Polysaccharide sulphated derivative from *Aconitum coreanum* induces cell apoptosis in the human brain glioblastoma U87MG cell line via the NF-κB/Bcl-2 cell apoptotic signaling pathway

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Received July 13, 2017; Accepted December 12, 2017

DOI: 10.3892/or.2017.6165

Abstract. In a previous study, our team preliminarily investigated the bioefficacy of an extracted polysaccharide from the medicinal plant *Aconitum coreanum* (ACP1). In the present study, we further evaluated the antitumor efficacy of an ACP1 sulphated derivative (ACP1-s) in the human brain glioblastoma U87MG cell line. Cell viability assay and flow cytometry results demonstrated that 400, 800 and 1,600 μg/ml ACP1-s induced cell growth inhibition and cell apoptosis. We then investigated the underlying molecular mechanism of the ACP1-s induced cell apoptosis and found that the NF-κB/Bcl-2 cell apoptotic signaling pathway was involved. Following treatment with ACP1-s, the expression of IκB in U87MG cells was significantly upregulated, whereas the level of NF-κB and the ratio of Bcl-2/Bax was significantly decreased. The level of cleaved caspase-3 was increased accordingly. When we introduced exogenous p65 protein into the U87MG cells, the ACP1-s-induced cell growth inhibition and cell apoptosis were partially neutralized, and the expression of the anti-apoptotic gene Bcl-2 was recovered accordingly. These findings suggest the potential value of ACP1-s as a novel therapeutic agent for the treatment of glioblastoma.

Introduction

Glioblastoma (GB) is the most common and malignant type of primary brain tumor in adults. The standard treatment of GB includes surgery, radiation therapy, chemotherapy and also combined treatment. However, no matter which treatment approach is used, the therapeutic efficacy of GB is far from satisfactory.

There are many types of natural medicines that possess potential antitumor efficacy in traditional Chinese medicine (TCM). The antitumor activities of TCM mainly include: i) inhibition of tumor proliferation and migration; ii) inhibition of cell cycle progression; iii) promotion of cell apoptosis; and iv) antiangiogenesis. Currently, more and more effective ingredients are gradually being purified from natural medicines and have been applied to treat various types of carcinomas, including glioma (1-5). Zhang *et al* (6) reported that shikonin significantly inhibited the cell proliferation, migration, invasion and the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 in human glioblastoma U87 and U251 cells. Cao *et al* (5) used a Chinese medicine formula named 'Pingliu Keli' (a mixture of *Lycium chinense*, *Dendrobium officinale* and *Arisaema heterophyllum*) to treat SHG-44 glioma cells and they found that the folk remedy significantly induced cell apoptosis *in vitro*.

Nuclear factor-κB (NF-κB) is a transcription factor regulating a wide array of genes mediating numerous important biological processes, such as cell proliferation, autophagy, DNA repair, motility and protection against apoptosis (7). Proteins of the inhibitory κB family (IκB) serve as inhibitors and regulators of NF-κB activity. Phosphorylation of IκBs results in their proteasomal degradation and the release of NF-κB for nuclear translocation and activation of gene transcription (8). The *in vitro* and *in vivo* studies have demonstrated that some natural products such as isoflavone, curcumin, resveratrol and lycopene exert inhibitory effects on human and animal cancers by targeting NF-κB and its regulated gene products, including c-myc, Bcl-2, Bcl-xL, MMPs and vascular endothelial growth factor (VEGF) (9-13).

Aconitum coreanum is one of the most important herbs, predominantly found in China, Korea and Japan. *A. coreanum* has long been considered as a traditional folk medicine with therapeutic effects against many disorders, such as migraine headache, cardialgia, facial distortion, infantile convulsion, epilepsy, tetanus, vertigo and rheumatic arthralgia (14). In our

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Key words: glioblastoma, polysaccharide, cell apoptosis

previous study, we succesfully extracted a polysaccharide from *A. coreanum* and preliminarily investigated its bioefficacy in regards to inhibiting the cell migration in breast cancer cells. However, whether it induces cell apoptosis in cancer cells has not been elucidated.

