Rosiglitazone and retinoic acid inhibit proliferation and induce apoptosis in the HCT-15 human colorectal cancer cell line

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Abstract. The aim of this study was to explore the effects of rosiglitazone (RSG) in combination with all-trans retinoic acid (ATRA) on the proliferation and apoptosis of the HCT-15 human colorectal cancer cell line. HCT-15 cells were divided into a blank control group, a vehicle control group and experimental groups (RSG only or ATRA only or RSG plus ATRA). Growth inhibition was examined using the MTT assay. Apoptosis and cell cycle progression were examined by flow cytometry. The expression of COX-2, MMP-7 and TIMP-1 was examined by immunocytochemistry. RSG alone inhibited HCT-15 cell proliferation in a concentration- and time-dependent manner ($P<0.05$). The combination of RSG and ATRA exhibited significant synergy ($q>1.15$). RSG or ATRA alone effectively increased the proportion of cells in the G0/G1 phase and decreased the proportion of cells in the S phase, thus inducing apoptosis ($P<0.05$). The combination of RSG and ATRA resulted in even stronger G1 cell cycle arrest ($P<0.05$). HCT-15 cells expressed COX-2, MMP-7 and TIMP-1, with positive expression rates in the control group of 66.79, 73.21 and 64.08%, respectively. After the combined application of RSG and ATRA, the positive rates significantly declined to only 19.33, 20.58 and 13.13%, respectively ($P<0.01$). In conclusion, the combination of RSG and ATRA reduced the expression of COX-2, MMP-7 and TIMP-1, caused cell cycle arrest at the G1 phase and induced apoptosis, which resulted in the inhibition of cell proliferation in the HCT-15 human colorectal cancer cell line.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily, first identified by Issemann and Green in 1990 in mouse cells (1). PPARs play important roles in the regulation of cell growth, differentiation and apoptosis. There are three known subtypes of PPARs, namely PPARα, PPARβ and PPARγ. These are encoded by separate genes with highly similar amino acid sequences, particularly in the DNA- and ligand-binding domains. Among the three subtypes, PPARγ has been the most extensively studied (2), as it possesses complex and diverse biological functions. In recent years, PPARγ agonists have attracted interest due to their antitumor effect. Mueller *et al* (3) demonstrated that PPARγ ligands inhibit the growth of prostate cancer, suggesting that the activation of PPARγ is a potential cancer therapeutic method. Nagamine *et al* (4) found that rosiglitazone (RSG) treatment increased the number of apoptotic cells in the human gastric cancer cell lines MKN-28, -45 and -74. Thus, there is growing evidence that PPARγ agonists exhibit significant antitumor effects (5-7).

All-trans retinoic acid (ATRA) is one of the more established types of retinoid drugs used in clinical chemotherapy and cancer prevention. ATRA inhibits the growth of various types of malignant tumor cells and is a commonly used differentiation-inducing reagent. PPARs are known to form heterodimers with the retinoid X receptor (RXR), bind to a specific DNA sequence-peroxisome proliferation response element, and regulate target gene transcription (8). Both *in vitro* and in transplanted breast tumors in nude mice, Elstner *et al* (9) found that ATRA assisted PPARγ ligands in inhibiting tumor cell growth and decreased bcl-2 levels, suggesting that the use of PPARγ agonists and retinoic acid activates the PPARγ/RXR heterodimer and may be an effective method for the treatment of a variety of tumors. In this study, we investigated the effect of the combined use of highly selective ATRA and the PPARγ agonist RSG on the proliferation and apoptosis of the HCT-15 human colorectal cancer cell line, and further explored the molecular mechanisms involved.

