·Basic Research·

Effects of salvianolic acid B on *in vitro* growth inhibition and apoptosis induction of retinoblastoma cells

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Abstract

• AIM: To observe the effects of salvianolic acid B (SalB) on *in vitro* growth inhibition and apoptosis induction of retinoblastoma HXO-RB44 cells.

• METHODS: The effects of SalB on the HXO-RB44 cells proliferation *in vitro* were observed by MTT colorimetric method. The morphological changes of apoptosis before and after the treatment of SalB were observed by Hoechst 33258 fluorescent staining method. Apoptosis rate and cell cycle changes of HXO-RB44 cells were detected by flow cytometer at 48 hours after treated by SalB. The expression changes of Caspase-3 protein in HXO-RB44 cells were detected by Western Blot.

• RESULTS: SalB significantly inhibited the growth of HXO-RB44 cells, while the inhibition was in a concentration-and time-dependent manner. The results of fluorescent staining method indicated that HXO-RB44 cells showed significant phenomenon of apoptosis including karyorrhexis, fragmentation and the formation of apoptotic bodies, *etc.* after 24, 48 and 72 hours co-culturing of SalB and HXO-RB44 cells. The results of flow cytometer showed that the apoptosis rate and the proportion of cells in S phase were gradually increased at 48 hours and 72 hours after treated by different concentrations of SalB. Western Blot strip showed that the expression of Caspase-3 protein in HXO-RB44 cells was gradually increased with the increase of the concentration of SalB.

• CONCLUSION: SalB can significantly affect on HXO-RB44 cells growth inhibition and apoptosis induction which may be achieved through the up-regulation of Caspase-3 expression and the induction of cell cycle arrest.

• KEYWORDS: salvianolic acid B; HXO-RB44 cell; apoptosis; Caspase-3 protein

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INTRODUCTION

Salvia is mainly used for the treatment of cardiovascular and cerebrovascular diseases in clinic. The water-soluble components of Salvia are tanshinol, protocatechuic aldehyde and Salvianolic aicd B (SalB), having the effects of anti-thrombosis, improving hemodynamics and antioxidant. Some researches also found that SalB has the effect of inducing apoptosis. SalB is a phenolic acid compound condensed by three molecules of Danshensu and one molecule of caffeic acid[1]. SalB represents the most characteristic constituent of Salvia miltiorrhiza Bge with a strong free radicals scavenger activity. This property may be useful in the treatment of some severe chronic diseases, where there is an imbalance of reactive oxygen species formation and where intracellular reactive oxygen and nitrogen species level can cause severe cell damage and even cell death. It has been reported that SalB has the effects of scavenging free radical, inhibiting lipid peroxidation and *etc.*[2-4].

There were other studies in recent years which confirmed that SalB has the effect of anti-tumor by inducing apoptosis [5-7]. In this study, human retinoblastoma cell lines HXO-RB44 were used as a model to observe apoptosis-inducing effect of SalB on HXO-RB44 cell, while the changes in apoptosis-related protein Caspase-3 expression were detected to investigate the mechanism of SalB on HXO-RB44 cells.

MATERIALS AND METHODS

Materials Human retinoblastoma cell lines HXO-RB44 were obtained from Institute of Cell Biology, Chinese Academy of Sciences. RPMI1640 medium was purchased from Hyclone Co. (USA). Fetal bovine serum purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.. Thiazolyl blue (MTT), salvianolic acid B, AnnexinV/PI double labeling kit, rabbit anti-human Caspases-3 monoclonal antibody and Hoechst 33258 were purchased from Sigma Co. (USA). The other reagents were domestic analytical reagent. The instruments were TE300 multifunctional inverted fluorescence microscope (Nikon Co. Japan), DC200 image acquisition system and image analysis software (Leica Co. Germany), Medel 550 microplate reader (BIO-RAD Co. USA), FAS Calibur flow cytometer (Becton Dickinson (BD) Co. USA), CO_2 incubator (Napco Co. USA) and Western Blot transfer device (Bio-Rad Co. USA).

