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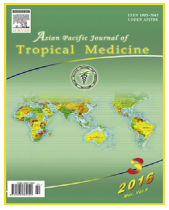

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Mechanism of all-transretinoic acid increasing retinoblastoma sensitivity to vincristine

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ABSTRACT

Objective: To explore the mechanism of all-transretinoic acid (ATRA) increasing retinoblastoma (RB) sensitivity to vincristine, and the inhibiting effect of vincristine combined with ATRA treatment on the SO-RB50 cell proliferation.

Methods: SO-RB50 cells were cultivated by routine culture method. Different concentrations of vincristine or ATRA were added into culture solution. After 48 h, cell counting kit-8 was used to detect the median inhibitory concentration (IC_{50}) of vincristine combined with ATRT treatment to SO-RB50 cells. SO-RB50 cells were divided into drug combination group, vincristine group, ATRA group and control group. Different drugs were added into the culture solution respectively for cell culture based on the IC_{50} value. Cell counting kit-8 was used to detect the cell proliferation every 24-h cultivation. After continuous determination for 6 d, data was processed to draw the cell growth curve. After drug use for 72 h, flow cytometry was used to detect the proportion of different cell cycles of SO-RB50 cells in each group. After drug use for 48 h, annexin V/propidium iodide method was used to detect the SO-RB50 cell apoptosis in each group.

Results: The IC_{50} value of vincristine treatment on the SO-RB50 cells was 0.11 μmol/L, and ATRT was 12.84 μmol/L. The cell growth curve in control group rose gradually along with the extended culture time, but after vincristine and ATRA treatment, the cell growth curve was smooth and steady. The cell increment was the least in drug combination group and its cell growth curve was the smoothest. There was significant difference in A_{450} 48 h and 72 h after treatment ($F_{grouping} = 77.316$, $P < 0.001$; $F_{time} = 86.985$, $P < 0.001$). Compared with control group, A_{450} value in drug combination group, vincristine group, ATRA group was significant lower ($P < 0.001$). Compared with control group, the G_2/M phase cell proportion in vincristine group was significantly increased, while the G_0/G_1 phase cell proportion was significantly decreased; the G_0/G_1 phase cell proportion in ATRA group was significantly increased, while the S phase cell proportion was significantly decreased ($F_{G0/G1} = 85.878$, $F_s = 56.455$, $F_{G2/M} = 85.878$, $P < 0.001$). After 48 h, there was significant difference in SO-RB50 cell apoptosis rate among groups ($F = 11.312$, $P < 0.05$). The apoptosis rate in drug combination group was significantly higher than that of other groups ($P < 0.001$).

Conclusions: ATRA can increase the sensitivity of SO-RB50 cells to vincristine. Vincristine combined with ATRA treatment can significantly increase the inhibiting effect on SO-RB50 cells, which may be related with promoting cell apoptosis and involving in cell cycle control.

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1. Introduction

Retinoblastoma (RB) is a kind of intraocular malignant tumor. Most infants are vulnerable to RB, accounting for 3%–4% of children's malignant tumors. RB has brought a great threat to the eyesight and life of children [1]. Survival rate of children with RB in developed countries is up to 95%, while it is only 50% worldwide [2,3]. Clinical treatments for RB mainly include chemotherapy, radiotherapy, surgery and other local treatments [4]. Among them, chemotherapy plays a very important therapeutic effect. However, the drug resistance of tumor cells easily leads to the chemotherapy failure [5,6]. Therefore, the research hotspot in this world is focused on looking for a kind of more sensitive chemotherapy [7]. All-transretinoic acid (ATRA) can effectively promote the cell differentiation and obviously inhibit the cell proliferation [8]. There are researches showing that ATRA can effectively increase the sensitivity of multiple tumor cells like breast cancer and liver cancer to chemotherapies. However, researches on increasing the sensitivity of RB chemotherapies are relatively less [9,10]. In this study, the mechanism that ATRA could increase the sensitivity of RB to vincristine and the inhibiting effect of vincristine combined with ATRA treatment on the SO-RB50 cell proliferation have been discussed.

2. Meterials and methods

2.1. Materials

2.1.1. Cell sources

SO-RB50 cell lines were provided by Pathology Lab of Zhongshan Eye Center, Zhongshan University. RPMI/1640 culture solution (containing 1% mass fraction of mycillin antibody and 10% volume fraction of fetal calf serum) was used to culture these cells. Culture condition: temperature (37 °C), saturation humidity and volume fraction (5% CO_2). Cells showed a suspended growth and cells in logarithmic phase were selected for detection.