In the present study, we prepared a polysaccharide sulphated derivative from *A. coreanum* (ACP1-s) and examined its inhibitory capacity on human malignant glioblastoma U87MG cells. We also revealed the molecular mechanism underlying ACP1-s-induced apoptosis. Our findings may contribute to the further understanding of the biological efficacy of polysaccharide, as well as highlight the possibility of ACP1-s as a novel therapeutic agent for the treatment of glioma.

Materials and methods

Preparation of A. coreanum polysaccharide. The *A. coreanum* polysaccharide (ACP) was prepared by our research team, as previously reported (15). Briefly, the roots of *A. coreanum* were grinded and defatted with ethanol. The residue was extracted with hot water, and the extract supernatant was then precipitated with ethanol. Crude polysaccharide precipitate was collected and dried under reduced pressure. After removing the proteins, the crude ACP was yielded by dialysis and lyophilisation. The crude ACP was further applied to a DEAE-cellulose column and a Sepharose CL-6B column to yield purified *A. coreanum* polysaccharide named ACP1.

Physicochemical characterization and sulphated modification of ACP1. We used high-performance gel permeation chromatography (HPGPC) to determine the homogeneity and molecular weight of ACP1. Monosaccharide compositions were identified and quantified using gas chromatography (Gas Chromatograph, GC-2010 Plus; Shimadzu, Beijing, China). Fourier transform infrared spectrum was measured with a Nicolet 5700 FT-IR spectrometer (Thermo Fisher Scientific, Co., Ltd., Shanghai, China) in the frequency range of 400-4,000 cm^{-1} (15).

The ACP1 with sulphated modification was designated as ACP1-s. ACP1-s was prepared according to the chlorosulfonic acid-pyridine method reported by Xu *et al* (16).

Cell culture. U87MG, a human brain glioblastoma cell line, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was routinely cultivated in MEM medium (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Beijing, China), 1X Non-essential amino acid (NEAA; Invitrogen, Carlsbad, CA, USA), 1% glutamine and 100 U/ml penicillin-streptomycin (HyClone Laboratories). The NE-4C neuroectodermal cells (ATCC) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin. All cells were cultured in a humidified incubator with 5% CO_2 at 37˚C.

Expression vector and cell transfection. The NF-κB eukaryotic expression vector pcDNA3.1-P65 and its control vector pcDNA3.1 were maintained in our laboratory. Before transfection, the U87MG cells were planted into a 6-well plate. After 24 h, when the cells reached 70% confluence, U87MG cells were transfected with an equal amount of pcDNA3.1-P65 or pcDNA3.1 plasmid using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific). Stable cells were selected using neomycin.

Cell viability assay. Cell viability was determined by MTT assay. U87MG and NE-4C cells were plated into a 96-well plate at a density of $1x10^4$ cells/well and treated with different concentrations of ACP1-s for 24 h. The medium was then replaced with 100 μl fresh medium containing 0.5 mg/ml MTT (0.5 mg/ml; Sigma-Aldrich, Shanghai, China). After 4 h of incubation, the supernatants were discarded and 150 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added. Optical density (OD) at 570 nm was measured using a microplate reader (BioTek Instruments, Beijing, China). The cell growth inhibition rate (IR) was calculated as the ratio between the OD of the ACP1-s treatment group and the OD of the control group. All of the experiments were performed in triplicate and repeated at least three times.

Apoptosis analysis. Cell apoptosis was determined using Annexin V-FITC and PI double staining flow cytometric analysis. Briefly, $1x10^6$ U87MG cells were treated with different concentrations of ACP1-s for 24 h. Then, the cells were collected and incubated with Annexin V-FITC/PI (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min in the dark and immediately analyzed with flow cytometry (FACScan; BD Biosciences) with the FlowJo FACS analysis software (FlowJo, LLC, Ashland, OR, USA). The cells in the different portions represented the different cell states as follows: late-apoptotic cells were presented in the upper right quadrant, viable cells were presented in the lower left quadrant, and early apoptotic cells were presented in the lower right quadrant.