Materials and methods

Materials and reagents. The HCT-15 human colorectal cancer cell line was purchased from the Shanghai Cell Library of the Chinese Academy of Sciences. RSG and retinoic acid (purity >99%) were purchased from Gaomeng Yanshan (Beijing, China), prepared as a 1 mmol/l stock solution in DMSO and stored at -20˚C. Immediately before use, the drugs were

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Abbreviations: ATRA, all-trans retinoic acid; PPARs, peroxisome proliferator-activated receptors; RSG, rosiglitazone

Key words: rosiglitazone, all-trans retinoic acid, proliferation, apoptosis

diluted to the desired concentrations with RPMI-1640 medium containing 10% fetal bovine serum (FBS). COX-2, MMP-7 and TIMP-1 rabbit anti-human polyclonal antibodies were purchased from Boaosen Biotechnology (Beijing, China). The SP staining and DAB kits were purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China).

Experimental grouping and treatments. The MTT assay was used to examine cell proliferation. The cells were divided into five groups: group I, blank control group (100 μl per well of medium); group II, vehicle control group (100 μl per well of culture medium containing DMSO); group III, RSG only group (100 μl per well of fresh medium with final concentrations of RSG of 6.25, 12.5, 25 or 50 μmol/l); group IV, ATRA only group, (100 μl per well of fresh medium with a final concentration of ATRA of 2 μmol/l); group V, RSG and ATRA combined treatment group (100 μl per well of fresh medium with final concentrations of RSG of 6.25, 12.5, 25 and 50 μmol/l, in combination with 2 μmol/l of ATRA).

Experimental methods

Cell culture. The HCT-15 human colorectal cancer cells, which are adherent cells, were cultured in conventional RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 mg/l streptomycin in a 5% CO_2 incubator at 37˚C.

MTT assay for cell proliferation. Cells in the logarithmic phase of growth were digested with trypsin and prepared as a $5x10^4$/ml single-cell suspension, then were seeded into 96-well plates at a density of 5,000 cells/well, each well containing a total volume of 100 μl. The medium was replaced on the following day, then the cells in each group were treated as described above. At the end of the treatment, 20 μl of MTT (5 g/l) was added to each well, and culturing was continued in the dark for 4 h. The culture supernatant was then removed and replaced with 150 μl of DMSO. After shaking for 10 min until the crystals dissolved, the absorbance (A) was measured at a wavelength of 570 nm with a microplate reader, and the inhibition rate of the tumor cells was calculated according to the formula: inhibition (%) = [1 - mean A_{570nm} of the experimental group/mean A_{570nm} of the control group] x 100. The IC_{50} was calculated as the half inhibitory concentration. The interaction of the two drugs was calculated as: q = E (a + b)/[Ea + (1 - Ea) x Eb], where E (a + b) is the inhibition rate of the two drugs combined and Ea and Eb are the inhibition rates of the drugs used alone. The effect of the two drugs was additional at q=0.85-1.15, synergistic at q>1.15, and antagonistic at q<0.85. The experiment was repeated three times, and the results are represented as the mean values.

Flow cytometry for the detection of cell cycle progression. The cells were collected after digestion, washed twice with PBS and centrifuged at 1,000 rpm for 5 min. The supernatant was discarded. The cells were then resuspended, fixed in ice-cold 75% ethanol and stored at 4˚C. After two washes in PBS, the cells were stained with propidium iodide (PI) and subjected to flow cytometric analysis of the percentage of cells in G0/G1, S and G2/M phases. Treatment with each drug concentration was conducted in triplicate.

Annexin V/PI and flow cytometry to detect apoptosis. The cells were resuspended in 100 μl of binding buffer, followed by the addition of 5 μl Annexin V-FITC and 10 μl PI. After mixing, the cells were incubated in the dark at room temperature for 15 min. Binding buffer (400 μl) was added to the reaction tube, and the cells were resuspended and analyzed using a flow cytometer. Treatment with each drug concentration was performed in triplicate.

