Rosiglitazone enhances 5-fluorouracil-induced cell growth inhibition in hepatocellular carcinoma cell line Hep3B

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[Abstract] Background and Objective: Rosiglitazone is a peroxisome proliferators-activated receptor γ (PPARγ) ligand, which inhibits tumor growth by activating PPARγ signaling pathways. Fluorouracil (5-FU) is one of the commonly used chemotherapeutic drugs. However, patients develop drug resistance of 5-FU over time. The aim of this study was to investigate whether rosiglitazone can enhance 5-FU-induced cell growth inhibition and to explore its potential mechanisms.

Methods: Cell viability was measured using MTT assay. Protein expression levels were detected by Western blot analysis. Small interference RNA was utilized to knock out PPARγ and PTEN in Hep3B cells. Results: After 48 h of treatment with 10, 20, and 40 μmol/L rosiglitazone, the viability of Hep3B cells was (78.0 ± 2.7)%, (37.3 ± 8.1)%, and (19.8 ± 2.2)%, respectively (compared with control group, P values were all < 0.001). After 48 h of treatment with 10 μmol/L 5-FU, the viability of Hep3B cells was about (82.8 ± 3.9)% when cells were treated with 10 μmol/L 5-FU in combination with either 10, 20 or 40 μmol/L rosiglitazone, the cell viability was (51.6 ± 5.4)%, (14.8 ± 4.2)%, and (8.5 ± 0.9)%, with corresponding q value of 1.36, 1.23, and 1.19, respectively. These data suggested that the two drugs had synergic effect in inhibiting Hep3B cell growth, which was further confirmed in an in vivo mice model. Subsequent investigations showed that rosiglitazone activated PPARγ signaling pathways and increased the expression of PTEN.

Conclusions: Rosiglitazone enhances 5-FU-induced cell growth inhibition of Hep3B cells.

Key words: Rosiglitazone, 5-fluorouracil, hepatocellular carcinoma

Primary liver cancer (PLC), also known as hepatocarcinoma, is one of the most common malignant cancers in China with high mortality, which greatly threatens the health and life of human beings. It is the second lethal malignant cancers in China and its incidence increases worldwide. Most patients have already been at advanced stage by the time of diagnosis and have lost opportunities of surgical operation. Traditional systemic chemotherapy for liver cancer has low curative rate with many toxic side effects, and is therefore not widely accepted by clinical practitioners. Recently, the application of new chemotherapeutic drugs and new combination regimens has been proved to be effective against advanced hepatocarcinoma. Fluorouracil (5-FU) is commonly used in liver cancer chemotherapy either alone or in combination with other agents. However, cancer cells develop drug resistance over time, which is an obstacle for effective chemotherapy targeting advanced liver cancer[1]. Thus, it is necessary to explore new chemotherapeutic regimens to overcome drug resistance in liver cancer cells, and to understand their molecular mechanisms. Rosiglitazone (Rosi) belongs to the medication class of thiazolidinediones and is used clinically as a sensitizer for anti-diabetic drugs with hypoglycemic effect. Rosiglitazone have shown to be one of peroxisome proliferators-activated receptor γ (PPARγ) ligands, which inhibits hepatocarcinoma cell growth and induces cell apoptosis by activating PPARγ signaling pathway. However, a complete picture of how resiglitazone works remains unknown[2]. Compared with other ligands of PPARγ such as troglitazone, ciglitazone, pioglitazone, the interaction between resiglitazone and
PPARγ is strongest and is able to exert a variety of biological consequences[3]. It has been reported that resiglitazone synergically induces cancer cell apoptosis with other chemotherapeutic agents and enhances chemotherapy effect in colon cancer HT-29 cells, which is mediated by regulating several factors in these cells[4]. However, the effect of resiglitazone in hepatocarcinoma chemotherapy has not been investigated. Clinical and basic scientific researchers are looking for new chemotherapeutic drugs or new combination regimens to treat hepatocarcinoma. This study aimed to determine the synergic effect of resiglitazone and 5-FU in inhibiting hepatocarcinoma cell growth and to explore potential molecular mechanisms, using in vitro cell cultures and an in vivo mice model.

