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A polysaccharide isolated from the medicinal herb *Bletilla striata* induces endothelial cells proliferation and vascular endothelial growth factor expression *in vitro*

Chunming Wang[1], Jiantao Sun[1,2], Yi Luo[1], Weihua Xue[1], Huajia Diao[1], Lei Dong[1], Jiangning Chen[1] & Junfeng Zhang[1,*]
[1]*State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China*
[2]*Department of Neurosurgery, Nanjing University School of Medicine Affiliated Gulou Hospital, Nanjing, China*
**Author for correspondence (Fax: +86-25-83324605; E-mail: jfzhang@nju.edu.cn)*

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Abstract

A polysaccharide from the traditional Chinese medicinal herb, *Bletilla striata* (Thunb.) Reichb. f., was isolated, purified and characterized. It induced the proliferation of human umbilical vascular endothelial cells and the expression of vascular endothelial growth factor up to 156% and 147% of control after 72 h, respectively.

Introduction

The herb, *Bletilla striata* (Thunb.) Reichb. f., has been widely used in Eastern Asian countries to treat alimentary canal mucosal damage, ulcer, bleeding, bruises and burns. Although the herb was reported to contain glucomannan (Tomoda *et al.* 1973), its monosaccharide content is ambiguous and the exact carbohydrate structure is unclear. Furthermore, little attempt has been made to explain its mechanism during the therapy, including which cell types the polysaccharide components may target and affect.

The proliferation of vascular endothelial cells (EC) is the initial and key step of angiogenesis, which plays a critical role in wound healing and tissue repair. Vascular endothelial growth factor (VEGF) is notably known as an endothelial cell-specific mitogen to regulate EC proliferation (Risau 1997). Drugs which stimulate EC growth are particularly welcome in chronic wound dress, surgical hemostasis and vascular tissue engineering. If, supposing a medicine is beneficial for wound and ulcer healing, it might contribute to EC growth. Indeed, a number of polysaccharides or glycoproteins, some of which have been used to heal gastric mucosal ulcers, were also reported to enhance EC proliferation and VEGF expression (Cominetti *et al.* 2004, Wang *et al.* 2004). In the present study, we purified and characterized a polysaccharide from the herb, *Bletilla striata*, and observed that it promoted EC growth and upregulated VEGF expression *in vitro*.

Materials and methods

Isolation, purification, sugar content and molecular weight of polysaccharides

Dry *Bletilla striata*, from Nanjing Medical Corporation (Nanjing, China), was homogenized and

dispersed in 80 °C double distilled water (dd water) for 4 h, and then filtered to remove impurities. Polysaccharides were precipitated by addition of three vol. 95% (v/v) ethanol and left to stand overnight. The resultant precipitate was collected by centrifugation, washed with 95% (v/v) ethanol, and resuspended in dd water. Then, 1/3 vol. chloroform/*n*-butanol (4:1, v/v) was added to precipitate proteins and the aqueous phase was subsequently collected, dialyzed at a cut-off of 3000–5000 Da, and lyophilized to give a crude extract. This crude was dissolved and applied to a DEAE-cellulose (3×45 cm) column. Fractions, in which polysaccharides were detected by the anthrone assay (Kawagishi *et al.* 1990), were obtained and applied onto a Sephadex G-200 (1.4×75 cm) column for further purification. Only a single peak was revealed by the anthrone assay and the target fractions were combined and lyophilized to afford pure BSP (*Bletilla striata* polysaccharide). The purity of this compound was confirmed by HPLC using a Bio-Gel TSK-40 column (Bio-Rad, USA) and a differential refractive index detector. Scanning from 400 to 200 nm was also performed to detect protein and nucleic acids.

Total sugar content was determined by a modified phenol/sulfuric acid method (Chaplin & Kennedy 1994). Uronic acids content was determined by the hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen 1973). The molecular weight of BSP was estimated by gel filtration using a Sephadex G–200 column (1.4×75 cm) in comparison with different molecular size markers of dextran (2×10^4–2×10^6).