Immunocytochemical detection of intracellular COX-2, MMP-7 and TIMP-1. Upon reaching confluence, the cells were fixed with 4% paraformaldehyde for 30 min, rinsed three times with PBS and permeablized in PBS with 0.1% Triton X-100 at room temperature for 40 min. After being rinsed three more times with PBS, the cells were incubated in 3% hydrogen peroxide at room temperature for 15 min, followed by an additional three rinses with PBS. The cells were then blocked with inactivated normal goat serum at room temperature for 15 min and then respectively incubated at 4˚C overnight with the following antibodies: rabbit polyclonal anti-human COX-2 (1:100), MMP-7 (1:100) and TIMP-1 (1:100). After being rinsed three times with PBS, biotinylated goat anti-rabbit IgG was added and the incubation was continued at 37˚C for 15 min. After another three rinses in PBS, the signal was visualized by DAB colorimetric reaction. The cells were counterstained with hematoxylin for 2 min, differentiated in acidic ethanol, dehydrated in an ascending series of ethanol, cleared in xylene and mounted with neutral gum. PBS containing no primary antibody was used as the negative control. Positive staining was determined by the appearance of brown or brownish yellow granules on the cell membrane or in the cytoplasm. At a high magnification (x200), COX-2, MMP-7 and TIMP-1 protein expression was observed under a microscope. The results were analyzed with the high-resolution color pathological image analysis system (Nikon TE2000-U).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). SPSS 13.0 software was used for statistical analysis, with the t-test for comparison between two groups and one-way ANOVA for comparison among multiple groups. $P<0.05$ was taken to indicate statistical significance.

Results

Growth inhibition detected by MTT assay. The cell growth inhibition rates of the single or combined application of different concentrations of RSG and ATRA in HCT-15 cells are shown in Table I.

For RSG treatment alone in HCT-15 cells, the IC_{50} values at 24 and 48 h were 48.84 and 33.33 μmol/l, respectively. When combined with ATRA, the IC_{50} values were 34.89 and 19.75 μmol/l at 24 and 48 h, respectively. RSG or ATRA alone exhibited a slight inhibitory effect on HCT-15 cell growth ($P<0.05$), whereas the combined application of RSG and ATRA showed stronger cell growth inhibition ($P<0.01$). With increasing drug concentrations and reaction times, the cell growth inhibition rate increased and the difference was statistically significant ($P<0.05$). RSG and ATRA showed significant synergy in combination (q>1.15 when 25 and 50 μmol/l of RSG were combined with ATRA).

Detection of cell cycle progression by flow cytometry. For RSG, ATRA, their combination and the control groups, the

Table I. Effect of RSG, ATRA and their combination on HCT-15 cell proliferation (mean ± SD).

Group	Concentration (μmol/l)	A_{570nm}		Inhibition rate(%)	
		24 h	48 h	24 h	48 h
Control group	-	0.91±0.10	1.05±0.09	-	-
R_1	6.25	0.81±0.03[a]	0.83±0.03[a]	11.23	20.35
R_2	12.50	0.70±0.11[a]	0.84±0.05[a]	22.71	19.54
R_3	25.00	0.59±0.04[a]	0.51±0.03[a]	35.64	41.02
R_4	50.00	0.46±0.04[b]	0.17±0.01[b]	51.88	73.76
A	2.00	0.85±0.11[a]	0.82±0.03[a]	6.25	21.65
R_1+A	6.25+2	0.86±0.02[c]	0.81±0.04[c]	5.11	22.77
R_2+A	12.5+2	0.72±0.05[c]	0.74±0.05[c]	20.36	29.72
R_3+A	25.0+2	0.54±0.09[c]	0.36±0.03[c]	40.62	66.08
R_4+A	50.0+2	0.32±0.09[d]	0.09±0.01[d]	65.01	91.22

[a]P<0.05, [b]P<0.01 vs. control group; [c]P<0.05, [d]P<0.01 vs. RSG single-drug group. R, rosiglitazone; A, all-trans retinoic acid.