Western blotting. Cells were lysed in RIPA lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) supplemented with cocktail protease inhibitor (Roche Diagnostics, Shanghai, China). Protein concentrations were determined by BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Equal amount of proteins were separated by 5-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The blots were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in TBST at 37˚C for 1 h. After that, the blots were incubated with diluted solution of monoclonal antibodies against NF-κB (1:1,000; #ab16502; Abcam, Shanghai, China), IκB (1:1,000; #ab32518; Abcam), Bcl-2 (1:1,000; #ab32124; Abcam), Bax (1:1,000; #ab32503; Abcam), cleaved caspase-3 (1:1,000; #9661; Cell Signaling Technology, Shanghai, China) and β-actin (1:3,000; #sc47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4˚C overnight. After being washed for 3 times in TBST, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG-HRP second antibody, #sc2004; and goat anti-mouse IgG-HRP second antibody, #sc2005; Santa Cruz Biotechnology) at room temperature for 45 min. After being washed for another 3 times in TBST, the blots were visualized by an enhanced chemiluminescence system (ECL; Thermo Fisher Scientific). Protein expression was determined

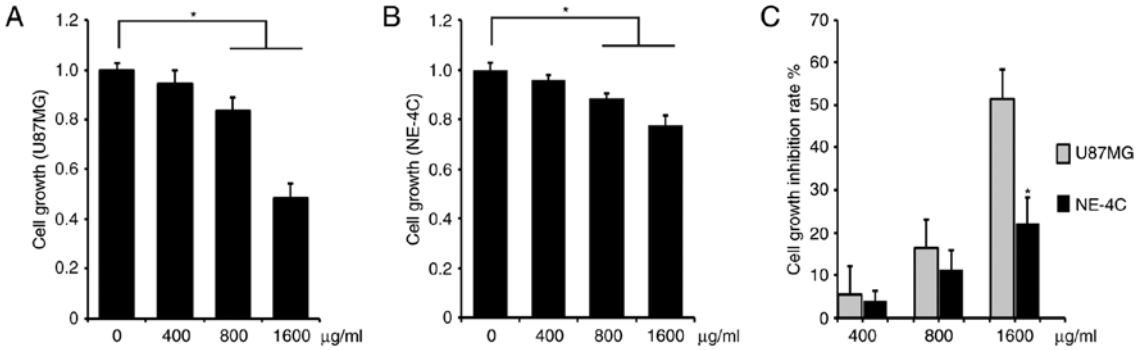

Figure 1. ACP1-s inhibits the cell growth of U87MG cells. U87MG and NE-4C cells were treated with 400, 800 or 1,600 μg/ml of ACP1-s for 24 h. Cell growth was determined by MTT assay. (A) Cell growth of U87MG cells. *P<0.05 as compared with the control group. (B) Cell growth of NE-4C cells. *P<0.05 as compared with the control group. (C) Comparison of the cell growth inhibition rate between the ACP1-s-treated U87MG cells and NE-4C cells. *P<0.05 as compared with the U87MG cells. ACP1-s, polysaccharide sulphated derivative from *A. coreanum*.

semi-quantitatively by densitometric analysis with Quantity One software (Bio-Rad Laboratories, Beijing, China).

Real-time PCR. Total cellular RNA was extracted using an Eastep® Super Total RNA Isolation kit (Promega, Beijing, China). RNA was converted to cDNA with SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Subsequently real-time PCR was performed using an ABI SepOnePlus Real-Time PCR system (Applied Biosystems, Beijing, China). The reaction system consisted of 20 μl containing an aliquot of first-strand cDNA as a template, 10 μl 2X SYBR Premixed buffer (Roche Diagnostics) and 2 μl forward and reverse primers. The primers were as follows: Bcl-2 sense, 5'-AAAGGACCTGATCATTGGGG-3' and antisense, 5'-CAACTCTTTTCCTCCCACCA-3' (17); β-actin sense, 5'-TCACCCACACTGTGCCCATCTACGA-3' and antisense, 5'-CAGCGGAACCGCTCATTGCCAATGG-3' (18). The PCR amplification process consisted of one cycle at 95˚C for 10 min, 30 cycles at 95˚C for 10 sec and 55˚C for 30 sec.

Statistical analysis. The SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all data analysis. Data shown represent mean ± SD. Student's t-test was used to compare statistical differences for variables among the treatment groups. Significance was defined as *P<0.05.

