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Induction of apoptosis in SGC-7901 cells by polysaccharide-peptide GFPS1b from the cultured mycelia of *Grifola frondosa* GF9801

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Abstract

The biological function of GFPPS1b, a novel polysaccharide-peptide isolated from cultured mycelia of *Grifola frondosa* GF9801, was well investigated. GFPS1b has anti-tumor activity and can significantly inhibit the proliferation of SGC-7901 cells, whereas slightly influences the growth of human normal liver cell line L-02. When treated with GFPS1b, SGC-7901 cells showed typical apoptotic morphological features such as the loss of villus and appearance of apoptotic bodies on the cell surface, volume reduction, and chromatin condensation, by scanning electron microscopy (SEM) and fluorescent microscopy (Hoechst 33342). The results of flow cytometry analysis and annexin V-PI assay showed that the SGC-7901 cell cycle was arrested in the G_2/M phase, the subdiploid peak of DNA characteristic of apoptotic was also observed, and the apoptosis ratio was about 15.08%. DNA isolated from SGC-7901 cells cultured with GFPS1b showed a typical DNA 'ladders' of apoptosis in agarose gel electrophoresis. Further investigation results showed that the apoptotic machinery of SGC-7901 induced by GFPS1b was associated with drop in mitochondrial trans-membrane potential, upregulation of Bax, downregulation of Bcl-2, and activation of caspase-3. Our finding suggests that GFPS1b could suppress SGC-7901 cell growth and reduce cell survival via arresting cell cycle and inducing apoptosis of tumor cells.

Keywords: *Grifola frondosa*; Polysaccharide-peptide; Apoptosis; Mitochondrial membrane potential; Bax; Bcl-2; Caspase-3

1. Introduction

Many edible mushrooms have physiological effects on humans and animals, affecting immune system, digestive system, cardiacvessel functioning, as well as having anti-cancer, anti-oxidation, and anti-hypertensive effects (Nadeem et al., 2002; Smith et al., 2002; Zaidman et al., 2005). Many reports revealed that polysaccharides or polysaccharide-peptide (protein) complexes, as the major bioactive ingredients of various mushrooms, exerted their anti-tumor action via activation of the immune response of the host organism, so they were regarded as biological response modifiers (BRMs) (Kima et al., 2004; Ruan et al., 2005). However, increasing references confirmed that the polysaccharides or their complexes could have cytotoxic or cytostatic effect on various tumor cell lines in vitro by inducing cell apoptosis, whereas less toxic to normal cells (Lavi et al., 2006; Lee et al., 2005; Yang et al., 2005; Li et al., 2004; Takako et al., 2004).

Grifola frondosa, an oriental fungus of high Basidiomycetes, has been widely consumed in China, Japan and other Asian countries. The fruit bodies and mycelia of *G. frondosa* have been reported to possess beneficial anti-tumor and other bioactive properties due to its possessing of many

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biologically active compounds (Kawagishi et al., 1990; Lee et al., 2003; Suzuki et al., 1984). GFPS1b, extracted and purified from the cultured mycelia of *G. frondosa* GF9801 screened and stored by our lab, is a novel bioactive heteropolysaccharide-peptide composed of D-glucose, D-galactose, and L-arabinose with a molar ratio of 4:2:1 and with approximately 16.60% protein, 4.3% uronic acid and molecular weight of 21 kDa. Its main backbone of GFPS1b consists of α-D-glucose (1 → 3), α-D-galactose (1 → 4), and α-D-glucose (1 → 3,6)-linkages with branches at *O*-6 of glycosyl residues composed of α-L-arabinose-(1 → α-glucose (4 → 1)) linked residues (Cui et al., 2006, 2007). Our previous studies showed that GFPS1b exhibited significant effect on the proliferation of the tumor cells in vitro, however, the anti-proliferative activity on other tumor cell lines and its relative mechanism of GFPS1b have yet not been elucidated clearly. In the present report, we investigated the GFPS1b anti-tumor activity by evaluating its effects on the gastric carcinoma cell line (SGC-7901) and its possible mechanism.

2. Material and methods

2.1. Preparation of GFPS1b sample

GFPS1b fraction of *G. frondosa* GF9801 was isolated and purified based on a previous method (Cui et al., 2007). Briefly, the mycelia of *G. frondosa* were extracted with 1 l of water for 2 h. The suspension was centrifuged to remove the insoluble part. The crude polysaccharide fraction was obtained by ethanol precipitation and dissolution with H_2O. GFPS1b was obtained by applying the water-soluble polysaccharide fraction to the DEAE-Sepharose fast-flow anionic resin (Pharmacia AP, Sweden) column and Sephadex G-100 (Amersham Biosciences, Sweden) using an AKTA purifier (Amersham Biosciences, Sweden).

2.2. Cell culture

SGC-7901, a gastric carcinoma tumor cell line, was obtained from Shanghai Cell Biology Institute, China. Cells were inoculated in RPMI 1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY), 100 mg/l streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell viability assay

Viability of cells was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay. Briefly, SGC-7901 and human normal liver L-02 cells (8×10^3/well) in their exponential growth phase were seeded into each well of a 96-well flat-bottomed culture plate. After incubation for 24 h, various concentrations of GFPS1b were added into each well and each concentration was repeated in four wells. After another 48-h incubation, the medium was aspirated and MTT was added to cells at a concentration of 0.5 g/l. Cells were incubated at 37 °C for another 4 h and the formazan product was solubilized with dimethylsulfoxide (DMSO). The absorbance was detected by the microplate reader (Multiskan MK3, Labsystems, Finland) at 570 nm, and measured values were expressed as mean ± SD.

2.4. Morphological studies of apoptotic cell

2.4.1. Scanning electron microscopy analysis

As described previously (You and Lin, 2002), SGC-7901 cells treated with certain concentrations of GFPS1b were washed with PBS, centrifuged, and pre-fixed with 2.5% glutaraldehyde at 4 °C for 2 h. The cells were then rinsed thoroughly in PBS and post-fixed in 1% OsO_4 at 4 °C for 1 h, and scanning electron microscopy was applied to investigate the effects of the pro-damage on GFPS1b-treated cell surface morphology.

