Inhibitory Effect of Benzyl Isothiocyanate on Proliferation in vitro of Human Glioma Cells

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Introduction

The treatment of malignant glioma is mainly relying on surgical excision at present, supported by postoperative radiotherapy and chemotherapy. Therefore, it is necessary to develop new medicine in order to improve the treatment efficacy. Recent studies have found that a large number of edible cruciferous plants, such as broccoli, cabbage and water celery, can reduce the risk of cancer. It has also been confirmed that the isothiocyanates compounds contained in these plants have anti-tumor ability. Up to now, researchers have studied more than 20 kinds of natural or synthetic isothiocyanate compounds and observed that they have a great inhibition effect on a variety of tumors. Recent epidemiological and experimental studies have also shown that isothiocyanate compounds possess the potential to be developed into chemotherapy medicine, but the exact mechanism of their anti-tumor activity has not yet been fully understood (Kim et al., 2012; Pawlik et al., 2012; Zandalinas et al., 2012). Therefore, this study intended to explore the inhibition effect of benzyl isothiocyanate (BITC) on human glioma U87MG cells, as well as its potential mechanism. It will provide a certain level of theoretical and scientific basis for future research.

Materials and Methods

Cell culture

Culture the human glioma U87MG cell lines in the DMEM complete medium (containing 10% FCS, 100 μg/mL streptomycin and 100 μg/mL penicillin) under the conditions of 37 °C, 5% CO₂. Take cells in the experimental logarithmic phase to perform test.

Effects of BITC on proliferation of human glioma U87MG cells

Culture the human glioma U87MG cells on a 96-hole plate (5×10³ cells / hole) under the conditions of 37°C, 5% CO₂ for 24h. After the cells are attached, add BITC medium with the final concentration of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM respectively into the cell samples; add the medium with an equal volume of PBS into the negative control group (six multiple-holes for each dose). Then, culture the cells in the incubator again for a continuous 72 h. After removing the supernatant carefully, add 200 μL plasma-free medium and 40 μL MTS respectively into the cell samples. Use aluminum foil to wrap the plate and culture for another 4h. Thereafter, extract the supernatant and use the enzyme-labeled meter to measure its OD value at 490 nm; calculate the medicine IC₅₀. Repeat the experiment for three times and take the mean value. The tumor growth inhibition rate (%) = (1 - the OD value of the medicine-treated group / the OD value of the negative control group) × 100%.

Effects of BITC on apoptosis of human glioma U87MG cells

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10⁵ cells / hole) under the conditions of 37 °C, 5% CO₂ for 24h. Then, add 10 μM and 20 μM BITC into the supernatant and use the enzyme-labeled meter to measure its OD value at 490 nm; calculate the medicine IC₅₀. Repeat the experiment for three times and take the mean value. The tumor growth inhibition rate (%) = (1 - the OD value of the medicine-treated group / the OD value of the negative control group) × 100%.
the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 μL cell suspension, Annexin-V-FITC and 10 μL PI and incubate the samples in dark environment for 10 min. Use the flow cytometer to perform test.

**Effects of BITC on cell cycling of human glioma U87MG cells**

Culture the human glioma U87MG cells on a 6-hole plate (1.5 × 10⁵ cells/hole) under the conditions of 37 °C, 5% CO₂ for 24 h. Then, add 2 μM and 5 μM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 μL cell suspension and add in 5 μL intracellular reactive oxygen detection probe DCFH-DA. Incubate the samples at room temperature in dark environment for 30 min. Then, use the flow cytometer to perform test.

**Effects of BITC on ROS expression of human glioma U87MG cells**

Culture the human glioma U87MG cells on a 6-hole plate (1.5 × 10⁵ cells/hole) under the conditions of 37 °C, 5% CO₂ for 24 h. Then, add 2 μM and 5 μM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 μL cell suspension and add in 5 μL intracellular reactive oxygen detection probe DCFH-DA. Incubate the samples at room temperature in dark environment for 30 min. Then, use the flow cytometer to perform test.

