THE EFFECT OF SCUTELLARIN ON APOPTOSIS AND THE EXPRESSION OF BCL-2 AND BAX IN GLIOMA C6 CELLS

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ABSTRACT

Objective*: To explore the inhibitory effect of Scutellarin(SCU) on prolifenation of rat glioma C6 cells, and to clarify its mechanism.*

Methods: *The C6 cells were cultured and divided into blank control group and 5, 10, 20, 40, 80 mg·L-1 SCU groups, the surival rates of C6 cells in various were examined by MTT assay; the morphology of C6 cells in various groups were observed by inverted microscope; the apoptotic rates of C6 cells were examined by Annexin V/PI staining; the expression levels of bax and bcl-2 proteins in the C6 cells in various group were detected by cell immunohistochemistry.*

Results: *The MTT results showed that compared with blank control group, the survival retes of C6 in 10, 20, 40 and 80 mg·L-1SCU at 48h were decreased (P<0.01);And the survival retes of C6 in 20, 40 and 80 mg·L-1SCU at 72h were decreased (P<0.01). The group, the C6 cells in SCU group was significantly decreased, some cells became inverted microscope observation results showed that compared with blank control round and volume reduced, and cell contour was not clear. The results of Annexin V/PI staining showed that the late and total apoptotic rates of C6 cells in 80 mg·L-1 SCU group were increased compared with blank control group (P<0.05). The cell immunohistochemistry results showed that compared with blank control group, the expression levels of bax protein in the C6 cells in 80 mg·L-1 SCU group were increased, and the bcl-2 protein were decreased; Compared with blank control group, the ratio of bax/bcl-2 in the C6 cells in SCU group was significantly increased (P<0.05).*

Conclusion: *SCU induce the apoptosis and suppress the tumor growth by upregulating the bax/bcl-2 in the C6 cells.*

Keywords: *Scutellarin, glioma, apoptosis, bax, bcl-2.*

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Introduction

Neuroglioma, also called gliocytoma, is one of the most common forms of human primary malignant brain tumorat present. It accounts for more than 50% of brain cancers, with about three new cases per 100,000 populations each year[1, 2], and the degree of malignancy is high, the incidence and recurrence rate are high, and endangers human life and health[3, 4]. The postoperative effect is not good. So far, there is still a lack of effective treatment. In the neuroglioma patients, fewer than 10% of them could survive beyond 5 years[5]. Once malignant glioma occurs, it will not only pose a great threat to the lives of patients, but also bring great economic burden to families. The neuroglioma pathogenesis is related to multiple processes as affected by dozens of regulatory factors[6]. Brain tumors pose the greatest threat to life and health, gliomas are the most common, and the postoperative effect is poor. So far, there is still a lack of effective treatment. Once malignant glioma occurs, it will not only pose a great threat to the lives of patients, but also bring great economic burden to families. Therefore, it is necessary to explore the traditional Chinese herbal medicine with unique efficacy and little side effects in the treatment of glioma.Scutellarin (SCU) is a flavonoid active ingredient[7], which can be isolated from the leaves

of Scutellaria baicalensis, Scutellaria barbata and Erigeron breviscapus. It has a good effect on many diseases[8, 9], especially in reducing cerebrovascular resistance, improving cerebral blood circulation, increasing cerebral blood flow, anti-platelet aggregation, anti-cancer and anti-virus. In recent years, the anti-tumor activity of SCU has been paid more and more attention. Research data have shown that the anti-tumor effect of SCU is significant[10-15].

However, the effect and mechanism of SCU on glioma cells have not been reported. In this study, after C6 cells were treated with SCU, the proliferation activity, cell morphology, apoptosis rate and the expression of Bax and Bcl-2 protein were detected to explore the effect of SCU on proliferation and apoptosis of C6 cells and its mechanism. It is helpful to develop a new anti-tumor drug scutellarin.

Materials and methods

Cell samples

Rat glioma cell line C6 was achieved from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). In a humidified atmosphere of 5% CO_2, all the cells were cultivated at 37°C in an incubator, containing Dulbecco's modified Eagle medium (DMEM) with 10% FBS (Gibco BRL, Grand Island, NY). Check the condition of cell adhesion on time and change the fluid in time.

Groups and drug administration

The C6 cells with good growth condition were divided into six groups: the concentrations of SCU were 0, 5, 10, 20, 40 and 80 $mg{\cdot}L^{-1}$.