Results

Physicochemical and structural characterization of ACP1 and ACP1-s. Physicochemical properties of ACP1 were reported in our previous study (15). Briefly, the carbohydrate content of ACP1 was 96.1%. HPGPC elution profile revealed that ACP1 was a homogeneous polysaccharide with an average molecular weight of 67.6 kDa. GC demonstrated that ACP1 was composed of arabinose, mannose and glucose with a molar ratio of 0.24:1:3.23. The FT-IR spectrum demonstrated that α- and β-configurations present simultaneously in ACP1. FT-IR spectrum also showed some characteristic absorption of ACP1, such as O-H bending, C-H stretching and C-O bending.

The sulphated derivative ACP1-s was prepared according to the CSA-Pyr method. The sulfur content of ACP1-s was 7.57% (w/w). The FT-IR of ACP1-s showed characteristic absorption bands of an asymmetrical S=O stretching vibration and a symmetrical C-O-S vibration associated with a C-O-SO_3 group, which indicated that ACP1 was successfully sulphated (15).

ACP1-s inhibits the cell growth of human brain glioblastoma U87MG cells. We investigated the cell growth inhibition capacity of ACP1-s for U87MG glioblastoma cells. As a control group, mouse neuroectodermal cell line NE-4C was investigated in parallel. We added 400, 800 or 1,600 μg/ml ACP1-s in the cell culture media and determined the cell proliferation by MTT assay. These three concentrations of ACP1-s inhibited the cell growth in both the U87MG and NE-4C cells, and the doses of 800 and 1,600 μg/ml ACP1-s exhibited significantly higher activities than that of the 400 μg/ml ACP1-s treatment (Fig. 1A and B; P<0.05). Then we compared the cell growth inhibition rate (IR) of ACP1-s between the U87MG and the NE-4C cells. We found that all three doses of ACP1-s generated higher IRs in the U87MG cells (5.4 vs. 3.9%, 16.5 vs. 11.4% and 51.4 vs. 22.2%; Fig. 1C), and the IR of the 1,600 μg/ml dose group was significantly higher in the U87MG cells (P<0.05).

ACP1-s induces cell apoptosis in U87MG cells. We examined the cell apoptosis of the ACP1-s-treated U87MG cells with flow cytometry. As shown in Fig. 2, all three doses of 400, 800 and 1,600 μg/ml ACP1-s significantly induced cell apoptosis compared to the control group (7 vs. 22.5 vs. 43.9%; Fig. 2B, P<0.05), and the cell apoptotic percentage of the 1,600 μg/ml dose group was the highest (Fig. 2).

ACP1-s induces cell apoptosis through the NF-κB/Bcl-2 signaling pathway. We analyzed the molecular mechanism underlying the ACP1-s-induced cell apoptosis using western blot analysis and real-time PCR methods. Western blot results indicated that after treatment with ACP1-s, the level of IkB was increased 2.1-fold and the level of NF-κB was accordingly reduced 5.2-fold (1,600 μg/ml dose group; Fig. 3A and B, P<0.05). The expression levels of NF-κB-regulated genes Bcl-2, Bax and caspase-3 were also altered after treatment with ACP1-s. The ratio of Bcl-2/Bax was reduced 3-fold and the level of cleaved caspase-3 was increased 5.8-fold (1,600 μg/ml dose group; Fig. 3A and B, P<0.05).

We then introduced exogenous p65 protein to delineate the specificity of the ACP1-s-activated NF-κB/Bcl-2 cell apoptotic