Methods

Cell culture RPMI-1640 culture solution including 10% fetal bovine serum and 100U/mL gentamicin was used to incubate HXO-RB44 cells in the 37℃, 50mol/L CO_2 incubator, and the culture solution was changed for passage every 24 hours. HXO-RB44 cells in log phase were randomly selected and divided into 5 parts, 4 of them were incubated in CO_2 incubator to incubate HXO-RB44 cells, and the culture solution was also changed for passage every 24 hours.

Determination of cell proliferation inhibition rate by MTT assay Log phase growth cells were prepared to 1×10^4/mL after trypsinization and inoculated into a 96-well culture plate. 100μL cell suspension was added into each well. The liquid in the hole was discarded after 12 hours culturing while sterile SalB with the concentration of 0.1, 0.4, 0.7 and 1.0mg/mL were added, respectively. The cell without SalB was used as blank control. After 24, 48 and 72 hours culturing; 20μL MTT with the mass concentration at 5mg/mL was added to each well. The liquid in the hole was discarded after 4-hour culturing, 150μL DMSO was added. After 10 minutes oscillation, the absorbance value (A) at the wave length of 490nm was determined by the microplate reader. The effects of SalB on cell proliferation inhibition rate (%) after 24, 48 and 72 hours were calculated as follows. Each group had 6 parallel complex holes, and the experiment was repeated 3 times. Cell proliferation inhibition rate (%) =1- ($D_{\text{the value of experimental group}}$-$D_{\text{the value of blank control group}}$)/ ($D_{\text{the value of control group}}$- $D_{\text{the value of blank control group}}$) ×100%.

Nuclear morphology observation by Hoechst33258 fluorchrome staining HXO-RB44 cells were treated by 0.7mg/mL SalB for 24, 48 and 72 hours respectively. The cells at different time points were washed and suspended by PBS. 5mg/L Hoechst33258 fluorescent dyes were added, dark reaction at room temperature for 10 minutes. Nuclear morphological changes were observed by fluorescence microscopy and the images were captured. The blank control was set up at the same time.

Cell cycle and apoptosis rate detected by flow cytometry HXO-RB44 cells in log phase were selected. When cells grew to 70% full scale, after digestion and passage, the old culture solution was discarded. The culture solution at different concentrations containing SalB was added and incessantly cultured for 48 hours. Flow cytometry was used to detect. HXO-RB44 cells treated by SalB were as the experimental group and those without drugs were as controls. The cells were collected after 5 minutes centrifugation at 1 000r/min and fixed for 2 hours by 70% ice ethanol. 0.5mL propidium iodide (PI) dye was added to each sample tube after centrifugation and stained in dark at 4℃ for 30 minutes. The cell cycle and apoptosis rate were detected by flow cytometry. The experiment steps were repeated 3 times.

Expression of Caspaes-3 protein detected by Western Blot HXO-RB44 cells in log phase were selected. When cells grew to 70% full scale after digestion and passage, the old culture solution was discarded. The culture solution containing 0.7mg/mL SalB was added and continued culturing for 48 hours and 72 hours. Total cellular protein was extracted from cell lysate. The same amount of sample was taken after quantification and SDS-PAGE electrophoretic separation was conducted. Then the protein was transferred to PVDF membrane. The film was confined in a confining fluid for 2 hours and was incubated overnight with Caspaes-3 and β-actin primary antibody at 4℃. And then, incubated for 1 hour with secondary antibody at room temperature after washing the film. After 1 minute chemiluminescence detection, the image was displayed and formed. The relative expression level of the protein was the ratio of the gray value of objective protein and the gray value of internal reference-β-actin. The experiment steps were repeated 3 times.

Statistical Analysis SPSS11.0 software was used to complete statistical processing. Experimental data were all presented as mean±standard deviation. Student's t-test was used to analyze and compare the data between groups. A $P<0.05$ was taken to indicate a difference of statistical significance.

