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Original paper

Effect of lentinan on proliferation and apoptosis of human astrocytoma U251 cells

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To investigate the effect of lentinan on proliferation and apoptosis of human astrocytoma U251 cells.
Lentinan was dissolved in Dulbecco's modified eagle medium complete medium to form different concentrations (0, 25, 50, 100, 200, 400, 500, 600 μg/ml). CCK8 was used to detect the effect of lentinan with different concentrations on proliferation of human astrocytoma U251 cells, and the expression of Ki-67 was detected by immunofluorescence. In addition, the effect of different concentrations of lentinan on apoptosis of human astrocytoma U251 cells was detected by flow cytometry.
Compared with the blank control group, 50 and 100 μg/ml lentinan significantly promoted proliferation of human astrocytoma U251 cells. When the concentration is more than 100 μg/ml, the cell activity gradually decreases, and the cell activity is the lowest when the concentration is 600 μg/ml. In addition, the low concentration lentinan (25, 50, and 100 μg/ml) had no significant effect on apoptosis of human astrocytoma U251 cells. However, lentinan above 200 μg/ml significantly promoted apoptosis of human astrocytoma U251 cells and had a concentration gradient effect, and the highest apoptosis rate was at 600 μg/ml.
Lentinan can effectively inhibit proliferation and promote apoptosis of human astrocytoma U251 cells.

Key words: lentinan, astrocytoma, U251 cells, cell proliferation, apoptosis.

Introduction

Astrocytoma is the most common primary central nervous system tumor. It is a malignant brain tumor with high genetic susceptibility and carcinogenic environmental factors, accounting for about half of all primary intracranial tumors [1, 2]. At present, surgical resection is the first choice for the treatment of glioma. However, because there is no apparent boundary between glioma and normal brain tissue, it is difficult to achieve complete cytological resection, with rapid recurrence, a high recurrence rate, short survival time, and poor prognosis. The response to adjuvant therapy such as radiotherapy and chemotherapy is also inadequate, and the incidence has been gradually increasing in recent years, causing a severe economic burden to society [3, 4]. Therefore, to seek a fair and effective comprehensive treatment to prolong the survival period and improve the quality of life is the focus of glioma research.

Lentinan is a kind of polysaccharide extracted from *Lentinus edodes*, which is widely used in clinics. As early as 1969, Chihara, a Japanese scholar, discovered the anti-tumor activity of lentinan. During

the nearly 50 years from basic research to clinical application, lentinan has been shown to be the active component of *Lentinus edodes* [5]. Studies showed that lentinan could inhibit tumor formation and regulate immune function. It is an ideal auxiliary anti-tumor drug [6]. At present, there are few studies on the inhibitory action of lentinan on glioma. In this study, the effect of lentinan on proliferation and apoptosis of human astrocytoma U251 cells was studied to explore the antitumor effect and mechanism of action of lentinan on glioma.

Materials and methods

Experimental materials

Lentinan (freeze-dried powder) was purchased from Nanjing Yiheng Pharmaceutical Co., Ltd. of China, and U251 cells were purchased from bncc337874.

Dulbecco's modified eagle medium (DMEM, kgm12800s, Kaiji Biology), trypsin ethylene diamine tetraacetic acid (EDTA) digestive solution (t1300, Solarbio), phosphate buffer saline (PBS, kgb5001, Kaiji Biology), penicillin mixed solution (p1400, Solarbio), CCK8 cell proliferation detection reagent (kga317, Kaiji Biology), inverted fluorescence microscope (mf53, Guangzhou Mingmei photoelectric Co., Ltd.), full-automatic enzyme labeling instrument (wd-2102b, Beijing Liuyi Biotechnology Co., Ltd., Ki-67 (ab92742, Abcam), Cy3 converged (cw0159s, cwbio), ready to use 4',6-diamidino-2-phenylindole (DAPI) dye solution (kga215-50, Kaiji Biology), fluorescence microscope (ckx53, Olympus).

