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Evaluation the Antitumor Effects of Exopolysaccharide Produced by Newly Isolated *Bacillus amyloliquefaciens* Strain C-1

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Exopolysaccharide (EPS) was extracted and purified from a newly isolated *Bacillus amyloliquefaciens* strain, C-1. The anti-tumor activities of this EPS were tested *in vitro*. C-1 EPS exhibited good and direct, dose-dependent *in vitro* anti-proliferative effects on 6 human cancer cell lines. The inhibition rates of 7 mg/ml EPS on tumor cells SGC-7901, HeLa, A549, 7721, 7402, and MCF-7 were 73.2%, 78.6%, 65.0%, 64.0%, 41.1%, and 57.7%, respectively. The anti-proliferative effect of EPS was associated with the direct induction of apoptotic cell death, rather than the cell cycle. It was also verified that the expression of anti-apoptotic Bcl-2 family proteins was decreased. These results collectively show that this EPS has considerable potential for use in medical compounds.

Key words: Exopolysaccharides; Anti-tumor activity; Cell apoptosis; *Bacillus amyloliquefaciens* C-1.

Reactive oxygen species (ROS) and oxygen-derived free radicals, generated by normal metabolic processes or from exogenous factors and agents, may contribute to a variety of pathological effects, including DNA damage, carcinogenesis, and cellular degeneration, and it may induce many diseases, including cancer, atherosclerosis, hyperlipidemia, and diabetes[1,2]. Most organisms possess antioxidant defense and repair systems that are insufficient to prevent damage. Therefore, it is essential to develop new agents with effective therapeutic functions that protect the human body from free radicals and slow the progress of many chronic diseases[3].

Exopolysaccharide (EPS) is one type of metabolite in many microorganism species, and it is always found in the growth environment. These biopolymers are secreted under special environmental conditions, such as with unique concentrations of nutrients in culture medium [4, 5]. They are usually water soluble, biodegradable, biocompatible, edible, and nontoxic to humans and the environment[6]. Recently, polysaccharides have attracted increasing attention in the biochemical and medical areas due to their immunomodulatory and anti-tumor effects that include immunostimulation, antioxidant, antitumor, and anti-inflammatory activities[7, 8]. For example, *Lycium barbarum* polysaccharide (LBP) induced apoptosis in QGY7703 cells[9], and *Lactobacillus acidophilus* cell-bond exopolysaccharide (cb-EPS) inhibited the proliferation of HT-29 colon cancer cells. Moreover, EPS possesses great pro-apoptotic activity in oncotherapy and adjuvant therapy[10]. Therefore, it is of great value to develop corresponding bacterial EPS-related bio-drugs or food adjuncts to counteract cancer.

Bacillus amyloliquefaciens is known for its antifungal and antimicrobial activities, while neither the EPS of *B. amyloliquefaciens* nor its antitumor activity has been previously reported[11, 12]. *B. amyloliquefaciens* strain C-1 was isolated

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from fruit samples, and it produced EPS during fermentation. The EPS was purified by ethanol precipitation, the Sevag method, ion-exchange chromatography, dialysis, and freeze drying. The physical and chemical properties of the EPS were determined. To obtain more insight into this EPS, its antioxidant activities were investigated using various *in vitro* assays. Additionally, the antitumor activities of C-1 EPS were evaluated in three ways (inhibition of human tumor cell growth, cell apoptosis, and cell cycle progression). Finally, the underlying mechanism of apoptotic cell death induction was preliminarily determined.

MATERIALS AND METHODS

Microorganism

Bacillus amyloliquefaciens strain C-1 (GenBank 16S rRNA accession no. JX028840) was isolated by the Food Microbiology Lab of the Nutrition and Food Safety Engineering Research Center of Shaanxi Province, Xi'an, China. It is now stored in the China Center for Type Culture Collection with the strain number CCTCC M 2012177. Tumor cells included MCF-7(human breast adenocarcinoma MCF-7), HeLa cells, 7901 (human gastric cancer SGC-7901), A549 (human lung cancer cell A549), 7721 (human hepatocellular carcinoma SMMC-7721), and 7402 (human hepatocarcinoma BEL-7402). Tumor cells and normal human gastric epithelial cells A1 were seeded in 96-well plates and incubated at 37°C with 5% CO_2 at 80% humidity.