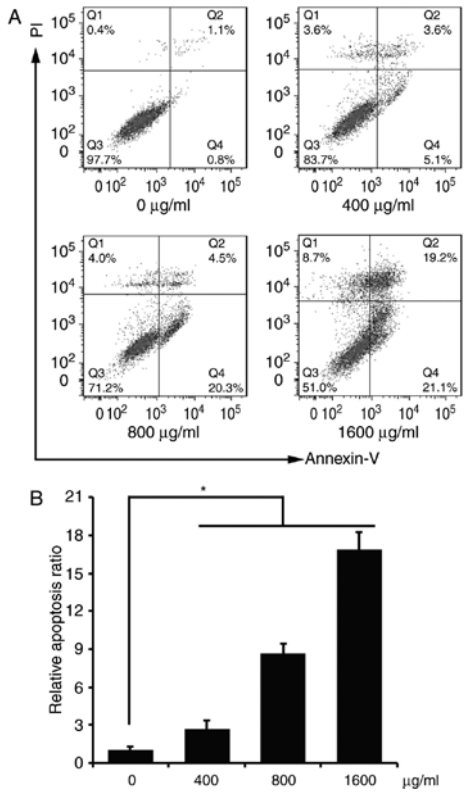

Figure 2. ACP1-s induces cell apoptosis in U87MG cells. U87MG cells were treated with 400, 800 or 1,600 μg/ml of ACP1-s for 24 h. Cell apoptosis was determined using Annexin V-FITC and PI double staining flow cytometric analysis. (A) The dot-plot chart of flow cytometry. (B) Comparison of the relative apoptosis ratios between the different dosage groups. *P<0.05 as compared with the control group. ACP1-s, polysaccharide sulphated derivative from *A. coreanum*.

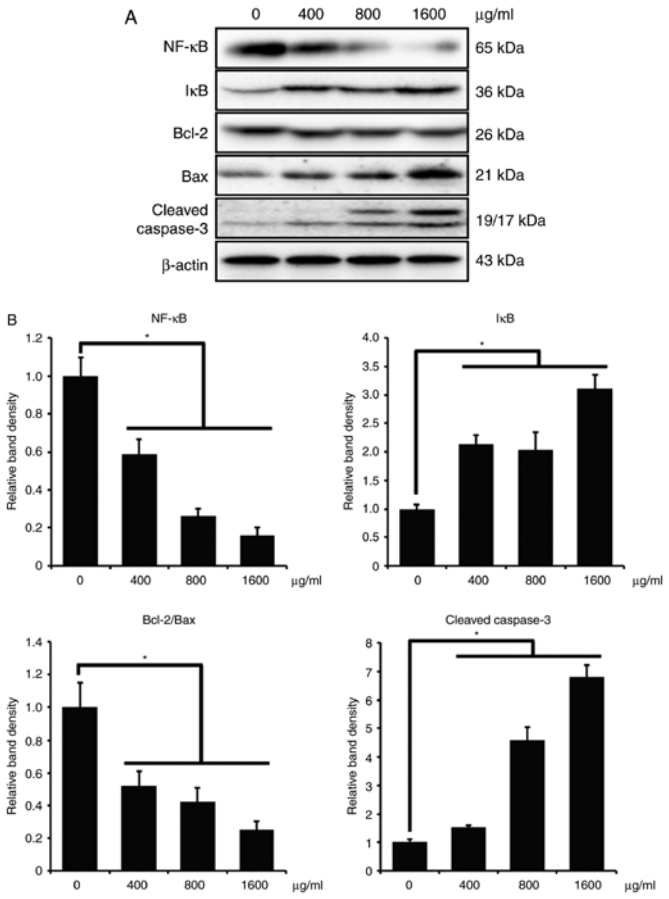

Figure 3. ACP1-s induces cell apoptosis through the NF-κB/Bcl-2 apoptotic pathway. U87MG cells were treated with 400, 800 or 1,600 μg/ml of ACP1-s for 24 h. Cells were lysed and the expression levels of cell apoptotic-associated molecules including NF-κB, IκB, Bcl-2, Bax and cleaved caspase-3 were examined by western blot analysis. (A) Western blot analysis. (B) Relative band density of the blots. *P<0.05 as compared with the control group. ACP1-s, polysaccharide sulphated derivative from *A. coreanum*.

signaling pathway. We first established a stable U87MG cell line that overexpressed p65 (Fig. 4A), and then treated the stable cells with ACP1-s for 24 h to investigate the cell growth and cell apoptosis. We found that after introducing p65 to the U87MG cells, the ACP1-s-induced cell growth inhibition and cell apoptosis were abolished (Fig. 4B and C; P<0.05).

Then, we used real-time PCR to further examine the Bcl-2 mRNA expression. Compared to the untransfected cells or the cells transfected with the control plasmid, overexpression of p65 protein in U87MG cells partly neutralized the ACP1-s-induced Bcl-2 inhibition (Fig. 5; P<0.05).