Cell culture

U251 cells were cultured in a medium containing fetal bovine serum (and DMEM with 10% double antibody. When the cell density reaches 80-90%, the cells need to be passaged. The supernatant was discarded, washed twice with 1 × PBS, digested with 0.25% pancreatin (containing 0.02% EDTA). After the cells became round, the supernatant was added to the culture medium to stop digestion. The cell suspension was collected into a 10 ml centrifuge tube and centrifuged at 1000 rpm for 3 min. Then the supernatant was discarded and added to the culture medium to resuspend the cells. Dilute the cells according to the experiment's needs, evenly spread them into the cell culture plate, make marks, and place them in the incubator for culture. In the experiment, the experimental operation was carried out 3 times.

Cell proliferation

Cells were digested, resuspended, counted, and plated, with a cell density of 8×10^3 cells/well. After the cells were attached to the wall, lentinan of different concentrations (0, 25, 50, 100, 200, 400, 500, 600 μg/ml, dissolved in DMEM complete medium) was used for 24 hours, and 96 healthy plate cells to be tested were replaced with the same medium, 10 μl/well; 10 μl/well of CCK8 reagent was added and incubated in an incubator for 3 hours. The absorbance value of each well was measured at 450 nm wavelength by an enzyme labeling instrument to calculate the survival rate.

Cell apoptosis

After digestion with trypsin, the cells were collected, the cell density was adjusted to $1 \times 10^6 – 3 \times 10^6$ cells/ml, and the cells were resuspended with binding buffer. Add 3 μl annexin V-FITC and 5 μl PI-PE solution to each tube after heavy suspension. After slightly mixing, the cells were incubated at room temperature in the dark for 10 minutes, and apoptosis was detected by flow cytometry.

Immunostaining

The cells were immersed in PBS three times and fixed with 4% polyoxymethylene (POM) for 15 minutes. Then 0.5% Triton X-100 was added for 20 minutes. After three times of PBS washing, 5% bovine albumin (BSA) was added and sealed at 37°C for 30 min. Absorb the sealing liquid around the tissue with absorbent paper, add enough fluorescent diluted primary antibody Ki-67 (1 : 100) to each slide, put it into a wet box, incubate at 4°C overnight. After the slide had rewarmed to room temperature, PBS was used to wash the slide three times, each time for 3 minutes. After the absorbent paper had absorbed the excess liquid on the slide, drop the diluted fluorescent secondary anti Cy3 (1:200), and incubate in the wet box at 37°C for 30 minutes. After that, DAPI was added dropwise and incubated in the dark for 5 min. The samples were stained, the excess DAPI was washed with PBS, the Petri dish was sealed with 50% glycerol, and then the images were observed under the fluorescence microscope.

Statistical analysis

All data were statistically analyzed with GraphPad prism 7, expressed as mean ±SD. T-test, one-way, and two-way analysis of variance (ANOVA) were performed. $P < 0.05$ was considered statistically significant.

Results

Effect of lentinan on viability of human astrocytoma U251 cells

In order to investigate the effect of lentinan (0, 25, 50, 100, 200, 400, 500, 600 μg/ml) with different

concentrations on proliferation of human astrocytoma U251 cells, we detected the cell activity using a CCK8 kit. The results are shown in Figure 1; compared with the blank control group, 50 and 100 μg/ml lentinan significantly promoted the viability of U251 cells ($p < 0.05$). When the concentration was more than 100 μg/ml, the cell activity gradually decreased, and the cell activity was the lowest when the concentration was 600 μg/ml ($p < 0.05$).

Effect of lentinan on Ki-67 expression in human astrocytoma U251 cells

In addition to CCK8, we also detected the expression of Ki-67 by immunofluorescence. As shown in Figure 2, compared with the control group, 50 and 100 μg/ml lentinan can regulate the relative expression of Ki-67 in human astrocytoma U251 cells. However, lentinan above 200 μg/ml can effectively inhibit the expression of Ki-67 in human astrocytoma U251 cells, and the expression of Ki-67 is the lowest at 600 μg/ml. The results showed that low-concentration lentinan could promote proliferation of human astrocytoma U251 cells, while high-concentration lentinan could inhibit proliferation of human astrocytoma U251 cells.

