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• LIVER CANCER •

Apoptosis of hepatoma cells SMMC-7721 induced by *Ginkgo biloba* seed polysaccharide

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Abstract

AIM: To study the apoptosis of hepatoma cells SMMC-7721 induced by polysaccharide isolated from *Ginkgo biloba* seed.

METHODS: *Ginkgo biloba* seed polysaccharide (GBSP) was isolated by ethanol fractionation of *Ginkgo biloba* seed and purified by Sephadex G-200 chromatography. The purity of GBSP was verified by reaction with iodine-potassium iodide and ninhydrin and confirmed by UV spectrophotometer, cellulose acetate membrane electrophoresis and Sepharose 4B gel filtration chromatography. The Scanning Electron Microscope (SEM) and Flow Cytometry (FCM) were used to examine the SMMC-7721 cells with and without GBSP treatment at 500 mg/ml for 36 h.

RESULTS: GBSP product obtained was of high purity with the average molecular weight of 1.86×10^5. Quantitative analysis of SMMC-7721 cells *in vitro* with FCM showed that the percentages of G_2-M cells without and with GBSP treatment were 17.01 ± 1.28 % and 11.77 ± 1.50% ($P<0.05$), the debris ratio of the cells were 0.46 ± 0.12 % and 0.06 ± 0.06 % ($P<0.01$), and the apoptosis ratio of cells was 3.84 ± 0.55 % and 9.13 ± 1.48 %($P<0.01$) respectively. Following GBSP treatment, microvilli of SMMC-7721 cells appeared thinner and the number of spherical cells increased markedly. Most significantly, the apoptosis bodies were formed on and around the spherical cells treated with GBSP.

CONCLUSION: GBSP could potentially induce the apoptosis of SMMC-7721 cells.

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INTRODUCTION

Ginkgo biloba L., also named after white-seed tree and gongsun tree, is one of the immemorial gymnosperm of the mesozoic era. It is regarded as a living fossil and is also best known for its pharmaceutical value. According to Pen-ts' so Kan-mu (i.e. Compendium of Materia Medica), *Ginkgo biloba* L. can help cure about 20 different diseases. More recently, it has been widely accepted that flavonoid and terpeneand are the effective components of leaves of *Ginkgo biloba*[1-5] for treating cardiovascular and nervous system diseases, scavenging free radicals and antioxidating, etc.[6-11]. Water-soluble polysaccharides of *Ginkgo biloba* leaves, endocarp, seeds and cultured cells were isolated and purified and their structures and some biological activities such as immunoregulation, antineoplastic action, scavenging free radicals and antioxidating were identified[12-16]. Furthermore, *in vivo* and *in vitro* induction of apoptosis of cancer cells by polysaccharides has been reported lately[17-22]. However, there was no report about the effects on apoptosis of tumor cells by polysaccharides isolated from *Ginkgo biloba* L. except from *Ginkgo biloba* endocarp[23]. In this study, high-purity polysaccharide was extracted from *Ginkgo biloba* seeds and the apoptotic effect of *Ginkgo biloba* seed polysaccharides (GBSP) on hepatoma cell line SMMC-7721 was investigated by scanning electron microscope (SEM) and flow cytometry (FCM).

MATERIALS AND METHODS

Materials

High-quality *Ginkgo biloba* seeds (*i.e.* milkwhite color, equal weight, and smooth surface without mildew) were purchased from Jianlian Chinese Traditional Medicine Store in Jinan, Shandong Province. Hepatoma cell line SMMC-7721 was obtained from Shanghai Cell Institute, China Academy of Sciences. Culture medium RPMI1640 was obtained from Gibco Co. (USA). Sephadex G-200, Sepharose 4B, monose as standard, calf serum and propidium iodide (PI) were obtained from Sigma Co. (USA).