2.4.2. Fluorescence microscopy observation

The cultures, plated onto glass coverslips in 6-well plates and exposed to GFPS1b at concentrations of 0, 30, 60 and 120 μg/ml for 48 h, were washed twice with PBS and stained with Hoechst dye 33342 (Sigma) for 15 min at room temperature. After washing with PBS, cover slips were mounted onto microscope slide and nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan) at 100×.

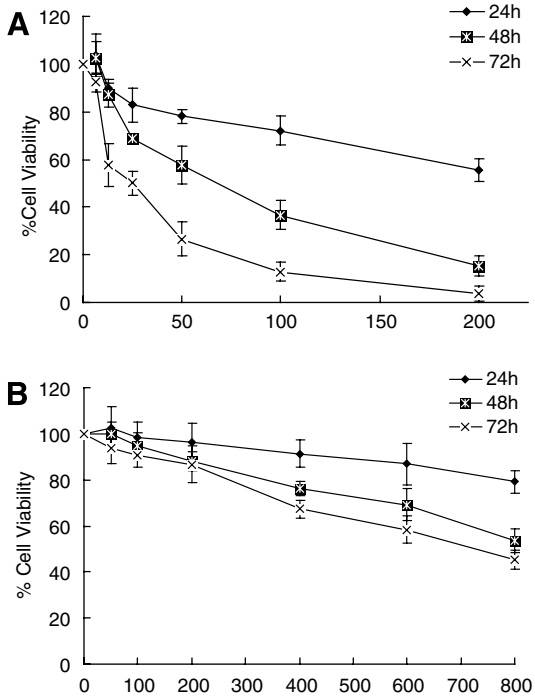

Fig. 1. GFPS1b inhibits SGC-7901 cells (A) and human normal liver cell L-02 proliferation (B) after the 24, 48, or 72 h treatment. Cell survival was evaluated by MTT assay. MTT was added into medium for an additional 4 h. The survival of cells was detected by measuring the absorbance at a wavelength of 570 nm. The cell survival was expressed as the optical density ratio of the treatment to control. Data represent the mean ± SD of three independent experiments.

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2.5. DNA fragmentation assay

SGC-7901 cells grown in 75 cm^2 culture flasks were treated with GFPS1b at concentrations of 0, 30, 60 or 120 μg/ml for 48 h. Following centrifugation at 200*g*, the harvested cells were lysed with a DNA extraction buffer (10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.5% TritonX-100) and incubated at 4 °C for 10 min. After centrifugation at 12,000*g* for 5 min, the lysate of cells was treated with 500 μg/ml DNase-free RNase at 37 °C for 1 h, followed by treatment with 500 μg/ml proteinase K at 50 °C for 30 min. Electrophoresis in 1% agarose gel was then performed. DNA ladders were visualized by UV illumination after staining with ethidium bromide.

2.6. Apoptotic assay with annexin V-PI dual staining using FACS

Cells (1×10^6) were incubated with GFPS1b for 24 h and then harvested. Specific binding of annexin V-FITC was performed by the incubation of cells for 15 min at room temperature in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing a saturating concentration of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Liao and Lieu, 2005). After incubation, the cells were pelleted and analyzed in FACScan analyzer (Becton Dickison Company, America). Annexin V^+/PI^- (lower right) cells were recognized as apoptotic cells, while Annexin V^+/PI^+ (upper right) cells were defined as necrotic (or late apoptotic). The data were analyzed using Cellfit Analysis Software (Becton Dickison Company, America).

2.7. Cell cycle analysis

The DNA and the proportion of cells in different phases exposed with GFPS1b were analyzed with flow cytometry (Jakopec et al., 2006). Briefly, 1×10^6 cancer cells were harvested and washed with PBS after GFPS1b treatment. The cells were fixed with cold 75% ethanol for 24 h, and then stained with PI solution consisting of 45 mg/ml PI, 10 mg/ml RNase A, and 0.1% Triton X-100. After 1 h incubation at 4 °C in the dark, fluorescence-activated cells were sorted in the FACScan flow cytometer, and the data were analyzed using Cellfit Analysis Software.

2.8. Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$)

The changes of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) in SGC-7901 cells, treated with different concentrations of GFPS1b for 48 h, were analyzed using a FACScan flow cytometer according to the method described by Sun et al. (2006). Briefly, after trypsinization, SGC-7901 cells were washed twice with PBS, and then the concentration