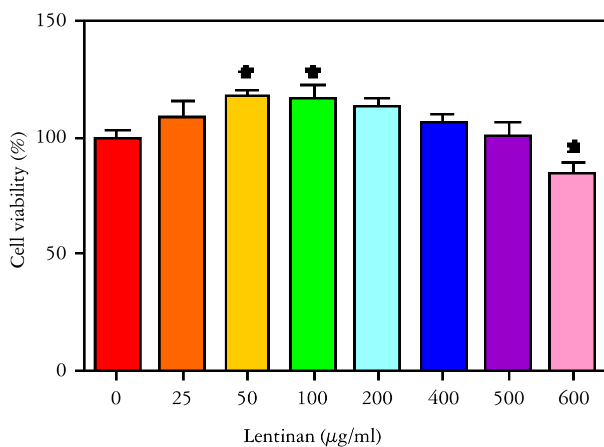

Fig. 1 CCK8 was used to detect the effect of lentinan with different concentrations on proliferation of human astrocytoma U251 cells. *P < 0.05, compared with the blank control group (0 μg/ml).

Effect of lentinan on apoptosis of human astrocytoma U251 cells

In order to investigate the effect of lentinan (0, 25, 50, 100, 200, 400, 500, 600 μg/ml) with different concentrations on apoptosis of human astrocytoma U251 cells, we detected the apoptosis rate by flow cytometry. Results are shown in Figure 3; compared with the blank control group, the low concentration lentinan (25, 50 and 100 μg/ml), had no significant effect on apoptosis of human astrocytoma U251 cells. In addition, more than 200 μg/ml lentinan significantly promoted apoptosis of human astrocytoma U251 cells and had a concentration gradient effect, and the highest apoptosis rate was at 600 μg/ml.

Discussion

Our study showed that lentinan can effectively inhibit proliferation of human astrocytoma U251 cells and promote apoptosis. A low concentration of lentinan can promote proliferation of human astrocytoma U251 cells. A high concentration of lentinan can inhibit proliferation of human astrocytoma U251 cells.