2.1.2. Key instruments and reagents

CO_2 incubator was from Japan SANYO Co.; ELIASA was from Beijing Perlong New counting Co., Ltd.; flow cytometry (FCM) was from America BD Co. RPMI/1640 culture medium and fetal calf serum were from America Hyclone Co.; ATRA was from America Sigma Co.; vincristine was from Zhejiang Hisun Pharmaceutical Company Ltd.; cell apoptosis detection kit was from Beijing 4A Biotech Co., Ltd.; cell counting kit-8 (CCK-8) was from Japan Dojindo Co.; cell cycle detection kit was from Hangzhou MultiSciences Biotech Co., Ltd.

2.2. Methods

2.2.1. IC_{50} value of SO-RB50 cells after vincristine and ATRA treatment detected by CCK-8

SO-RB50 cells in logarithmic phase were inoculated into 96-well culture plates evenly, with 5000/hole. Different mass concentrations of 0.005, 0.010, 0.050, 0.100, 0.500 and 1.000 μmol/L of vincristine and 2.50, 5.00, 10.00, 20.00 and 40.00 μmol/L of ATRA were added respectively. A total of 5 holes were set up for each concentration. After being cultured for 48 h, 10 mL of CCK-8 reagent was added into each hole for 2 h incubation at 37 °C. ELIASA method was used to detect the A_{450} value at 450 nm [11].

2.2.2. Cell proliferation detected by CCK-8

The cultured cells were divided into drug combination group, vincristine group, ATRA group and control group. SO-RB50 cells in logarithmic phase were inoculated into 96-well culture plates, with 2.5×10^4/mL of density. A total of 200 μL was added into each hole. IC_{450} of vincristine (0.11 μg/mL) or ATRA (12.84 μmol/L) was added respectively according to the grouping. Five holes were set up in each group. CCK-8 was used to detect the A_{450} value of cells in each group at 450 nm every 24 h for 6 continuous days. Then the average value was calculated and the cell growth curve was drew [12].

2.2.3. Cell cycle detected by FCM

SO-RB50 cells in logarithmic phase were inoculated into six-well culture plates, with 2.5×10^4/mL of density. Each hole was inoculated with 2 mL. IC_{450} of vincristine or ATRA was added respectively. A total of 72 h after drug treatment, PBS was used for washing for 2 times, with centrifugation for 5 min (1000 r/min and 12 cm of centrifugal radius). A total of 1×10^6 cells were collected in each group and were fixed with ethyl alcohol with 70% of volume fraction. Temperature was controlled at 4 °C and treatment was performed after overnight. After PBS washing for once, 100 μL of buffer solution (containing 100 μg/mL of RNA enzyme and 0.2% volume fraction of Triton X-100) was added for 30 min incubation at 37 °C; then 400 μL of propidium iodide (PI) staining solution was added for 30 min incubation at 4 °C, with keeping out of the sun. FCM was performed to detect the cells of different cell cycles. Cell proportion was calculated [13].

2.2.4. Apoptosis rate of cells detected by annexin V/PI

SO-RB50 cells in logarithmic phase were inoculated into six-well culture plates, with 2.5×10^4/mL of density. Each hole was inoculated with 2 mL. IC_{450} of vincristine or ATRA was added respectively. A total of 72 h after drug treatment, PBS was used for washing for 2 times, with centrifugation for 5 min (1000 r/min). A total of 1×10^6 cells were collected in each group. A total of 100 μL of binding buffer solution was added for cell resuspension. Then 10 μL of annexin V-FITC and 5 μL of PI were added into culture solution for mixing, with incubation for 30 min at 4 °C. Afterwards, incubation for 15 min was performed with keeping out of the sun and 400 μL of binding buffer solution was added once more. FCM was used to make a comparison of the ratio of apoptotic cells [14].

2.3. Statistical method

SPSS 13.0 statistical package was used for experimental data analysis. The data information in this study showed a normal distribution by Shapiro–Wilk test. Data was expressed by mean ± SD and was detected by Levene homogeneity test of variances. Grouping was based on the balanced grouping and multilevel experimental design method. Block design two-way analysis of variance (ANOVA) was adopted to compare the overall difference of A_{450} value changes in different time in drug combination group, vincristine group, ATRA group and control group. *SNK-q* test was used for multiple comparisons among

groups. One-factor ANOVA was used to compare the cell percentage and apoptosis rate in different cell cycles in each group. When $P < 0.05$, the difference had statistical significance.