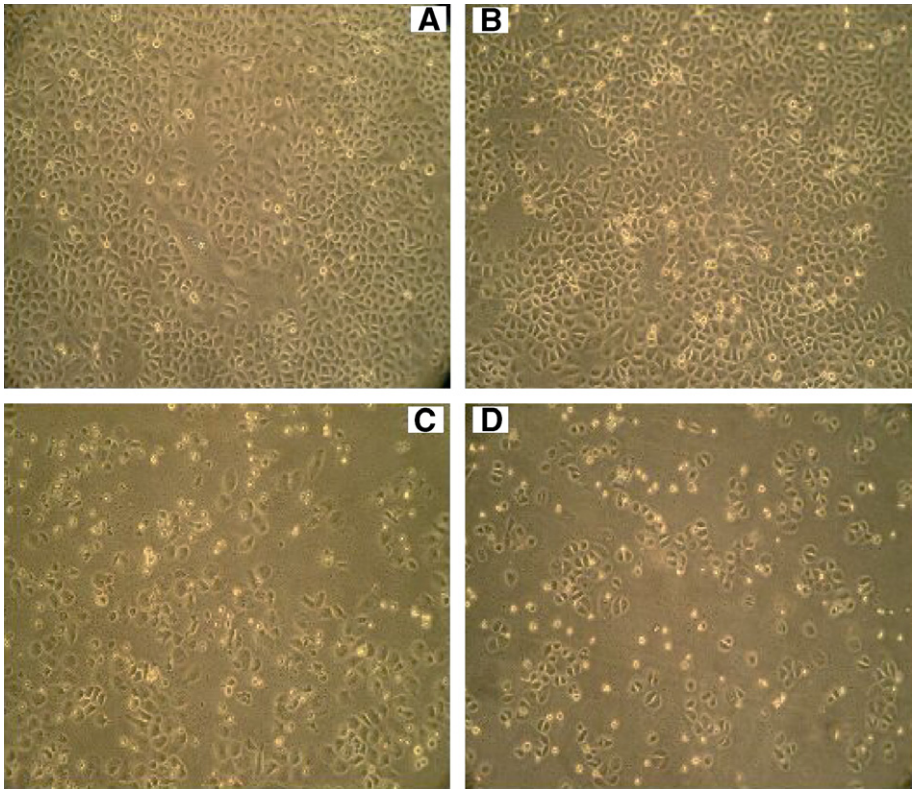

Fig. 2. Morphological changes of SGC-7901 cells observed by phase-contrasted microscopy after treated with (A) control, (B) 30 μg/ml, (C) 60 μg/ml; (D) 120 μg/ml of GFPS1b for 48 h (×100). The control group cells showed a typical polygonal and cobblestone monolayer appearance (A). Significant decrease of the number of SGC-7901 cells treated with GFPS1b (C, D) was observed compared to the control group. Furthermore, the cells began to have morphological changes, showed round-shaped, and poorly adhered to the culture flasks (C, D).

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of cell suspension was adjusted to 1×10^6 cells/ml. One hundred microliter rhodamine 123 (Rh123) solutions (20 μg/ml) was added to the harvested cells and incubated at 37 °C in the dark for 30 min. Then the cells were washed with PBS again and stained with propidium iodide (PI) solution (100 μg/ml), rinsed with phosphate-buffered saline twice, and subjected to flow-cytometric analysis immediately. All data were collected, stored, and analyzed with the use of Cellfit Analysis Software.

2.9. Immunohistochemical analysis

After a 48-h exposure to GFPS1b 0, 60, and 120 μg/ml, the expressions of Bcl-2, Bax and p53 proteins in SGC-7901 cells were analyzed using flow cytometry (Aggarwal and Gupta, 1998). SGC-7901 cells were washed and sequentially fixed with 1% paraformaldehyde for 15 min at room temperature and by 70% methanol for 45 min at 4 °C, then washed, and finally incubated with FITC-conjugated anti-53, FITC-conjugated anti-Bcl-2, and FITC-conjugated anti-Bax. FITC-labeled mouse IgG was used as isotype-matched background controls. Following incubation, the cells were washed twice with PBS, and 5000 cells were acquired using FACScan. SGC-7901 cells were gated. The percentage of positive cells expressing fluorescence intensity of p53, Bax and Bcl-2 was measured by mean fluorescence channel number.

2.10. Western blot analysis

Protein extracts of SGC-7901 cells were treated with or without GFPS1b were prepared by lysing cells in the buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris–Cl, 1 mM phenylmethylsulfonyl fluoride) with occasional vortexing. Samples were then centrifuged for 15 min at 15,000*g*. Protein concentration in supernatant was determined. For each sample, 60 μg/lane of protein was loaded on 12% polyacrylamide gels and transferred to TotalBlot PVDF membranes (Amresco Inc., USA), which were blocked with 5% fat-free milk and immunostained with anti-Bcl-2 antibody (diluted 1:1000), anti-bax antibody (diluted 1:1000), anti-p53 antibody (diluted 1:1000) or anti-actin (diluted 1:500). Thereafter, membranes were incubated for 1 h with HRP-labeled secondary antibodies: sheep anti-mouse (diluted 1:2500) and donkey anti-rabbit (diluted 1:5000), and then developed by an ECL system according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

2.11. Caspase-3 activity assay

As caspase-3 activation is a key step in the regulation of apoptosis, activity of caspase-3 was detected by using a Colorimetric assay kit (B.D. Company, USA) according to the manufacturer's protocol. In brief, 2×10^6 cells were collected following treatment with vehicle or various concen-

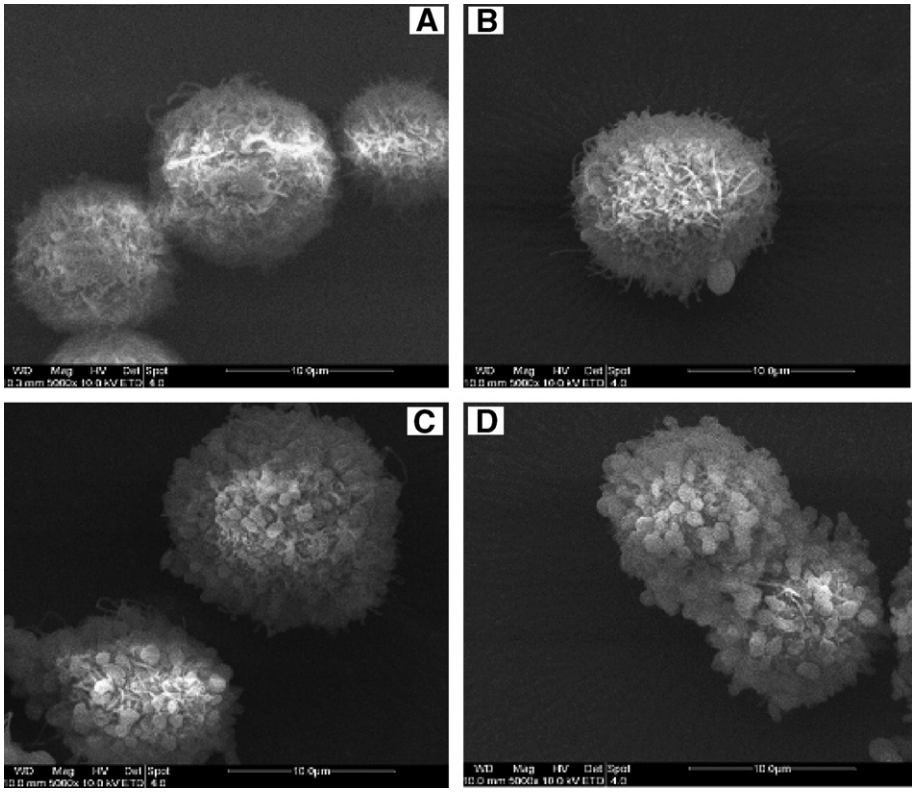

Fig. 3. Scanning electron micrographs of SGC-7901 cells showing the surface modification of the cells treated without (A) or with ((B) 30 μg/ml, (C) 60 μg/ml, (D) 120 μg/ml) of GFPS1b for 48 h. Extensive membrane blebbing is shown at longer recovery times. The microvilli on surfaces of the control group cells (A) were kept intact, and the cells of GFPS1b treated groups provided the morphological changes of pre-apoptotic characteristics, such as loss of microvilli, appearance of apoptotic bodies (B–D).

