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Anti-tumor activity and related mechanism study of *Bacillus Polymyxa* transformed *Panax ginseng* C. A. Mey

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ABSTRACT

Panax ginseng C. A. Mey has a history of drug use for thousands of years, and ginsenoside is the main active ingredient. Whether the conversion products of ginseng by *Bacillus polymyxa* can enhance the anti-tumor activity is to be further studied. This paper aims to study the anti-tumor activity of transformed ginsenoside products by *Bacillus polymyxa* in vivo and vitro, the results were as follows: In vitro, apoptosis mainly occurred in the late stage for breast cancer cells MCF-7, and the conversion products had better anti-tumor effect than ginseng group ($P < 0.05$). In vivo, Compared with the model group, the low, medium and high dose group could inhibit the growth of H22 tumor, improve the organ index of mice, promote the H22 tumor apoptosis ($P < 0.05$), increase the proliferation activity of lymphocyte cells and $CD4^+/CD8^+$ ratio, improve the contents of IgG, IgM, IgE, TNF-α, IFN-γ, IL-2, IL-6, IL-12, decrease TGF-β that can inhibit IL-2 in serum. The conversion products could enhance cell immunity, organ immunity, humoral immunity and anti-tumor effect. In particular, TNF-α activity was increased while TGF-β was reduced. Positive drug 5-FU had the effects of improving cell and organ immunity, but also had certain side effects.

1. Introduction

Malignant tumors are still a worldwide problem. Ginsenosides, the main active ingredient of *Panax ginseng* C. A. Mey, which have a significant degree of anti-tumor effect. They could play an anti-tumor role by inducing tumor cell apoptosis, inhibiting tumor angiogenesis, enhancing immunity and up-regulating tumor suppressor gene p53 [1–3]. Ginsenoside Rg3 could inhibit the expression of VEGF in human liver cancer cells under hypoxic condition, and inhibit the proliferation of tumor cells [4]. Ginsenoside Rh1 could inhibit the expression of matrix metalloproteinases in human liver cancer cells by inhibiting the activity of activated protease1 [5]. Oh [6] found that ginsenoside CK could promote the release of cytochrome C and form an apoptosis cycle when studying the mechanism of ginsenoside CK induced apoptosis. Liu [7] used ginsenoside Rg1 pyrolysis products in transplanted tumor model of H22 hepatocarcinoma in mice and found that saponins had anti-tumor effect in vivo. Furthermore, ginsenoside monomer Rd could suppress human breast cancer cell and gastric cancer [8], ginsenoside Rg1 had an inhibiting effect on colon cancer in vivo and in vitro [9], ginsenoside Rg5 could suppress breast cancer cell [10].

In addition to ginsenoside monomer, ginsenoside derivatives also have anti-tumor activity. Bi [11] modified the natural compound 25-OH-PPD in ginseng, found that its derivatives could inhibit human lung cancer cell, breast cancer cell and colon cancer cell. Chen [12] used fungi *Absidia corymbifera* to transform 20(S)-protopanaxadiol, its products had a significant inhibitory effect on prostate cell. Moreover, ginsenoside derivatives transformed by microbial could also inhibit human lung cancer [13], gastric cancer, oral cancer and human fibrosarcoma [14].

The content of ginsenoside in ginseng is low, especially rare ginsenoside content is lower, how to obtain ginsenoside has become the focus of research. Microbial conversion method is low cost, easy to control conditions, at the same time, fewer by-products, as the hot spot of transformation of ginsenosides to improve the anti-tumor activity. Studies found that using different ginsenoside monomers as substrates can be transformed to ginsenoside Rh2, CK, Rd, Rg3, Rc, Rg1, F1, F2, C-Mc, and C–Y respectively, by microbial conversion [15–31]. A new compound was also obtained by transformation of *Absidia corymbifera AS 3.3387* with 20 (R) protopanaxadiol as substrate [32].

Bacillus polymyxa is a class of probiotics of Gram-positive bacteria, which is widely distributed in nature, has the function of promoting [33] and improving immunity [34,35], and is classified as one of the commercially available strains by the US Environmental Protection Agency (EPA), China's Ministry of Agriculture classified it as a safety-

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free identification of a bacteria as well. With the gradual deepening of anti-tumor research on microbes, the application of *Bacillus polymyxa* in anti-tumor will also become a new research direction.

2. Materials and methods

2.1. Materials and instruments

Bacillus polymyxa was from our laboratory, preserved in the China General Microbiological Culture Collection Center (CGMCC No.7250). The fresh four-year-old ginseng was collected from the Medicinal Plant Garden in Jilin Agricultural University. Ginsenoside standards were purchased from Jilin University (Jilin, China). RPMI-1640 medium was from U.S. Hyclone Company. Kits for IL-2, IL-6, IL-12, TNF-α, IFN-γ, TGF-β, IgG, IgE and IgM ELISA were purchased from Nanjing Jiancheng Bioengineering Institute, PE anti-mouse CD3, PE/Cy5 anti-mouse CD8 and FITC anti-mouse CD4 were from U.S. BioLegend Company, Annexin V-FITC, was from U.S. BD Company, CMC-Na, ConA, and MTT were purchased from U.S. Sigma–Aldrich Company, Hanks balanced solution, trypsine were from Gibco Company, 5-fluorouracil was purchased from Jinyao Pharmacy Company, fetal bovine serum was purchased from Tianhang Biological Engineering Company(batch number: 20150914). Culture flasks were from U.S. Corning Company. MCF-7 cells line was from College of Life Science, Northeast Normal University. Hepatoma cells line H22 was from Jilin Cancer Hospital. Flow cytometry(Merck, China), Microplate reader(Bio-Rad, U.S.).

