0.0001, ** P = 0.003. (D) Cells were assayed for changes in cell proliferation via MTT assay after 72 h treatment with PPAR γ ligands and antagonist. Cell proliferation is expressed as % of control and is representative of two similar experiments. Seven replicates per data point were utilized. * P < 0.0001, ** P = 0.0177.

Figure 6.

Lipid associated gene activation after PPAR γ ligand treatment. Ap2 (FABP4) luciferase reporter gene activation after PPAR γ activation is depicted in A-F. Cells were treated for 24 h and analyzed via reporter gene assay. There were nine replicates per group. Error bars are enclosed within the lines of the Figure at a 95% CI. * $P < 0.0001$. Representative of two experiments. Figure 5G demonstrates semi quantitative RT-PCR analysis of upregulation of FABP4 (ap2) and adipsin after PG-J2 treatment for 24 h in the CA-9-22 cells.

Figure 7. PPAR receptor expression in head and neck cancer specimens. (A–F) Scatter and box-whisker plots of PPAR γ , α , and δ expression in head and neck cancer specimens and normals from our genomic database. The horizontal dotted lines represent cutoff for baseline expression of PPAR subsets. PPAR γ and δ were significantly downregulated in the specimens as described in the text. (G) Western blot analysis of nine tumors for PPAR γ . Six of the nine tumors were scored + for expression with equal protein loading of all specimens.

Table 1p21 Upregulation With 10 μ M Pioglitazone Treatment of Ca-9-22 Cells

Timepoint	p21 promoter fold change over untreated control ($\pm$ std dev)
0 h	1.00 ($\pm$ 0.41)
4 h	1.07 ($\pm$ 0.12)
6 h	1.08 ($\pm$ 0.04)
8 h	1.16 ($\pm$ 0.13)
12 h	1.17 ($\pm$ 0.22)
24 h	3.54 ($\pm$ 0.52)
48 h	5.68 ($\pm$ 0.41)