Biomedicine & Pharmacotherapy 103 (2018) 738–745

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Polysaccharide from *Rhizopus nigricans* inhibits the invasion and metastasis of colorectal cancer

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ARTICLE INFO

Keywords:
Extracellular polysaccharide
Rhizopus nigricans
Metastasis
Invasion
Angiogenesis

ABSTRACT

Polysaccharide (EPS1-1) extracted from fermentation liquor of *Rhizopus nigricans* possesses antitumor and immune-enhancing activities. The study was the first to investigate the anti-metastasis effects of EPS1-1 *in vitro* and *in vivo*. Results suggested that EPS1-1 dose-dependently suppressed the migration, invasion and adhesion abilities of CT26 cells. Furthermore, EPS1-1 dramatically inhibited the enzyme activity and expression levels of matrix metalloproteinases (MMPs) in CT26 cells, as well as the tube formation of HUVECs. Similar results were observed in the lung metastasis mice which were administrated with EPS1-1 for 14 d. EPS1-1 could inhibit angiogenesis by decreasing the expression of vascular endothelial growth factor (VEGF) and microvessel density (MVD) in lung tissue. Moreover, vimentin, as a marker molecule in epithelial-mesenchymal transition (EMT) which is closely related to metastasis, was found to be down expression by EPS1-1 in CT26 cells and lung tissue. These results suggested that EPS1-1 could suppress metastasis *in vitro* and *in vivo* by inhibiting invasion and angiogenesis, which provides potential application to against colorectal cancer metastasis.

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality worldwide caused by metastasis [1]. The new cases and death of colorectal cancer were almost 1.2 million and 600,000 throughout the world per year, respectively [2]. Although the improvement of treatment strategies has contributed the increase of overall survival rates in the early stages, about 40–50% of patients with CRC died of metastasis [3]. Tumor metastasis is a multi-step process where cancer cells break away from primary tumor site, then infiltrate into the surrounding tissue and enter into lymphatic or blood vessels to form secondary tumors at a distant site [4].

Adhesion of tumor cellular is a common development process of metastasis. Cell adhesion molecules, such as E-cadherin and integrin, were proved very important in the adhesion process [5]. It has been reported that extracellular matrix (ECM) is composed of highly variable and dynamic components which can regulate the behavior of cells [6]. Increasing degradation of ECM is a hallmark event of metastasis, which makes cancer cells to penetrate in the ECM for successful metastasis [7]. Although there is a wide range of biological functions of MMPs, a vital role is the degradation and remodeling of ECM to provide the basis by the peripheral tissue for invasion and metastasis. MMP-9 and MMP-2, the important members of soluble MMPs, are thought to be over-expression in many tumor types. VEGF promotes proliferation of endothelial cell and vascular permeability in angiogenesis [8]. Increasing of angiogenesis and microvessel density that represent a poor prognosis are closely correlated with the expression of VEGF [9]. In addition, the expression level of Vimentin was positively relative to malignant tumor type and associated with invasive ability of cancer cells [10]. Most CRC originate from intestinal epithelium after premalignant lesions called adenomas which are transformed to CRC. At the early stage of CRC, the primary tumor can be removed by surgical resection. Conventional therapy is not suitable for most patients with CRC distant metastases whose survival rate in 5-year is very poor of $< 10\%$ [11]. These patients with CRC distant metastases could be achieved in the chemotherapeutic treatment. However, a substantial proportion of these patients do not seem to benefit from the chemotherapy [12]. Thus, to find an effective drug with less toxicity to inhibit metastasis is a curial

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https://doi.org/10.1016/j.biopha.2018.04.093
Received 19 December 2017; Received in revised form 9 April 2018; Accepted 13 April 2018
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challenge for the treatment of colorectal cancer.

Bioactive polysaccharides have been widely studied and used in food and drug industries because of their therapeutic properties and relatively low side effects. The antitumor [13], immunomodulatory [14] and antibacterial [15] activities of polysaccharides have been well researched and used in biochemical and medical fields. Furthermore, Increasing studies have revealed that polysaccharides possess anti-metastatic activity. For example, polysaccharide from *Ascophyllum nodosum* was proved to inhibit the migration and adhesion of B16 melanoma cells by reducing the expression of N-cadherin and enhancing the expression of E-cadherin in a concentration-dependent manner [16]. Besides, SIP-SII, a sulfated *Sepiella maindroni* ink polysaccharide, could markedly decrease B16F10 pulmonary metastasis in mice models [17]. Moreover, natural polysaccharides could inhibit the metastasis by suppression angiogenesis [18] and the activity of MMPs [19]. These studies revealed that polysaccharides might be promising a new drug or adjuvant medicine for anti-metastatic and chemotherapeutic therapy.

Previous studies have reported that the structural characterization of natural polysaccharide (EPS1-1) which was isolated from the fermentation liquor of *Rhizopus nigricans* with average molecular weight of 9682 Da [20]. EPS1-1 could inhibit the proliferation of HCT-116 and tumor growth in S180 and CT26 bearing mice [21,22]. Moreover, EPS1-1 could significantly enhance the activities of macrophages and improve immunity of immunosuppressed mice [23]. These results indicate that EPS1-1 possibility possesses the anti-metastatic and anti-angiogenic activities. In this research, we first evaluated whether EPS1-1 could inhibit metastasis *in vitro* and *in vivo* and its possible mechanisms.