Production and purification of EPS

For EPS production, LB medium with 1% glucose (pH 7.5) was used. A 2% inoculum of overnight *B. amyloliquefaciens* C-1 culture was subcultured and grown for 72 h at 30°C at 200rpm. To extract EPS, 1l of fermented *B. amyloliquefaciens* C-1 culture supernatant was concentrated, and crude EPS was precipitated with pre-cooled 95% ethanol[4, 13]. The EPS was then deproteinized with Sevag reagent and dialyzed (molecular weight cutoff, 8-14 kDa) against distilled water. The dialyzed solution was concentrated and lyophilized. The dried EPS was stored at room temperature for further use.

In vitro proliferative activity against tumor cells

The antitumor activity of C-1 EPS was determined as previously described[10]. Six tumor cell lines (MCF-7, HeLa, 7901, A549, 7721, and 7402) and 1 normal cell line were seeded and incubated overnight. Next, 7 mg/ml C-1 EPS was added to the tumor cells and incubated for 48 h at 37°C with 5% CO_2 and 80% humidity. Then, 20 µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Fluka) solution (5 mg/ml stock) was added, and the cultures were incubated for another 4 h. The cell mixtures were centrifuged, and the precipitates were dissolved in 100 µl dimethyl sulfoxide (DMSO; Merck). The plates were then gently agitated for 5 min. Finally, the absorbance was measured at 490 nm with a multiple microplate reader (Bio-Tek Instruments Inc., Highland Park, USA). PBS was used as a negative control. The percentage of inhibition was calculated as [(B-A)/ A× 100%], where A and B are the absorbance values of the control group and treated cells, respectively. To test whether the inhibition of cancer cells' proliferation by C-1 EPS was dose-dependent, 0.3, 0.6, 1.2, 3.5, 5.25, or 7 mg/ml EPS was added into the culture medium of selected SGC7901 and HeLa cells, and the inhibition rate was obtained using the method described above. All experiments were performed in triplicate.

Flow cytometry (FCM)/apoptosis detection by FACS analysis

Apoptosis detection was conducted using the Annexin V-FITC apoptosis kit (Zhuhai Joincare Biosciences Co,. Ltd.). SGC-7901 cells were chosen for this test. Briefly, 0.3 mg/ml (low concentration), 0.6 mg/ml (medium concentration), and 1.2 mg/ml (high concentration) EPS were added into the cell culture medium as a treatment. The treated and untreated cells were harvested and washed twice with pre-cooled PBS. The cell pellets were re-suspended in 500 µl of 1× binding buffer at a concentration of 1-5×10^5 cells per ml. Next, 5 µl of Annexin V-FITC (Annexin V-fluorescein isothiocyanate) and 10 µl PI (propidium iodide) were added into the cell suspension, and the mixture was gently vortexed. The stained samples were incubated for 5-15 min in darkness and analyzed with a BD FACSCanto II flow cytometer (BD Biosciences, US) within 1 h. The ratio of apoptotic or necrotic cells was counted, and the apoptosis of SGC7901 induced by EPS addition was analyzed. All data were analyzed using the FACSCanto Clinical software supplied with the instrument[14].

Cell cycle analysis

DNA content and cell cycle distribution was analyzed with a cell cycle staining kit (Multisciences Biotech Co,. Ltd). SGC-7901 cells were treated with 0.3, 0.6, or 1.2 mg/ml C-1 EPS for 48 h, then harvested at times corresponding to 2×10^5 to 1×10^6 cells. One milliliter of DNA staining solution (PI) and 10 µl of permeabilization solution were added to the cells, and vortexed for 10 sec, then incubated for 30 min at room temperature. For each determination, the DNA content of cells was measured using a BD FACSCanto II flow cytometer (BD Biosciences, US). Apoptotic cells were considered to constitute the sub-G1 population. And the cell cycle distribution was determined[14].

