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Full Length Research Paper

Apoptosis induced by GanoPoly in human gastric cancer cell line SGC-7901 cells

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In order to investigate polysaccharide effect on the cultured human gastric cancer cells (SGC7901), DNA ladder, flow cytometry and western blot were used to examine the morpholog, proliferation and apoptosis of human gastric cancer SGC-7901 cells when they were affected by polysaccharide. Results show that multiplication of the cultured cells was restrained by treating with polysaccharide, especially with long time and higher polysaccharide concentration. The cultured cells performed typical apoptosis both in morphological characters and DNA fragmentation after been treated in 48 h with 10 mg/ml polysaccharide. There were about 40% of the cultured cells present in apoptosis, but the expression of BCL-2 protein was down-regulated, while the concentration of BAX protein was up-regulated. This research indicated that polysaccharide can induce human gastric cancer cells apoptosis and restrain the cells multiplication, which can be used for human gastric cancer's active therapy.

Key words: Apoptosis, polysaccharide, human gastric cancer cells.

INTRODUCTION

Gastric cancer is a very common malignant tumor worldwide and it has the second highest mortality rate among all cancers. Surgery and radiation therapy or conventional chemotherapy treatment saved a lot of lives, but too many people developed metastasic gastric cancer, which is not curable with current treatment measures (Lin et al., 2007). Compounds that block or suppress the proliferation of tumor cells by inducing apoptosis are considered to be potential antitumor agents (Frankfurt and Krishan, 2003).

Ganoderma lucidum is a favourite medication in oriental medicine for centuries and the body of its fruit is called "Lingzhi" in China and "Reishi" in Japan. For hundreds of years, this mushroom has been regarded as a traditional Chinese medicine or a folk medicine, used for the prevention and treatment of many human diseases. The major bioactive components in *G. lucidum* are polysaccharides, ganoderic acid (triterpene) and adenosine. Among them, polysaccharides have a primary effect for their biological activity and therapeutic use (Maruyama et al., 1989; Hsu et al., 2002; Gao et al., 2002). The most attractive property of *G. lucidum* is its anti-tumor effect (Jiang et al., 2004; Gao et al., 2005). Later, the active component was demonstrated to be polysaccharides in *G. lucidum*, which can enhance human's immune system (Sliva et al., 2002; Chen et al., 2004). *G. lucidum* polysaccharides were demonstrated to possess anti-angiogenic property by our research group, and anti-angiogenesis might be a new mechanism for anti-tumor effect of *G. lucidum* polysaccharides (Cao and Lin, 2004). The active components of polysaccharides are all glucans, which have a similar structure with sclera-glucan, but vary in their water solubility as well as the degree and nature of their side-chains. What is more, material from *G. lucidum* has showed high activity at a dosage of 10 mg/kg against a Sarcoma 180 tumor in mice (Sutherland, 1990).

Cordyceps, one of the most valued traditional Chinese medicines, consists of the dried fungus (*Cordyceps sinensis*) that is growing on the caterpillar larva. It is also called "summer-grass and winter worm" because of its

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appearance in different seasons. The parasitic complex of the fungus and the caterpillar is found in prairie at 3500 – 5000 m elevation. It is widely used in China to strengthen the kidney's function and soothe human's breath and also for the treatment of alleviated fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart diseases and liver disease (Zhu et al., 1998). Recent studies have demonstrated the multiple effective pharmacological actions of Cordyceps including anti-oxidation (Li et al., 2001, 2002), which enhance the immune system (Koh et al., 2002) and hypoglycemic activities (Kiho et al., 1999). However, polysaccharides in Cordyceps are often associated with these pharmacological activities.

In this study, GanoPoly (GP) was composed of the polysaccharides of *Ganoderma lucidum*, Cordyceps, hedgehog hydnum and Coriolous Dersicolor Quel. The emphases of this study were to examine the *in vitro* antitumor activities of GP in human gastric cancer cell line SGC-7901 cells through the activation of apoptosis, which was proven by the induction of internucleosomal DNA fragmentation and the annexin V-FITC/PI binding study using flow cytometry. The activation of apoptosis by GP was probably caused by altering the expression level of the Bcl-2 family by Western blotting analysis.