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trations of GFPS1b (60, 120 μg/ml) for 12, 24 and 48 h, respectively. Control or treated cells were washed and lysed in 50 ml of cold lysis buffer and incubated in ice for 10 min. The cell lysate was centrifuged at 15,000 rpm for 10 min at 4 °C. Reaction buffer/DTT was then added to the supernatant. After incubation at 37 °C for 1 h with DEVD-pNA serving as substrate, the absorbance was measured at the wavelength of 405 nm using an ELISA microreader. Caspase-3 activity was denoted as *U* and calculated from the standard curve generated with pNA solutions of different concentrations.

2.12. Statistical analysis

Data are represented as mean ± SD, statistical differences between control and treated groups were determined by Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Anti-proliferation of GFPS1b on the SGC-7901 cell line

To investigate the growth inhibitory activity, the viability of GFPS1b treated SGC-7901 cells and human normal liver L-02 cells were measured by the colorimetric 3-[4,5-dimethyLthiazol-2-Yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. When the SGC-7901cells were treated with various concentration of GFPS1b, growth was significantly inhibited at dose- and time-dependent manners (Fig. 1). The proliferation of normal human liver L-02 cells was slightly influenced at the similar time and dose. However, the cells treated with the higher concentration (>400 μg/ml) were inhibited significantly, which indicated that GFPS1b also was relate safe to the normal human liver L-02 cells.

3.2. Effect of GFPS1b on the morphology of SGC-7901 cells

The morphological examinations of the SGC-7901 cells were illustrated using phase contrast microscope (Fig. 2). The control group cells showed a typical polygonal and cobblestone monolayer appearance (Fig. 2A). Significant decrease of the number of SGC-7901 cells treated with GFPS1b (60, 120 μg/ml) for 48 h was observed compared to the control group. Furthermore, the cell treated with GFPS1b for 48 h began to have morphological changes; showing round-shaped cells poorly adhered to the culture flasks (Fig. 2C and D). Scanning electron microscopy showed that numerous microvilli on surfaces of the control group cells were kept intact, and the cells of GFPS1b treated groups provided the morphological changes of pre-apoptotic characteristics, such as loss of microvilli, blebbing formation, and appearance of apoptotic bodies (Fig. 3). The changes of nuclear morphology of SGC-7901 with GFPS1b treatment for 48 h were analyzed under a fluorescence microscope by Hoechst-staining (Fig. 4). The

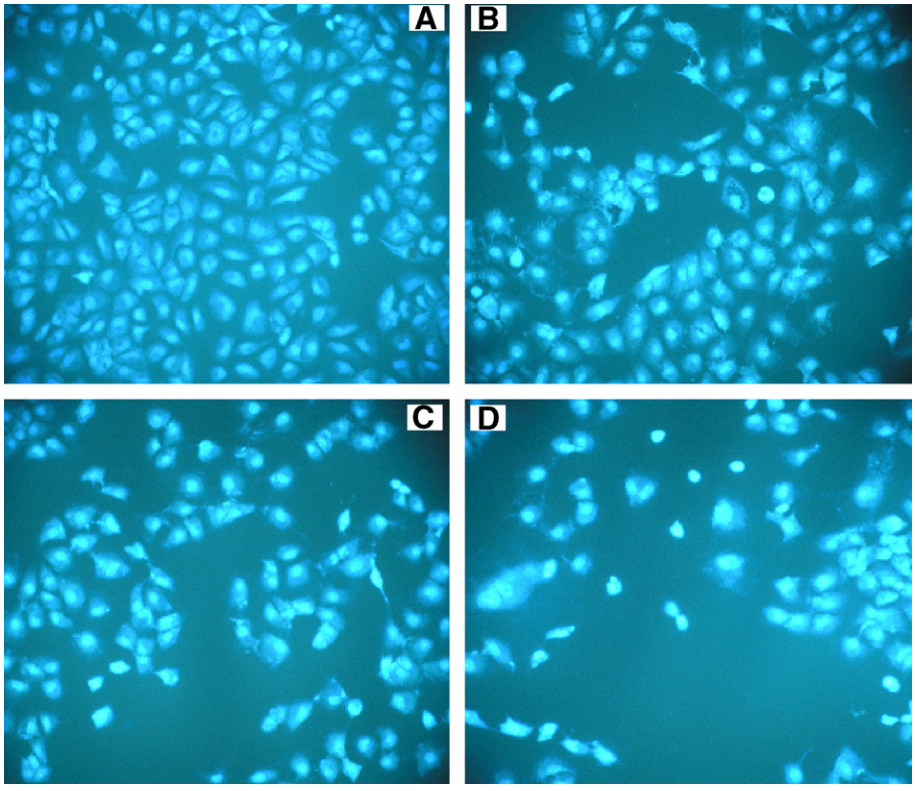

Fig. 4. Fluorescence micrographs of SGC-7901 cells stained with Hoechst 33342 (×100). Cells were treated with 0 μg/ml (A); 30 μg/ml (B); 60 μg/ml (C); 120 μg/m (D); GFPS1b for 48 h. And they show that GFPS1b induces apoptosis in SGC-7901 cells, which is characterized by nuclear condensation or nuclear fragmentation.