Thiazolyl blue tetrazolium bromide (MTT) assay

Cells were seeded into 96-well culture plates, and each well was inoculated with 3×10^4cells. Meanwhile, each well with 100 μL of culture medium was added with MTT (1μL, 5mg/mL; Thermo Fisher Scientific, Waltham, MA) to incubate at 37°C in 5% CO_2 for 4 hours. The reaction was terminated by removal of the supernatant and addition of 100μL of DMSO each well. The goal is to get the crystal compounds to be ablated by vibration. The optical density of each well was measured at 570 nm.

Observation of C6 cells by inverted microscope

C6 cells were collected by planting on a 24-well plate, and the culture medium was added (the proportion of FBS was 10%). The C6 cells were incubated in a CO_2 incubator at 37°C and 5%, and the medium was changed at the next day.

After the C6 cells were completely adhered to the wall, the blank control group and SCU group (the concentration of SCU in the medium was 80 $mg{\cdot}L^{-1}$) were established and incubated for 72 hours under the same condition. The biological characteristics of tumor cells were observed by inverted microscope.

Flow cytometry (FCM) assay

The number of cells in logarithmic phase was 2×10^5/well. The apoptosis rate of cells was divided into two groups: control group and SCU group (the concentration of SCU in culture medium was 80 $mg{\cdot}L^{-1}$). The 6 well plate with cells was incubated in the incubator for 72 hours under the conditions of 5% CO_2 and 37°C.

When the time is up, the cells are digested with trypsin and collected, and the cells are washed 2-3 times with phosphate-buffered saline (PBS) buffer (pH 7.2~7.4) precooled in advance at 4°C.

Then, according to the experimental procedure of the instructions, the fresh 190 μ L buffer is absorbed (the cell is re-suspended at 1:4 by binding buffer), and the cell concentration is adjusted to 1×10^6 ml-1, in the mixed cell fluid into annexin V-FITC and PI (the concentration is 20 $\mu g\ mL^{-1}$).

The volumes of the two groups were 5 μL and 10 μL respectively, and the apoptosis rate of C6 was determined by exposure to water at 0°C for 10-15 min. Then, FCM was used to calculate the apoptotic cells.

Immunohistochemical method

C6 was obtained by planting in 24 well plate, and the number of cells was 1×10^4 mL-1. The 24 well plate was covered with slides. The immunohistochemical experiment was divided into two groups: control group and SCU group (the concentration of SCU in culture medium was 80 $mg{\cdot}L^{-1}$). The slides containing C6 in 24 well plates were fixed with methanol. The fixed slides were washed twice by PBS, and the slides were treated with 3% H_2O_2 for 20 minutes.

After washing the slides containing C6 with PBS again, the slides containing C6 were sealed with calf serum sealing solution for 30 minutes. Calf serum was removed and treated with first antibody bax (Sigma, 1:50) and bcl-2 (Sigma, 1:50).

The reaction was carried out at 4°C. On the second day, the liquid pipette was used to absorb the

first antibody (bax (1:50) and bcl-2 (1:50)), continued to add the prepared PBS and washed on the shaker for 3 times, the time was 5min. After flicking the tablets, the second antibody was added for 1 hour.

After the reaction, the second antibody was removed and the glass slides were washed with PBS for 3 times, each time was 5 min. First of all, the color was developed by DAB kit, then re-dyed with hematoxylin, dehydrated with prepared gradient ethanol, then transparent xylene solvent and sealed with neutral resin, and different staining parts were selected to observe the staining.

The absorbance value of positive products was calculated by using Image-Pro Plus software. Finally, the calculated average value was used for statistical analysis.

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). The analysis of variance was used to analyze the discrepancies between multisamples, while Student t test was adopted to make a comparison of the differences between two groups. Pvalue of less than 0.05 was ofstatistical significance.

Results

SCU inhibited the survival of C6 cells

Compared with the control group, the cell survival rate of C6 48h treated with different concentrations of SCU (10 mg·L^{-1}, 20 mg·L^{-1}, 40 mg·L^{-1} and 80 mg·L^{-1}) decreased gradually with the increase of SCU concentration, while that of C6 72h treated with different concentrations of SCU (20 mg·L^{-1}, 40 mol·L^{-1} and 80 mg·L^{-1}, respectively) decreased gradually with the increase of SCU concentration. 48h IC50=76.53 mg·L^{-1}, 72h IC50=20.05 mg·L^{-1}. See Table 1.