MATERIALS AND METHODS

GP were purchased at New Zealand Alpha Group International, while methyl thiazolyl tetrazolium (MTT), ethidium bromide (EB), RNase A, propidium iodide (PI) and trypsin were purchased from Sigma Chemical Co (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), low melting point agarose and normal melting point agarose were from GIBCO (Grand Island, NY, USA). The apoptosis ladder detection kit was from Wako Pure Chemical Industries (Osaka, Japan), while Bcl-2 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents used were obtained from Sigma (USA). However, all chemicals and reagents were of analytical grade.

Cell culture

The human gastric cancer cell line (SGC-7901) was obtained from the China Center for Type Culture Collection (Wuhan, China) and the cells were cultured by DMEM with 10% (*v*/*v*) heat-inactivated FBS, 100 µg/ml streptomycin and 100 unit/mL penicillin in 100 ml culture flasks in a humidified atmosphere at 37°C with 5% CO_2. Single cell detachment was achieved with incubation at 37°C with trypsin–EDTA.

Cell viability assay

Cell viability was measured by 3-(4,5)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the SGC-7901 cells were treated with GP at concentrations of 4.0, 6.0, 8.0 and 10.0 mg/ml for 12, 24, 36, 48, 60 and 72 h, respectively. After the MTT labeling reagent was added and incubated for 4 h at 37°C, they were incubated for 12 h with the 10% sodium dodecyl sulphate (SDS) solution and the absorbance (at a test wavelength of 540 nm and a reference wavelength of 690 nm) was measured by a microtiter plate reader (Molecular Devices, Silicon Valley, CA, USA). The optical density (OD) was calculated as the difference between the absorbance from the reference and test wavelengths, while the percentage of viability was calculated as follows:

(Viable cells)% = (OD of drug - treated sample/OD of untreated sample) × 100.

Morphological examination

The cells were harvested and washed 3 times with PBS (Phosphate buffer saline) after being incubated with different concentrations of GP for 48 h and were stained with 10 µg/ml Hoechst 33258 for 5 min. As a result, apoptotic morphology was observed by a fluorescence microscope (BX51, Olympus, Shinjuku-ku, Tokyo, Japan) after Hoechst 33258 staining.

Analysis of DNA fragmentation

The cells were harvested and washed 3 times with PBS after being incubated with different concentrations of GP for 48 h. DNA was extracted with the apoptosis ladder detection kit, analyzed by 1.0% agarose gel electrophoresis, and then stained with EB.

Annexin V FITC/PI binding study using flow cytometry

Cultured SGC-7901 cells (10^6) were treated with GP for 48 h and were then washed with cold PBS and centrifuged at 1500 rpm for 5 min at 4°C. The cells were resuspended in 1× annexin-hepes buffer and washed twice. Similarly, the pellets were resuspended in the same buffer (100 ml), while annexin V-FITC (5 mg) and propidium iodide (1 mg) was added to the cell suspension. After 15 min of incubation in a dark place at room temperature, analysis was done by flow cytometer (Becton Dickinson FACS caliber single laser flow cytometer) immediately. Flow cytometric reading was taken using 488 nm excitation and band pass filters of 530/30 (for FITC detection) and 585/42 nm (for PI detection). Data analysis was performed with Cell Quest software program (n = 4).

Western blotting

For Western blotting, the cells were first treated for 24 h with different concentrations of GP, 2 × 10^6 cells that were washed twice with ice-cold PBS, lysed for 30 min at 4°C and then the debris was removed by centrifugation for 15 min at 12 000 ×*g* at 4°C. The equivalent amount of protein (20 µg) was separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filters were hybridized first with monoclonal antibody (anti-Bcl-2) at a dilution of 1:1000 for 2 h, followed by extensive washes with PBS and TBST (Tris Buffered Saline supplemented with 0.1% Tween-20) twice. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 for 1 h and washed with TBST. As a loading control, β-actin was also detected. Moreover, the immunoreactive proteins were detected using an ECL Western blotting detection system (Beyotime Institute of Biotechnology, Haimen, China).