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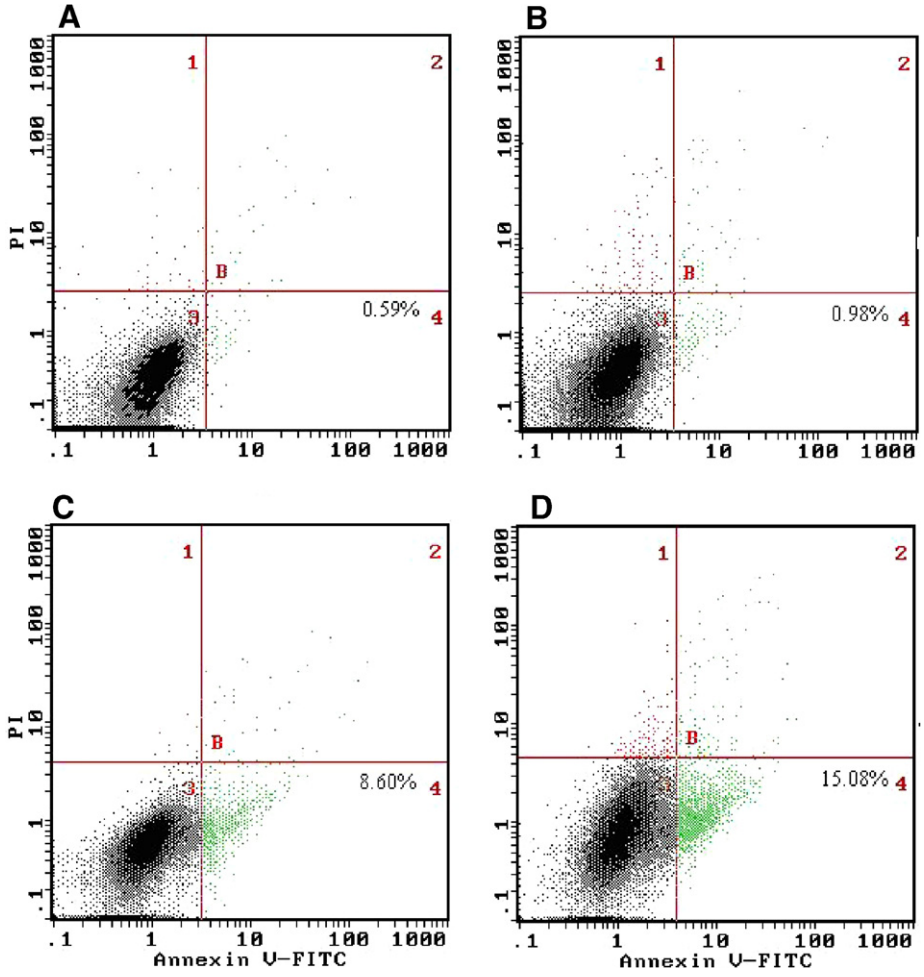

Fig. 5. Apoptosis induced by GFPS1b on SGC-7901 cells. SGC-7901 cells were treated for 0, 30, 60 and 120 μg/ml of GFPS1b with 48 h. And then they were stained with FITC-conjugated Annexin V and PI for flow cytometry. As shown, the cell populations shown in the lower right (Annexin V^+/PI^-) represents apoptosis cells, upper right (Annexin V^+/PI^+) represents necrotic cells.

nuclei of the treated cells have nuclear shrinkage and condensed chromatin.

3.3. Detection of apoptotic features by Annexin V-PI dual staining

Based on the morphological changes of SGC-7901 cells after treated with GFPS1b for 48 h treatment, we further demonstrated the occurrence of GFPS1b-induced apoptosis by Annexin V-PI staining methods. Fig. 5 showed that the proportion of apoptotic cells (lower right) significantly elevated in GFPS1b (60, 120 μg/ml)-treated cells from 0.59% in untreated cells to 8.60–15.08%. However the necrotic cells (upper right) was no appreciable change after exposure to GFPS1b (Fig. 5). These results suggested that apoptosis not necrosis contributed to the GFPS1b-induced death of SCG-7901 cells.

3.4. DNA fragmentation assay

We examined the nuclear DNA fragmentation using agarose gel electrophoresis and the results are shown in Fig. 6. After the treatment with vesicle, no DNA ladder pattern was observed. However, it is indicated that the exposure of SGC-7901 cells to GFPS1b might cause DNA fragmentation characteristic of apoptosis.

3.5. Effect of GFPS1b on cell cycle distribution and apoptosis ratio of SGC-7901

To determine the effect of GFPS1b on the cell cycle in SGC-7901 cells for 48 h, the percentage of cells in each phase of cell cycle in control and treated cells were determined by FACS and the results were showed in Fig. 7. We observed that SGC-7901 accumulated in G_2/M phase after treatment of GFPS1b for 48 h (11.9%, 12.5% and 20.9%, respectively). On the other hand, GFPS1b caused G_0/G_1 phase attenuation after 48 h treatment (52.3%, 47.7%, and 38.0%). Synchronously, as compared to control, the sub-G1 groups, which indicated apoptotic-associated chromatin degradation, significantly increased after cells cultured with GFPS1b (1.4%, 5.0%, 13.4%) at the concentration of 30, 60, 120 μg/ml, which was in accordance with the results of Annexin V-PI dual staining. These results suggested that

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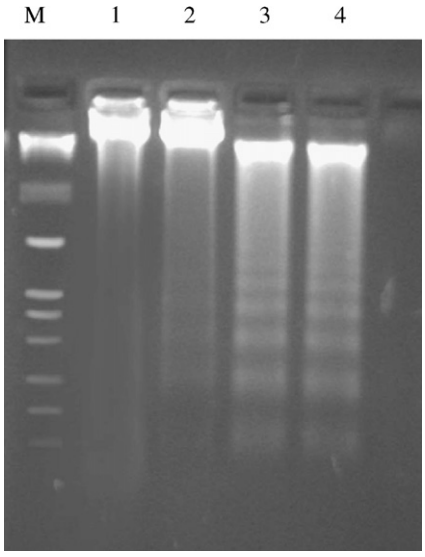

Fig. 6. DNA fragmentation assay was examined to confirm the apoptosis change of SGC-7901 cells after treatment with GFPS1b. SGC-7901 cells were exposed to GFPS1b at various doses for 48 h. M: DL2000 DNA Marker; lane 1: untreated control cells; lane 2: 30 μg/ml GFPS1b-treated cells; lane 3: 60 μg/ml GFPS1b-treated cells; lane 4: 120 μg/ml GFPS1b-treated cells.

GFPS1b may induce cell cycle arrest in G_2/M phase and apoptosis in SGC-7901 cells with a dose-dependent manner.