3. Results

3.1. IC_{50} value of vincristine and ATRA treatment for SO-RB50 cells

The SO-RB50 cells were treated with different concentrations of vincristine working solutions, respectively, and the cell proliferation inhibition rate was measured and IC_{50} value of vincristine calculated. The SO-RB50 cells were treated with different concentrations of ATRA, respectively, and the cell proliferation inhibition rate was measured and IC_{50} value of ATRA calculated. The results were shown in Table 1.

3.2. Cell proliferation in each group

Vincristine and ATRA treatment was performed respectively for SO-RB50 cells for 6 d. The change in cell growth rate with time in control group was significantly larger than that in vincristine group, ATRA group and drug combination group, and that in drug combination group was the minimum. There were significant difference in cell proliferation rates among the 4groups 48 h and 72 h after drug treatment ($P < 0.001$). A total of 48 h after drug treatment, compared with vincristine group, ATRA group and control group, A_{450} value in drug combination group was significantly lower ($P < 0.001$) (Table 2).

3.3. Proportion of SO-RB50 cells in different cell cycles 72 h after drug treatment

There were significant differences in proportion changes of SO-RB50 cells in G_0/G_1 phase, G_2/M phase, S phase 72 h after treatment of different drugs ($F = 130.565$, $F = 114.290$, $F = 57.435$; $P < 0.001$). The G_0/G_1 phase cell proportion in vincristine group was significantly reduced; G_2/M cell proportion was significantly increased ($P < 0.001$). The G_0/G_1 phase cell proportion in ATRA group was significantly higher than that in control group; S phase cell proportion was significantly lower than that in control group ($P < 0.001$). The results were shown in Table 3.

3.4. Apoptosis rate of SO-RB50 cells in each group 48 h after treatment of different drugs

A total of 48 h after different drug treatment, there was significant difference in apoptosis rate of SO-RB50 cells among four groups ($P < 0.05$). Compared with control group, the apoptosis rate in drug combination group, vincristine group and ATRA group were significantly higher ($P < 0.001$). Compared with vincristine group and ATRA group, the apoptosis rate in drug combination group was significantly higher ($P < 0.001$).

Table 1

IC_{50} values of vincristine and ATRA for SO-RB50 cells.

Vincristine group	Concentrations (μg/mL)	0.005	0.010	0.050	0.100	0.500	1.000	IC_{50} value
	Cell proliferation inhibition rate (%)	12.52	22.57	39.73	53.68	67.00	73.93	0.11 μg/mL
ATRA group	Concentrations (μmol/L)	2.50	5.00	10.00	20.00	40.00	IC_{50} value	
	Cell proliferation inhibition rate (%)	8.57	31.23	49.86	65.04	70.01	12.84 μg/mL	

Table 2

Cell proliferation in each group.

Group	1 d	2 d	3 d	4 d	5 d	6 d
Drug combination group	0.392 ± 0.026	0.482 ± 0.029	0.531 ± 0.035	0.546 ± 0.032	0.550 ± 0.036	0.564 ± 0.031
Vincristine group	0.392 ± 0.026	0.594 ± 0.030[a]	0.667 ± 0.039[a]	0.724 ± 0.032[a]	0.733 ± 0.030[a]	0.740 ± 0.035[a]
ATRA group	0.392 ± 0.026	0.612 ± 0.037[a]	0.798 ± 0.017[a]	0.831 ± 0.018[a]	0.831 ± 0.018[a]	0.831 ± 0.018[a]
Control group	0.392 ± 0.026	1.079 ± 0.023[a]	1.380 ± 0.022[a]	1.479 ± 0.023[a]	1.782 ± 0.025[a]	2.397 ± 0.026[a]
F	–	77.316	86.985			
P	–	<0.001	<0.001	<0.001	<0.001	<0.001

Compared with drug combination group, [a]$P < 0.001$.

Table 3

Proportion of SO-RB50 cells in different cell cycles 72 h after drug treatment.

Group	Sample size	Cells in different cell cycles		
		G_0/G_1	G_2/M	S
Drug combination	3	69.373 ± 0.747[ab]	15.522 ± 1.255[b]	15.101 ± 0.556[ab]
Vincristine	3	71.268 ± 0.138[ab]	14.512 ± 0.895[b]	14.214 ± 0.801[ab]
ATRA	3	87.452 ± 2.224[a]	5.872 ± 1.399[a]	6.680 ± 0.942
Control	3	75.804 ± 0.726	15.939 ± 0.636	7.921 ± 0.305
F		85.878	64.547	56.455
P		<0.001	<0.001	<0.001

Compared with control group, [a] $P < 0.001$; compared with ATRA group, [b] $P < 0.001$ (one-way ANOVA, *SNK-q* test).