Discussion

Medicinal plants have a long history of use in the fight against diseases, and some medicinal plants now have been developed into important drugs (19). There is growing interest in the use of medicinal plant-derived drugs to combat human tumors in recent years. It is estimated that currently more than 70% of anticancer drugs have a natural origin (20).

Glioblastoma (GB) is the most damaging tumor of the brain. The usual survival at identification of GB is only approximately 1 year due to the therapeutic resistance and tumor relapse after removal by surgery (21). Currently, the drugs of choice for first-line therapy of GB include the methylating agent temozolomide (TMZ) and chloroethyl-derivatives of nitrosourea: carmustine, nimustine, lomustine and fotemustine (22). Although these drugs improve clinical outcomes, chemoresistance remains one of the major problems (23). Therefore, the development of novel drugs is crucial to the treatment of GB.

Natural medicines have fewer side-effects compared with conventional anticancer drugs. More recently, several effective ingredients have been purified from medicinal plants and have been used to kill glioma cells. For example, curcumin, resveratrol and elemene can induce cell apoptosis, inhibit cell proliferation, regulate the cell cycle and inhibit cell migration and invation in glioma cells (24-29).

Natural polysaccharides possess many beneficial health properties. Recent research has indicated that polysaccharides and their sulphated derivative are able to activate many cell signaling events that closely correlate with tumor development (30-33). Therefore, polysaccharides from medicinal plants may be a resource repository for the search for novel therapeutic agents against cancer. In our previous study, we succesfully extracted a polysaccharide from *A. coreanum* (ACP1) and prepared its sulphated derivative (ACP1-s). We verified that ACP1 can significantly inhibit the cell migration of human breast cancer MDA-MB-435s cells *in vitro* and affect the dynamic remodeling of the cell actin cytoskeleton. Moreover, we demonstrated that

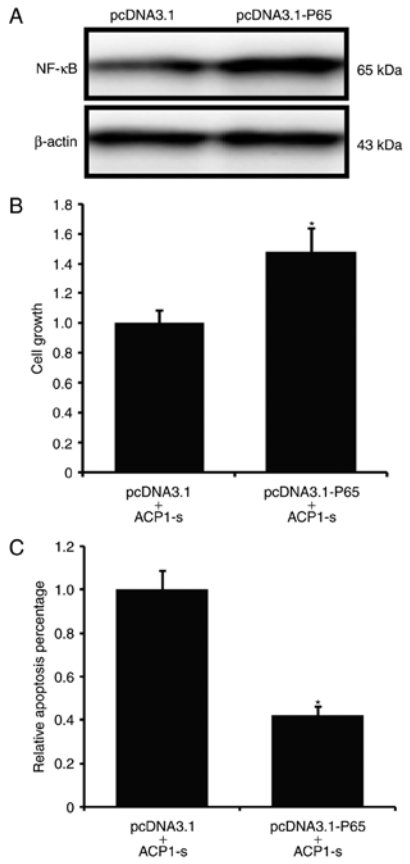

Figure 4. The introduction of exogenous p65 protein partly neutralizes the ACP1-s-induced cell apoptosis. U87MG cells were transfected with the p65-expressing plasmid or the control plasmid. The stable cells that overexpress exogenous p65 protein were selected by neomycin. The stable cells were treated with 400, 800 or 1,600 μg/ml of ACP1-s for 24 h, and the cell growth and cell apoptosis were examined again. (A) Preparation of the stable U87MG cells that overexpress exogenous p65 protein. The expression of p65 was determined by western blot analysis. (B) Cell growth of the ACP1-s-treated stable U87MG cells. (C) Cell apoptosis of the ACP1-s-treated stable U87MG cells. *P<0.05 as compared with the control group. ACP1-s, polysaccharide sulphated derivative from *A. coreanum*.