Compositional and structural analysis

To analyse the constituent monosaccharides, BSP was hydrolyzed with 1 M trifluoroacetic acid (TFA) at 110 °C for 3 h and the hydrolyzed products were divided into two aliquots. One was analysed by bidirectional TLC on silica gel plates eluting with *n*-butanol/ethyl acetate/2-propanol/acetic acid/water/pyridine (35:100:60:35:30:30, by vol.). The other aliquot was converted into their corresponding alditol acetates with hydroxylammonium chloride and acetic anhydride in pyridine for GC as detailed elsewhere (Chaplin & Kennedy 1994). Several types of standard monosaccharides were used as references in both TLC and GC.

To retrieve structure information of BSP, FT-IR spectra, ^{13}C NMR and assistant ^{1}H NMR spectra measurements were carried out.

Isolation and culture of cells

Primary human umbilical vein endothelial cells (HUVEC) were obtained by treatment of umbilical veins with 1 mg collagenase IV/ml by using the method of Jaffe *et al.* (1973) and maintained in M199 medium supplemented with 10 mg fetal bovine serum/ml, 20 μg endothelial cell growth supplement/ml, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37 °C. HUVEC from passages 5–7 were used in all experiments and, were seeded in 24-well culture plates at 4×10^4 cells/ml and allowed to adhere overnight before treatment.

Cell proliferation assays and VEGF detection

The cells were incubated with BSP in phosphate buffered saline (PBS) from 20 to 200 μg/ml. PBS and recombinant human $VEGF_{165}$ (R&D systems, USA) were served as negative and positive controls, respectively. Cell proliferation was estimated using the MTT colorimetric assay. At various times, the medium was aspirated and the remaining cells in each well were further incubated with 0.5 mg MTT/ml for 5 h. Then, MTT was extracted with 500 μl dimethyl sulfoxide (DMSO) and the color change in the extract was measured at 575 nm.

After being treated with 80 μg BSP/ml for different times, HUVEC cells were disrupted by using a cell lysis buffer as described by Cominetti *et al.* (2004). Then, ELISA assays were performed using the Quantikine immunoassay kit for human VEGF (R&D) according to the manufacturer's instructions. The absorbancies were read on a Tecan Safire microplate detection system (Tecan, Switzerland) at 450 nm. All the above measurements were repeated for six times.

Results and discussion

Isolation, purification and molecular weight of polysaccharides

BSP gave a single symmetrical narrow peak by HPLC. Neither proteins nor nucleic acids were detected by scanning from 400 to 200 nm. The purified polysaccharide contained 96.9% (w/w) of total sugar but no uronic acids. It had a molecular weight of ~135 kDa as indicated by gel filtration chromatography.

Composition and characterization of BSP

Results from both TLC and GC revealed that glucose and mannose were the only two monosaccharides in BSP. GC analysis in Table 1 shows that the relative mole ratio of mannose and glucose is ~2.4:1 according to their corresponding peak areas.

FT-IR spectrum of BSP is shown in Figure 1. The characteristic absorption at 895.6 cm^{-1} suggests that BSP contain β-glucosyl residues and 810.2 cm^{-1} peak reveals the existence of mannose. The pyranoses can be evidenced by the strong absorption at 1030.6 and 1148.5 cm^{-1}. Lack of absorption between 1650 and 1550 cm^{-1} excludes the possibility of amino groups (Mathlouthi & Koenig 1986).

