Therapeutic mechanism of ginkgo biloba exocarp polysaccharides on gastric cancer

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AIM: To study the therapeutic mechanism of Ginkgo biloba exocarp polysaccharides (GBEP) on gastric cancer.

MATERIALS AND METHODS

Patients
A total of 30 patients with gastric adenocarcinoma (20 males aged from 28 to 81 years old, 10 females aged from 52 to 75 years old) were all ascertained by the pathological examination in Jiangsu Provincial Subei People Hospital or the First People Hospital of Yangzhou, China. Their Karnofsky scores were all above 60. All the patients did not receive any other anti-tumor treatments recently.

Cell line
Human gastric cancer cells (SGC-7901) purchased from Department of Cellular and Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology Academia Sinica, were sub-cultured every 2 or 3 days.

Reagents
GBEP was extracted from exocarp of ripe ginkgo biloba. The content of polysaccharides was higher than 80 %. RPMI 1640 was from GibcoBRL(Maryland, USA). MTT and trypsin (1:250) were from Sigma (ST. Louis, USA). Monoclonal mouse anti-human c-myc and bcl-2 were purchased from Antibody Diagnostica Inc., USA. Monoclonal rabbit anti-human c-fos was from Santa Cruz (Santa Cruz, USA).

Methods
Influence of GBEP on gastric carcinoma patients GBEP capsules are composed of GBEP dry powder and a certain proportion of excipient, and 0.25 g per capsule. Each patient with gastric carcinoma was treated with oral GBEP capsule, 2 pills each time, twice a day, for over 30 d. Changes of tumor size were measured by electron gastroscope. The inhibitory rates (IR) were calculated according to the formula: IR=(tumor area before treatment - tumor area after treatment) ÷ tumor area before treatment×100 %, which is used in the assessment of therapeutic effects. The assessment followed the clinical assessment standards for solid tumors made by WHO, which are classified as complete response (CR), partial response (PR), stable disease (SD) or no change (NC), and progressive disease (PD). The effective rate equals CR plus PR. At the meantime tumor biopsies were obtained for ultrastructural examination by transmissional electron microscope (HV-300). Images captured by transmissional electron microscope were analysed, and the nucleocytoplasmic ratio as well as the surface density of heterochromatin in the tumor cells were calculated before and after treatment.

MTT experiment SGC-7901 cells growing exponentially were digested by 0.25 % trypsin for 1-2 minutes, then washed in Hanks’ balanced salt solution (HBSS) for 2 times, and RPMI
1640 containing 10% new born bovine serum medium was added to adjust the cell density to 1×10^6 cells/L. After addition of the final cell suspensions of 100 μl/well, 96-well plates were put into an incubator containing 5% CO2 and incubated at 37°C for 24 hours. Then, 100 μl RPMI 1640 containing different concentrations of GBEP was added to each well. Each concentration had 3 wells, and the control was added with 100 μl RPMI 1640. They were cultured for 24 hours, 48 hours and/or 72 hours. Fresh medium was changed per 24 hours, and GBEP was added. Four hours before the end of culture, 10 μl MTT (the final concentration was 5 μL/L) was added, and cultured for 4 hours. Optical density (OD) values for each well were measured at 570 nm with the enzyme linked immunosorbent assay meter. The inhibitory rates were calculated according to the formula: IR=[1-(the mean of treated group)/(the mean of control group)]×100%.

**Measurement of cell apoptosis** SGC-7901 cells growing exponentially were digested with 0.25% trypsin for 1-2 minutes. After that, the cells were washed 2 times with PBS buffer (pH 7.2), counted and mixed with RPMI 1640 containing 10% new born bovine serum to create a final cell density of 2×10^5 cells/L. Four ml final cell suspension was added into each culture bottle, and cultured for 24 h in the condition of 5% CO2 at 37°C. Then the culture bottles were randomly mixed with different concentrations of GBEP or a positive control drug adriamycin. The negative control group was mixed with an equal volume of RPMI 1640. Then, all the culture bottles were cultured for another 48 h. After that, they were digested and washed. At last they were fixed with alcohol and kept at 4°C. The tumor cells were fixed with alcohol and kept in citrate buffer for at least 1 hour after washed in PBS to create a final cell density of 1×10^6 cells/L, they were then centrifuged and mixed with 1 800 μl solution A (trypsinization solution). After 10 minutes, they were mixed with 1 500 μl solution B (RNASE) for 20 minutes, then mixed with 1 500 μl solution C (PI) and filtered by a nylon net after 15 minutes. Finally, the apoptotic rate of the cells was examined.