**Effects of BITC on SOD activity of human glioma U87MG cells**

Culture the human glioma U87MG cells on a 6-hole plate (1.5 × 10⁵ cells/hole) under the conditions of 37 °C, 5% CO₂ for 24 h. Then, add 2 μM and 5 μM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 μL cell suspension and add in 5 μL intracellular reactive oxygen detection probe DCFH-DA. Incubate the samples at room temperature in dark environment for 30 min. Then, use the flow cytometer to perform test.

**Effects of BITC on GSH expression of human glioma U87MG cells**

Culture the human glioma U87MG cells on a 6-hole plate (1.5 × 10⁵ cells/hole) under the conditions of 37 °C, 5% CO₂ for 24 h. Then, add 2 μM and 5 μM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 μL cell suspension and add in 5 μL intracellular reactive oxygen detection probe DCFH-DA. Incubate the samples at room temperature in dark environment for 30 min. Then, use the flow cytometer to perform test.

**Results**

**Effect of BITC on proliferation of human glioma U87MG cells**

The MTS method was applied to observe the effect of BITC on the in-vitro growth of human glioma U87MG cells. The experimental results showed that: after BITC taking effect on human glioma U87MG cells for 72 h, cell proliferation was inhibited and exhibited the dose-response relationship; its IC₅₀ was equal to 15.2 μM (Figure 1). After the cell lines being treated by 2 μmol/L BITC for 24 h, the inhibition rate was less than 5%; after being treated by 5 μmol/L BITC for 24 h, the inhibition rate was less than 15%. The U87MG cells were in the non-toxic and low-toxic concentration, respectively. Therefore, BITC with the concentration of 2 μmol/L and 5 μmol/L were used to test the cell cycle, the ROS, SOD and GSH expression/activity and the caspase-3 activity, in order to eliminate the interference of cell growth inhibition and apoptosis on the above mentioned experiments.

**Effects of BITC on apoptosis of human glioma U87MG cells**

The Annexin-V-FITC and PI double staining test showed that after treating the cells with BITC for 24 h, the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, the cells shall be lysed. Perform centrifugation at 4 °C and 10,000g/10min; extract the supernatant to obtain the total protein of tissues. Perform electrophoresis in 12% SDS polyacrylamide gel; then, transfer the protein samples to a PVDF membrane. Use 4 °C, 5% skimmed milk to seal the samples and rest for overnight. Add first antibody (1:500) and β-actin (1:5000) into the samples, respectively. Then, incubate the samples at 4 °C for overnight. Use IgG (1:2000) that is labeled with horseradish peroxidase to incubate the samples at room temperature for 1h. Develop and show image.
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Figure 2. The Apoptosis-induced Effect of BITC on the U87MG Cell Line. A: The flow cytometry results in apoptosis effect-induced of U87MG cells; B: The apoptosis effect of BITC on the U87MG cell line. Bars indicate SD. n=3. *P<0.05 compared with control group

Effects of BITC on cell cycling of human glioma U87MG cells

The measurement result of the flow cytometer showed that after treating the cells with 2 μM and 5 μM BITC for 24 h, the cell cycle distribution of the tumor was changed (P < 0.05). Specifically, the proportion of phase G2/M was increased, while the proportion of phase G0/G1 was decreased (P < 0.05) (Figure 3), suggesting that BITC can arrest the cell cycle of U87MG cells at phase G2/M.

Effects of BITC on ROS expression of human glioma U87MG cells

The result of the flow cytometer showed that after treating the cells with 2 μM and 5 μM BITC for 24 h, the ROS expression of the tumor cells was significantly increased; specifically, the ROS expression of the 2 μM group and 5 μM group was 3.76-fold and 6.07-fold, respectively (P < 0.05) (Figure 4).

Effects of BITC on GSH expression and SOD activity of human glioma U87MG cells

The result showed that after treating the U87MG cells with 2 μM and 5 μM BITC for 24 h, the GSH expression and SOD activity expression of the tumor cells were significantly decreased. The GSH expression of the 2 μM group and 5 μM group was 71.3% and 44.9% of that of the control group, respectively (P < 0.05); the SOD activity of the 2 μM group and 5 μM group was 63.5% and 21.8% of that of the control group, respectively (P < 0.05) (Figure 5).

Effects of BITC on caspase-3 activity of human glioma U87MG cells

The result showed that after treating the U87MG cells with 2 μM and 5 μM BITC for 24 h, the caspase-3 activity of the tumor cells was significantly decreased (P < 0.05) (Figure 6).