Figure 2 shows the ^{13}C NMR spectrum of BSP. Three anomeric signals at δ 103.5, 102.3, and 101.6 ppm, and chemical shifts at δ 61.8, 61.2, and 62.3 ppm for C-6 signals were successfully separated. Chemical shifts between 66.8 and 81.4 ppm present signals of carbon rings. In addition, results from the ^{1}H NMR (data not shown) suggest one characteristic signal for α-anomeric proton at δ 5.20 ppm. Thus, we presume that signals at δ 103.5 and 61.2 can be assigned to a β-glucopyranosyl residue with O-1

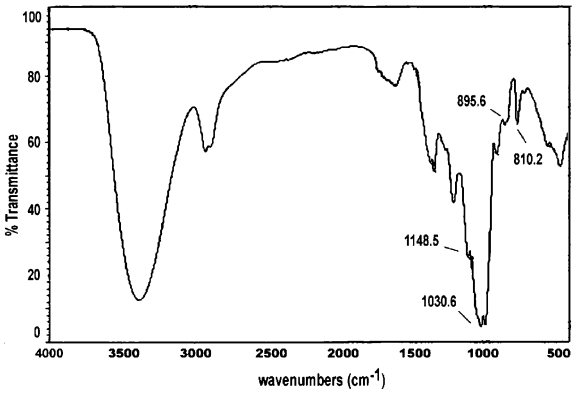

Fig. 1. IR spectra of BSP. Measurements were carried out with KBr pellets on a Nexus 870 FT-IR spectrophotometers (Thermo Electron, USA) at room temperature.

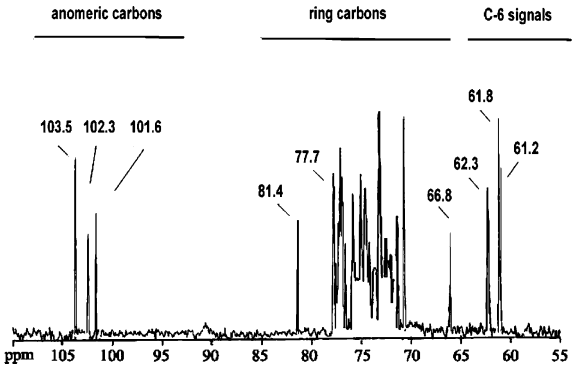

Fig. 2. ^{13}C NMR spectra of BSP recorded on a Bruker DRX-500 NMR spectrometer (Bruker, Germany) at 60 °C. The samples were dissolved in D_2O with concentration of 8.0 mg/0.5 ml and chemical shifts are referenced against internal TSP-d_4 acid at −1.80 ppm.

substituted, and chemical shifts at δ 101.6 and 102.3 can be attributed to two O-substituted C-1 signals of β- and α-mannopyranosyl, respectively. Furthermore, signals at δ 66.8 and δ 81.4 indicate that α-mannopyranosyl has both O-4 and O-3 substitutes, and the signal at δ 77.7 reveals

Table 1. Monosaccharides composition[a] of polysaccharide from *Bletilla striata.*

Monosaccharides	Xylose	Mannose	Glucose	Galactose
R_T for references (min)	9.53	16.26	19.74	21.35
R_T for samples (min)	–	16.16	19.62	–
[b]Peak area for samples (%)	–	4.07	1.68	–

[a]Analysis of BSP monosaccharides by GC (see Methods). Myo-inositol hexa-acetate was used as internal standard.
[b]The peak areas of monosaccharides are their molar ratios.

its linkage to O-substituted C-4 of β-mannopyranosyl (databases at http://www.dkfz-heidelberg.de/spec/sweetdb/, Agrawal 1992). On the basis of the above results, a possible structure of BSP is proposed in Figure 3.

Cell proliferation and VEGF expression

The reason to choose HUVEC cells to evaluate the bioactivities of BSP was based on the hypothesis of relationship between EC growth and the polysaccharide. Firstly, the proliferation of EC is essential for various kinds of wound healing and tissue repair, which fall into the therapeutic scope of *Bletilla striata* in folk medicine. Secondly, among the many types of EC, HUVEC is one of the most widely used and easily acquired. Consequently we employed HUVEC to design cell proliferation assays for BSP. The cell viability after 72 and 120 h incubation with BSP is shown in Figure 4. Results indicate that the proliferation of HUVEC cells was enhanced by BSP at 40, 60, 80, 100, and 120 μg/ml between

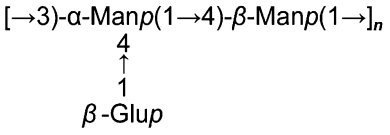

Fig. 3. The proposed structure of the polysaccharide.