RESULTS

Inhibitory effect of salvianolic acid B on HXO-RB44

Table 1 Cell cycle and the apoptosis rate of HXO-RB44 cell treated with salvianolic acid B

Group	Cell cycle (%)			Apoptosis (%)
	G0/G1	S	G2/M	
Control	61.07±1.27	22.85±1.43	15.29±1.95	2.78±0.32
0.1mg/mL	56.16±2.03	29.12±1.66	13.34±1.86	3.76±1.21
0.4mg/mL	49.69±2.44	33.58±2.17	15.63±1.55	6.28±0.74[a]
0.7mg/mL	41.28±1.87	41.04±1.98	16.14±1.46	8.75±1.85[a]
1.0mg/mL	40.34±1.57	43.51±1.65	15.02±1.41	9.01±1.61[a]

[a]$P<0.05$ *vs* control group.

cell proliferation MTT assay was used to detect the inhibitory effect of SalB on HXO-RB44 cell proliferation. The results showed that SalB significantly inhibited the growth of HXO-RB44 cells in a concentration-and time-dependent manner. There was statistically significant difference when compared with the control group ($P<0.05$, Figure 1).

Effects of salvianolic acid B on HXO-RB44 cell apoptotic morphology Hoechst33258 fluorescence staining showed that HXO-RB44 cells in control group had uniform chromatin and regular nuclear morphology. After treated by 0.7mg/mL SalB for 24, 48 and 72 hours, apoptotic features including apoptotic cellappearance, cell volume shrinkage, intracellular vacuole occurrencechromatic agglutination and chromatorrhexis and *etc.* could be observed (Figures 2A-D).

Effects of salvianolic acid B on HXO-RB44 cell apoptosis and cell cycle After 48-hour treatment of different concentration of SalB on HXO-RB44 cells, typical hypodiploid apoptotic peak detected by flow cytometry was gradually increased with the increase of drug concentration. The apoptosis rate of cells treated by 0.4, 0.7 and 1.0mg/mL SalB was significantly higher than that of the control group ($P<0.05$, Table 1). Compared with the negative control group, the cells in SalB treated group had stronger blocking effects in S phase.

Caspase-3 protein expression in HXO-RB44 cell before and after salvianolic acid B treatment detected by Western Blot The result of HXO-RB44 cell treated by SalB after 48 hours showed that cell total Caspaes-3 protein expression significantly increased with the increase of SalB concentration, and the protein bands are pretty clear (Figure 3).

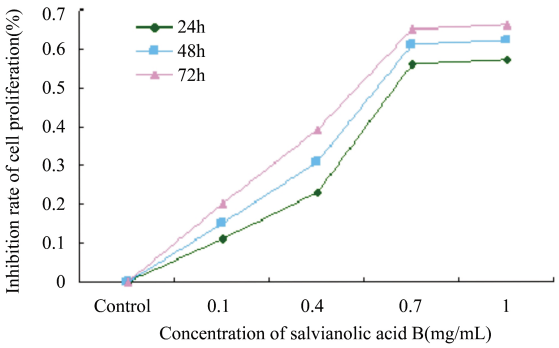

Figure 1 Growth inhibition of HXO-RB44 cell treated with salvianolic acid B.

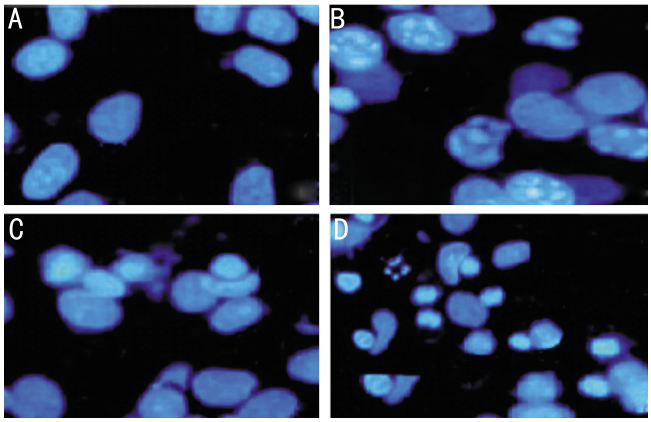

Figure 2 Morphological change of HXO-RB44 cell apoptosis treated with 0.7mg/mL salvianolic acid B A: control group; B: 24-hour drug group; C: 48-hour drug group; D: 72-hour drug group.

