The PPARγ Agonist Rosiglitazone Inhibits Glioma Cell Proliferation and Migration in vitro and Glioma Tumor Growth in vivo

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ABSTRACT

Peroxisome proliferator-activated receptor-γ (PPARγ) has been implicated in the growth inhibition of a number of cancer cells. In the present study, we investigated the antitumor effect of the PPARγ agonist rosiglitazone in U87MG human glioma cells. Rosiglitazone treatment in vitro reduced cell proliferation without induction of cell death in a dose- and time-dependent manner. Rosiglitazone decreased cell migration and mRNA level of MMP-9. Rosiglitazone treatment also induced marked changes in glioma cell morphology. Oral administration of rosiglitazone in animals with subcutaneous U87MG glioma cells reduced tumor volume. Subsequent tumor tissue analysis showed that rosiglitazone decreased the number of PCNA-positive staining cells and MMP-9 expression and induced apoptosis of tumor cells. These data suggest that rosiglitazone exerts antineoplastic effect in U87MG cells and may serve as potential therapeutic agent for malignant human gliomas.

Key words: PPARγ, rosiglitazone, proliferation, migration, glioma tumor growth, apoptosis, MMP-9, human U87MG glioma cells

INTRODUCTION

Glioblastomas are the most common primary brain tumors in adults. These malignant astrocytic tumors exhibit a high proliferation rate and an aggressive growth pattern and acquire resistance against many therapeutic interventions (Ohgaki and Kleihues, 2005). Despite aggressive treatment including surgery, radiation, and chemotherapy, most patients die of the disease, with median survival of one year (DeAngelis, 2001).

The peroxisome proliferator-activated receptors (PPARs) are a family of ligand activated transcription factors belonging to the nuclear receptor superfamily (Willson et al., 2001). Three different isoforms have been identified, PPARα, PPARβ, and PPARγ, each with distinct physiological functions (Isssemann and Green, 1990; Dreyer et al., 1992). PPARs are the primary targets of numerous natural and synthetic compounds including phthalate plasticizers, long-chain fatty acids, and pharmacologic drugs. Among them, PPARγ is of particular interest, because it has been implicated in many human diseases including type II diabetes, atherosclerosis, hypertension, inflammation, and cancer.

Although PPARγ agonists can promote either cytoprotection or cytotoxicity, depending on the cell
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Type and/or the ligand used (Clay et al., 2000; Rosen and Spiegelman, 2001; Nikitakis et al., 2002; Na and Surh, 2003), they can induce growth arrest, apoptosis, and terminal differentiation in a number of different cancer cells (Sarraf et al., 1998; Clay et al., 2002; Yamakawa-Karakida et al., 2002; Zander et al., 2002; Chen et al., 2003; Grommes et al., 2005; Piva et al., 2005). In support of the in vitro studies using cell lines, there are many reports showing inhibition of tumor growth in tumor-bearing rodent models treated with PPARγ agonists (Fujisawa and Horikoshi, 2000; Houseknecht et al., 2002; Grommes et al., 2006). Therefore, PPARγ ligands may offer potential new therapy for the treatment of tumors.

Rosiglitazone, a synthetic PPARγ agonist, is already in clinical use as an antidiabetic drug. Rosiglitazone did not induce cell death and apoptosis in human hepatoma cells (Yamamoto et al., 2001), whereas it suppress cell proliferation and cause apoptosis in glioblastoma cell lines (Morosetti et al., 2004) and human neuroblastoma cell lines (Valentiner et al., 2005). Thus, effectiveness of the PPARγ agonists on cancer cell growth differed depending on the cell line.

The present study was undertaken to examine the effect of rosiglitazone on glioma growth in vitro and in vivo. In this study, rosiglitazone inhibited proliferation of human glioma cells and caused dramatic regression of subcutaneously implanted mouse gliomas.