Isolation and purification of GBSP

Ginkgo biloba seeds of 200 g were crushed into fine particles and extracted with 3000 ml of distilled water for 8 hours at 75 ℃ for 3 times. The extracts were pooled, concentrated to 30 % of the original volume in a rotary evaporator at 45 ℃ and then centrifuged at 3000 rpm for 15 min. The supernatant was collected and added with 3 volumes of 95 % ethanol to precipitate the polysaccharide. Following centrifugation at 4000 rpm for 15 min, the polysaccharide pellet was dissolved in appropriate volume of distilled water completely, dialyzed with distilled water and decontaminated by means of Sevag to remove protein. The polysaccharide was then freeze-dried, re-dissolved in salt solution and purified further by Sephadex G-200 chromatography. The purity of the resulting GBSP was analyzed by Sepharose 4B gel filtration chromatography and cellulose acetate membrane electrophoresis[24].

Culture of SMMC-7721 cells and treatment with GBSP[25-28]

The SMMC-7721 cells were grown to logarithmic phase of proliferation, washed 3 times with culture medium RPMI1640 and collected at a concentration of 10^6 cells /ml. This cell suspension was then aliquoted into 6 culture bottles and cultured at 37 ℃ and 5 % CO_2 (CO_2 incubator, MCO-17AC, SANYO, Japan) for 24h. For cultures that were prepared for

SCM test, cover slips were placed into bottles in advance. After the cells stuck on the walls of culture bottles, GBSP solution made up with culture medium was added into 3 of 6 culture bottles at the final concentration of 500 mg/ml. The other 3 culture bottles were added with equal volume of culture medium. The cells were cultured for further 36 h under the same conditions.

Flow Cytometry[29-31]

Supernatants of the cultures were discarded and SMMC-7721 cells with and without GBSP treatment were collected by digestion with pancreatin followed by centrifugation. PI was added to the cells for 15 min to label DNA. FCM (FACS/420, Becton Dickinson, USA) was used to analyze cell cycles and apoptosis ratios.

Scanning Electron Microscopy[32-33]

Supernatants of the cultures were discarded and SMMC-7721 cells stuck on the cover slips with and without GBSP treatment were examined by SEM (S-570, Hitachi, Japan).

RESULTS

Characterization of GBSP

One of the objectives of this work was to obtain high-purity GBSP product from the G*inkgo biloba* seeds. The purity of GBSP was first tested by reactions with iodine-potassium iodide and ninhydrin respectively. The results of these two reactions were negative, indicating absence of starch and protein in the GBSP product obtained. The reaction of GBSP with Molish reagent was positive, indicating that the GBSP product was composed of monose. The GBSP solution was then analyzed by UV absorption (UV-1200, The Second Beijing Optical Instrument Manufactory, China). As shown in Figure 1, there were no peaks at wavelengths of 260nm and 280nm on the UV spectrum, indicating that the GBSP product was not contaminated with nucleic acid and protein. The purity of GBSP was further analyzed by Sepharose 4B gel filtration chromatography and cellulose acetate membrane electrophoresis. The elution profile of Sepharose 4B was a single symmetrical peak (Figure 2). The cellulose acetate membrane electrophoresis displayed a single stripe of GBSP on the membrane. These indicated that the present GBSP was of high purity. The average molecular weight of the GBSP was 1.86×10^5 based on the linear calibration curve derived from Sephadex G-200 chromatography, as shown in Figure 3.

G_2-M cell ratio with and without GBSP treatment

The percentages of G_2-M cells without and with GBSP treatment were 17.01%±1.28% and 11.77%±1.50%($P<0.05$) respectively (Figures 4, 5 and Table). This indicated that GBSP inhibited the proliferation of SMMC-7721 cells.