Group	Cell viability (%)	
	48 h	72 h
Blank control	100±6.21	100±9.04
SCU (mg·L^{-1})		
5	89.92±2.79	89.36±6.03
10	83.11±1.86[1)]	88.84±2.39
20	76.92±6.02[1)]	67.28±6.31[1)]
40	53.59±4.57[1)]	26.24±2.74[1)]
80	45.54±6.72[1)]	9.16±0.32[1)]

Table 1: MTT detection of C6 cell survival rate in each groups (n=5, $\bar{x}\pm s$), *P<0. 01compared with blank control group.

Observation on the morphology of C6 cells by inverted microscope

The morphology of C6 cells was observed by inverted microscope. The results showed that the growth state of C6 cells in the blank control group was good, the cells were closely arranged, the cells were long fusiform, the shape was full and the outline was clear. In the drug group, the volume of C6 cells became smaller, the interval widened, and the number of C6 cells decreased. At the same time, the cells appeared spherical or even exfoliated. See Figure 1.

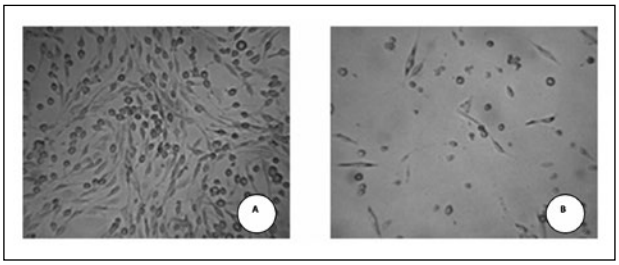

Figure 1: Morphology of C6 cells in each group under inverted microscope (×200).
A: Blank control group; B: SCU group (80 mg·L^{-1}).

SCU promoted apiptosis of C6 cells

In this experiment, Annexin V/PI double staining was used to detect the apoptosis rate of C6 cells. As shown in Figure 2, C6 cells were treated with 80 mg·L^{-1} SCU for 72 hours. Flow cytometry showed that the late apoptosis rate and total apoptosis rate of C6 cells in the SCU group were significantly higher than those in the control group (P<0.05).

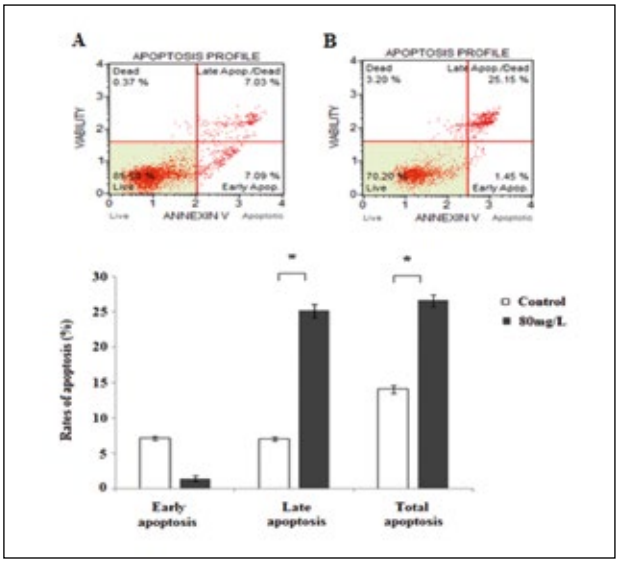

Figure 2: The apoptotic rates of C6 cells in each group.

SCU regulated the expression of BAX and BCL-2 protein in C6

According to the immunohistochemical results, Figure 3 and Table 2 showed that compared with the

blank control group, the expression of bax protein in SCU group was up-regulated, and the difference was not statistically significant (P>0.05). Compared with the blank control group, the expression of bcl-2 protein in SCU group was down-regulated, and bax/bcl-2 was larger, the difference was statistically significant (P<0. 05).

