INHIBITORY EFFECT ITS MECHANISM OF SCUTELLARIN PROLIFERATION ON GLIOMA CELLS

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ABSTRACT

Introduction: *To study the inhibitory effect of Scutellarin(SCU) on proliferation of glioma C6 cells and to explore its mechanism.*

Materials and methods: *Then C6 cells were cultured and divided into control and 0, 5, 10, 20, 40, 80 mg·L-1 SCU groups. The morphology of C6 cells was observed by inverted microscope; The survival rates of C6 cells was examined by MTT assay; The apoptotic rates of C6 cells were examined by Annexin V/PI staining; The expression of Caspase-3 and Caspase-9 proteins in C6 cells were detected by Western blotting.*

Results: *The MTT results showed that compared with levels blank control group, the survival rates of C6 glioma cells in SCU groups were decreased. The results of inverted microscope observation showed that the cell number in 80 mgL-1 SCU group was significantly decreased, and the cell volume was smaller. The C6 cells were shrinkage, shedding, contour blur, adherent cell outline. The results of AnnexinV/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. The western blotting results showed that compared with the control group, the expression levels of caspase-3 and caspase-9 protein in 20,40 and 80 mg·L-1 SCU groups were significantly increased.*

Conclusion: *Compared with the blank control group, SCU can up-regulate the expression of caspase-3 and caspase-9 protein, inhibit tumor growth and induce apoptosis.*

Keywords: *Scutellarin, glioma, caspase-3, proliferation.*

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Introduction

Glioma is the most common brain tumor, which often exists in malignant form. Because it grows in the brain, it poses the greatest threat to human life and health. In the course of its treatment, the therapeutic effects of comprehensive therapy and temozolomide therapy are not ideal. At the same time, the incidence of cancer is increasing year by year, and the recurrence rate after surgery is high. Some experimental results show that the imbalance between tumor cell proliferation and apoptosis leads to unlimited tumor growth[1-6]. Inducing apoptosis of tumor cells has become a new target of new anticancer drugs. and the apoptosis rate can be used as a new index to evaluate the anti-tumor effect[7]. Some studies have found that SCU has protective effects on the immune and magical system. At the same time, it can enhance immunity, anti-inflammation, anti-oxidation, protect liver, anti-microorganism, anti-tumor, improve microcirculation and anti-platelet aggregation[8-21]. Among these pharmacological activities, its ability to act on cells, inhibit cell proliferation and induce apoptosis of tumor cells has become a hot topic in tumor therapy research at home and abroad. At the same time, the anti-tumor activity

of SCU has attracted more and more attention of medical researchers due to its remarkable efficacy, small side effects and low price[22].

It is precisely because SCU has the above advantages that researchers have conducted multi angle and multi mechanism research on its clinical anti-tumor treatment. The purpose of this study was to investigate the inhibitory effect of SCU on the proliferation of C6 cells and the role of SCU in inducing tumor cell apoptosis from the aspects of cell proliferation activity, cell morphology, apoptosis rate and protein expression after SCU acted on C6 cells, and to preliminarily explore the mechanism of SCU regulating caspase-3 protein expression and inducing apoptosis of glioma cells.

Materials and methods

Cell samples

Rat glioma cell line C6 was achieved from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The Dulbecco's modified Eagle medium (DMEM) culture medium containing 10% FBS (Gibco BRL, Grand Island, NY) was preheated in a 37°C incubator, and the C6 cells were removed from the liquid nitrogen tank.

After shaking in a water bath at 37°C, the C6 cells were centrifuged for 5 min, and re-suspended with 1 mL culture medium in a petri dish, then added 9 mL fresh medium, mixed gently, and cultured in a 5% CO_2, 37°C incubator. After the cells grew to 80% and 90%, the cells were subcultured at 1:3.

Groups and drug administration

After incubating and freezing enough C6 cells, C6 cells in logarithmic growth phase were collected and divided into six groups: the SCU concentration was 0, 5, 10, 20, 40, and 80mg/ L, respectively.