Materials and Methods

Reagents and cell culture

Rosiglitazone and 5-FU were purchased from Cayman Chemical company and were dissolved in dimethyl sulfoxide (DMSO) respectively with the final concentration of 0.1%. Mouse monoclonal anti-human PPARγ and phosphatase and tensin homologue deleted on chromosome ten gene (PTEN), rabbit polyclonal anti-human phospho-Akt (Ser473) primary antibodies, and horseradish peroxidase conjugated sheep anti-mouse or rabbit secondary antibodies were purchased from Santa Cruz, USA. Human hepatocellular carcinoma cell Hep3B was kindly provided by Professor Wen Li in Surgery Laboratory at the First Affiliated Hospital, Sun Yat-sen University. Cellswere cultured at 37°C in a 95% humidified atmosphere containing 5% CO2 and in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) containing 10% fetal bovine serum (VVS), 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were passaged when reached 80%–90% of confluence. Cells at logarithmic growth phase were used for experiments.

MTT assay

Hep3B cells at logarithmic growth phase were adjusted to a cell concentration of 1×10⁶ cells/well with DMEM medium containing 10% fetal serum, seeded into a 96-well plate with 200 μL/well, and cultured for 8 h at 37°C in an incubator with 5% CO2. After cell adherence, the culture mediums were then replaced with 200 μL fresh medium containing either various concentrations of resiglitazone or 10 μmol/L 5-FU, or both. Control cells were incubated with 200 μL DMEM medium containing 0.1% DMSO only. For MTT assays, wells without any cells were set as blank control. After cells were routinely incubated with various concentrations of drugs for 48 h, 20 μL MTT (5 g/L) were added into each well and cells were incubated for additional 4 h. Then the supernatants were removed and 200 μL DMSO was added into each well, followed by gentle oscillation of the plates for 10 min to dissolve the crystallizations. Subsequently, the absorbance at 570 nm (A570 value) was measured using a microplate reader. Cell viability and growth inhibition were calculated as described below: cell viability rate = A570 value of the drug treated group / A570 value of the control untreated group × 100%; growth inhibition rate = 1 – cell viability. The interaction between two drugs was judged according to a method describe by Jin et al.[5]. Briefly, a q value was obtained by comparing within the dose-effect curves of the growth inhibition by combined treatment of two drugs to the growth inhibitions by the treatment of single drugs. That is, q = growth inhibition by the combined treatment of two drugs / the expected inhibition based on the growth inhibitions by the treatment of single drugs = Eₐ + Eₖ / (Eₐ + Eₖ + Eₐ × Eₖ). Two drugs have additive effects if 0.85 ≤ q ≤ 1.15, are synergic if q > 1.15, and are antagonistic if q < 0.85.

Total protein extraction and Western blot analysis

The treated cells were rinsed for 3 times with cold PBS and incubated with 200 μL cold protein lysis buffer at 4°C for 30 min. The undissolved cell debris was removed by spinning out at 15 000×g for 10 min. A total of 50 μg of total protein were separated in SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was blocked in a blocking buffer containing 20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.1% Tween-20 and 5% dry milk for 1 h and incubated overnight with appropriate primary antibodies at 4°C. After being washed for 3 times, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies for another 1 h at room temperature. The proteins were visualized using enhanced chemiluminescence (ECL).

Small interfering RNA (siRNA)

The siRNAs targeting human PPARγ and PTEN were designed and synthesized by Guangzhou Ribobio Company. Cells were seeded into 6-well plates and cultured with antibiotic-free medium for 24 h to totally adhere and reach 50% confluence. The culture medium was then replaced with 1.5 mL fresh antibiotic-free medium and the cells were transfected using Lipofectamine 2000 according to the manufacturer’s instruction. Briefly, 10 μL Lipofectamine 2000 was gently mixed with 250 μL opti-MEMI and incubated at room temperature for 5 min. In the meantime, 10 μL targeting siRNA or scramble siRNA were mixed with 250 μL of opti-MEMI to reach a final concentration of siRNA.
at 100 nmol/L. Subsequently, the two mixtures were combined and incubated at room temperature for 20 min before dropping into the cell culture medium. Cells were cultured for additional 48 h.