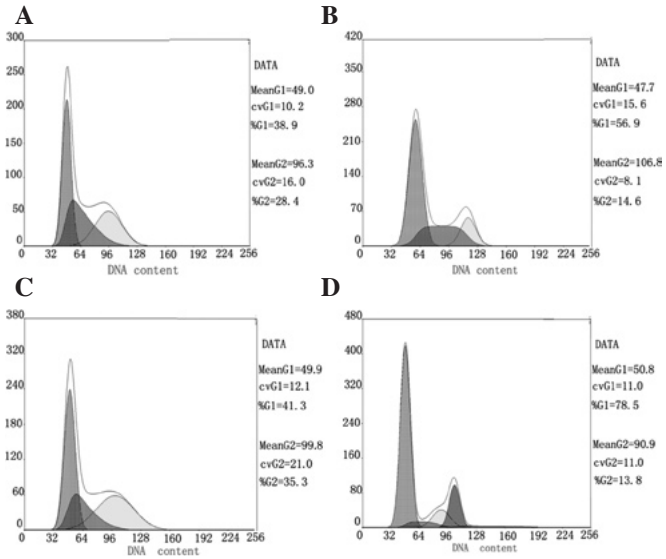

Figure 1. Cell cycle detection by flow cytometry. (A) Control group. (B) RSG 25 μmol/l. (C) ATRA 2 μmol/l. (D) RSG 25 μmol/l + ATRA 2 μmol/l.

percentages of cells in the G0/G1 phase were 56.9, 41.3, 78.5 and 38.9%, respectively; S phase cell percentages were 28.5, 27.4, 7.7 and 32.8%, respectively; G2 phase cell percentages were 14.6, 31.3, 13.8 and 28.4%, respectively. The results showed that, compared to the control group, the single-drug treatment groups had an increased proportion of G0/G1 phase cells and a decreased proportion of S phase cells (P<0.05). The combination treatment group had an increased proportion of G1 phase cells and decreased S phase cells (P<0.01). The results suggest that RSG and ATRA alone or in combination cause G1 cell cycle arrest, and that the combination enhances this G1 phase arrest (Fig. 1).

Detection of apoptosis by flow cytometry. For RSG, ATRA, their combination and the control groups, the apoptosis rates were 27.4, 14.5, 41.5 and 12.7%, respectively. The results showed that, compared to the control group, RSG and ATRA alone or in combination induced the apoptosis of HCT-15 cells, and the difference was statistically significant (P<0.05). The combination treatment was also statistically significant compared to the single-drug treatments (P<0.05) (Fig. 2).

SP immunocytochemistry. COX-2, MMP-7 and TIMP-1 were all expressed in the HCT-15 cells (Table II). Under a light microscope, brown particles were located in the cytoplasm of the tumor cells. In the control group, the expression was strong. A higher number of positive cells was noted and more brown granules in the cytoplasm and darker staining were observed. The positive rates of COX-2, MMP-7 and TIMP-1 protein expression in the control cells were 66.79, 73.21 and 64.08%, respectively (Fig. 3). In the experimental groups, the expression was rather weak, characterized by fewer positive cells and lighter staining. In the RSG group, the positive rates of COX-2, MMP-7 and TIMP-1 expression were 50.02, 49.78 and 43.37%, respectively. In the ATRA group, the positive rates were 46.67, 48.58 and 48.89%, respectively; significantly lower than in the control group (P<0.05). In the combination group, the positive rates of COX-2, MMP-7 and TIMP-1 expression were 19.33, 20.58 and 13.13%, respectively; significantly lower than in the control group (P<0.01) (Fig. 3).

Discussion

With the advancement of research on the genetic and molecular mechanisms of malignant tumors, drugs that target specific structures, functional areas, molecular groups, enzymes and signal transduction pathways in tumor cells have been increasingly used in the clinical setting. These targeted therapies have become important treatment modalities in addition to conventional surgery, chemotherapy and radiotherapy, and have achieved encouraging results (10).