Western blot analysis

SGC-7901 cells were treated with 0.3, 0.6, and 1.2 mg/ml C-1 EPS for 48 h, washed twice with PBS, mixed with RIPA lysis buffer (Santa Cruz, CA, USA), and placed on ice for 30 min. The mixture was centrifuged to collect proteins in the supernatant. The protein concentration was determined by the Bradford method. For each sample, 30 µg of protein was loaded and run on 10% SDS-PAGE gels, transferred to PVDF membranes and blocked for 2 h. The membranes were then incubated with primary antibodies against Bcl-2, Bax and NADPH (Beijing Biosynthesis Biotechnology Co., Ltd) at 4°C overnight. After washing, the blots were incubated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Beijing Biosynthesis Biotechnology Co., Ltd) at 37°C for 2 h. Detected proteins were visualized by enhanced chemiluminesence.

Statistical analysis

The data were analyzed by ANOVA, and $p < 0.05$ was selected prior to the experiments to reflect statistical significance. Unless otherwise stated, all results are expressed as the mean ± SD ($n \geq 3$). All the analyses were conducted using the General Linear Model (GLM) procedure of SAS Version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Inhibitory effect on human tumor cells

In recent studies, it was demonstrated that polysaccharides from microorganisms have direct antiproliferative effects on human cancer cells[8, 10, 15]. Thus, increasing attention has been paid to the screening and development of functional polysaccharides for the eventual elimination of tumorigenic cells and rebalancing of normal cellular homeostasis[16]. In this work, the effects of C-1 EPS extracts on 6 human tumor cell lines were tested, and significant inhibitory activities against proliferation were obtained. However, there was no obvious inhibitory effect on a normal cell line (human gastric epithelial cells A1). The tumor cells were treated with 7 mg/ml EPS. The results shown in Fig. 1A indicated that the corresponding inhibition rates against 7901, HeLa, A549, 7721, 7402, MCF-7and A1 cells were 73.2%, 78.6%, 65.0%, 64.0%, 41.1%, 57.7% and 2.1%, respectively. Compared with the control, these data demonstrated that C-1 EPS has a potent suppressive effect on the growth of tumor cells *in vitro* ($p<0.01$).

To learn whether cell proliferation inhibition by C-1 EPS was dose-dependent, various concentrations of EPS (0.3-7 mg/ml) were added into the culture medium of 7901 and HeLa cells. As shown in Fig. 1B, the EPS induced a distinct dose-

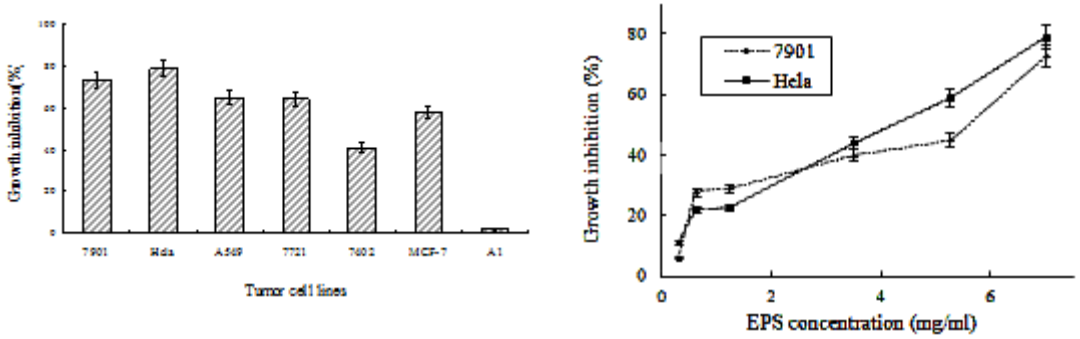

Fig. 1. The growth inhibition of different human cells by 7 mg/ml C-1 EPS*in vitro* (A) and the dose-dependent effect of EPS on the growth of 7901 and HeLa tumor cells (B)