**MATERIALS AND METHODS**

**Reagents**

Rosiglitazone was obtained from Cayman (Ann Arbor, MC, USA) and dissolved in dimethyl sulfoxide (DMSO). Hoechst 33258, propodium iodide (PI) and 3-(4,5-dimethylthiazol-s-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical (st. Louis, MO, USA). Antibody for proliferating cell nuclear antigen (PCNA) was purchased from Cell Signaling Technology (Beverly, MA, USA) and antibody for matrix metalloproteinase-9 (MMP-9) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies for immunohistochemistry were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

**Cell culture**

U87MG human glioma cells were obtained from American Type Culture Collection (ATCC, MD, USA), incubated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Invitrogen, Carsbad, CA, USA) supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% fetal bovine serum, and maintained at 37°C in a humidified incubator containing 5% CO2. In experiments for proliferation and cell death measurement in vitro, cells were exposed to rosiglitazone in serum-free medium.

**Measurement of cell proliferation and cell death**

Cell proliferation was determined by MTT assay and counting the number of viable cells. For the MTT assay, cells were cultured in a 24-well culture plate and treated with rosiglitazone or vehicle for the indicated times. The cells were washed and incubated with MTT (0.5 mg/ml) for 2 h at 37°C. The formazan granules were dissolved in DMSO, and the absorbance was measured with a microplate reader at 570 nm.

For counting of cell number, cells were harvested, suspended in 4% trypan blue solution and the number of cells was counted by using a hemocytometer under light microscopy. In these experiments, cells failing to exclude the dye were considered nonviable.

**Migration**

Migration of cells was measured using transwell (costar, MA, USA). Transwell inserts with an 8 μm pore size were coated with a final concentration of 50 ng/ml collagen. Cells (5×10⁴) were suspended in DMEM containing rosiglitazone or vehicle (DMSO). And then cells were plated in the wells of the upper compartment of the chamber and the wells of the lower compartment were filled with DMEM containing 10% fetal bovine serum. After incubation, cells on the bottom side of the membrane were fixed with 4% paraformaldehyde, stained with Hoechst 33258, and counted.
Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of total RNA were reverse transcribed by using a oligo (dT) primer and reverse transcriptase (Promega, Madison, WI, USA) and then the synthesized cDNA was used as a template for the PCR reaction. PCR primers were used to amplify MMP9 (forward 5'-GGAGTACTCGACCTGTACCA-3' and reverse 5'-GTGAAGCGGTACATAGGGTA-3') and GAPDH (forward 5'-TCCATGACAACTTTGGTATCG-3' and reverse 5'-TGTAGCCAAATTCGTTGTCA-3'). PCR was carried out under the following conditions: the initial denaturation 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s, and extension for at 72°C for 30 s and additional extension for at 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

Observation of cell morphology

Cells were cultured with normal culture medium containing 50 μM rosiglitazone and cell morphology was observed by phase-contrast microscopy. To evaluate if the rosiglitazone-induced change in morphology was reversible, cells were treated with 50 μM rosiglitazone for 4 days and then further cultured with rosiglitazone-free growth medium for 3 days.

In vivo tumor growth assay

U87MG cells (2×10^6) were injected subcutaneously into the right hind leg of 4-week male Balb/c nude mouse. After injection, rosiglitazone (5 mg/kg, n=5) or vehicle (DMSO, 0.1% final concentration, n=5) was administered by oral gavage daily and the animals were weighed weekly. After 7 weeks, the tumors were excised and tumor volume was calculated using the equation: tumor volume (mm^3) = (length×width^2)×π/6. Tumor was fixed in formalin, embedded in paraffin, and sectioned by standard methods and processed for histological examination and immunohistochemistry. All animal experiments were conducted according to the international guidelines and NIH guidelines for the laboratory animal care.

Immunohistochemistry

Tumor sections were blocked with 8% BSA in PBS and incubated with mouse anti-PCNA or mouse anti-MMP-9 at 4°C overnight. Sections were washed and incubated with secondary antibodies at room temperature for 60 min. After washing, counterstaining was carried out with PI. Sections were viewed under a fluorescent microscope (Leica, Wetzlar, Germany).