Figure 3 Protein expression of Caspase-3 in HXO-RB44 cells exposed to salvianolic acid B by Western Blot 1: control group; 2:0.1mg/mL group; 3: 0.4mg/mL group; 4: 0.7mg/mL group; 5: 1.0mg/mL group.

DISCUSSION

In this study, tumor development is closely related to the free radical reaction especially reactive oxygen. Appropriate free radical *in vivo* is beneficial to human body. However, the significant increase of oxygen free radicals within tumor can result in tumor proliferation and differentiation. SalB can inhibit tumor cell proliferation and differentiation by inhibiting the generation of reactive oxygen, which is one of its anti-tumor mechanisms [8-10]. Furthermore, SalB can inhibit tumor cell growth by inhibiting tumor cell extracting nucleic acid from the host cell [11].

The results of this study showed that SalB could effectively

inhibit HXO-RB44 cell proliferation. The inhibition rate of cell proliferation was increased with the increase of SalB concentration and treatment time. Cell proliferation is achieved by the operation of the cell cycle while G1/S phase and G2/M phase are the two important regulating points of cell cycle. In this experiment, after 48-hour treatment of different concentration of SalB for HXO-RB44 cells, flow cytometry detection found that cells in S phase of the drug group gradually increased in a concentration-dependent manner. This indicated that SalB could block the cell cycle of HXO-RB44 cells in S phase and could induce cell growth inhibition and death through inhibiting cells entering the G2 phase. The mechanism may be related to the inhibition of the synthesis of nucleic acid and tumor cells using oxygen free radicals.

Apoptosis is an active cell death process regulated by gene. In this experiment, the results of Hoechst33258 fluorescent staining showed that the number of apoptotic HXO-RB44 cells increased with the increase of drug concentration, while the phenomenon of cell shrinkage, karyorrhexis, formation of apoptotic bodies and mitotic count were reduced with the increase of drug concentration. Flow cytometry also showed that after 48-hour treatment of SalB, apoptosis of HXO-RB44 cells was significantly increased. While the higher the drug concentration, the more obvious cell apoptosis rate increased. This suggested that SalB promoted apoptosis in HXO-RB44 cells. The mechanism may be related to that SalB could increase the ratio of HXO-RB44 cells in S phase and block the cell cycle in S phase, which further causes programmed cell apoptosis. The occurrence and regulation of apoptosis is an extremely complex system whose mechanism is not completely clear. However, it has been proved to be regulated by multiple genes. Caspase-3 gene and caspase-3 protein play important roles in the regulation of apoptosis [12-14]. Under normal circumstances, Caspase-3 exists in the form of inactive zymogen in cytoplasm. When cells undergo apoptotic stimulus, Caspase-3 is activated by a series of reactions and thus induced cell apoptosis [15]. Plenty of studies have confirmed that the expression rate of Caspase-3 protein is not the same. For instance, expression of caspase-3 protein in prostatic cancer is missing [16, 17], expression in lung cancer tissue [18], gastric cancer tissue [19] and primary hepatocellular carcinoma [20, 21] is far below the lateral cancer and normal tissue. Similarly, expression of caspase-3 protein in breast cancer tissue shows significant down regulation comparing with benign breast lesions, and is negatively correlated with the histological grading of breast cancer [22]. This experiment demonstrated that the positive expression rate of caspase-3 protein in HXO-RB44 cells was very low. The down-regulation and missing of Caspase-3 which is the core protease of apoptosis may cause malignant tumor to evade the self-regulation of collective apoptosis during its formation, thus be able to continue proliferation and form malignant tumor ultimately. This experiment detected the expression of Caspase-3 protein in HXO-RB44 cells before and after the treatment of SalB by Western Blot. The results showed that the expression of Caspase -3 protein in HXO-RB44 cells was significantly increased after the treatment of SalB in a concentration-dependent manner. This indicated that SalB could up-regulate the expression of Caspase-3 protein in HXO-RB44 cells and thus induce HXO-RB44 apoptosis.

In conclusion, SalB is able to inhibit HXO-RB44 cell growth. The mechanism may be related to the up-regulation of the expression of intracellular Caspase-3, blocking cell cycle at S phase, inducing apoptosis, and thus inhibiting the proliferation of tumor cells.

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