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An arabinogalactan from flowers of *Panax notoginseng* inhibits angiogenesis by BMP2/Smad/Id1 signaling

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ABSTRACT

Angiogenesis plays an essential role in tumor development. Blocking angiogenesis in tumor has become a promising tactic in limiting cancer progression. Here, an arabinogalactan polysaccharide, RN1 was isolated from flowers of *Panax notoginseng*. Its structure was determined to possess a backbone of 1,6-linked Gal*p* branched at C3 by side 1,3-linked Gal*p*, with branches attached at position O-3 of it. The branches mainly contained 1,5-linked, 1,3,5-linked, terminal Arabinose and terminal Galactose. RN1 could inhibit microvessel formation in the BxPC-3 pancreatic cancer cell xenograft tumor in nude mice. The antiangiogenesis assay showed that RN1 could reduce the migratory activity of endothelial cells and their ability of tube formation on matrigel, but no effect on endothelial cells growth. Further studies revealed that RN1 could inhibit BMP2/Smad1/5/8/Id1 signaling. All those data indicated the RN1 had an antiangiogenic effect *via* BMP2 signaling and could be a potential novel inhibitor of angiogenesis.

1. Introduction

Angiogenesis is a physiological process involving the growth of new blood vessels from existing vessels. It is now widely recognized as one of the hallmarks of cancer, a crucial step in the transition of tumors from a dormant state to a malignant state, and playing an essential role in tumor growth, invasion and metastasis (Folkman & Shing, 1992; Hanahan & Weinberg, 2011). Due to its essential roles in tumor, controlling tumor-associated angiogenesis has become a promising tactic in limiting cancer progression (Weis & Cheresh, 2011).

Our group has been actively involved in the search for new modulators of angiogenesis from natural products. We have reported that sulfate polysaccharide WSS25 (Qiu, Yang, Pei, Zhang, & Ding, 2010) inhibited angiogenesis by binding to bone morphogenetic protein 2 (BMP2), a pro-angiogenesis protein. However, in the animal safety experiment, we found that WSS25 could increase the chances of internal bleeding at its high concentration. Thus development of safe and effective potential drug is essential.

The flowers of *Panax notoginsen* (FPN) has been wildly used as a traditional Chinese medicine and food additives. Recent study showed that the extract of flowers from *Panax notoginsen* had strong anti-proliferative effects on colorectal cancer cells (Ng, 2006). Here, in the course of a screening program, RN1, firstly isolated and purified from FPN, was selected for its ability to inhibit angiogenesis *in vitro* and *in vivo*. To our knowledge, the structure of arabinogalactan RN1 and its antiangiogenesis activity from FPN have not been reported previously.

2. Materials and methods

2.1. Materials

The dried flowers of *Panax notoginseng* were purchased from shanghaikangqiao Co., Ltd., Shanghai, China and were identified by Prof. Ping Li (Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China). Monosaccharide standards (galactose, glucose, mannose, arabinose, xylose, rhamnose, galacturonic acid) were all from Fluka, Switzerland. Trifluoroacetic acid (TFA), 1-phenyl-3-methyl-5-pyrazolone (PMP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and was from sigma-Aldrich, USA. Dextran standards were purchased from Pharmacia Co., Sweden. Acetonitrile and Dimethyl

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Sulfoxide (DMSO) were purchased from E. Merck, Germany. DEAE-Cellulose 32 was from Whatman Co., U.K. Matrigel with growth factors (354234) was purchased from BD Biosciences, USA. Other reagents were analytical grade.

2.2. General analysis

Total sugar content was determined by the phenol–sulfuric acid method using galactose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of protein was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Monosaccharide composition of polysaccharides was determined by a PMP-HPLC method according to our previous report (Wang et al., 2014).

2.3. Extraction, isolation and purification of polysaccharides

Extraction of crude polysaccharides was performed by the previous procedure (Wang et al., 2014). In brief, the dried flowers of *Panax notoginseng* was extracted with boiling water for 5 h (5 times). The combined supernatant was concentrated and treated with 15% trichloroacetic acid at 4 °C for 4 h to remove the protein. After neutralization and centrifugation, the supernatant was dialyzed (3500 Da, MWCO), concentrated and precipitated with three volumes of 95% EtOH. The crude polysaccharide RN was fractionated on a DEAE-cellulose column (Cl—, 120 cm × 6 cm), eluted with distilled water and further 0.1 M NaCl. The fraction with 0.1 M NaCl elution was collected, concentrated and lyophilized to obtain polysaccharide RN1. The relative molecular weight of RN1 was determined by HPGPC with series-connected Ultrahydrogel TM 2000 and Ultrahydrogel TM 500 columns and it was estimated to be 20.5 kDa.

