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Codonopsis lanceolata polysaccharide CLPS inhibits melanoma metastasis via regulating integrin signaling

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ABSTRACT

β1 integrin-mediated migration plays a fundamental role in melanoma metastasis. Here, we evaluated the molecular mechanisms underlying the anti-metastatic capacity of a polysaccharide fraction (CLPS) from *Codonopsis lanceolata*. CLPS inhibited *in vivo* melanoma metastasis in a B16F10 pulmonary metastasis model, impaired β1 integrin-mediated B16F10 melanoma cell migration *in vitro* evaluated by Transwell assay, reduced affinity between β1 integrin and ligand protein GST-FNIII$_{9-10}$. Also, CLPS attenuated the adhesion-dependent formation of focal adhesion and blocked β1 integrin/FAK/paxillin signaling axis. These findings illustrate a better understanding underlying the anti-metastatic activity of CLPS, indicating a potential treatment strategy for melanoma metastasis.

1. Introduction

Metastatic melanoma, also known as Stage IV melanoma, is an aggressive and highly metastatic cancer with a rapid systemic dissemination through lymph nodes to distant tissues or organs [1]. Most treatment strategies for patients suffering from metastatic melanoma have only a very low rate of complete regression, and the 5-year survival rate for the metastatic melanoma patients is less than 15% [2,3]. Integrins are transmembrane glycoprotein receptors, mediating cell–cell and cell–matrix interaction. Recent reports [4,5] showed that integrins play a crucial role in cell adhesion, survival, differentiation, and proliferation. Integrins can bind multiple extracellular matrix (ECM) molecules, such as fibronectin, vitronectin, collagen, fibrinogen or laminin, thereby establishing a direct link between ECM and cytoskeleton, and controlling cell motility [6]. Recent reports [7–9] have revealed the crucial roles of β1 integrin in melanoma progression and metastasis. Thus, metastatic potential of melanoma can be influenced by blocking β1 integrin function and integrin-mediated signaling pathways [10,11].

Codonopsis lanceolata is a traditional folk medicine widely used in Korea, Japan and China, which possesses various biologically active components, including polyphenols, tannins, alkaloids, saponins, essential oils, triterpene and steroids [12]. It also exhibits several pharmacological activities [13], such as antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory activity. Moreover, accumulated evidence from contemporary pharmacological experiments have demonstrated that the extract of *C. lanceolata* possesses remarkably anti-cancer effects [14,15]. Nonetheless, the underlying mechanisms that account for anti-cancer effects of *C. lanceolata* remain largely unclear and require further investigation. Here, we investigated the inhibitory effects and mechanisms of *C. lanceolata* polysaccharide on the metastasis and integrin-mediated migration of murine melanoma B16F10 cells. These findings may reveal the molecular targets affected by *C. lanceolata* polysaccharide and its potential therapeutic implications in treating the melanoma metastasis.

2. Materials and methods

2.1. Materials and chemicals

The roots of *C. lanceolata* were purchased from a local pharmaceutical market in Jilin Province, China. The roots were identified by Prof. Xiaoxu Gao (Beihua University, Jilin, China) according to the identification standard of Pharmacopeia of the People's Republic of China, and the specimen was deposits in College of Forestry, Beihua University (voucher specimen number: 2015-Dangshen-011).

Antibodies against β1 integrin, FAK, paxillin, vinculin, phospho-FAK and phospho-Paxillin were purchased from Santa Cruz Biotechnology. Glutathione-Sepharose 4B beads, Sepharose CL-6B and ECL Western blotting chemiluminescent detection reagents were purchased from GE Healthcare Life Sciences. DEAE-cellulose and TIRTC-conjugated phalloidin were purchased from Sigma. Dul-

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becco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. All other chemical reagents were analytical grade.

2.2. Polysaccharide CLPS preparation

The roots of *C. lanceolata* were extracted with 80% (v/v) ethanol for 24 h to remove small molecule compounds. The residues were further extracted with hot water for three times. The whole extract supernatant was combined, filtered and concentrated, and then the crude polysaccharide was precipitated by treating the supernatant with three volumes of ethanol. Crude polysaccharide precipitate was collected by centrifuge and dried under reduced pressure. Proteins in the sample were removed by the Sevag method. The supernatant was collected, dialyzed and lyophilized to yield total *C. lanceolata* polysaccharides. Polysaccharide sample was further purified by ion exchange chromatography using a DEAE-cellulose column, eluted with distil water and a NaCl linear gradient ($0 \rightarrow 2$ M). Fractions were collected and monitored by phenol–sulfuric acid method, and then collected polysaccharide samples were further purified by gel filtration chromatography using a Sepharose CL-6B column eluted with 0.15 M NaCl. The major fraction of purified *C. lanceolata* polysaccharide was collected and coded as CLPS.