Determination of apoptosis

Tumor sections were examined for apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP transferase nick-end labeling (TUNEL assay). Sections were examined using the in situ Cell Death Detection kit (Roche Applied Science, IN, USA) according to the manufacturer's instructions. The same samples were counterstained with Hoechst 33258 to label nuclear. Slides were mounted and observed under a fluorescent microscope.

Statistical analysis

The data are expressed as means±SEM and the difference between two groups was evaluated using a paired Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. A probability level of 0.05 was used to establish significance.

RESULTS

Effect of rosiglitazone on proliferation and migration in vitro

Cells were exposed to various concentrations of rosiglitazone for various times and proliferation was measured by MTT assay and cell counting. Rosiglitazone inhibited cell proliferation in a dose- and time-dependent manner (Fig. 1).

In addition to proliferation, the ability of U87MG cells to invade the surrounding and intact tissues characterizes the malignancy state of gliomas. Therefore, we next examined the effect of rosiglitazone on cell migration. Migration was measured for 24 and 48 hr in the presence of 20 and 100 μM rosiglitazone using a transwell chemotactic assay. Rosiglitazone reduced migration in a dose- and time-dependent manner (Fig. 2A). To explore if
Fig. 1. Dose- and time-dependent effects of rosiglitazone on cell proliferation. Cells were exposed to various concentrations of rosiglitazone for 1~5 days. Cell proliferation was estimated by MTT reduction assay (A) and counting of cell number (B). Data are mean±SEM of four independent experiments performed in duplicate.

Reduced migration by rosiglitazone is associated with MMP-9 levels, its mRNA level was evaluated in cells treated with rosiglitazone. As shown in Fig. 2B, the MMP-9 mRNA level was markedly suppressed by rosiglitazone.

Effect of rosiglitazone on cell morphology
To examine if rosiglitazone causes morphological changes of U87MG cells, cells were cultured in the medium containing serum with or without rosiglitazone. After 3 days of culture, cells showed cluster formation in the medium without rosiglitazone, but the cluster formation was inhibited in the medium with 50 μM rosiglitazone. In addition, the cells were changed from their dominantly elongated bipolar nature to a round shape after 3 days of rosiglitazone treatment (Fig. 3A and B). To examine if these morphological changes were reversed by replacement with rosiglitazone-free medium, the cells treated with rosiglitazone for 4 days were cultured in control medium without rosiglitazone for 3 days. As shown in Fig. 3C, the cells were changed to morphology of control cells, suggesting that the morphological changes were reversible.

Effect of rosiglitazone on glioma tumor growth in vivo
To determine the anti-tumor effect of rosiglitazone in vivo, U87MG cells were injected subcutaneously into Balb/c nude mouse. After injection, rosigli-
Zone (5 mg/kg) or vehicle was given by oral gavage daily. Although there was variability, all vehicle-mice developed subcutaneous tumors with volume of approximately 320 mm$^3$ after 7 weeks. In each case, the tumor volume in the rosiglitazone-treated mice was significantly smaller than in vehicle-mice. Rosiglitazone produced approximately 75% reduction in tumor growth compared with the vehicle-mice (Fig. 4A∼C). There was no difference in body weight between two groups (Fig. 4D).

**Effect of rosiglitazone on proliferation and apoptosis in vivo**

To assess whether rosiglitazone inhibits tumor proliferation in vivo, PCNA expression was evaluated. Rosiglitazone decreased the proliferative rate of subcutaneously implanted U87MG glioma cells (Fig. 5A and C), supporting the results obtained in vitro. We obtained evidence that rosiglitazone also induced apoptosis. While U87MG glioma cells from the vehicle-mice showed almost no TUNEL-positive cells, tumor cells from mice treated with rosiglitazone showed a marked increase in TUNEL-positive cells (Fig. 6).