Statistical analysis

Data are presented as mean ± SD. The differences among the

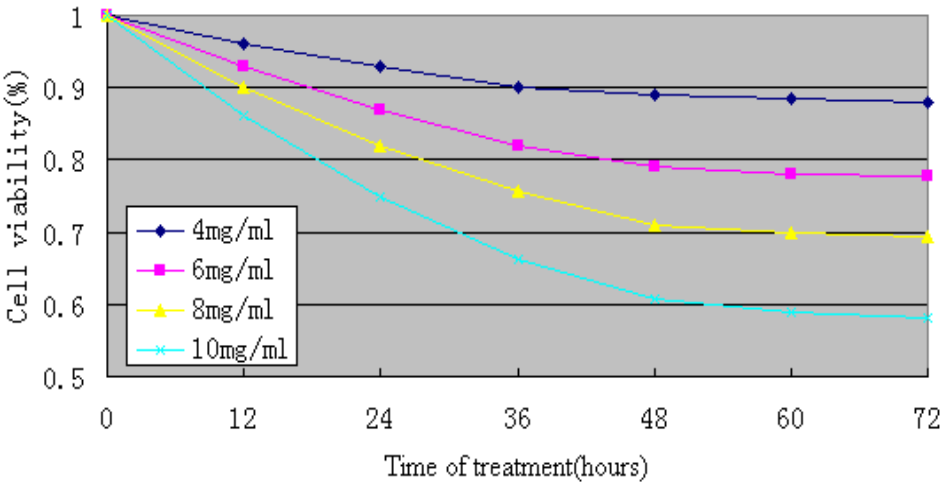

Figure 1. Effects of GP restrained SGC-7901 cells proliferation. GP caused a time- and dose-dependent growth inhibition in SGC-7901 cells. Data are given as percentage of controls (mean ± SD of four independent experiments). Statistical significance ($p < 0.05$) of growth inhibition was shown in 4 – 10 mg/ml GP for SGC-7901 cell line compared to untreated controls.

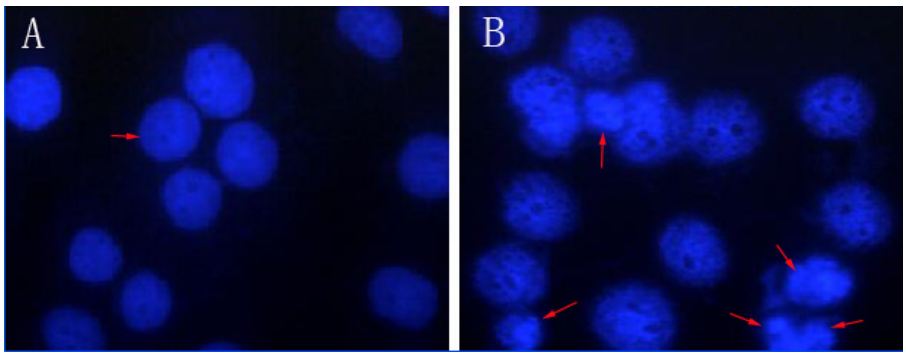

Figure 2. Morphology changes of SGC-7901 cells stained with Hoechst 33258 in fluorescence microscope (×400). A: Control group; B: 10 mg/ml GP treated for 48 h.

groups were analyzed using one-way ANOVA with Scheffe's test. A P-value, less than 0.05 was considered to be statistically significant.

RESULTS

Effect of GP on SGC-7901 cells multiplication

The potential effect of GP to gastric cancer cell line (SGC-7901) is shown in Figure 1. Different color line represents different concentrations of GP. From four lines in Figure 1, GP can restrain the SGC-7901 cell's proliferation ability within 48 h compared to the untreated cells (taken as 100% viable), but the inhibition ability of GP decreased after responding for 48 to 72 h.