PPARs have diverse biological functions, including the regulation of lipid and glucose metabolism. PPARs have been found to induce tumor cell differentiation and apoptosis and to inhibit tumor angiogenesis. In particular, PPARs are closely related to gastrointestinal tumors. Recent studies have found

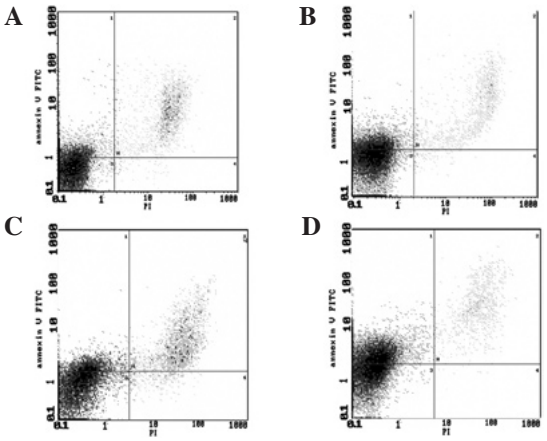

Figure 2. Detection of apoptosis by flow cytometry. (A) Control group. (B) RSG 25 μmol/l. (C) ATRA 2 μmol/l. (D) RSG 25 μmol/l + ATRA 2 μmol/l.

that PPARγ is expressed not only in adipose tissue, where it is involved in lipid metabolism (11), but also in a variety of tumors (12). Upon activation by its specific ligands, PPARγ inhibits the growth of tumor cells (13-16). Although the exact mechanism has not been fully elucidated, it is thought to be related to the induction of apoptosis and cell cycle arrest (17).

Our results showed that the application of the PPARγ agonist RSG inhibited the proliferation of HCT-15 human colon cancer cells. At a low dose of 6.25 μmol/l, the inhibition was not significant; however, when the dose was increased from 25 to 50 μmol/l, the inhibition was gradually increased, achieving a significant difference compared to the vehicle control group. This inhibition of proliferation showed a marked dose-effect relationship, and when RSG was combined with ATRA the inhibition was markedly enhanced. The IC_{50} values for the HCT-15 cells at 24 and 48 h were 48.84 and 33.33 μmol/l, respectively, when RSG was applied alone, and 34.89 and 19.75 μmol/l, respectively, when RSG was combined with ATRA. These results revealed that the combined drug treatment exerted a stronger effect than the single drug treatment in inhibiting HCT-15 cell proliferation. Moreover, within a certain concentration range, this effect was dose- and time-dependent. In addition, after intervention with RSG, ATRA or their combination, the percentage of cells in the G1 phase increased and the percentage of cells in the S phase decreased; cells were prevented from entering the division cycle and instead entered a resting state. The apoptosis assay also showed that, compared to the control group, RSG, ATRA or their combination induced the apoptosis of HCT-15 cells, and their combined use had a stronger effect than the single treatments. The results revealed that RSG in combination with ATRA arrested HCT-15 cells in the G1 phase, halted mitosis and increased the percentage of tumor cell apoptosis. Therefore, the combination of RSG and ATRA inhibits proliferation and induces the apoptosis of HCT-15 human colon cancer cells, consistent with a previous report (18).

Table II. COX-2, MMP-7 and TIMP-1 protein expression in each group (mean ± SD).

	Positive rate (%) in each group			
	RSG (25 μmol/l)	ATRA (2 μmol/l)	RSG + ATRA (25 + 2 μmol/l)	Control group
COX-2	50.02±3.10[a]	46.67±2.58[a]	19.33±1.21[b]	66.79±6.10
MMP-7	49.78±2.09[a]	48.58±2.78[a]	20.58±1.04[b]	73.21±6.62
TIMP-1	43.37±2.01[a]	48.89±3.19[a]	13.13±1.09[b]	64.08±4.28

[a]P<0.05, [b]P<0.01 vs. control group.