Establishing a xenograft tumor model in Balb/c nude mice implanted with human hepatocellular carcinoma Hep3B cells

Four-week-old Balb/c nude mice (Animal Certification number: SCXK (Guangdong) 2009-0011) were purchased from Experimental Animal Center of Sun Yat-sen University. Half of the mice are male and half of them are female at SPF level. Hep3B cells were diluted in PBS to a final concentration of $1 \times 10^7$ cells/mL and then subcutaneously inoculated in nude mice at 0.1 mL per mouse. The diameter of xenografts reached 0.6 mm 15 days after implantation. These nude mice were then randomly divided into 4 groups with 5 mice in each group. Mice in the control group were intraperitoneally injected once a day with 0.1% DMSO (1 mL/kg). Mice in the rosiglitazone group were injected with rosiglitazone (20 mg/kg) daily. Mice in the 5-FU group were injected with 5-FU (10 mg/kg) daily. Mice in the rosiglitazone combined with 5-FU group were injected with rosiglitazone (20 mg/kg) and 5-FU (10 mg/kg) daily. The size of tumors was measured every two days for 2 weeks and the tumor volume ($V$) was calculated using the following formula: $V = \frac{1}{2} \times \text{length} \times \text{width}^2$.

Statistical analysis

Experimental data were presented as mean ± standard deviation (SD). ANOVA test was used for univariate analysis by SPSS 11.0 statistical software, whereas the Student-Newman-Keuls test was used for multiple comparisons. A value of $P < 0.05$ was considered as significantly different.

Results

Rosiglitazone enhanced inhibition of Hep3B cell growth induced by 5-FU

After treatment with 5, 10, 20, 40, and 80 μmol/L rosiglitazone for 48 h, the viability of Hep3B cells was $(89.1 \pm 3.6)\%$, $(78.0 \pm 2.7)\%$, $(37.3 \pm 8.1)\%$, $(19.8 \pm 2.2)\%$, and $(3.7 \pm 2.2)\%$, respectively. Significant difference was observed between the individual drug-treated group and the control group (compared with control group, $P$ value was 0.002, < 0.001, < 0.001, < 0.001, and < 0.001, respectively). The cell viability was $(82.6 \pm 3.9)\%$ after 48 h of treatment with 10 μmol/L 5-FU alone. When Hep3B cells were treated with the combination of 5-FU and rosiglitazone, the cell viability decreased dramatically. The viability of Hep3B cells was $(51.6 \pm 5.4)\%$, $(14.8 \pm 4.2)\%$, and $(8.5 \pm 0.9)\%$ when encountered 10 μmol/L 5-FU along with either 10, 20, and 40 μmol/L rosiglitazone, respectively. The corresponding $q$ values were 1.36, 1.23, and 1.19. Therefore, these two drugs are synergic in inhibiting the growth of Hep3B cells.

Rosiglitazone enhanced anti-tumor effect of 5-FU by activating PPARγ signaling pathway

Rosiglitazone is biologically active by acting as a PPARγ ligand. To understand the mechanism of synergic anti-tumor effect of 5-FU plus rosiglitazone, we silenced the expression of PPARγ using siRNA, which blocks the binding of PPARγ to rosiglitazone. As shown in Figure 1A, the endogenous PPARγ was repressed with transient siRNA transfection in Hep3B cells. In these PPARγ knockout cells, 20 μmol/L rosiglitazone was unable to inhibit the cell growth either alone or synergically with 10 μmol/L 5-FU (Figure 1B). These results suggest that rosiglitazone enhances anti-tumor effect of 5-FU by activating PPARγ signaling pathway.

Activation of PPARγ signaling increased the expression level of PTEN

In subsequent experiments, we found that the expression level of PTEN changed in neither the 5-FU treated group nor the control group (0.1% DMSO). By contrast, rosiglitazone alone or its combination with 5-FU significantly increased PTEN expression and inhibited the downstream phosphorylation of Akt (Figure 2A). To confirm the upregulation of PTEN is due to the activation of PPARγ signaling pathway, we utilized siRNA to knock down PPARγ expression. After blocking PPARγ signaling pathway by silencing PPARγ, neither rosiglitazone nor its combination with 5-FU was able to upregulate PTEN expression. In addition, no effect was observed in the scramble siRNA treated group, suggesting that rosiglitazone upregulated the expression level of PTEN by activating PPARγ signaling pathway (Figure 2B).