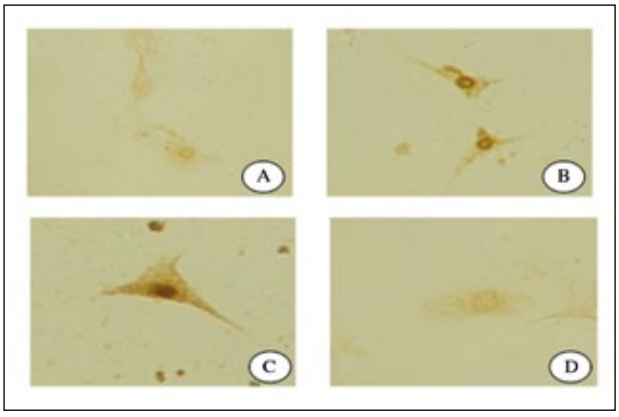

Figure 3: Bax and bcl-2 protein expressions in C6 cells of each group.
A, B: bax; C, D: bcl-2; A, C: Blank control; B, D: SCU group (80 mg·L^{-1}).

Group	bax	bcl-2	bax/bcl-2
Blank control	45.79±7.60	75.84±40.01	0.60±0.18
SCU(mg·L^{-1})			
80	54.95±24.91[1)]	52.46±12.38[1)]	1.04±0.47[1)]

Table 2: Bax and bcl-2 protein expression levels in C6 cells in various groups ($\bar{x}$±s).
$^{*}P<0.05$ compared with blank control group.

Discussion

In recent years, researchers have found that SCU has a good anticancer effect, and its anticancer activity has been paid more and more attention. As one of the flavonoids, SCU has the characteristics of anti-tumor, but its anti-tumor mechanism is not clear. To prepare for the development of a new antineoplastic drug SCU and explore its anti-tumor pathway. First of all, we demonstrate whether SCU inhibits the growth of C6 cells.

MTT method was used to determine the survival rate of C6 cells treated with, SCU (5, 10, 20, 40 and 80 mg·L^{-1} for 48h and 72h. The survival rate of C6 cells decreased with the increase of SCU concentration, and showed a concentration-time dependent response (Table 1). Microscopic observation: in the control group, C6 cells were fusiform, with clear boundaries, a large number of full cells and closely arranged together; SCU (80 mg·$^{L-1}$) when C6 cells were treated with C6 cells for 72 hours, we observed that the decrease of the number of cells caused a decrease in cell density, a small number of cells became smaller and gradually became spherical, and the cell boundary was blurred (Figure 1).

The above two experiments showed that SCU could inhibit the growth of C6, and the inhibitory effect of high concentration of SCU was more obvious with the increase of the concentration of SCU (5, 10, 20, 40 and 80 mg·L^{-1}). Recent experimental results have shown that tumor cells can stimulate therapeutic resistance by inducing cell growth pathway and/or inhibiting cell death pathway[16-18]. Objective to study the inhibitory pathway of SCU on C6 cells treated with, SCU (80 mg·L^{-1}). The reaction time was 72 hours. After the reaction, the apoptosis rate was measured by Annixin V/PI method. Apoptosis results: compared with the blank group, the late stage and total apoptosis rate of C6 cells in SCU group increased, which confirmed that SCU could induce apoptosis of C6 cells. Among the various ways to induce tumor apoptosis, mitochondrial pathway has been in a very important position. Bax and bcl-2 proteins are two key proteins that cause apoptosis. A large number of experimental results have confirmed that insulin-like growth factor 1 (IGF-1) can play a role in apoptosis[19-21]. At the same time, it can inhibit the death of different kinds of cells and promote their growth and differentiation[22, 23]. Foreign researchers such as Buerke M and Murohara T have found that IGF-1 can inhibit apoptosis, and a certain concentration of IGF-1 can regulate the expression of Bax and Bcl-2 in mouse preosteoblast-like MC3T3-E1 cells at the same time[24]. In the immunohistochemical experiment of C6 cells, as shown in figure 3 and Table 2, we can observe that as the expression of BcL-2 protein decreases, the concentration of SCU used in the experimental group increases, and when the concentration of SCU in each treatment group increases, the expression of bax protein is up-regulated, and the bax/bcl-2 ratio is also up-regulated. In this way, apoptosis factors have the upper hand, leading to apoptosis of tumor cells.

Conclusion

The results of this anti-tumor experiment of SCU suggest that in the process of proliferation of C6 cells treated with SCU, when the concentration of SCU increases, the proliferation rate of C6 cells slows down, and even the cell boundary is blurred, shrunken and dropped, perhaps because the pro-ap-

optotic factor bax/bcl-2 is up-regulated, and then activate the mitochondrial pathway to induce tumor cell apoptosis and inhibit cell growth.

However, if we want to use SCU as an effective antineoplastic drug in the treatment of clinical tumor patients, we still need to further study and explore the exact anticancer mechanism of SCU.

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