GP causes of apoptosis

In order to investigate whether the apoptosis of SGC-7901 cell was caused by GP or not, the cell morphological changes and DNA fragmentation were observed under fluorescence microscope. The result is shown in Figure 2. GP with 10 mg/ml concentration was treated with SGC-7901 cell for 48 h and can induce cell's typical apoptotic morphological changes, including cytoplasmic blebbing, condensation aggregation of nuclear chromatin and the formation of apoptotic bodies. The nucleosomal size DNA fragmentation (DNA ladder), one of the most obvious biochemical event in the early stages of apoptosis, was also shown in SGC-7901 cells after being treated with GP. DNA fragmentation can be observed obviously after 36 and 48 h, in which no DNA ladder appeared in the control group (Figure 3).

Annexin V FITC/PI binding study using flow cytometry

The apoptosis rate of SGC-7901 cells induced by GP for 48 h was checked under flow cytometry (Figure 4), while

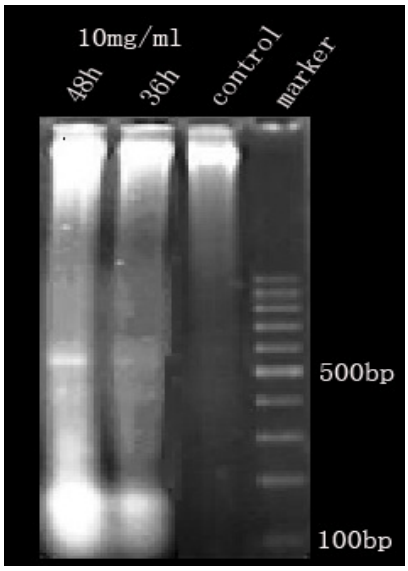

Figure 3. DNA fragmentation analysis of SGC-7901 cells treated with GP. Cells were incubated with GP at various concentrations for 36 and 48 h. Genomic DNA was extracted from the cells, electrophoresed in 1.0% agarose gel and visualized with ethidium bromide staining.

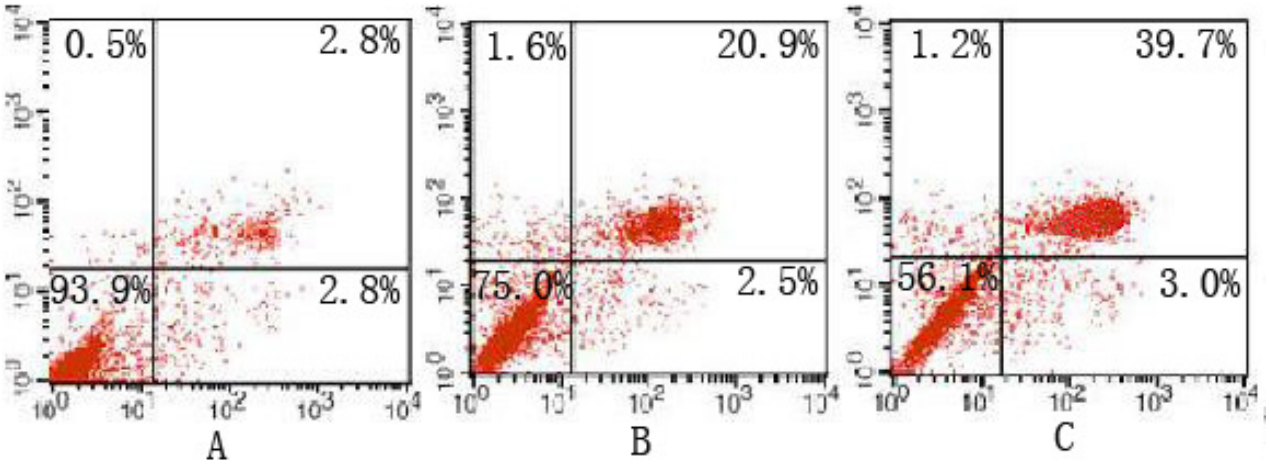

Figure 4. Effect of GP on the annexin-V/PI binding of SGC-7901 cells. Apoptosis was induced by GP on SGC-7901 cells under flow cytometry. A: Control group; B: 10 mg/ml GP treated for 36 h; C: 10 mg/ml GP treated for 48 h.