Table 4
Apoptosis rate of SO-RB50 cells in each group 48 h after drug treatment.

Group	Apoptosis rate (%)
Drug combination group	34.50 ± 1.86[ab]
Vincristine group	27.47 ± 2.46[ac]
ATRA group	27.33 ± 1.38[ac]
Control group	7.18 ± 0.17[bc]
F	11.312
P	<0.001

Compared with control group, [a]$P < 0.001$; compared with ATRA group, [b]$P < 0.001$; compared with drug combination group, [c]$P < 0.001$ (one-way ANOVA, *SNK-q* test).

There was no significant difference in the apoptosis rate between vincristine group and ATRA group ($P = 0.919$) (Table 4).

4. Discussion

In recent years, the therapeutic goal for RB have changed from increasing the survival rate of children to the living quality of children. The treatment mode has also changed gradually from eyeball extraction to conservative treatment [15,16]. Chemotherapies in clinic could increase the retention rate of eyeballs in a certain degree, but the drug resistance of tumor cells to the traditional drugs of chemotherapies would easily lead to the treatment failure [17]. At present, research hotspots are focused on looking for effective chemosensitizer.

There were researches showing that ATRA could effectively increase the chemical sensibility of multiple tumors [18,19]. Through experiments *in vitro* and *vivo*, researchers found that ATRA could effectively induce the liver cancer stem cell differentiation and increase the sensitivity of tumor cells to *cis*-platinum [20]. Through phase II clinic trial, it was found that ATRA combined with paclitaxel or *cis*-platinum could increase the efficacy of patients with advanced non-small cell lung cancer [21]. There were reports showing that ATRA combined with paclitaxel could relieve the metastatic or recurrent breast cancer in a certain degree and bring better tolerance for patients [22,23]. In this study, the effect of the sensitivity of ATRA to vincristine, the typical chemotherapy drug for RB, was mainly discussed. Research results showed that ATRA combined with vincristine treatment had obvious inhibiting effects and apoptosis-promoting effects on the SO-RB50 cell proliferation, and the efficacy of the combined drugs was obviously better than single drug use of vincristine. That was to say, ATRA could obviously increase the sensitivity of SO-RB50 cells to vinblastine.

Vitamin A metabolism *in vivo* could produce ATRA and ATRA combined with cell receptor could regulate and control the gene expression, and then promote the cell differentiation and inhibit the cell proliferation [24]. There were researches showing that ATRA could effectively inhibit the proliferation of multiple tumors, like RB, glioma, leukemia, and neuroblastoma [25]. In this research, CCK-8 detection method was adopted to prove the fact that ATRA could inhibit the cell growth, after playing effects, G_0/G_1 phase SO-RB50 cells were obviously increased; while S phase SO-RB50 cells were obviously decreased. ATRA could make cell stasis at G_0/G_1 phase and then slower the cell growth speed, which was in accordance with the previous reports [26,27]. Vincristine was a kind of cell mitosis inhibitor, action mechanism of which was that vincristine combined with microtubulin specifically could block the microtubule formation and result in the stasis of cell division at the metaphase [28]. This research indicated that after vincristine treatment, SO-RB50 cells at G_0/G_1 phase were obviously reduced; while SO-RB50 cells at G_2/M phase were obviously increased, which was in accordance with the previous reports that vincristine could produce periodic blocking to neuroblastoma [29,30]. Its mechanism was that vincristine was a key factor resulting in the cell cycle changes. Vincristine and ATRA could effectively play inhibiting effects on cell proliferation for different phase of cells. In addition, in this study, also it was also found that ATRA could increase the apoptosis rate of cells, which indicated that ATRA might induce the cell apoptosis and then play inhibiting effects on cell proliferation.

In conclusion, through experiments *in vitro* in this study, it was proved that ATRA can effectively increase the sensitivity of SO-RB50 cells to vincristine, which provide theoretical basis for exploring new drug combination scheme for neuroblastoma. The possible mechanism of that includes inducing the cell apoptosis, and regulating and controlling the cell cycle. In addition, further research is necessary to discuss other possible action mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

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