Table 1 The cycles and apoptosis ratios of SMMC-7721 cell with and without GBSP treatment

GBSP treatment (mg/ml)	Number of culture bottles tested	G_2-M cell ratio (%)	Apoptosis cell ratio (%)	Debris ratio (%)
0	3	17.01±1.28	3.84±0.55	0.46±0.12
500	3	11.77±1.50[a]	9.13±1.48[b]	0.06±0.06[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* control group (*t* test)

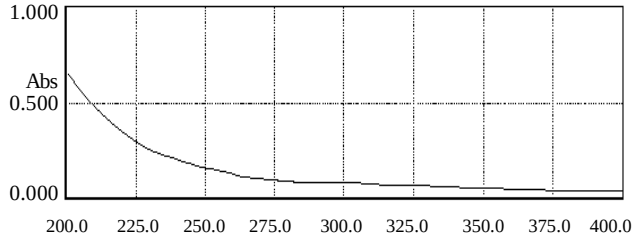

Figure 1 The UV spectrum adsorption curve of GBSP from 200nm to 400nm

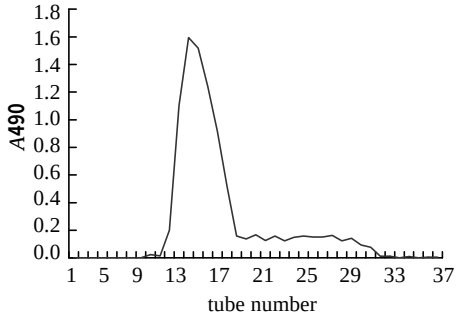

Figure 2 The profile of Sepharose 4B gel filtration chromatography

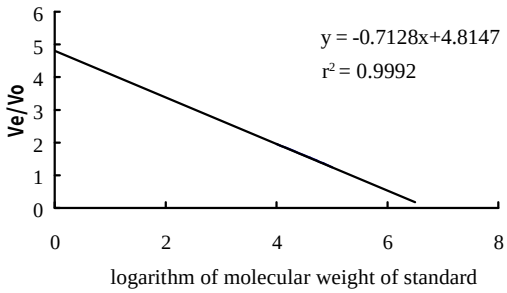

Figure 3 Standard linear calibration curve of Sephadex G-200 chromatography

Apoptosis ratio of the SMMC-7721 cell with and without GBSP treatment

The apoptosis ratio of SMMC-7721 cells without GBSP treatment was 3.84±0.55 % (Figure 4 and Table 1). After GBSP treatment, the apoptosis ratio increased to 9.13±1.48 % ($P<0.01$ *vs* control group) (Figure 5 and Table 1). The debris ratio of the cells without and with GBSP treatment was 0.46± 0.12 % and 0.06±0.06 %, respectively ($P<0.01$) (Figures 4, 5 and Table 1). These results showed that GBSP could induce and promote the apoptosis of SMMC-7721 cells, rather than kill SMMC-7721 cells directly.

Morphology of the SMMC-7721 cell with and without GBSP treatment

The morphology of SMMC-7721 cells with and without GBSP treatment was studied by SEM. The majority of SMMC-7721 cells without GBSP treatment was of shuttle shape (Figure 6) and small proportion of cells was of spherical shape. Close examination revealed dense microvilli on the surface of the cells, and occasionally, 2 to 3 protuberances were also observed on the cell surface. After the treatment of GBSP, these microvilli became thinner, protuberances disappeared (Figure 7) and number of spherical cells increased markedly. These spherical cells shrunk

so that their volume decreased and wrinkles were formed on their surface. Most significantly, apoptosis bodies were formed on and around the spherical cells (Figure 7). These observations suggest that GBSP could induce apoptosis in hepatoma cell line SMMC-7721, consistent with the results of FCM analysis.

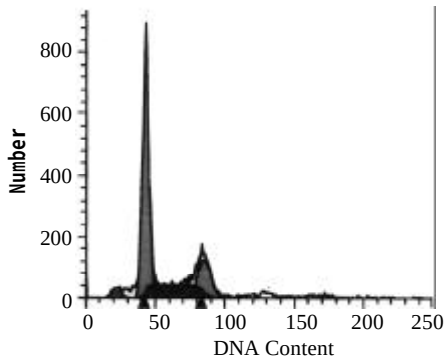

Figure 4 In the absence of GBSP treatment, the apoptosis ratio of SMMC-7721 cells was 3.84±0.55 %, the cells debris was 0.46 ±0.12 % and the percentage of G2-M cells was 17.01±1.28 %.