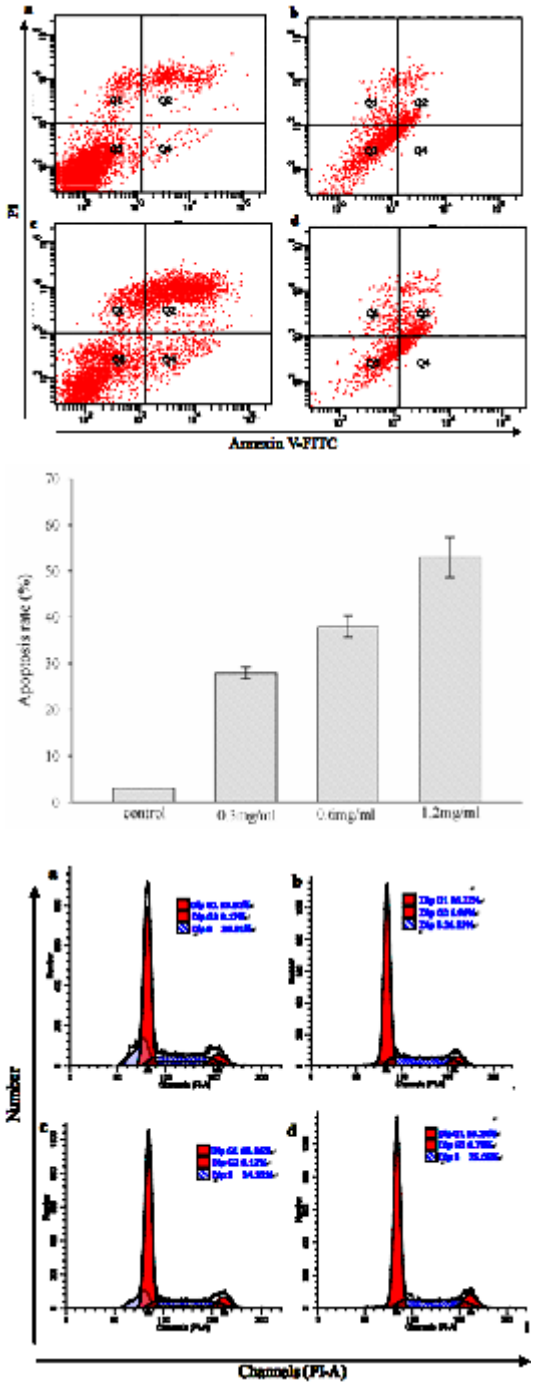

Fig. 2. The effect of C-1 EPS on apoptosis (A) and the apoptotic rate comparison (B) and cell cycle distribution (C) in SGC7901 cells by flow cytometry after Annexin V-FITC/PI staining. Cells were treated with EPS at 0 (a), 0.3 (b), 0.6 (c) and 1.2 mg/ml (d) concentrations for 48 h. Q1, necrotic cells; Q2, late apoptotic or necrotic stage; Q3, control cells; Q4, early apoptotic stage

dependent diminution of cell viability. In cell samples treated with 0.3 mg/ml EPS, a 6% inhibition rate for SGC7901 cells and an 11% inhibition rate for HeLa cells were obtained. The corresponding inhibition rates with 7 mg/ml EPS treatment were 73% and 79%. It has been reported that the negatively charged phosphate groups on EPS can bind to receptors on the surface of immune cells with high affinity, effectively activating an immune response[17]. Therefore, the high antitumor activity of EPS is most likely due to its high affinity for the receptors on immune cells. This interpretation can explain why C-1 EPS also showed high and broad-spectrum antitumor activities *in vitro*.

Inducing of apoptosis and its mechanism

One key mechanism associated with the control of cancer cell growth is apoptosis. The induction of apoptosis has been considered a potential approach to the treatment of cancer[18, 19]. To determine whether the growth inhibitory effect of C-1 EPS on SGC7901 was associated with the induction of apoptotic cell death, FITC-conjugated Annexin V and PI (FL2-H) staining were used as criteria to distinguish viable cells ($Annexin^-/PI^-$), early apoptotic cells ($Annexin^+/PI^-$), late apoptotic cells ($Annexin^+/PI^+$), and necrotic cells ($Annexin^-/PI^+$). The population of cells shifted from viable cells to early- and late-stage apoptotic cells after treatment with EPS. The rate of apoptosis was calculated as the sum of early and late apoptotic cells. The proportion of apoptotic cells increased in an EPS concentration-dependent manner. As shown in Fig. 2A and 2B, incubation of SGC7901 cells with 0.3, 0.6 and 1.2 mg/ml EPS for 48 h caused a 28%, 38% and 53% increase in apoptotic rates, respectively, compared to control cells. Experiments were also conducted to evaluate whether EPS-induced cytotoxicity was associated with a disturbance in SGC7901 cell cycle regulation. As shown in Fig. 2C, no obvious effect was detected on the percentage of cells in the cell cycle, including in G0/G1 and S phases. Therefore, it appears that C-1 EPS may inhibit 7901 cell proliferation by directly affecting cell morphology other than the cell cycle; a similar result was reported for the apoptosis of HT-29 colon cells induced by cb-EPS[10].