2.2. Anti-tumor experiments in vitro

2.2.1. Preparation of the conversion products of ginsenoside by Bacillus polymyxa

Three sterile test tubes were taken and ginseng powder was added to them, each test tube for 500 mg, sealed with test tube plug, dried and sterilized for 2 h at 80℃ in the oven and cooled to room temperature in the clean bench. Then 1 ml (OD_{600} = 0.5) *Bacillus polymyxa* solution and 4 ml sterile water were added by pipetting. The same operation was performed in three other tubes (Replace *Bacillus polymyxa* solution with sterile water). After transferring, the tubes were sealed and the samples were cultured in constant temperature shaking incubator for 8 days. Three sterilized penicillin vials were taken and ginsenoside monomer (Rb1,Rb2,Rb3,Rd,Rc,Rg1,Rg2) were placed in them, each vial for 5.00 mg. Adding 2 ml sterile water, then adding 150 μl *Bacillus polymyxa* solution respectively, and the samples were shaken for 8 days at 120 rpm/min, 28℃. The above conversion products were extracted by ultrasonic extraction.

2.2.2. Apoptosis testing

The logarithmic growth phase of breast cancer cells MCF-7 were seeded in 6-well plates at 1×10^6 cells/well, 2 ml per well. 0.3 ml sample drug was added into each well(Concentration were 250, 125, 100, 62.5, 31.25 μg/ml). After 80% of the cells adhered to the wall of the culture flask, digested with 0.25% trypsine. Each group of the cells were collected and blowed into single cell suspension, respectively. Then, They were centrifuged at 800 rpm/min for 5 min and washed with PBS solution, centrifuged again. The supernatant was discarded and the cells were resuspended in 500 μl Buffer. RnaseA was added to a final concentration of 0.25 mg/ml, 37℃ for 30 min, then 5 μl of PI dye was added and stained for 30 min at room temperature. Apoptosis assay was performed according to the kit by flow cytometry.

2.3. Anti-tumor experiments in vivo

2.3.1. Sample preparation

The preparations of conversion products of ginseng by *Bacillus polymyxa*, ginseng group, bacterial fluid group and original powder group were similar to that of 2.2.1, and all of the these were diluted with CMC-Na to the concentration of drug for pharmacological experiments.

2.3.2. Experimental animals

ICR mice (female) weighed 19–22 g were purchased from Jilin University, and all feeds were provided by Jilin University as well. Sufficient food and drinking water were provided to the mice, free feed, housed at room temperature. The female mice were randomly divided into 9 groups (N = 16) as follows: tumor model group,blank control group (negative control group), positive control group, conversion products of ginseng group (low, medium and high dose group), ginseng group, bacterial fluid group, original powder group.

2.3.3. Establishment of tumor model

After H22 ascites tumor mice were grown to a certain extent, killed at the cervical spine and the tumor solution was removed under sterile condition. The tumor solution was diluted with physiological saline to the cell concentration of 1×10^7 cells/ml, 0.2 ml tumor solution was received to the right breast of each group of mice. The normal control group were injected with the same amount of saline.

2.3.4. Administration and feeding

The mice were injected with tumor solution a week later, intragastric administration. The control group and the tumor model group were inoculated with normal saline (0.4 m/d), the positive control group was injected with 5-Flu (20 mg/kg), low dose group (3.9 mg/0.4 ml/d), medium dose group (7.8 mg/0.4 ml/d) and high dose group (15.6 mg/0.4 ml/d), ginseng group (7.8 mg/0.4 ml/d), bacterial fluid group (0.4 ml/d), original powder group (7.8 mg/0.4 ml/d). Each group of mice was given the drug continually by weight for 10 days (0.4 ml/20 g).

2.3.5. Immune organ index

The mice were dosed 10 days later and then killed. The tumor, heart, kidney, thymus and spleen were taken out. The rate of tumor inhibition, heart index, kidney index, spleen index and thymus index were calculated, the materials were saved quickly and made into specimens. The rate of tumor inhibition (%) = (1 − the tumor weight of the treatment group/the tumor weight of the control group) × 100%,organ index (mg/g) = 1000 × organ weight (mg)/body weight of mice(g).

2.3.6. HE staining of spleen and tumor tissue of H22 mice

The solid tissues were made into 4 μm paraffin sections, spread sequentially in 30–40% alcohol for 3 min and 38℃ water for 5 min, and then they were picked up, stored in an oven at 37℃ overnight, finally kept at room temperature. The sections were immersed in xylene to remove paraffin for three times, 15 min each time. Then immersed in 100%, 95%, 80% alcoholrespectively for twice, 2 min each time, washed by distilled water. Immersed in hematoxylin to dye for 6 min, washed by distilled water. Immersed in 0.1% hydrochloric-alcohol solution to color separation for several seconds, washed by distilled water. Backed to blue by 0.5% ammonia, washed by distilled water for 3 times. Immersed in the eosin stain for several seconds, washed by distilled water. And then immersed in 80%, 95%, 100% alcohol for several seconds respectively, dehydrated for 2 min by 100% alcohol. After that, dried for 10 min at 64℃, 1 drop of neutral balsam was added and coverslips were covered. Morphological changes of spleen and tumor cells were observed under optical microscope.

2.3.7. Tumor tissue apoptosis of H22 mice

In the sterile conditions, the tumor tissues were placed in sterilized petri dishes, a little trypsine was added, and then tissues were incubated at room temperature. The tumor tissue cells were blown down by pipetting, a little cell culture medium was added, and transferred them to sterile small centrifuge tubes after mixed. They were centrifuged at 1000 × *g* for 5 min, washed with PBS solution, and then counted.