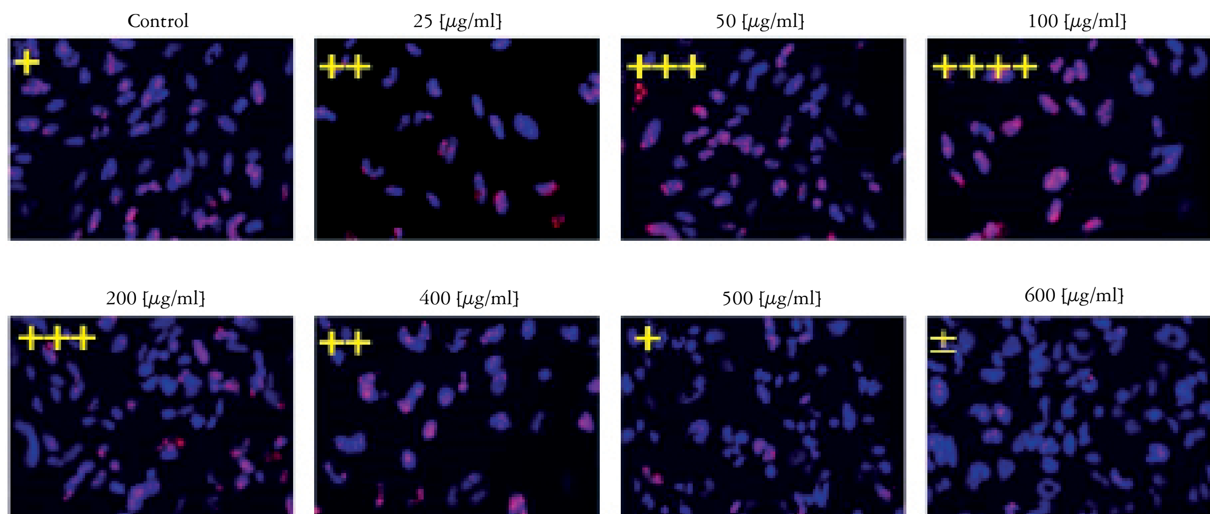

Fig. 2 Expression of Ki-67 in human astrocytoma U251 cells was detected by immunofluorescence. Observe the specific fluorescence intensity of the specimen. Generally, „+" can be used to indicate: (–) no fluorescence; (±) extremely weak, doubtful fluorescence; (+) fluorescence is weak, but clearly visible; (++) fluorescence is bright; (+++ –++++) fluorescence shines. The specific fluorescence staining intensity of the specimen to be tested is above „++," and various controls show (±) or (–). It can be judged as positive

However, the low concentration of lentinan had no significant effect on apoptosis of human astrocytoma U251 cells, while the high concentration of lentinan had a positive correlation with apoptosis of human astrocytic U251 cells.

Lentinan, as an immunomodulator, plays a role by improving the function of T cells and macrophages, promoting proliferation of T and B lymphocytes and enhancing the activity of NK cells, activating the toxic effect of macrophages and the production of tumor necrosis factor to kill tumor cells [7, 8]. Lentinan has gradually become a research hotspot in cancer treatment due to its relative safety and relatively minor side effects. The effect of lentinan in chemotherapy of lymphoma, lung cancer, gastric cancer, colon cancer, and other tumors has been gradually recognized [9–11]. Recent studies have shown that lentinan can not only stimulate the immune system of the body to play an anti-tumor role but also directly kill tumor cells. In clinical application, lentinan is often used as an immunomodulator in the adjuvant treatment of tumors [7, 8, 12].

Astrocytoma is the most common primary malignant tumor in the nervous system. It is characterized by rapid growth, substantial invasion, a high recurrence rate, and mortality, which seriously affect patients' quality of life [3]. Long non-coding ribonucleic acids (lncRNAs) have been recognized as markers in several cancers and play essential roles in glioblastoma (GBM). The effects of the X inactive specific transcript (XIST) gene may promote cellular malignancy of GBM through the miR-448/ROCK1 axis, which will provide a new understanding of GBM pathogenesis and progression [13]. However, at present, the surgical resection of glioma is still the preferred treatment method. After the operation, it is assisted with radiotherapy, chemotherapy, immunotherapy, and other comprehensive treatments, but the overall treatment effect is not satisfactory [14]. To find the best treatment combination of gliomas is the common goal of researchers.

It has been found that lentinan injection combined with cisplatin in the treatment of malignant pleural effusion has the effect of improving the quality of life, increasing the efficiency and reducing the toxicity of patients, obviously reducing fever, chest pain, and other symptoms, and it can be used as an essential auxiliary drug for chemotherapy [15]. The combination of S-1 and cisplatin is the standard first-line treatment for advanced gastric cancer. The combination of lentinan and S-1/cisplatin can improve the overall survival rate of gastric cancer patients [16]. By inhibiting the invasion of B16-BL6 and HCT-8 cells, lentinan can significantly inhibit the growth and metastasis of transplanted melanoma and colon cancer cells and can also reduce the accumulation of selenium in the liver and kidney tissue of mice. Lentinan, as a drug carrier, can significantly improve the antitumor activity of selenium [17].