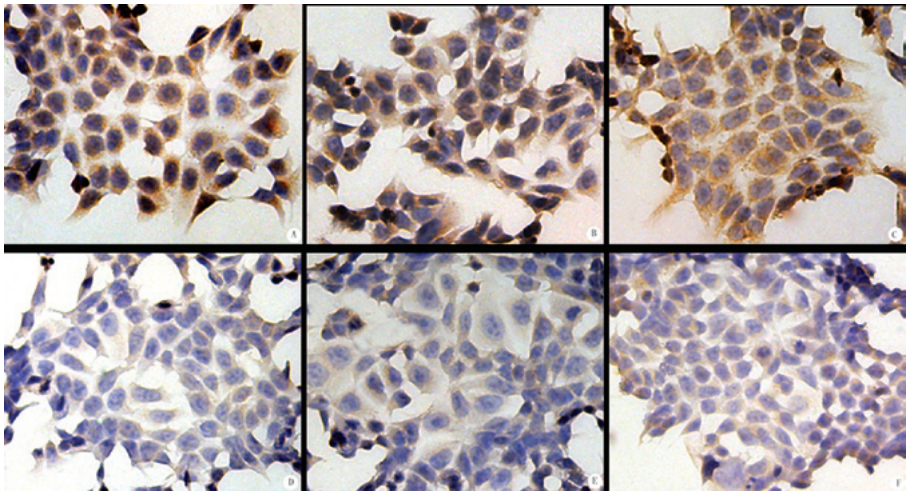

Figure 3. (A) COX-2, (B) TIMP-1 and (C) MMP-7 protein expression in HCT-15 cells without drug treatment. (D) COX-2, (E) TIMP-1 and (F) MMP-7 protein expression in HCT-15 cells treated with RSG 25 μmol/l + ATRA 2 μmol/l. Magnification, x200.

Many studies have shown that COX-2 overexpression is closely related to cancer occurrence and development in the digestive system. COX-2 promotes cell adhesion, inhibits cell apoptosis, induces tumor angiogenesis and promotes tumor invasion and metastasis (19). MMP-7 is the smallest of the MMP family members and its expression is related to the occurrence and development of various malignant human tumors, such as the invasion and metastasis of colorectal cancer (20,21). As MMP inhibitors, the primary function of TIMPs is to counter the activity of MMPs, by which they limit the degradation of the matrix. Tumor invasion and metastasis are probably due to the inhibition of TIMP secretion in tumor cells (22). Our results showed that COX-2, MMP-7 and TIMP-1 were expressed at a high level in the control group, with the positive rates of 66.79, 73.21 and 64.08%, respectively. RSG or ATRA treatment alone decreased the expression levels of COX-2, MMP-7 and TIMP-1; RSG and ATRA in combination further significantly decreased the expression of COX-2, MMP-7 and TIMP-1. This result revealed that the combined use of RSG and ATRA significantly inhibited the expression of COX-2, MMP-7 and TIMP-1, genes related to tumor cell growth, invasion and metastasis, therefore the potential for tumor cell growth, development, invasion and metastasis was greatly reduced. The mechanisms behind the anti-proliferative and growth inhibitory effect of the combination of RGS and ATRA leading to apoptosis in the HCT-15 human colorectal cell line may involve the down-regulation of COX-2, MMP-7 and TIMP-1 expression.

In the present study, through a series of *in vitro* experiments, we investigated whether the PPARγ agonist RSG and ATRA inhibited the proliferation and growth of HCT-15 human colorectal cancer cells *in vitro*, and determined that the combination of the two targeted drugs was more effective. Through the detection of genes related to tumor growth and metastasis, the possible molecular mechanism behind the inhibition of HCT-15 human colorectal cancer cell growth by the two drugs was investigated, and was found to potentially involve the down-regulation of the expression of COX-2, MMP-7 and TIMP-1. However, the correlation between TIMP-1 and MMP-7 in HCT-15 cells and their related signaling pathways requires further investigation in future studies.

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