**Analysis of protein content** Cell culture was carried out as previously described. The SGC-7901 cells were centrifuged (2 000 ω/minute for 5 minutes) after washed in PBS, mixed with monoclonal mouse anti-human c-myc, or bcl-2, or rabbit anti-human c-fos, and kept at 4°C for 45 minutes. The cells were washed in PBS and mixed with sheep anti-mouse or sheep anti-rabbit IgG and kept at 4°C for another 45 minutes. After washed in PBS and centrifugation, the cells were mixed with 300 μl PBS in sediment and the rate of positive protein for c-myc, bcl-2 and c-fos gene was measured by flow cytometry.

**RESULTS**

**Effect of GBEP capsules on gastric cancer cells**

Compared with that before treatment, the tumor area was apparently reduced, which was further proved by electron gastroscopy, and the inhibitory rate of GBEP on tumors was 53.5%. According to the standards proposed by WHO for the short-term therapeutic effectiveness of solid tumors, there were 2 cases of CR (6.7%), 20 PR (66.7%), 5 SD (16.7%), 3 PD (10%) in the 30 cases, and the total effective rate was 73.4%. Images captured by transmissional electron microscope showed that most of the cancer cells had sufficient euchromatin but deficient heterochromatins in the nuclei, the cancer cells had sufficient free ribosomes and deficient glycogens in the cytoplasm before treatment. After treatment with GBEP, most of the cancer cells had sufficient heterochromatins in the nuclei. Some cancer cells became pyknosis. Heterochromatin margination was seen in some of the cancer cells (in the course of apoptosis). Some euchromatins were dissolved, mitochondria were swollen, and rough endoplasmic reticulum was dilated.

**Inhibition of GBEP on human gastric cancer SGC-7901 cells**

GBEP could inhibit SGC-7901 cell proliferation following 24-72 hours treatment in vitro at 10-320 mg/L. Compared with the control group, the inhibition of SGC-7901 cell proliferation by GBEP was dose- and time-dependent (P<0.01) (Figure 2).
**Effects of GBEP on human gastric cancer SGC-7901 cell apoptosis**

DNA contents of human gastric cancer SGC-7901 cells were analysed by flow cytometry. The results showed that GBEP could induce apoptosis in SGC-7901 cells at a certain degree (Figure 3).

**Effect of GBEP on expression of c-myc, bcl-2 and c-fos genes in SGC-7901 cells**

Protein contents of human gastric cancer SGC-7901 cells were analysed by flow cytometry. The results showed that GBEP could inhibit the expression of c-myc and bcl-2 genes, but enhance the expression of c-fos in SGC-7901 cells (Table 1).

| Group       | Dose (mg/L) | Rate of positive protein sign (%) |
|-------------|-------------|-----------------------------------|
|             |             | c-myc    | bcl-2    | c-fos    |
| RPMI 1640   | -           | 22.05    | 19.35    | 12.68    |
| GBEP        | 40          | 20.33    | 15.29    | 17.35    |
| GBEP        | 80          | 12.50    | 11.74    | 24.96    |
| GBEP        | 160         | 7.34     | 7.17     | 45.26    |
| Adriamycin  | 2           | 9.67     | 9.31     | 68.01    |

**DISCUSSION**

Gastric cancer is one of the most common malignant tumors in China. Surgical treatment is the main therapy of it. Anti-tumor drugs still play an important role in comprehensive therapy. Now cytotoxic compounds remain the main part of the chemotherapy drugs. The main defects of the cytotoxic compounds are the poor therapeutic effects on solid tumors, higher toxic side-effects and easy occurrence of drug resistance. Many Chinese drugs can enhance the immune function of the body. When used in the treatment, they showed less toxic side-effects but lower inhibitory rate on tumors.

Polysaccharides are big molecules linked by monosaccharides. The sugar-chain of polysaccharides can regulate cell proliferation, differentiation, growth and aging. They showed definite therapeutic effectiveness in anti-tumor therapy, and the ability to enhance body’s immune function, as well as a lower toxic side-effect[7-10]. For example, mushroom polysaccharides have already been used as a drug to regulate the organism reaction in clinical therapy and to prevent tumors in Japan. Umbellate pore fungus polysaccharides which were developed and used in clinical therapy in China, could reduce side-effects of chemotherapy and enhance the effects of chemotherapy against tumors.