Upregulation of PTEN was involved in the synergic anti-tumor effect of rosiglitazone and 5-FU

To further explore if upregulation of PTEN is involved in the synergic anti-tumor function of rosiglitazone plus 5-FU, we examined the effect of rosiglitazone on Hep3B cell proliferation after inhibiting the expression of PTEN by
siRNA. As shown in Figure 3, rosiglitazone or the combined treatment along with 5-FU inhibited the proliferation of normal Hep3B cells, but not that of PTEN-siRNA treated cells. The viability of Hep3B cells was (84.5 ± 3.8)\% in the rosiglitazone treated group and (60.6 ± 3.1)\% in the group treated with the combination of rosiglitazone and 5-FU. No effect was observed in the scramble siRNA treated group compared with the untreated group (Figure 3B). These results demonstrate that the upregulation of PTEN is involved in the synergic anti-tumor effect of rosiglitazone and 5-FU.

**Rosiglitazone enhances 5-FU induced inhibition of Hep3B xenograft tumor growth in mice**

As shown in Figure 4, the size of tumors was (2015 ± 91), (1437 ± 101), (1025 ± 89) and (201 ± 69) mm³ in untreated control group, 5-FU treated group, rosiglitazone treated group, and combination of rosiglitazone and 5-FU treated group respectively 14 days after intraperitoneal injection of corresponding drugs. The tumor growth was
significantly inhibited in mice treated with the combination of two drugs (P < 0.001, vs. untreated control group; P < 0.001, vs. 5-FU treated group; and P = 0.003, vs. rosiglitazone treated group). The q value on the 14th day was 1.41, suggesting that the combination of two drugs has synergistic effect in inhibiting tumor growth.

**Discussion**

PPARγ is a ligand-activated nuclear hormone receptor and its encoding gene is located on human chromosome 3p25. PPARγ associates with retinoic acid receptor α to form heterodimers, which subsequently interacts with PPRE (peroxisome proliferating response element) and regulates the transcription of target genes. PPARγ is implicated in lipid metabolism, immune response, cell differentiation and so on. In addition, in vitro experiments showed that PPARγ ligands, binding to PPARγ, inhibit hepatocarcinoma cell growth\(^6,7\).

Chemotherapy is one of the effective ways to treat various cancers and has also been proved to be effective against advance liver cancer, especially for patients with portal vein thrombosis. The biggest challenge for chemotherapy is the development of drug resistance by cancer cells. Clinically used chemotherapeutic drugs kill cancer cells by inhibiting cell proliferation and inducing cell apoptosis. However, malignant cancer cells become drug resistant after they are exposed to anti-proliferation and anti-apoptosis drugs for a while. Cancer cells can even develop resistance to a wide range of anti-cancer drugs acting upon different mechanisms\(^8\). 5-FU is a commonly used chemotherapeutic drug against many cancers including hepatocarcinoma. 5-FU has to be transformed inside the cells into two major cytotoxic metabolites in order to inhibit tumor growth. One metabolite is 5-fluorouridine triphosphate (FUTP), which prevents dUTP from incorporating into RNA and interferes with RNA synthesis. The other metabolite is 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) synthesized via uridine kinase, which inhibits the activity of thymidylate synthase and blocks the conversion of dTMP from dUMP, thereby inhibiting DNA
PTEN gene is identified as a tumor suppressor gene located in human chromosome 10q23.3. PTEN, a phosphatase that reduces the level of Akt phosphorylation, can inhibit the growth of cancer cells by inhibiting PI3K/Akt signaling pathway. The combination of resiglitazone and 5-FU increases the expression level of PTEN and reduces Akt phosphorylation. When the expression of PTEN is knocked down, the synergistic anti-cancer effect of resiglitazone and 5-FU significantly decreases. Our results thus suggest that the upregulation of PTEN is involved in the synergistic increase of anti-cancer effect of 5-FU and resiglitazone. It has been reported that high expression level of PTEN increases cis-platin-induced apoptosis in human cervical carcinoma cells and enhances its anti-cancer function, supporting the idea that upregulation of PTEN can increase tumor sensitivity to chemotherapeutic drugs.

In summary, resiglitazone enhances the effect of 5-FU against hepatocarcinoma by activating PPARγ signaling pathway and by upregulating the expression level of tumor suppressor gene PTEN.

References