2.4. Methylation analysis

The vacuum-dried polysaccharide (10 mg) was methylated for 3–4 times based on previous methods (Hakomori, 1964). The methylated polysaccharide was hydrolyzed and then reduced with sodium borohydride and acetylated. The partially methylated alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) with a Shimadzu QP-5050A apparatus equipped with a DB-1 capillary column (0.25 mm × 30 m). Mass spectra of the derivatives were analyzed using Complex Carbohydrate Structural Database of Complex Carbohydrate Research Centre (http://www.ccrc.uga.edu/).

2.5. NMR analysis

For NMR analysis, polysaccharides (30 mg) were exchanged and dissolved in 0.5 ml of D_2O. The ^1H, ^{13}C NMR spectra, two-dimensional spectra (HMBC, HMQC and COSY) were measured at room temperature with acetone as internal standard. NMR spectra were recorded on a Varian Mercury 500 NMR spectrometer.

2.6. Partial acid hydrolysis

RN1 (200 mg) was first hydrolyzed in 0.05 M TFA at 100 °C for 1 h and then evaporated to remove TFA. After dialysis, the retentate was lyophilized to obtain the degraded polysaccharide RN1N1. RN1N1 was further hydrolyzed in 0.1 M TFA at 100 °C for 1 h, then evaporated and dialyzed. The retentate was freeze-dried, giving RN1N2. The monosaccharide composition, molecular weight and NMR analysis were performed for the degraded polysaccharides.

2.7. Cell lines and culture conditions

Human microvascular endothelial cells (HMEC-1) were purchased from Prime Gene Bio-Tech Co. Ltd., Shanghai and maintained in MCDB131 (Gibco BRL, U.S.A.) medium containing 15% FBS (v/v), 2 mM L-glutamine, 10 ng/ml EGF (Shanghai Prime Gene Bio-Tech Co. Ltd., Shanghai, China) and 100 U/ml penicillin, 100 μg/ml streptomycin. Human pancreatic cancer cell lines BxPC-3 were purchased from the Cell Bank in the Type Culture Collection Center in Chinese Academy of Sciences, Shanghai, China. These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured in an incubator at 37 °C under a humidified atmosphere containing 5% CO_2.

2.8. Cell proliferation (MTT) assay

Cell proliferation was measured by an MTT tetrazolium assay. Briefly, HMEC-1 (4×10^3 cells/well) cells were seeded into 96-well tissue culture plates and cultured with or without RN1. After 72 h, tetrazolium salt was added and the cells were incubated at 37 °C for another 4 h. The insoluble violet formazan product was solubilized by the addition of 150 μl of DMSO. The color absorbance was recorded at 490 nm using a Bio-Rad 3350 micro plate reader. The effect of RN1 on cell viability was calculated in terms of percent of control, which was arbitrarily assigned a value of 100% viability.

2.9. Tube formation assay

A HMEC-1 cells capillary-like tube formation assay was performed to determine the effect of the RN1 on angiogenesis *in vitro*. A total of 5×10^4 HMEC-1 cells were seeded on top of matrigel-coated (40 μl per well) wells of 96-well tissue culture plates containing 0.5 mg/ml, 1 mg/ml of RN1. The plate was then incubated at 37 °C and the formation of the capillary-like tubes was observed after 8 h. The wells were imaged using a Nikon microscope. Quantification of tube formation was assisted by Image-Pro Plus software.

2.10. Wound healing assay

To assess the effect of RN1 on mobility of HMEC-1 cells, a wound healing assay was performed. A total of 5×10^5 HMEC-1 cells were seeded in 6-well plates and incubated in FBS-free MCDB131 for 24 h. An artificial wound was then created, and the cells were washed and supplied with new medium containing 1% FBS and various concentrations of RN1. The migration of cells through the wound area was examined after 0 h, 6 h, 12 h and 24 h.

2.11. Western blotting

Total proteins were extracted and the concentration was determined using the bicinchoninic acid protein assay kit (Beyotime). The total cellular protein extracts were separated by electrophoresis on SDS-PAGE gels and blotted onto the nitrocellulose membrane (Millipore). Blots were incubated with antibodies raised against Id-1 (Santa Cruz), phospho-Smad1/5/8 (Cell Signaling Technology), β-actin (Santa Cruz). Protein bands were detected by incubation with HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence reagent (Pierce).

2.12. Xenograft model and immunohistochemistry

Five-week-old female athymic nude (nu/nu) mice were purchased from Shanghai Laboratory Animal center of the Chinese Academy of Sciences. The study was approved by our Institution