Thiazolyl blue tetrazolium bromide (MTT) assay

Cells were seeded into 96-well culture plates, and each well was inoculated with 8 × 104 cells. Incubate in 5% CO_2 and 37°C incubator for 24 hours, then change the culture medium containing different concentrations of SCU the next day, with 3 multiple holes in each group, and set up a blank control group. After 48 hours of culture, the reaction was terminated. Meanwhile, each well with 100 μL of culture medium was added with MTT (1μL, 5mg·L-1; 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 150μL of DMSO each well. The goal is to get the crystal compounds to be ablated by vibration. After 15 mins reaction, the optical density of each well was measured at 570 nm.

Observation of C6 cells by inverted microscope

Inoculate C6 cells into 5×10^3 cells in 24 well plates, and add medium (the proportion of FBS is 10%). C6 cells were cultured in the incubator at 37°C and 5% CO_2, and the prepared fresh medium was changed the next day. After the C6 cells completely adhered to the wall, the blank control group and 80 mg·L-1 SCU group were established and incubated for 48 hours under the same conditions.

The control cells and SCU cells were placed under the inverted microscope to observe the number, morphology and biological characteristics of glioma C6 cells.

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 80 mg·L-1 SCU group. The 6 well plate with cells was incubated in the incubator for 48 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 μg 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 10-15min. Then, FCM was used to calculate the apoptotic cells.

Western blot

C6 was obtained by planting in 24 well plates, 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: a control group and SCU group (the concentration of SCU in culture medium was 80 mg·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 FBS sealing solution for 30 minutes. FBS was removed and treated with first antibody caspase-3 (Sigma, 1:50) and caspase-9 (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 (caspase-3 (1:50) and caspase-9 (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 the Student t test was adopted to make a comparison of the differences between two groups. P-value of less than 0.05 was of statistical 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 (10mg·L-1, 20mg·L-1, 40mg·L-1 and 80mg·L-1) decreased gradually with the increase of SCU concentration. See Table 1.

Group	A	Survival rate (%)
Blank Control	0.995	100
Scutellarin (mg/L) 10	0.972	97.7
20	0.862	86.7
40	0.753	75.7
80	0.487	48.9

Table 1: MTT detection of C6 cell survival rate in each group (n = 3).

Observation on the morphology of C6 cells by inverted microscope

After the C6 cells with good growth conditions were treated with SCU group, the morphology of C6 cells in the blank control group and SCU group was observed by inverted microscope.

As shown in Figure 1, we see that the growth state of C6 cells in the blank control group was good, under the Microscope, the cells are closely arranged and grow in a fusiform shape, and most of the cells are full in shape and clear in outline. In SCU group, the number of C6 cells decreased significantly, the volume became smaller and the septum widened. At the same time, the contraction of C6 cells was spherical or even shedding, and the boundary of C6 cells was blurred.

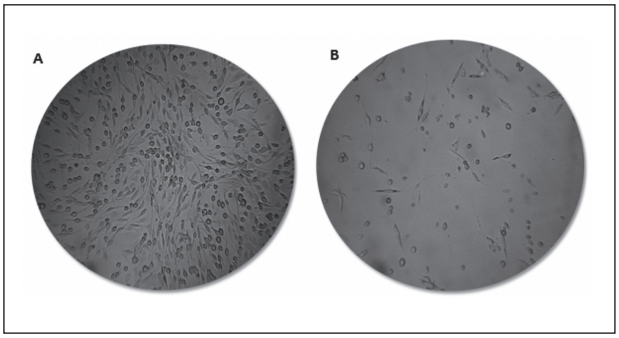

Figure 1: Morphology of C6 cells in each group under inverted microscope (×200).
A: Blank control group; B: SCU group (80mg·L^{-1}).

SCU promoted apoptosis 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 48 hours.

Flow cytometry showed that the late and total apoptotic rates of C6 cells in the 80mg·L-1SCU group were significantly higher than those in the control group.

