Experimental Cell Research 353 (2017) 63–71

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Thiazolidinediones abrogate cervical cancer growth

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ARTICLE INFO

Keywords:
Cervical cancer
PPAR
Thiazolidinedione
Pioglitazone
Head and neck cancer

ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPAR γ) is activated by thiazolidinedione drugs (TZDs) and can promote anti-cancer properties. We used three TZDs (pioglitazone, rosiglitazone, and ciglitazone) to target cervical cancer cell lines and a nude mouse animal model. Each agent increased activation of PPAR γ, as judged by a luciferase reporter gene assay in three HPV-associated cell lines (CaSki, SiHa, and HeLa cells) while decreasing cellular proliferation in a dose-dependent manner. They also promoted Oil Red O accumulation in treated cell lines and upregulated the lipid differentiation marker adipsin. Interestingly, xenograft HeLa tumors in nude mice treated with 100 mg/kg/day pioglitazone exhibited decreased growth compared to control mice or mice treated with standard cervical chemotherapy. In conclusion, TZDs slow tumor cell growth in vitro and in vivo with decreases in cell proliferation and increases in PPAR γ and adipsin. These agents may be interesting treatments or treatment adjuncts for HPV-associated cancers or perhaps even precancerous conditions.

1. Introduction

Cervical cancers are HPV associated malignancies and the second most common cause of cancer associated death worldwide [1]. HPV is also increasingly recognized as a risk factor for other cancers, notably head and neck oropharynx cancers [2]. These tumors are treated with surgery and other modalities including chemo-radiotherapy, however, many patients experience disease progression despite aggressive treatment. New modalities of treatment, such as targeted therapies, differentiation therapies, or gene therapy approaches are necessary for progress in the treatment of this malignancy. One attractive option for disease treatment could be the exploitation of nuclear receptor based differentiation therapies. In the past it has been demonstrated that nuclear receptor activation with topical and systemic retinoid may offer progress for prevention of this malignancy [3–6]. Further, treatment paradigms for nuclear receptors exist as anti-estrogen and anti-androgen therapies targeting breast and prostate cancer, respectively [7,8]. Therefore, a logical progression would be consideration of other types of nuclear receptor activation paradigms for cancer treatment.

Peroxisome proliferating activated receptors (PPAR) are nuclear receptors of the orphan class including steroid receptors, thyroid receptors, and a variety of other receptors (e.g. retinoid receptors) [9]. These receptors function as pro-differentiation, anti-proliferation complexes for transcriptional activation in a variety of cells. PPAR agonists cause PPAR and other orphan nuclear receptors to heterodimerize, often with a retinoid-X-receptor to form a transcription factor. The PPAR γ receptor subclass can be targeted with commercially available drugs for potential cancer treatment [10]. Thiazolidinedione (TZD) drugs activate PPAR γ and are FDA- approved agents used in the treatment of diabetes [11–13]. These drugs have been shown to have anti-cancer effects [14–16] and attributable to their tolerable toxicity, are potential agents for cancer prevention [17]. Currently, a variety of PPAR agonist classes have been studied preclinically in several epithelial derived carcinoma and adenocarcinoma cell lines [18–20]. In tumor cells, PPAR γ activation promotes S phase arrest [21], terminal cellular differentiation [22,23], and caspase 3 activation [24]. However, PPAR agonists, including non-thiazolidinedione PPAR γ agonists, have additional effects outside of the PPAR γ signaling axis [25,26].

In the present study, we demonstrated three TZDs (pioglitazone, rosiglitazone, and ciglitazone) functionally activate PPAR γ in three HPV associated cervical cancer cell lines. We also demonstrated these agents decreased proliferation and stimulated lipid accumulation in vitro. We were able to show pioglitazone, a commercially available PPAR γ activator, could significantly reduce cervical tumor growth in nu-/nu- mice. In conclusion, we feel that the use of pioglitazone may be useful in cervical cancer, at least partially due to PPAR γ signaling.

Abbreviations: PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; ETYA, eicosatetraynoic acid; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; AAALAC, American Association for the Accreditation of Laboratory Animal Care

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http://dx.doi.org/10.1016/j.yexcr.2017.02.020
Received 24 June 2016; Received in revised form 13 February 2017; Accepted 15 February 2017
Available online 20 February 2017
0014-4827/ © 2017 Published by Elsevier Inc.

2. Materials and methods

2.1. Cell culture

SiHa (HPV 16+), CaSki (HPV 16+), and HeLa (HPV 18+) cancer cells were cultured in DMEM (SiHa and CaSki) or RPMI 1640 (HeLa) with L-glutamine supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) in a humidified atmosphere of 5% CO^2 at 37 °C. Where indicated, cells were grown in serum free media containing only L-glutamine. SiHa and CaSki cells were obtained from ATCC and HeLa cells were obtained from Dr. Wantao Chen at Shanghai Jiao Tong University School of Medicine and were phenotyped as HeLa cells (STR genotyping by the Fragment Analysis Facility at Johns Hopkins University).