Discussion

Malignant glioma, also known as brain cancer, is the most common intracranial tumor, having an extremely high mortality. In recent years, although the various
treatment approaches for malignant glioma, including surgical operation, radiotherapy, chemotherapy and targeted therapy, have been undergoing a rapid development, there are still a series of problems, such as high recurrence rate, low survival rate and high treatment difficulty, to be further addressed. The survival cycle of the patients is merely about 14 months, and only 5% of the patients can survive for more than 5 years (Tabatabai et al., 2012). The high recurrence rate and low survival rate of malignant glioma is closely related to the invasive growth of the tumor and the special structure of the brain. Glioma is often occurred under the cortex, featured with infiltrative growth. It may transfer to a wide range of locations and often invades several brain lobes, as well as the deep structure. Sometimes, it can even injure the contralateral hemisphere via callosum and grow together with healthy brain tissues, so that the tumor is difficult to be completely removed by surgery. Meanwhile, for some patients, the tumor locations are inaccessible or serious consequences may occur during the postoperative phase, making the surgery operation impossible (Fidler, 2011). Therefore, chemotherapy of glioma has drawn increasingly more attention nowadays. This study observed that BITC has an inhibition effect on the proliferation of U87MG cells; it can also arrest the cell cycle and induce the apoptosis of the human glioma U87MG cells, indicating that BITC has the anti-tumor effect.

Apoptosis is a kind of biological phenomenon of programmed cell death. Anti-tumor medicine plays the anti-tumor effect by inducing the apoptosis of tumor cells. Cell cycle arrest and oxidative stress are the main causes of apoptosis. The nature of cell proliferation is to realize DNA replication constantly through cell cycle; when cell cycle arrest occurs, the proliferation of tumor cells will be inhibited (Deeb et al., 2012). This study showed that BITC can arrest the cell cycle at phase G2/M, so that cells cannot enter phase G0/G1 and phase S to conduct DNA synthesis and repair. As a result, the tumor cells cannot enter the next cycle, and thereby, the proliferation of tumor cells is inhibited and the cell apoptotic program is initiated.

Superoxide Dismutase (SOD) is an important oxygen free radical scavenger in the enzymatic defense system, which is able to catalyze the transformation of hyperoxide into oxygen and enzyme of hydrogen peroxide via disproportionation reaction. Glutathione (GSH), which is formed by the integration of glutamic acid, cysteine and glycine, is a kind of tripeptide containing mercapto. It possesses the functions of anti-oxidation and detoxification. Both of these two elements play an important role in the biochemical defense system of human body, able to remove free radical in the body. As an important anti-oxidant, they can protect the mercapto in the molecules of many proteins and enzymes and eliminate the harmful substances produced by the organisms in the metabolism process. Meanwhile, they can also resist or block the cell injury caused by oxygen free radicals and repair the injured cells. However, SOD and GSH may limit the effect of the anti-tumor medicine (Deng et al., 2012; Kohsaka et al., 2013). This study showed that BITC can reduce SOD activity and GSH expression, which is conductive for strengthening the anti-tumor activity.

Oxidative stress is another cause of tumor cell apoptosis (Gayathri R et al., 2009). Oxidative stress can activate the cascade reaction of the cysteine aspartate acid specific protein kinase and the caspase-3 activity of the cells, which may lead to DNA breaks. As the tumor cell cycle is arrested at phase G2/M, both the synthesis and repair capacity of DNA is relatively low, and thus, apoptosis occurs in tumor cells (Martinez-Outschoorn et al., 2012; Noureen et al., 2013). In this study, it was observed that BITC may increase ROS expression in the tumor cells, suggesting that BITC can activate caspase-3 activity by affecting the cell cycle and inhibiting the SOD activity and the GSH expression, so as to accelerate the apoptosis of human glioma U87MG cells. Its mechanism may be related to the fact that BITC can cause oxidative stress to tumor cells.

In summary, BITC, which can induce apoptosis through oxidative stress, is expected to become a new anti-tumor medicine. However, further studies are needed to investigate the mechanism of BITC for arresting cell cycle and its impact on intracellular signal transduction, in order to provide an experimental basis for clinical application.

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