2.3. Physicochemical characterization of CLPS

Homogeneity and molecular weight of CLPS was determined by Agilent 1200 HPLC system. The contents of carbohydrate, protein and uronic acid were determined by phenol–sulfuric acid assay, Bradford method and *m*-hydroxydiphenyl method, respectively. Fourier transform infrared spectrum was recorded with a Nicolet 6700 FT-IR spectrometer in the frequency range of 400–4000 cm^{-1}. Monosaccharide compositions in CLPS were identified by gas chromatography (GC) as described [16] using HP5890 series II GC system with HP-5 column and flame ionization detector.

2.4. Effect on in vivo metastasis

Male C57BL/6 mice were randomly divided into three groups (10 mice per group). Then murine melanoma B16F10 cells were injected into the tail vein of mice on day zero. CLPS was intraperitoneally administered at concentrations of 200 or 400 mg/kg for 12 days. The lungs were excised, and the metastatic nodules were counted.

2.5. Direct cytotoxicity of CLPS

B16F10 cells were routinely cultured in DMEM supplemented with 10% FBS in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin. Cells in 200 μl of DMEM were seeded into 96-well plates at a density of 1×10^4 cells per well and cultured overnight. After treated with different concentrations of CLPS for 48 h, the medium was replaced with 100 μl fresh medium containing 0.5 mg/ml MTT. The supernatants were removed after 4 h incubation, and 150 μl of DMSO was added. Absorbance at 570 nm was determined using an ELISA reader.

2.6. Transwell assay

B16F10 cells incubated with CLPS were placed in 8-μm pore diameter 24-well Transwell filter inserts pre-coated with fibronectin. The complete medium containing 10% FBS was added as the chemoattractant in the lower well. After incubation for 12 h, the non-migrated cells inside Transwell inserts were removed carefully. Then the migrated cells were fixed and stained with crystal violet. Migrated cell number was counted under microscopy from five random fields.

2.7. Live cell imaging

B16F10 cells were seeded on a fibronectin-precoated culture dish and treated with or without CLPS. For live cell imaging, B16F10 cells were placed in a 37 °C heating chamber with CO_2 supply, and images were captured at 10 min intervals with a CCD camera for 6 h under a Nikon microscope. All images were quantitatively analyzed by NIH ImageJ software.

2.8. Immunofluorescence for focal adhesion

B16F10 cells incubating with or without CLPS were allowed to adhere on a glass slide pre-coated with fibronectin. Then, after fixed and permeabilized, cells were incubated with vinculin antibody. After gently washing in PBS, cells were incubated with Alexa Fluor 488 conjugated secondary antibody and then mounted on slides. All images were obtained with a Nikon Eclipse 80i fluorescence microscope. The number of focal adhesion was counted using Image J software.

2.9. Ligand binding assay

The inhibitory effect of CLPS on the binding between β1 integrin and GST-FNIII9-10 proteins was determined as described previously [1]. Briefly, GST-$FNIII_{9-11}$ fusion proteins were purified by glutathione-Sepharose 4B beads from transformed *E. coli* strain BL-21 competent cells. After incubation with $MnCl_2$ (1 mmol/L), B16F10 cells were lysed on ice. The supernatants of cell lysates were incubated with anti-β1 integrin antibody (AB1952P) from Millipore for immunoprecipitation, and then protein A-Sepharose beads were added into the reaction mixture. The immunoprecipitate beads were treated with or without CLPS. After gently washing, the beads were incubated with GST-$FNIII_{9-11}$. The beads were collected by centrifugation, and the binding of GST-$FNIII_{9-11}$ was determined by immunoblotting.

2.10. Immunoblotting

After washed with ice-cold PBS, cells were lysed on ice. Then, after removing insoluble debris by centrifugation, supernatants were collected and quantified the protein concentration by enhanced BCA protein assay kit. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk and then probed with indicated primary antibodies. Protein bands were visualized by using ECL Western blotting chemiluminescent detection reagents. NIH ImageJ software was used for quantitative analysis.

2.11. Statistics

Data were shown as mean ± SD. Statistical significance was determined by Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Physicochemical properties of CLPS

Physicochemical properties of CLPS were summarized in Table 1. Content of total carbohydrate and uronic acid were 98.2% and 8.6%, respectively. CLPS had a negative response to the Bradford assay. Also, no absorption was detected in CLPS solution at 280 nm of UV spectrum, indicating that proteins did not exist in