SGC-7901 cells were treated in 10.0 mg/ml GP for 36 and 48 h, and was then stained with FITC-conjugated Annexin V and PI for flow cytometry. In Figure 4, the cell populations shown in the lower right (Annexin V+/PI-) represent apoptosis cells, while those at the upper right (Annexin V+/PI+) represent necrotic cells. Moreover, the spontaneous apoptosis rate of the control group cells was 2.8%. With 10 mg/ml GP treated for 36 and 48 h, the apoptosis rate of SGC-7901 cells was 20.9 and 39.7%, respectively.

Result of Western blotting

The results of the Western blot analyses of Bcl-2 and Bax protein in SGC-7901 cells with the GP and control group are shown in Figure 5, whilst the GP stimulated the expression of Bax in a dose-dependent manner (Figure

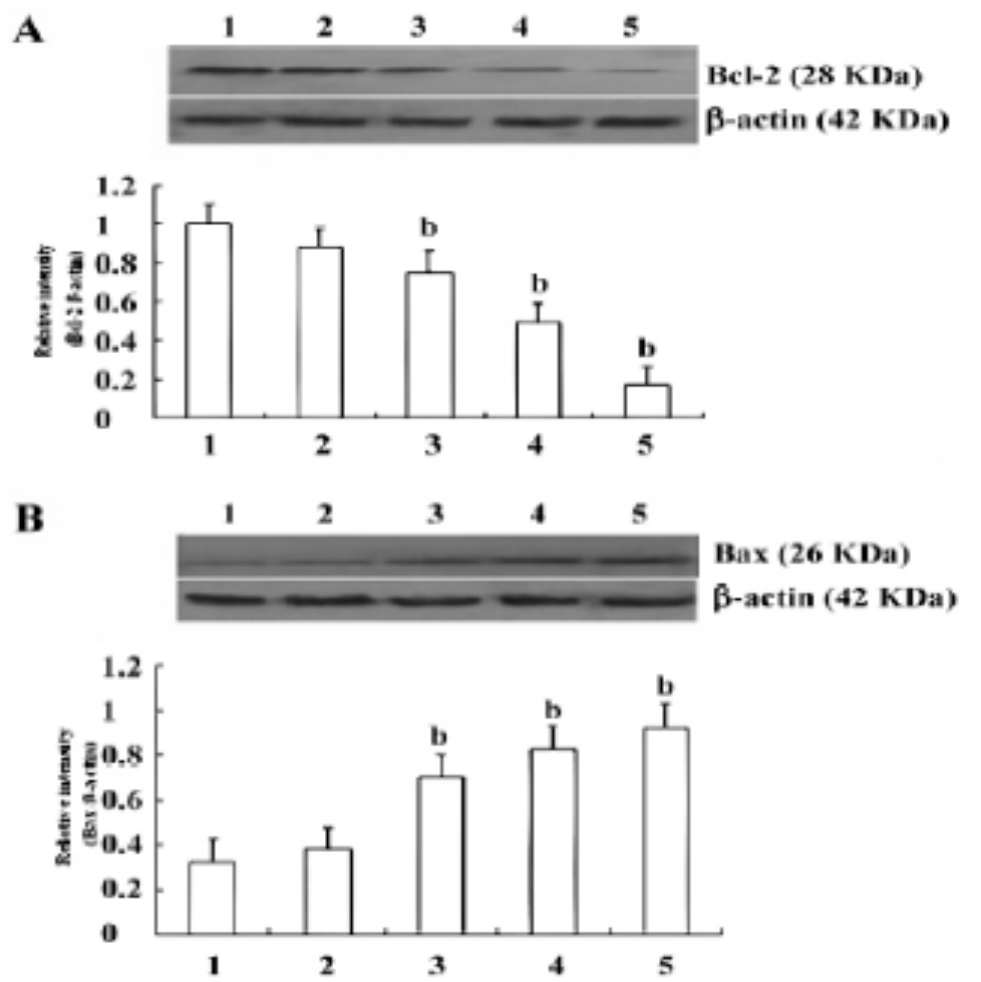

Figure 5. Western blot analysis and quantitative analysis of GP on expression of Bcl-2 (A) and Bax (B). The SGC-7901 cells were treated with 10 mg/ml (S) for 48 h, and cells with culture medium only were included as a control group (1). The graph represents optical density of Bcl-2 and Bax protein bands, normalized with the expression of β-actin. $^{b}P<0.05$ compared to the untreated control.