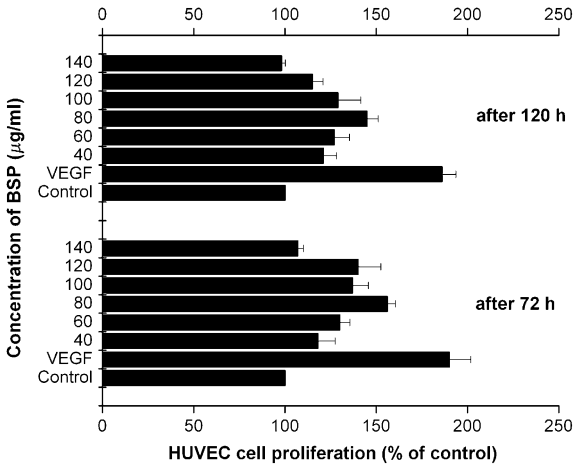

Fig. 4. Human umbilical vascular endothelial cell proliferation stimulated by polysaccharide from *Bletilla striata* at different concentrations after 72 and 120 h *in vitro*. Data are presented as mean ± standard deviation ($n = 6$), and compared with the control (as 100%).

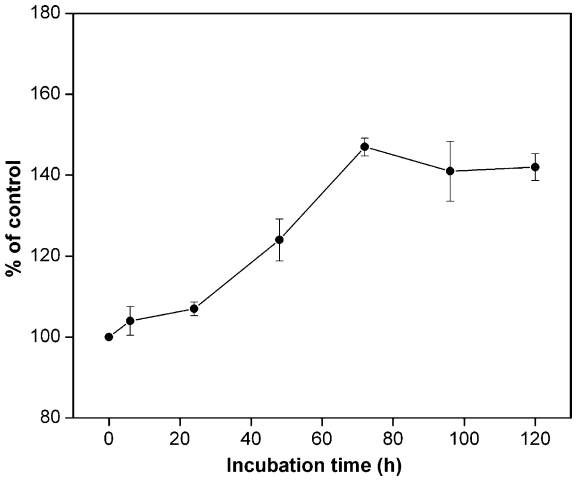

Fig. 5. Vascular endothelial growth factor analysis *in vitro* by ELISA assay in human umbilical vascular endothelial cells after being treated with polysaccharide from *Bletilla striata* at 80 μg/ml. Data are presented as mean ± standard deviation ($n = 6$), and compared with the control (as 100%).

24 to 120 h *in vitro*. The maximum increase of 56 ± 4.5% of HUVEC was achieved with 80 μg BSP/ml over 72 h incubation, which exceeds the proliferation of EC stimulated by a disintergrin-like protein (Cominetti *et al.* 2004), or insulin (Yamagishi *et al.* 1999), giving evidence for a major effect of BSP on HUVEC proliferation.

VEGF is one of the most important growth factors related to cell cycle control and has the ability to promote EC growth. Figure 5 shows the VEGF production induced by BSP determined by ELISA in HUVEC cells. The result suggests that BSP at 80 μg/ml can up-regulate 47 ± 2.2% VEGF expression after incubation for 72 h, which is consistent with the cell proliferation data shown in Figure 4.

We noticed that both the cell proliferation and VEGF expression induced by BSP are not dose-dependent and 80 μg/ml seems to be the peak value. A similar phenomenon has been documented in many other studies, such as EC proliferation and VEGF expression induced by insulin (Yamagishi *et al.* 1999), and EGF expression and RGM-1 cell proliferation stimulated by polysaccharide extract from *Angelica sinensis* (Ye *et al.* 2001). This may be due to limiting amounts of possible receptors for the substances or the adaptation of the cells in response to the stimulating action of drugs. However, more detailed mechanisms still remain to be elucidated in next studies.

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