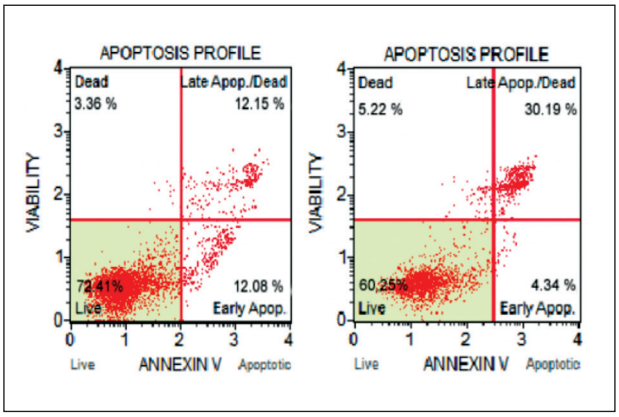

Figure 2: The apoptotic rates of C6 cells in each group.
A: Blank control; B: SCU group (80 mg·L^{-1}).

Western blot

According to the results of Western blotting hybridization, Figure 3 and Figure 4 showed that compared with the blank control group, the expression of caspase-3 protein in SCU group was up-regulated. Compared with the blank control group, the expression of caspase-9 protein in SCU group was also up-regulated.

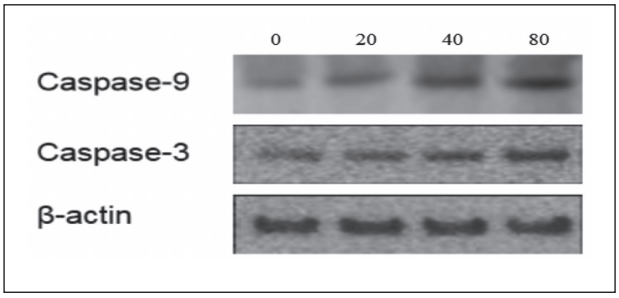

Figure 3: Caspase-3 and caspase-9 protein expressions in C6 cells of each group.

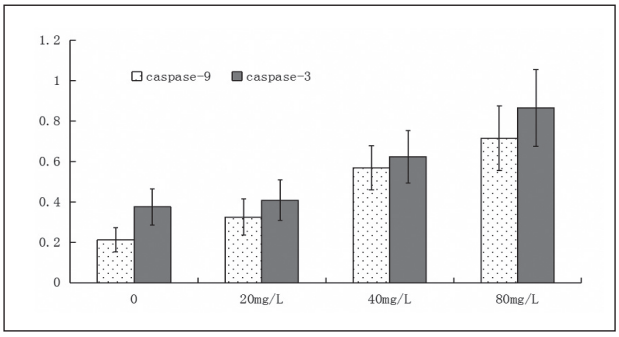

Figure 4: Caspase-3 and caspase-9 protein expression levels in C6 cells in various groups ($\bar{x}\pm s$).
$^{*}P<0.05$ compared with blank control group.

Discussion

Some research results show that malignant glioma has therapeutic resistance due to the activation of the growth pathway or inhibition of the apoptosis pathway in the growth process, resulting in accelerated proliferation of malignant glioma[23-27]. Studies have shown that the anti-tumor effect of traditional Chinese medicine can be exerted mainly through cytotoxicity, immune enhancement or biological response regulation. Among a variety of natural active ingredients of traditional Chinese medicine, some of them can kill cancer cells. They can play an anti-tumor role by directly killing tumor cells, while other active ingredients can play an anti-tumor role by improving the body's own immunity and resistance[28-32]. At present, some studies have revealed that traditional Chinese medicine components can also inhibit tumors by activating the reticuloendothelial system and complement, inducing a variety of cytokines and so on. The existing anti-tumor research directions of traditional Chinese medicine mainly include: promoting apoptosis, inducing tumor cells to differentiate into normal cells, immunomodulatory effect on tumor-bearing animals, and affecting tumor cell division and proliferation by inhibiting DNA, microtubules and their related enzymes.