5B). Moreover, the expression of Bcl-2, an anti-apoptotic protein, decreased in a dose-dependent manner (Figure 5A).

DISCUSSION

The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug (Frankfurt and Krishan, 2003). Gastric cancer is a very common disease that can cause high death worldwide (Lin et al., 2007). Many investigations have been carried out all over the world to discover naturally-occurring compounds which can suppress or prevent the process of carcinogenesis (Lee et al., 2002; Cheng et al., 2004). Natural compounds fit into a mechanism-oriented approach that targets the entire pathways and sets of intracellular events rather than a single enzyme, such as many synthetic drugs did. This offers a less specific, but perhaps more effective strategy for cancer therapy by inducing the combination of effects that may counteract the metabolic alterations related to cancer promotion (Paola and Riccardo, 2005). For example, a capsicum and green tea mixture has been reported to exhibit 100 times greater potency with respect to anticancer activity in a number of cell lines, than that of green tea alone on a weight basis (Morre and Morre, 2003). In this study, GanoPoly (GP) was composed of the polysaccharides of *Ganoderma lucidum*, Cordyceps, hedgehog hydnum and Coriolous Dersicolor Quel. Here, it was shown that GP was able to inhibit the growth of the human gastric cancer cell line (SGC-7901) in a certain time and in a dose-dependent manner (Figure 1).

Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and the activation of specific cysteine proteases known as caspases (Lee et al., 2000). In the present study, DNA fragmentation with a

ladder pattern (the characteristic of apoptosis) was observed in SGC-7901 cells treated with GP (10.0 mg/mL) for 48 h (Figure 3). We found that the growth inhibitory activity of GP was associated with the induction of apoptosis in SGC-7901 cells. Data from an apoptosis assay showed that GP induced obvious apoptosis in SGC-7901 cells and presented a dose-dependent manner of apoptosis-specific morphological changes (Figure 2). In annexin-V FITC/PI binding study, a noticeable phenomenon was that the apoptotic rate contrarily increased to GP% after treatment with 10.0 mg/ml GP (Figure 4). This result showed that GP inhibited the proliferation of SGC-7901 cells through inducing cell apoptosis.

We investigated the effect of GP on the levels of 2 members of the Bcl-2 family, the pro-apoptotic Bax and the anti-apoptotic Bcl-2, which regulate mitochondrial apoptosis (Ho et al., 2006). When Bax was over expressed in cells, apoptotic death in response to death signals was accelerated so as to take action as agonist, whereas when Bcl-2 was over expressed, it heterodimerized with Bax and death was repressed; thus, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis (Zhang et al., 2006). Through the exposure to GP, we found an increase of Bax level in the SGC-7901 cells, which paralleled a down regulation of Bcl-2 level (Figure 5). This indicates that the mitochondrion is an important target for GP actions.

Cell apoptosis is a programmed death process, which is induced and controlled by many complicated factors, such as blockage of the cell cycle, changes of expression of correlative apoptosis genes and the elevation of caspase activity (Waxman and Schwartz, 2003). Many anticancer drugs perform their curative effect by inducing apoptosis of tumor cells through those pathways. In conclusion, the data reported here indicate that GP inhibits growth and proliferation by inducing apoptosis of SGC-7901 cells. This apoptosis was mediated by the down regulation of the Bcl-2 level and the up-regulation of the Bax level in SGC-7901 cells. Conclusively, the results of the present study provide supportive data for the anticancer potential of GP.

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