Apoptosis is a biological process requiring activation of several signaling cascades[18]. Bcl-2 family proteins play critical roles

in the regulation of apoptosis by pro-apoptotic factors such as Bax, Beclin-1, and GRP78 or anti-apoptotic molecules like Bcl-2 [21]. It was reported that cb-EPS dramatically induced Beclin-1 and GRP78 and affected Bcl-2 and Bax regulation during apoptosis of HT-29 cells[10]. Crude fucoidan induced the apoptosis of mouse breast cancer 4T1 cells in a similar manner[21, 22]. To investigate the possible mechanism underlying the induction of cell apoptosis by EPS, the expression of Bcl-2 and Bax in SGC-7901 cells was examined after treatment with EPS. Western blotting showed that C-1 EPS significantly decreased the expression of Bcl-2; however, it increased the expression of Bax. As shown in Fig. 3, the ratio of Bcl-2/Bax was clearly decreased, which is essential for apoptosis. This result demonstrated that C-1 EPS has pro-apoptotic effects on SGC-7901 cells. Previous studies have indicated that incubation of polysaccharides together with tumor cells could change the expression of apoptosis-inducing signals within tumor cells, which explains the *in vitro* antiproliferative effect of polysaccharides[10, 20, 21, 22].

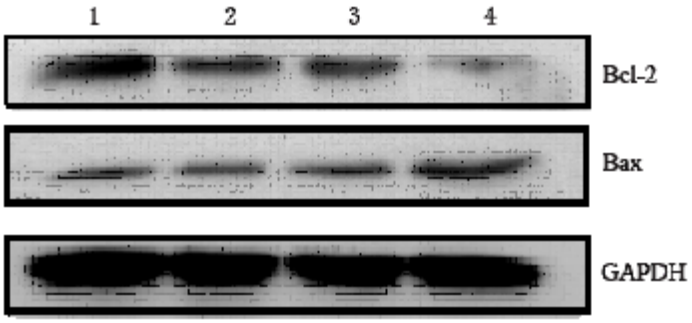

Fig. 3. Expression of Bcl-2 and Bax in SGC-7901 cells detected by western blot after 48-h treatment with C-1 EPS. Expression of GAPDH was used as an internal control. Lane 1, control cells; lanes 2-4, 7901 cells treated with 0.3, 0.6, or 1.2 mg/ml EPS, respectively

In *Bacillus* strains, EPS is a major component of the extracellular matrix that holds differentiated cell chains together to form highly organized colony and pellicle architecture. EPS synthase proteins are controlled by SinIR, sigma^H, Spo0A and ylbF/YmcA. In *B. amyloliquefaciens* strain C06, the *epsA-O* operon may contribute to EPS production[20]. *B. amyloliquefaciens* is known for its antifungal and antimicrobial activities deriving from ^{3}-polyglutamic acid, surfactin, iturin, fengycin, glucosidase, and other factors[11, 12, 20]. Neither the EPS of *B. amyloliquefaciens* nor its antitumor activity has previously been reported[11, 12]. Here, we report a newly isolated *B. amyloliquefaciens* strain, C-1, with strong production of an EPS that exhibited high and general antiproliferative activity against human cancer cells and induced cell apoptosis *in vitro*. Exposure of normal cells to the EPS of *B. amyloliquefaciens* C-1 did not show a cytotoxic effect and did not induce apoptosis (data not shown). Therefore, it seems that the anti-proliferative and apoptosis-inducing effects of C-1 EPS against human cancer cells are target-specific. A possible mechanism is that C-1 EPS inhibits expression of Bcl-2 and increases expression of Bax, which activates apoptotic cell death. Studies to investigate the target molecules of this EPS should evaluate various characteristics of human cancer cells to be useful in medical research.

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