3.6. Effect of GFPS1b on mitochondrial transmembrane potential ($\Delta\Psi_m$) in SGC-7901 cells

Mitochondria play an essential role in the propagation of apoptosis, and the disruption of the mitochondrial membrane potential is a critical step occurring in cells undergoing apoptosis. We evaluated whether treatment with the GFPS1b had any effect on the mitochondrial membrane potential ($\Delta\Psi_m$) and plasma membrane integrity, by double-staining control and GFPS1b treated cells with PI and Rh123 (Fig. 8). Under the experimental conditions, the control SGC-7901 cells were PI negative and strongly stained by Rh123 (PI^-Rh123^+, homeostasis, $\Delta\Psi_m$ high) and retained 98.9% of the whole cells. After treatment with GFPS1b for 48 h, the percentages of PI negative and low-staining (PI^-Ph123^-, apoptosis, $\Delta\Psi_m$ low) group increased markedly in a dose-dependent manner. No significant differences were observed in the cell death group of PI^+Rh123^- between the control and GFPS1b treated groups. These results indicated that GFPS1b target the mitochondria in treatment-induced apoptosis without altering plasma membrane permeability in SGC-7901 cells. This finding supports the report that alterations of the mitochondrial functions may play a major role in the apoptotic process, particularly in cell death induced by polysaccharide-fractions agents (Yang et al., 2005; Zhang and Huang, 2006).

3.7. Effect of GFPS1b on expression of relative gene-proteins p53, Bcl-2 and Bax in SGC-7901 cell

Based on the apoptosis analysis by determination of cells with DNA contents on cell arrest and apoptosis, we examined the p53 and Bcl-2 family genes in GFPS1b-induced apoptosis, the expressions of p53, Bax and Bcl-2 proteins were evaluated using immunohistochemical method and the differences in these protein levels were analyzed by changes in intensity/numbers assessed by flow cytometry (Fig. 9A–C). As shown in Fig. 6, GFPS1b treated SGC-7901 cells significantly increased the expression of Bax protein expression level but decreased the expression of Bcl-2 protein, whereas treatment of GFPS1b showed no appreciable change in the expression of p53 protein. To further verify whether GFPS1b-induced SGC-7901 cells apoptosis was involved in the expression of Bcl-2, Bax and p53 proteins, Western blot experiment for evaluating p53, Bcl-2/Bax protein was carried out (Fig. 9D). The results illustrated that in the same dosage and incubation time, GFPS1b could down-regulate Bcl-2 protein level and up-regulate Bax protein level, and also did not influence the expression of p53 protein.

3.8. Enhancement of caspase-3 activity in GFPS1b-induced SGC-7901 cells

Caspase-3 plays a critical role in apoptosis and its activity has been suggested as an index of apoptosis (Cohen, 1997). The caspase-3 enzyme activity stimulated by GFPS1b was measured using fluorometric detection. Caspase-3 activity assay showed an enhancement of enzymic activity starting from 12 h after GFPS1b application, which was evident at 24, 48 and 72 h (Table 1). GFPS1b activated caspase-3 in a time and concentration dependent manner ($P < 0.01$).

4. Discussion

The ability to induce cell apoptosis is an important property of the candidate anti-cancer drugs (Frankfurt and Krishan, 2003). In the past years, increasing number of references revealed that polysaccharides, proteo-polysaccharides originated from mushrooms and other materials exhibited the direct action on the proliferation of the tumor cells in vitro at certain concentrations. PSP, PSK, *Lycium barbarum* polysaccharide (LBP) and *Phellinus linteus* polysaccharide had been revealed to show the inhibition of the tumor cells proliferation by inducing the cell apoptosis (Lee et al., 2005; Li et al., 2004; Lin et al., 2003; Takako et al., 2004; Zhang et al., 2005; Yang et al., 2005).

Here we showed GFPS1b, a novel polysaccharide-peptide isolated from the cultured mycelia of *G. frondosa* GF9801, was able to significantly inhibit the growth of SGC-7901 cell line in a time- and concentration-dependent manner. In addition, GFPS1b could had slight influence on the human normal liver L-02 cells at the same concentra-

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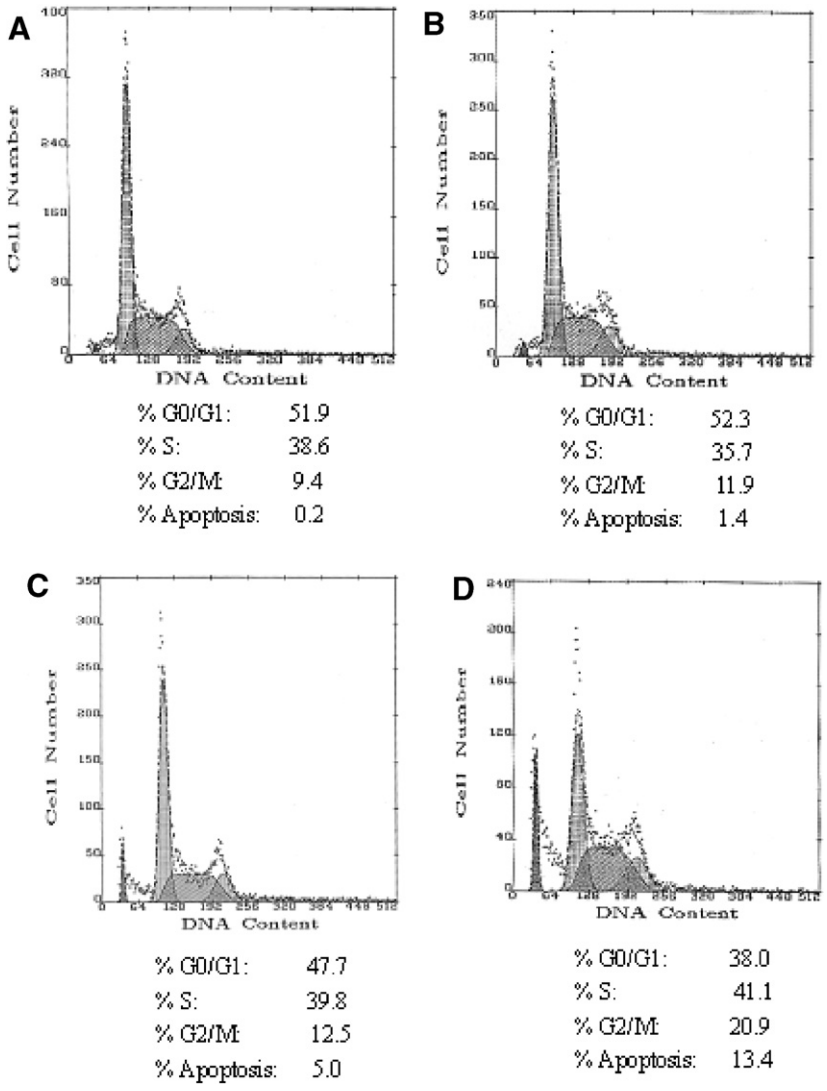