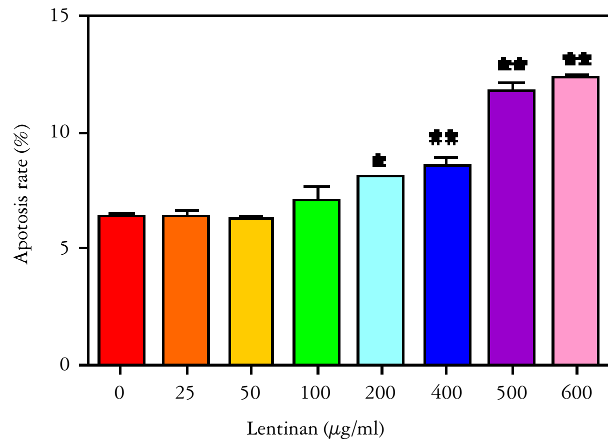

Fig. 3 Flow cytometry was used to detect the effect of lentinan with different concentrations on apoptosis of human astrocytoma U251 cells. $*P < 0.05$, compared with the blank control group (0 μg/ml)

The role of lentinan in glioma is rarely studied in the world, and the role of lentinan in gliomas has not been confirmed. U251, C6, U87, and other cell lines are commonly used in in vitro experiments on astrocytoma [18]. In this study, the effects of different concentrations of lentinan on proliferation of human astrocytoma U251 cells were investigated. It was found that a low concentration of lentinan could significantly promote proliferation and up-regulate relative expression of Ki-67, but had no significant effect on apoptosis of human astrocytoma U251 cells. In contrast, a high concentration of lentinan can inhibit proliferation and promote apoptosis of human astrocytoma U251 cells, and has a concentration gradient effect, which proves that lentinan can effectively inhibit proliferation of human astrocytoma U251 cells and promote apoptosis. Low concentration of lentinan can promote proliferation and upregulate the relative expression of Ki-67 in human astrocytoma U251 cells, which may be related to the mechanism of action of lentinan. At the same time, we think that the effect of different concentrations on the experimental results may be due to the fact that lentinan can prevent or inhibit the mitosis of U251 cells, decrease the number of cells in S phase and increase the number of cells in G0/G1 phase, so that U251 cells are blocked in G1 phase, thus inhibiting their proliferation. However, the specific mechanism needs to be confirmed in the next step.

However, there are still many limitations in our research. Firstly, like other traditional Chinese medicines, lentinan is challenging to penetrate the blood-brain barrier of the central nervous system. Applying the results of in vitro experiments to clinical treatment is still challenging. The advantages of new formulations of traditional Chinese medicine in the treatment of glioma have gradually emerged, and

the research level of targeted Chinese medicine in glioma is also increasing [19, 20]. Secondly, the mechanism of the effect of lentinan on proliferation and apoptosis of human astrocytoma U251 cells is still unclear. In addition, the sample size of this study is small, which may have a particular impact on the results. Furthermore, due to the limitation of experimental conditions, we used only one astrocyte line in our experiment, and only aimed at the control between different concentrations and blank groups, which may have a certain impact on the experimental results. We hope that in the future research, we can further explore the effect of lentinan on human astrocytes on the basis of current experiments, in order to reach more valuable conclusions.

To sum up, lentinan can effectively inhibit the proliferation of human astrocytoma U251 cells, promote cell apoptosis, and has a concentration gradient effect, which provides the experimental basis for the auxiliary treatment of glioma. However, the mechanism of lentinan's role in glioma, how to penetrate the blood-brain barrier, and how to function in vivo need more in-depth research.

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The authors declare no conflict of interest.