**Effect of rosiglitazone on MMP-9 expression in vivo**

The expression of MMP-9 closely correlates with the invasive and metastatic potentials of gliomas (Wild-Bode et al., 2001; Rao, 2003). Therefore, we evaluated the effect of rosiglitazone on the invasion of glioma tumor in vivo by detecting the expression of MMP-9. Immunohistochemical assay showed that MMP-9 was highly expressed in glioma tumor of the vehicle-mice and its expression was almost completely suppressed by rosiglitazone treatment (Fig. 5B and D).

**DISCUSSION**

PPAR-γ agonists have been shown to inhibit cell growth and potentially induce apoptosis in several carcinoma cell lines including those derived from breast, colon, lung, prostate, pancreatic and renal cancer (Elstner et al., 1998; Kubota et al., 1998; Sarraf et al., 1998; Tsubouchi et al., 2000; Eibl et al., 2001; Inoue et al., 2001; Clay et al., 2002; Yu et al., 2006). Recent studies have demonstrated the growth inhibitory effect of the PPARγ ligands on some glioma cell lines (Zander et al., 2002; Strakova et al., 2004; Grommes et al., 2005; Cho et al., 2006). Recently, Grommes et al. (2006) demonstrated that in vivo treatment of pioglitazone, a synthetic PPARγ agonist, inhibits glioma growth and invasion in animal models.

By contrast to anti-proliferative effect of PPARγ ligands, it has been reported that some PPARγ ligands can enhance colonic tumor formation in transgenic mice (Lefebvre et al., 1998; Saez et al., 1998; Pino et al., 2004; Yang et al., 2005). Given these conflicting data on whether PPARγ activation could induce growth inhibition or promotion of tumor
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Fig. 4. (A ∼ C) Effect of rosiglitazone on growth of subcutaneous U87MG gliomas in Balb/c nude mouse. U87MG cells (2×10^6) were injected subcutaneously into the right hind leg of 4-week male nude mouse. After injection, rosiglitazone (5 mg/kg) or vehicle (DMSO, 0.1% final concentration) was administered by oral gavage daily. After 7 weeks, the tumors were excised and tumor volume was calculated using the equation: tumor volume (mm^3)=length×width^2×π/6.

(D) Effect of rosiglitazone on body weight. Data in (C, D) are mean±SEM of five animals in each group.

formation, a better understanding of the mechanism of action of PPARγ in glioma is need.

Rosiglitazone is the most potent and selective PPARγ agonist in synthetic thiazolidinediones (Murphy and Holder, 2000). Although it has been reported that rosiglitazone inhibits proliferation of glioma (Morosetti et al., 2004), neuroblastoma (Valentiner et al., 2005; Cellai et al., 2006), lung carcinoma (Han and Roman, 2006), adrenocortical tumor (Betz et al., 2005; Ferruzzi et al., 2005) and malignant melanoma cells (Freudlsperger et al., 2006) in vitro, its anti-tumor effect was not explored in animal glioma model in vivo.

The present study demonstrates that rosiglitazone treatment in vitro inhibited proliferation of human glioma U87MG cells in a dose- and time-dependent manner (Fig. 1). But rosiglitazone did not cause cell death even at concentration of 100 μM as evidenced by trypan blue exclusion and annexin-V binding assay (data not shown). These data suggest that rosiglitazone inhibits glioma cell growth without induction of cell death. Similar results are reported
Fig. 5. Effect of rosiglitazone on cell proliferation and MMP-9 expression in subcutaneous U87MG gliomas. Animals were sacrificed at 7 weeks after injection of U87MG cells and tumor tissues were sectioned. Sections were incubated with mouse anti-PCNA (A) or mouse anti-MMP-9 (B) at 4°C overnight. Sections were washed and incubated with secondary antibodies at room temperature for 1 hr. Sections were viewed under a fluorescent microscope. PI staining served as counterstaining. Quantitative results of PCNA-positive cells (C) and MMP-9 expression (D) were shown. Data are mean±SEM of five animals in each groups. *p<0.05 compared with vehicle group.

In non-small-cell lung cancer (Keshamouni et al., 2004). In the present study, we did not explore the underlying mechanisms of rosiglitazone-induced inhibition of cell growth. However, it has been demonstrated that PPARγ agonists inhibit growth of various cancer cells through arrest in G1 phase by modulating the expression of genes involved in cell cycle (Takashima et al., 2001; Kim et al., 2003; Strakova et al., 2004).