2.2. PPAR γ reporter gene assay

Cell lines were transiently transfected with a thymidine kinase luciferase containing reporter plasmid with a PPAR γ response element (PPRE), TK-PPREx3-Luc, or Gal-PPAR Ligand Binding Domain (LBD) plasmids, kind gifts from Dr. Ronald Evans (The Salk Institute, San Diego, CA). The PPREx3 plasmid contains three repeats of the PPAR γ response element, 5'-TCGACAGGGGACCAGGACAAAGGTCACGTT-CGGGAGTCGAC, from the Acyl-CoA oxidase gene. The Gal-PPAR LBD fusions were made by fusing the PPAR γ ligand binding domain to the C-terminal end of the yeast GAL4 domain. Cells were treated with the general PPAR activator ETYA (Cayman Chemical, Ann Arbor, MI), as well as the thiazolidinediones pioglitazone (LKT, St. Paul, MN), rosiglitazone (LKT), or ciglitazone (BIOMOL, Plymouth Meeting, PA) for 24 h in serum-free media (or DMSO vehicle $< 0.1\%$). Cell lysates were analyzed on a Tristar dual injection flash luminometer (Berthold Technologies, Oak Ridge, TN). Co-transfection with a β-galactosidase (β-gal) reporter plasmid accounted for transfection efficiency and acted as an internal standard. Each experiment was repeated in triplicate and nine replicates per data point.

2.3. MTT assay

Cell proliferation was determined MTT incorporation. Cells were plated at 5×10^3 cells/well in 96-well tissue culture plates. Treatments were added at day zero in serum-free media. At day zero, three, or five, MTT was added to the culture media at 0.5 mg/mL and incubated at 37 °C for four hours, solubilized in isopropyl alcohol/DMSO and read the absorbance at 560 nm. Six replicates per data point were utilized and experiments repeated thrice.

2.4. Western blot analysis

HeLa cells were plated in 25 cm^2 flasks and treated the following day with 10 μM PPAR γ activators in serum free media for 48 h. Whole cell lysates were made using standard RIPA buffer technique and shearing cells by passing lysates through a 29-gauge needle. Protein quantification was performed BCA assay (Pierce Biotechnologies, Rockford, IL). Eighty micrograms of protein per lane was separated on a 4–12% BisTris NuPage Gel (Life Technologies). Proteins were transferred to Immobilon-PSQ 0.2 μm PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for one hour with 5% milk in TBS/0.1% Tween-20 then blotted with a primary antibody to cyclin D1 (Cell Signaling Technology, Danvers, MA) followed by a horseradish peroxidase conjugated secondary antibody. Proteins were visualized with a chemiluminescence assay system (Pierce Biotechnologies). Membranes were stripped and re-probed with β-actin (Affinity BioReagents, Golden, CO).

2.5. Trypan blue exclusion assay

Cells were plated at 1×10^5 cells/well in 12-well tissue culture plates. The following day, pioglitazone and/or chemotherapeutic agents were added to the wells in serum free media. Cells were harvested via trypsinization on day three and stained with 0.4% trypan blue at 1:1 cell suspension/trypan blue. Stained cells were incubated at room temperature five minutes and counted on a hemocytometer. Percentage of viable cells is the number of viable (unstained) divided by the number of total viable and dead (stained) cells.

2.6. Oil Red O lipid staining

HeLa cells were plated in 12-well plates and treated with DMSO (vehicle control), ETYA, pioglitazone, rosiglitazone, or ciglitazone at 10 μM in 1% FBS for eight days, retreating at 48 h. At the treatment conclusion, cells were fixed in 10% formalin in PBS for five minutes, rinsed with PBS to remove formalin, then rinsed in 50% isopropyl alcohol. Filtered working Oil Red O solution (60% of 0.5% (w/v) Oil Red O in isopropyl alcohol and 40% PBS) was added to cells and incubated for five minutes. Oil Red O stain was dissolved from the fixed cells in 75% isopropyl alcohol in PBS containing 4% SDS and measured on a spectrophotometer at 518 nm. Oil Red O was quantified both by mg protein and cell number.

2.7. RT-PCR

HeLa cells were plated in 25 cm^2 flasks at 5×10^5 cells per flask. At 80% confluency, the cells were treated with 10 μM TZDs or DMSO vehicle control. 24 or 48 h later, the cells were lysed and total RNA harvested using the PerfectPure Cultured Cell RNA kit (5Prime, Gaithersburg, MD). Human fat, used as a control for adipose tissue markers, was obtained anonymously under IRB approved tissue bank protocols at the University of Minnesota. Total RNA from human fat was isolated using Trizol Reagent (Life Technologies). cDNA was created from 2 μg of total RNA from each sample with the Superscript III Reverse Transcriptase (Life Technologies). PCR for adipose differentiation markers was performed using mRNA specific primer sets for each marker and the Platinum SuperMix polymerase (Life Technologies). PCR products were separated on 2% agarose gels and documented with BioRad Gel Doc.

2.8. Animal care

All animal studies were conducted in a facility accredited by the AAALAC Animal use protocols were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. Female athymic nu-/nu- mice at 6–8 weeks of age were used for the xenograft studies (NCI, Fredrick, MD) housed five to a cage in sterile, microisolator cages.

2.9. In vivo tumor growth

Groups of five female athymic nu-/nu- mice were inoculated subcutaneously on the right flank with 0.5, 1, or 5×10^6 HeLa cells in 100 μL serum free RPMI 1640. Tumor diameters were measured weekly and mice were weighed to monitor health. Tumor volumes were calculated using the formula; $V=4/3\pi r^3$ where r=average radius from three measurements of tumor diameter. In the second experiment, to test the efficacy of two doses of pioglitazone, additional groups of five female, nu-/nu- mice were inoculated with HeLa cells subcutaneously on the right flank. At day 18, animals were separated into three groups of five animals so the groups each had equivalent tumor volumes. Pioglitazone (powdered 45 mg Actos tablets) was mixed into powdered rodent chow to provide dosages of zero, 25, or 100 mg/kg/day. Animals were weighed and tumor diameters were measured weekly.

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