References

1. Wu X, Li Y, Wan X, et al. Down-regulation of neogenin accelerated glioma progression through promoter methylation and its overexpression in SHG-44 induced apoptosis. PLoS One 2012; 7: e38074.
2. Zhang CP, Li HQ, Zhang WT, et al. Clinical manifestations and imaging characteristics of gliomatosis cerebri with pathological confirmation. Asian Pac J Cancer Prev 2014; 15: 4487-4491.
3. Scharl S, Straube C, Meyer B, et al. Critical consideration of the European Association for Neuro-Oncology (EANO) guideline on the diagnosis and treatment of adult astrocytic and oligodendroglial gliomas. Strahlenther Onkol 2017; 193: 984-988.
4. Van den Bent MJ, Chang SM. Grade II and III Oligodendroglioma and astrocytoma. Neurol Clin 2018; 36: 467-484.
5. Chihara G, Maeda Y, Hamuro J, et al. Inhibition of mouse sarcoma 180 by polysaccharides from Lentinus edodes (Berk.) sing. Nature 1969; 222: 687-688.
6. Wang Y, Jin H, Yu J, et al. Quality control and immunological activity of lentinan samples produced in China. Int J Biol Macromol 2020; 159: 129-136.
7. Deng S, Zhang G, Kuai J, et al. Lentinan inhibits tumor angiogenesis via interferon γ and in a T cell independent manner. J Exp Clin Cancer Res 2018; 37: 260.
8. Liu HJ, Qin Y, Zhao ZH, et al. Lentinan-functionalized Selenium nanoparticles target tumor cell mitochondria via TLR4/TRAF3/MFN1 pathway. Theranostics 2020; 10: 9083-9099.
9. Wang XE, Wang YH, Zhou Q, et al. Immunomodulatory Effect of lentinan on aberrant T subsets and cytokines profile in non-small cell lung cancer patients. Pathol Oncol Res 2020; 26: 499-505.
10. Liu Y, Zhao J, Zhao Y, et al. Therapeutic effects of lentinan on inflammatory bowel disease and colitis-associated cancer. J Cell Mol Med 2019; 23: 750-760.
11. Zhang M, Zhang Y, Zhang L, Tian Q. Mushroom polysaccharide lentinan for treating different types of cancers: A review of 12 years clinical studies in China. Prog Mol Biol Transl Sci 2019; 163: 297-328.
12. Yoshino S, Yoshida S, Maeda N, et al. [Clinical evaluation of the combination treatment of intrapleural or intraperitoneal administration of lentinan and OK-432 for malignant effusion]. Gan To Kagaku Ryoho 2010; 37: 2798-800.
13. Li HL, Han PH, Pan DQ, et al. LncRNA XIST regulates cell proliferation, migration and invasion of glioblastoma via regulating miR-448 and ROCK1. J Biol Regul Homeost Agents 2020; 34: 2049-2058.
14. Weller M, van den Bent M, Tonn JC, et al. European Association for Neuro-Oncology (EANO) Task Force on Gliomas. European Association for Neuro-Oncology (EANO) guideline on the diagnosis and treatment of adult astrocytic and oligodendroglial gliomas. Lancet Oncol 2017; 18: e315-e329.
15. Congcong Q, Hengting Z, Shuhui L, et al. Evaluation of efficacy and safety for lentinan in the control of the malignant pleural effusions via intrapleural injection. Am J Med Sci 2019; 358: 400-411
16. Ina K, Furuta R, Kataoka T, et al. Chemo-immunotherapy using lentinan for the treatment of gastric cancer with liver metastases. Med Sci (Basel) 2016; 4: 8.
17. Liu YR, Sun B, Zhu GH, et al. Selenium-lentinan inhibits tumor progression by regulating epithelial-mesenchymal transition. Toxicol Appl Pharmacol 2018; 360: 1-8.
18. Souza PO, Bianchi SE, Figueiro F, et al. Anticancer activity of flavonoids isolated from Achyrocline satureioides in gliomas cell lines. Toxicol In Vitro 2018; 51: 23-33.
19. Zhang Y, Hu Y, Li M, et al. The traditional Chinese medicine compound, GRS, alleviates blood-brain barrier dysfunction. Drug Des Devel Ther 2020; 14: 933-947.
20. Zhang X, Liu T, Fan X, Ai N. In silico modeling on ADME properties of natural products: classification models for blood-brain barrier permeability, its application to traditional Chinese medicine and in vitro experimental validation. J Mol Graph Model 2017; 75: 347-354.

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