Although PPARγ agonists reduces migration of various cancer cells (Ferruzzi et al., 2005; Grommes et al., 2006; Zhang et al., 2006), it is
unclear whether rosiglitazone inhibits migration of glioma cells. In the present study, we observed that rosiglitazone treatment induces an inhibition in migration and mRNA levels of MMP-9 (Fig. 2).

Although it has been shown that rosiglitazone exerts a variety of effects on cell behaviors, such as inhibition of cell proliferation, induction of apoptosis, and inflammatory response, little is known about the possibility that rosiglitazone changes cell morphology in glioma cells. The present finding suggested that rosiglitazone could induce morphological change in glioma cells. The underlying
mechanism of these morphological changes remains to be defined. However, these changes may be attributed to a change of actin structure as suggested in human pancreatic cancer cells (Motomura et al., 2004). The rosiglitazone-induced changes in morphology were reversed by the replacement of rosiglitazone-containing medium with rosiglitazone-free-medium, suggesting that the action of rosiglitazone did not induce irreversible cell damage.

Proliferation and/or migration in glioma cells might be associated with morphological changes. However, the present study showed that inhibition of proliferation and migration by rosiglitazone was observed at 24 hr of treatment, but the morphological changes were induced after 3 days, suggesting that the morphological changes may not be involved in the inhibition of proliferation and migration. Although these data do not provide the evidence that the changes in cell morphology by rosiglitazone contributes directly to the inhibition of proliferation and migration, a possible molecular event during the process changing cell morphology may be involved in reduction of proliferation and migration.

To evaluate the anti-tumor effect of rosiglitazone in vivo, tumor growth after subcutaneous implantation of U87MG cells was monitored in nude mice, and the effect of rosiglitazone was determined. The present study demonstrated that oral rosiglitazone treatment reduced tumor volume by approximately 75% (Fig. 4). Reduction in tumor volume by rosiglitazone treatment in vivo was associated with inhibition of cell proliferation (Fig. 5A and C) and induction of apoptosis (Fig. 6). In the present study, the effect of rosiglitazone on cell death was different between in vitro and in vivo treatment. Rosiglitazone treatment in vivo induced apoptosis, whereas in vitro treatment did not affect cell death. Therefore, the apoptotic response produced by rosiglitazone in vivo does not seem to involve a direct effect on tumor cells.

Matrix metalloproteinases (MMPs) are a family of endopeptidases excreted by a number of cell types including cancer cells, capable of cleaving several macromolecules of the extracellular matrix. MMP-9 and MMP-2 are known to play an important role in angiogenesis, tumor growth and metastasis mainly through their degradation of the extracellular matrix that may result in tumor and endothelial cell migration due to loss of cell-matrix contacts and cell-cell contacts (Stetler-Stevenson, 1999; John and Tuszynski, 2001). MMP-9 is the most abundant MMP in gliomas (Forsyth et al., 1999) and is elevated during tumor progression because of its secretion in glioma cells (Choe et al., 2002; Rao, 2003). It has been reported that PPARγ agonists inhibit MMP-9 expression in various cancer cells (Liu et al., 2005; Panigrahy et al., 2005; Grommes et al., 2006). In the present study, mRNA levels of MMP-9 and its expression were inhibited by rosiglitazone treatment in vitro and in vivo, respectively (Figs. 2B and 5B and D). These data may suggest that rosiglitazone reduces invasion and metastasis of glioma tumors.

In conclusion, rosiglitazone treatment in vitro inhibited proliferation and migration in U87MG cells. Oral administration resulted in consistent regression of subcutaneous glioma tumors through inhibition of proliferation and induction of apoptosis in nude mice. Rosiglitazone down-regulated MMP-9 in vitro and in vivo. The data suggest that PPARγ agonists such as rosiglitazone may serve as potential anti-tumor agents in human glioma therapy.

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