In the course of this study, we conducted an in-depth study on the anti-tumor mechanism of SCU, and prepared for the development of a new anti-tumor drug SCU. In the course of the experiment, we first confirmed that SCU can inhibit the growth of glioma cells. In the MTT experiment, we found that the survival rate of C6 cells decreased with the increase of the concentration of scutellarin. We use an inverted microscope to observe the morphology of C6 cells in the blank control group and scutellarin group. From figure 1, we can find that the number of C6 cells in the SCU group is significantly less than that in the blank control group, and the cell spacing increases obviously, the cell changes from long fusiform to round, and the outline of the cell is gradually blurred, so it is not easy to distinguish the boundary. Then we detected the apoptosis of C6 cells in the blank control group and SCU group by Annexin V/PI double staining. From figure 2, we saw that the late and total apoptotic rates of C6 cells treated with SCU was significantly higher than that in the blank control group. From the results of the above experiments, we confirmed that scutellarin can inhibit the growth of glioma C6 cells and promote its apoptosis. Apoptosis is a programmed and autonomous death process of cells controlled by genes, which plays an important role in the normal growth, development and structural remodeling of the body[33, 34]. Apoptosis is the main way to eliminate mutation and damage cells. At present, many apoptosis-related genes have been studied and found, such as p53, bcl-2, caspases and so on. Among these families, Caspases is an evolutionarily conserved cysteine-dependent protein endonuclease family and the substrate for the post-hydrolysis of specific aspartic acid residues.

Caspases have always been related to the induction of apoptosis, which is a steady-state and non-lytic regulated mode of cell death, which supports the collaborative disassembly and clearance of old and damaged cells. Caspases members are one of the central links in the process of apoptosis and the common pathway of apoptotic signal transduction. In recent studies, Caspase mediates the mechanism

of cell lysis (necroptosis, a lysis-regulated cell death mode driven by receptor-interacting protein (Rip) kinase). In addition, in recent years, the mechanism of inflammatory Caspase promoting cell petrogenesis (pyroptosis) has been discovered, which is another major mode of lytic cell death. Apoptotic Caspase is functionally subdivided into induced Caspase (caspase 8, 9 and 10) and effector Caspase (caspase 3, 6 and 7). Caspase-8 is an important molecule in the apoptosis pathway, caspase-8 is the key protein in the endogenous apoptosis pathway[35-38], and caspase-3 is the key executive molecule, which plays a role in many pathways of apoptosis signal transduction. Caspase-3 normally exists in the cytoplasm in the form of zymogen (32KD). In the early stage of apoptosis, it is activated. The activated Caspase-3 consists of two large subunits (17KD) and two small subunits (12KD), cleavage the corresponding cytoplasmic nuclear substrate, and eventually lead to apoptosis. However, in the late stage of apoptosis and dead cells, the activity of Caspase-3 decreased significantly[39, 40]. Some studies have shown that abnormal activation of Caspase plays an important role in tumorigenesis, autoimmune diseases and infectious diseases. In the experiment, we also carried out the western blot experiment of C6 cells. In the results of figures 3 and 4, we can observe that the protein expression of caspase-3 and caspase-9 is up-regulated with the increase of the concentration of scutellarin. This proves that caspase-3 is activated at the early stage of cell apoptosis, and then the corresponding cytoplasmic and nuclear substrates are cut, and finally the effect of inducing cell apoptosis is produced to inhibit the survival number and growth state of cells.

Conclusion

Through the results of this experiment, we preliminarily confirmed that SCU can inhibit the growth of glioma C6 cells and determine that SCU can induce apoptosis of C6 cells. At the same time, western blot results showed that the apoptosis of C6 cells may be due to the up-regulation of caspases family members caspase-3 and caspase-9 protein expression induced by SCU, and then activated the mechanism of caspase-mediated cell lysis, but the interaction mechanism among caspases family members under the action of scutellarin is not clear, which requires further research. We believe that with the deepening of scientific research and the continuous efforts of scientific researchers, we will eventually have a clearer understanding of the mechanism of SCU induced tumor cell apoptosis, and ultimately make our research contributions to the development of new effective anti-tumor drugs.

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