Fig. 7. Cell-cycle (apoptosis ratio, G0/G1, S, G2/M) analysis of SGC-7901 cells exposed without (A) or with GFPS1b ((B) 30 μg/ml, (C) 60 μg/ml, (D) 120 μg/ml) for 48 h. The cell-cycle distribution was based on 2 N and 4 N DNA content for DNA content analysis using Cellfit software.

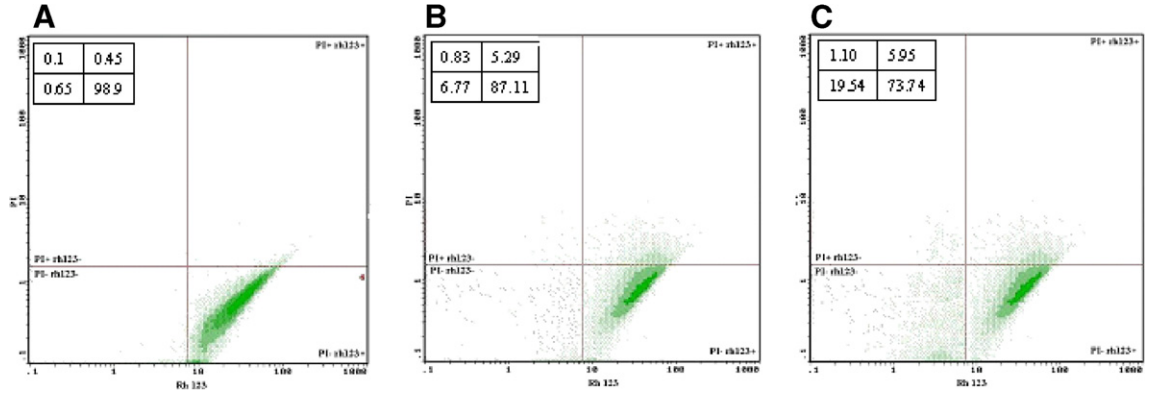

Fig. 8. Effects of GFPS1b on the mitochondrial transmembrane potential ($\Delta\Psi_m$) in SGC-7901 cells ((A) Control, (B) 60 μg/ml, (C) 120 μg/ml) for 48 h was monitored flow cytometrically. Cells were double-stained with Rhodamine-123 and PI for 30 min, respectively. After treatment with GFPS1b for 48 h, the percentages of PI negative and low-staining (PI^-Ph123^-, apoptosis, $\Delta\Psi_m$ low) group, representing the apoptotic cell group, increased markedly in a dose-dependent manner.

tion applied on SGC-7901 cells (Fig. 1). These results were in agreement with the previous reference showing that PSP could selectively inhibit the growth of tumor cells and human normal cells (Yang et al., 2005).

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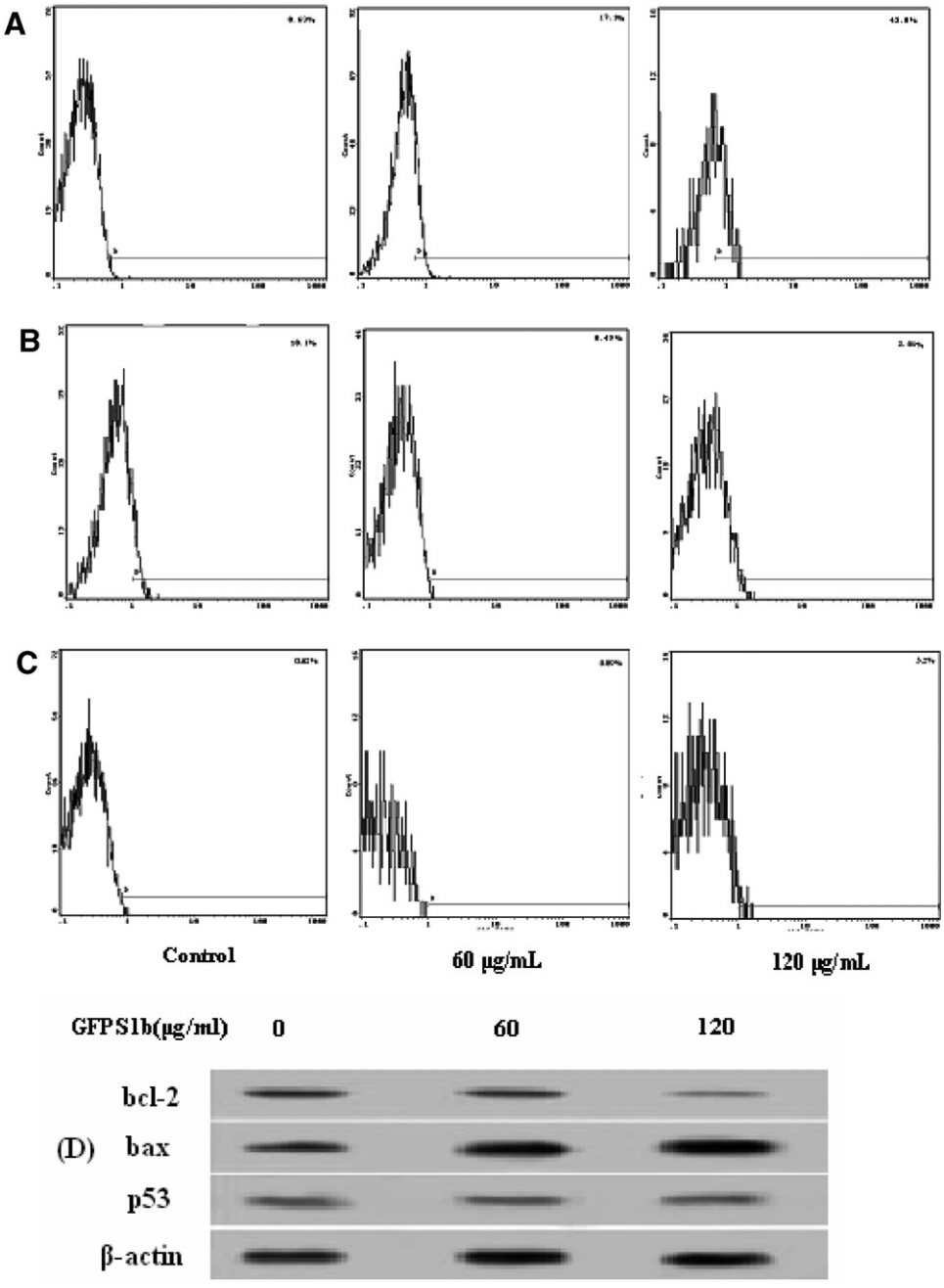