We performed this clinical experiment by treating 30 gastric cancer patients with oral GBEP capsules. The images captured by electron gastroscope showed the average inhibitory rate of its capsules on gastric tumor was 53.5 %. The effective rate was 73.4 %. It indicated that GBEP had good clinical therapeutic effectiveness on gastric cancer.

Apoptosis is an active cellular process whereby individual cells are triggered to undergo self-destruction. Recent studies showed apoptosis played a main role in the prevention and treatment of tumors[11-13]. Anti-tumor effect of many chemotherapy drugs could induce apoptosis in tumor cells[14,15]. Cell apoptosis was regulated by genes[16,17]. We have known that apoptosis regulators can be divided into two kinds, namely apoptosis-inducing genes and apoptosis-inhibitory genes. Up-regulation of apoptosis-inducing gene expression could elevate the sensitivity of cells to factors or signals inducing apoptosis, and trigger apoptosis in this way. Up-regulation of apoptosis-inhibitory gene expression could reduce the sensitivity of cells to factors or signals inducing apoptosis, and apoptosis could be inhibited or delayed in this way. Bcl-2 was an important apoptosis-inhibitory gene[18,19], it included a nucleus molecule that can block cell apoptosis, prolong cell lives, accelerate DNA repairing, and thus promoting tumor genesis and development. So it could down-regulate cell apoptosis[20-22]. This clinical study and examination of cellular ultrastructures showed clues of apoptosis induced by GBEP in human gastric cancer cells. Using techniques of cell culture in vitro and flow cytometry, the contents of DNA and protein of human gastric cancer SGC-7901 cells were analysed. The results showed GBEP could increase SGC-7901 cell apoptosis rate, down-regulate bcl-2 at concentrations of 40-160 mg/L. It indicated that one of the therapeutic mechanisms of GBEP on gastric cancers might be that it induced tumor cell apoptosis. It also indicated that bcl-2 was involved in this process.

Malignant cells are similar to undifferentiated embryonic cells in morphology, function and metabolism. When tissue changes into malignancy, many phenotypes of the cells go back to the embryonic cell phenotypes, which is called de-differentiation or retro-differentiation. Malignant cells can be induced to differentiate towards normal cells in the presence of differentiation-inducer. Many malignant cells can approach to normal cells, even transform into normal cells completely, which is called re-differentiation or reversion. Change of cells from normal to malignancy is a break of the balance between proliferation and differentiation. Uncontrollable proliferation and de-differentiation are the characteristics of most malignant tumors. Differentiation-inducers can decelerate proliferation, enhance differentiation, thus creating a new normal balance. Like apoptosis, proliferation and differentiation are regulated by genes. C-myc is an important gene involved in the control of cell proliferation, and could up-regulate cell cycle progression, and induce cell proliferation[26-32]. C-fos gene is considered as an early response gene, and its expression level was in proportion to the differentiation degree of gastric cancer[33]. This clinical study and results of the cell ultrastructural examination showed clues of apoptosis induced by GBEP in human gastric cancer cells. The results of MTT experiment in vitro showed GBEP could inhibit the proliferation of human gastric cancer SGC-7901 cells. The results measured by flow cytometry showed GBEP could down-regulate the expression of c-myc gene and up-regulate the expression of c-fos genes in SGC-7901 cells at the concentrations of 40-160 mg/L. It indicates that inhibition on cell proliferation and induction on cell differentiation might be involved in the therapeutic mechanism of GBEP on gastric cancer. C-myc and c-fos genes might contribute to the regulation of proliferation and differentiation.

**ACKNOWLEDGMENTS**

We are very grateful to Drs. Huo-Ying Shi, Li-Ming Yuan…
and Wei-Dong Zhou, Center of Electroscope, Yangzhou University, and Mei-Zao Le, Department of Pathology, Bayi Hospital, Nanjing. China for their technical assistance in ultrastructural analysis; Drs. Zhi-Jiang Wu, Lü-Rong Men, Department of Cellular and Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Academica Sinica for their technical assistance in flow cytometry.

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Edited by Zhang JZ and Wang XL