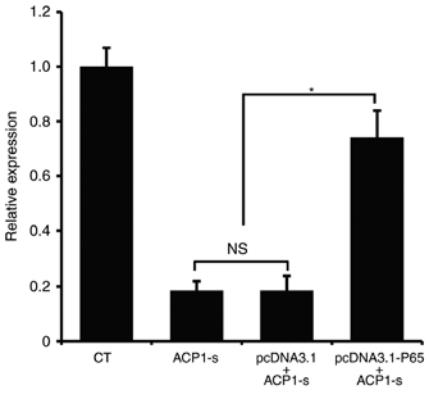

Figure 5. The exogenous p65 neutralizes the ACP1-s-induced Bcl-2 suppression. The stable U87MG cells were treated with ACP1-s for 24 h, and the total cellular RNA was extracted. The Bcl-2 mRNA expression in the ACP1-s-treated U87MG cells was examined by real-time PCR. *P<0.05 as compared with the p65-unexpressed groups; NS, not significant. ACP1-s, polysaccharide sulphated derivative from *A. coreanum*.

ACP1-s possesses higher biological activity compared with ACP1. In the present study, we investigated the anti-glioma activity of ACP1-s in the human brain glioblastoma cell line U87MG. Cell viability assay and flow cytometry results demonstrated that 400, 800 and 1,600 μg/ml ACP1-s induced cell growth inhibition and cell apoptosis through the NF-κB/Bcl-2 cell apoptotic pathway. To explore the cytotoxicity of ACP1-s to normal cells, a mouse neuroectodermal cell line NE-4C was employed as a control in the cell viability assay. Our result demonstrated that the cell growh inhibitory rate (IR) of the NE-4C cells was much lower than that of the U87MG cells (22.2 vs. 51.4%; Fig. 1), which means that ACP1-s has slighter cytotoxicity to normal neuronal cells.

NF-κB is highly activated in GB, and the NF-κB pathway is one of the most related pathways in the natural medicine induced cell apoptosis process (34). Cheng *et al* (35) found that a polysaccharide obtained from highland barley inhibited the cell proliferation of human colon cancer HT-29 cells through the activation of c-Jun N-terminal kinase (JNK) and the inhibition of NF-κB. Zhang *et al* (36) showed that a polysaccharide from *Lentinus edodes* decreased the cell proliferation of hepatocellular carcinoma cell lines HepG2 and H22 through the inhibition of NF-κB, Stat3 and survivin signaling. Bcl-2 family members regulate the mitochondrial pathway of apoptosis by complex interactions, which dictate the integrity of the outer mitochondrial membrane (37). The ratio between Bcl-2 and Bax is important in regulating the release of cytochrome *c* from mitochondria, which then activates caspase-3 and induces apoptosis (38). Additionally, Bcl-2 is a target gene for NF-κB, and there are multiple NF-κB binding sites on the Bcl-2 promoter (39). In the present study, our data showed that IκB was activated after treatment with ACP1-s and the level of NF-κB was accordingly reduced. Expression levels of NF-κB-regulated genes Bcl-2, Bax and caspase-3 were also altered following treatment with ACP1-s. These results indicated that ACP1-s induced the cell apoptosis of U87MG glioma cells through a NF-κB-mediated mitochondrial apoptosis. The p65 compensation experiment also confirmed our hypothesis.

Limited delivery of therapeutics across the blood-brain barrier (BBB) makes GB one of the most dreaded cancers in chemotherapy. Although the alkylating agent TMZ can cross BBB, its efficacy is limited in GB patients (40). Some natural medicines exhibit excellent brain penetration and efficacy against brain disorders, such as ferulic acid and ligustilide (41). Therefore, natural medicine-based therapies have a bright prospect for improving the efficacy of current GB treatment. In the present study, although the *in vitro* anti-glioma activity of ACP1-s was preliminarily confirmed, whether ACP1-s can cross the BBB has not been elucidated. *In vitro* model BBB transport and the animal experiments are warranted.

In conclusion, we extracted a polysaccharide from medicinal plant *Aconitum coreanum* and prepared its sulphated derivative. We preliminarily investigated the anti-glioma bioefficacy of ACP1-s using the U87MG cell line and revealed the apoptotic molecular mechanism. Our findings suggest the potential value of ACP1-s as a novel therapeutic agent for the treatment of glioma.

Acknowledgements

We thank Dr Dehai Yu and Dr Haijun Li of the First Hospital of Jilin University for kindly providing advice for the cell experiments.

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