2. Materials and methods

2.1. Materials

Transwell chambers were from Corning coster (8 μm, 24-well). Matrigel was from BD biosciences. TRIzol, enhanced chemiluminescence (ECL) kits and RIPA lysate were purchased from Invitrogen (Carlsbad, CA) and Beyotime (Jiansu, China), respectively. Anti-MMP-9 and anti-VEGF antibodies were obtained from Abcam (Cambridge, UK). Anti-Vimentin and anti-β-actin antibodies were the products of Cell Signaling Technology (Beverly, MA, USA).

2.2. Preparation of extracellular polysaccharide (EPS1-1)

Rhizopus nigricans is isolated from straw and preserved in Laboratory of Biomass Resources, Shandong University (Jinan, China). The fermentation liquor of *R. nigricans* was collected for concentration after cultivating in Potato Dextrose Agar at 28 °C of 120 rpm for 10 d. According to the method of Wenqian Yu et al. [20], the concentrated liquor was mixed with three times volume of 95% ethanol at 4 °C for 24 h to further deproteinze. The sediment was dissolved and decolorized with D301R. The crude polysaccharides were collected and loaded into a DEAE-Sepharose Fast Flow column (1.6 cm × 20 cm). The fraction of major peak was collected and named EPS-1. Lyophilized EPS-1 powder was dissolved and fractionated using Sephadex G-75 column chromatography (1.6 cm × 60 cm). Finally, we got the extracellular polysaccharide named EPS1-1.

2.3. Cell culture and animals

CT26 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (100 μg/ml), then maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Male BaLb/c mice (18 ± 2 g) were obtained from Animal Centre of Shandong University. Mice were kept at room temperature (12-h light/12-h dark, 25 ± 1 °C, 55 ± 5% relative humidity). All experiments were performed in compliance with the relevant laws and approved by the Ethics Committee of the Shandong University with the approval/protocol number of SYDWLL-2018-12, which is according to the regulations for the Administration of Affairs Concerning Experimental Animals of China.

2.4. Invasion and migration assays

Migration and invasion abilities of CT26 cells were detected using Transwell invasion as previously described [16]. Serum-free medium (100 μl) was seeded in the upper chamber which contained different concentrations of EPS1-1 (0, 0.1, 0.2 and 0.4 mg/ml) and CT26 cells (3×10^5/ml). The medium contented 10% FBS was added to the lower chamber. After a 24 h of incubation at 37 °C, cells in the upper side were removed. While cells that migrated through the polycarbonate membrane were washed by PBS and dyed with crystal violet. After dyed, cells were photographed under microscope and at least 5 images were selected. To analyze invasion ability of CT26 cells, upper surface of chambers were coated with 40 μl Matrigel (0.78 mg/ml) per well. The following steps were same to the migration assay after matrigel dryed.

2.5. Cell adhesion assay

The adhesion assay was performed according to the method [24]. A 96-well-plate was coated with 50 μl Matrige per well and air-dried overnight. Following treatment with EPS1-1(0, 0.2 and 0.4 mg/ml) for 24 h, CT26 cells were suspended by RPMI 1640 medium without serum and added into the pretreated 96-well-plate (100 μl, 1×10^5/ml). After incubation at 37 °C for 1 h, cells adhered to Matrigel were washed by PBS and measured by MTT. The untreated group was set as negative group and inhibitory rate of adhesion was calculated under the below formula:

Inhibitory rate = (Negative group - EPS1-1 treatment group) / Negative group × 100%

2.6. Endothelial cell tube formation assay

Tube formation assay was according to the method of DeCicco et al. [25]. HUVECs tube formation was conducted to measure the effect of EPS1-1 on the angiogenesis *in vitro*. A 96-well-plate was coated with 40 μl of Matrigel which was solidified at 37 °C for 2 h. Then, HUVEC cells (100 μl, 1×10^5 /ml) were suspended with RPMI 1640 medium which contained EPS1-1 (0 and 0.2 mg/ml) and added to each well. After incubation for 24 h, photographs from five randomly selected fields were taken by microscope (Olympus, Tokyo, Japan). The number of integrated tube was measured and analyzed.

2.7. Gelatin zymography

Gelatin Zymography was progressed with the method as previously described [26]. CT26 cells were pretreated with EPS1-1 (0, 0.1, 0.2 and 0.4 mg/ml) for 24 h and lysed with ice-cold RIPA buffer to get the total protein. The protein concentrations were determined with a BCA protein assay kit (Thermo Scientific Pierce). Equal amounts of proteins from the extracts were loaded into a 10% SDS-PAGE gel containing 1% gelatin. The gels were incubated with renaturation buffer (pH 7.5, 2.5% Triton X-100 in D.W) for 30 min at room temperature and then incubated in developing buffer (50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, and 150 mM NaCl) at 37 °C for 24 h. The gelatinolytic activity of MMPs was visualized by staining the gels with Coomassie blue G-250 for 30 min and washed by decolored solution. The results were photographed by UV transilluminator.

2.8. Real time quantitative polymerase chain reaction (qPCR)

CT26 cells were pretreated with EPS1-1 (0, 0.2 mg/ml) for 24 h, and

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