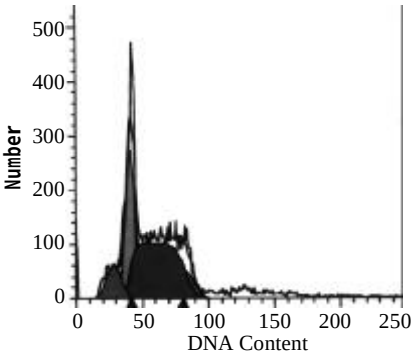

Figure 5 In the presence of GBSP treatment, the apoptosis ratio of SMMC-7721 cells was 9.13±1.48 %, the cells debris was 0.06±0.06 %,and the percentage of G2-M cells was 11.77±1.50%.

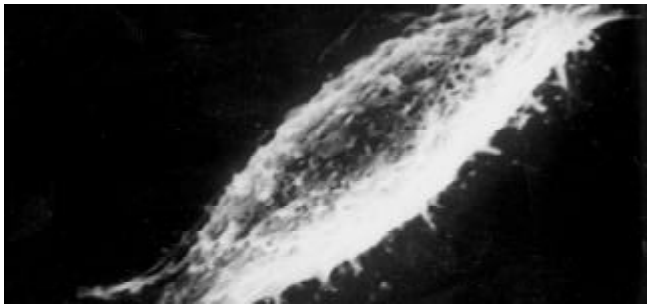

Figure 6 The majority of SMMC-7721 cells were of shuttle shape without GBSP treatment (×2500)

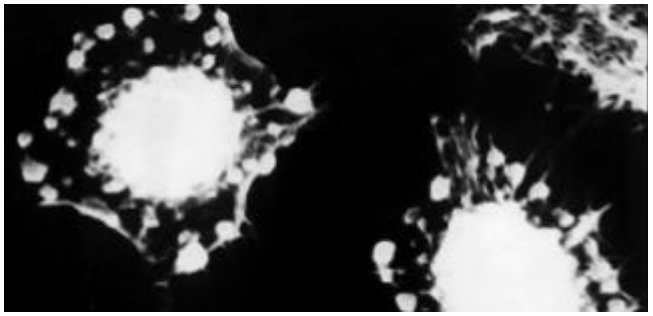

Figure 7 There were more spherical SMMC-7721 cells after GBSP treatment (×2200). These cells shrunk and apoptosis bodies were formed on and around them.

DISCUSSION

The expansion of neoplasma is associated with inhibition of apoptosis of tumor cells. There is evidence that high expression of the inhibition gene bcl-2 led to inactivation of the tumor suppression gene p53[34-45]. When apoptosis was inhibited, tumour cells would not be eliminated in time *in vivo* and, therefore, would proliferate and diffuse more rapidly. At present, drugs such as VP-16, ADR, MTX, and Hydroxyl urea etc. are commonly used in clinical chemotherapy. These agents interfere with growth, metabolism and proliferation processes of the cells so that the apoptosis of tumour cells can be induced [46-48]. However, like most of chemotherapical medicines, they have cytotoxic side-effects which cause damage to normal cells while killing neoplasma cells.

Experiments showed that ingredients of Chinese herbal medicine could induce apoptosis in tumour cells. For example, β-elemi-olefin, arabinose-cytidine and etoposide could induce apoptosis in leukemia cell line[49-55]. It was also reported that polysaccharides isolated from Chinese herbs could inhibit the growth of tumour by activating immune system *in vivo* rather than killing tumour cells directly[56,57]. In this *in vitro* study, we found that GBSP could effectively inhibit division of the SMMC-7721 cells and, meanwhile, induced apoptosis in hepatoma cell line SMMC-7721. Although *Ginkgo biloba* seed has been mainly used for treatment of asthm for over hundred years, it has not been known to have anti-tumour activity. The results of our present study provided valuable data for exploring the clinical application of GBSP for cancer therapy.

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