Fig. 9. Expression levels of p53, Bax and Bcl-2 in SGC-7901 cells treated without (control) and with GFPS1b (60, 120 μg/ml) were monitored. Cells were labeled with primary anti-p53 antibody, anti-Bax-2 antibody and anti-Bcl-2 antibody and FITC-conjugated secondary IgG antibody. Bax (A), Bcl-2 (B) and p53 (C) level was monitored flow cytometrically. Western blot assay (D) of Bcl-2, Bax, p53 and Actin protein expression in SGC-7901cell line. Protein (60 μg/lane) from cell lysates was electrophoresed in SDS-PAGE gels, transferred to TotalBlot PVDF membranes, and probed with anti-bcl-2, anti-bax, anti-p53 and anti-actin antibodies, respectively.

The proliferation inhibition of SGC-7901 was the result of apoptosis induction, as evidenced by the cell morphological analyses of the GFPS1b-treated SGC-7901 cells showing the apoptotic characteristics, such as appearance of apoptotic bodies, nuclear shrinkage and condensed chromatin, in contrast to control cells. Flow cytometry data of PI single staining and annexin V-PI dual staining confirmed that GFPS1b could cause the apoptotic ratio in a concentration-dependent manner. DNA fragmentation is very typical of the apoptotic process, with generation within the nucleus of a series of multiplets of a 180 bp subunit. In present studies, typical DNA ladder of apoptosis after the GFPS1b treated SGC-7901 cells were obtained, which further indicated that GFPS1b could induce the apoptosis of SGC-7901 cells. In the cell-cycle analysis, significant increase of cell population in G2/M phase was observed at 30, 60 and 120 μg/ml.

Flow cytometry was used to investigate the loss of mitochondrial membrane potential which had been shown to play an essential role in mediating apoptosis (Desagher and Martinou, 2000; Kroemer et al., 1998). Double-staining

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Table 1
Effects of GFPS1b on activity of Caspase-3 in SGC-7901 cells

Concentration (μg/ml)	Caspase-3 activity (U)		
	12 h	24 h	48 h
Control	35.42 ± 3.57	37.76 ± 2.45	37.59 ± 4.72
60	39.91 ± 2.14*	60.39 ± 3.81**	97.24 ± 5.49**
120	52.29 ± 4.08**	86.64 ± 3.16**	112.87 ± 4.51**

Each value represents mean ± standard deviation of three separate experiments. Significant differences with control were designated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

experiments showed that the decrease in $\Delta\Psi_m$ was with high concentration (60, 120 μg/ml) of GFPS1b, which indicated that the mitochondrial apoptotic death-signal pathway plays a critical role in GFPS1b-induced apoptosis in gastric carcinoma tumor SGC-7901 cells.

Apoptosis is a tightly regulated process and its mechanisms involve in mainly two signaling pathways, including cell death receptor pathway and mitochondrial pathway (Reed, 2001). In the mitochondrial pathway, a variety of death signals triggers the release of several pro-apoptosis proteins. Among the numerous factors known to modulate cancer-related apoptosis, Bcl-2 family and p53 are the most extensively characterized mechanistically. Bax is a crucial mediator and as a tumor suppressor (Yamaguchi et al., 2003), it mediates the p53-induced apoptosis and increases sensitivity to the drug-induced apoptosis. Meanwhile, Bcl-2 is an anti-apoptosis protein. When it is activated or prevalent, apoptosis is prohibited (Tsujimimoto and Shimizu, 2000). And relative expression levels of Bcl-2/Bax were reported to determine the sensitivity to apoptosis (Groc et al., 2001; Danial and Korsmeyer, 2004). By the results of immunohistochemical method and western blot assay, we found that GFPS1b up-regulated the Bax protein expression and down-regulated the Bcl-2 protein expression in SGC-7901 cells, whereas influenced slightly on the expression of p53 protein. Possibly the mechanism of GFPS1b-induced apoptosis in SGC-7901 cells does not include the functional p53' modulation, which also needs to be studied further.

Caspase have been shown to be activated during apoptosis in many tumor cells. Related references revealed that whatever the mitochondrial pathway and cell death receptor pathway, both finally activate caspase-3, which is essential for DNA fragmentation, the morphological change associated with apoptosis, and its activation represents a key and irreversible point in the development of apoptosis (Janicke et al., 1998). To obtain further insight into the GFPS1b action, we detected the activity of caspase-3 in SGC-7901 cells. Results showed that GFPS1b could activate capsase-3 in treated SGC-7901 cell. This experiment suggested that the possible mechanism of GFPS1b-induced apoptosis in SGC-7901 cells also involved caspase-3 activation and the following cascades of reactions.

In conclusion, we have first combined morphological and biochemical techniques to demonstrate that GFPS1b has potent anti-cancer activity, arrest cell cycle at G2/M phase and induce apoptosis in SGC-7901 cells. And the induction of apoptosis was closely associated with drop in mitochondrial transmembrane potential, down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3. Nevertheless, further detailed studies are required to clarify the cellular signaling process by which GFPS1b induces apoptosis in SGC-7901 cell.

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