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Original article

Inhibition of EGF-induced migration and invasion by sulfated polysaccharide of *Sepiella maindroni* ink *via* the suppression of EGFR/Akt/p38 MAPK/MMP-2 signaling pathway in KB cells

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ABSTRACT

SIP-SII, the sulfated *Sepiella maindroni* ink polysaccharide (SIP), has been manifested to possess anti-tumor and anti-metastasis activity *in vivo* and *in vitro*. In the present study, we evaluated its inhibitory effect on the epidermal growth factor (EGF)-induced migration and invasion of human epidermoid carcinoma cell (KB cell line) as well as the related signaling pathways. The results of MTT assay indicated that SIP-SII inhibited the proliferation of KB cells in a concentration and time dependent manner. Notably, the attenuation of cell growth by SIP-SII was enlarged in the presence of EGF. The wound healing assay and transwell invasion assay were used to evaluate the effect of SIP-SII on the EGF-induced migration and invasion of KB cells and the results showed that SIP-SII markedly attenuated the EGF-induced migration and invasion. Besides, the EGF-induced matrix metalloproteinase-2 (MMP-2) expression was also suppressed by SIP-SII. However, SIP-SII showed no significant inhibition of the EGF-induced matrix metalloproteinase-9 (MMP-9) expression. Further research revealed that SIP-SII decreased the EGF-induced phosphorylation of epidermal growth factor receptor (EGFR), Akt and p38, but no significant suppression on EGF-induced phosphorylation of extracellular signal-regulated kinase 1 and 2 (Erk1/2) and c-Jun N-terminal kinases (JNK) by SIP-SII treatment was observed. The involvement of EGFR/Akt/p38 pathway was confirmed by evidence that SIP-SII would enlarge the inhibitory effect of the specific signal pathway inhibitors. These results indicate that SIP-SII has the potential to be used as the inhibitor of tumor metastasis especially for cancers characterized by over-activation of EGF/EGFR signaling.

1. Introduction

Epidermal growth factor receptor (EGFR), also known as ErbB 1, belongs to the ErbB family of receptor tyrosine kinases. EGFR is a 170 kDa transmembrane glycoprotein that is mainly composed of an extracellular domain, a transmembrane region and a tyrosine kinase domain in the cytosol [1]. Upon ligand binding, EGFR forms homo- or hetero-dimers followed by tyrosine kinase domain activation, leading to the initiation of downstream signaling cascades including phosphatidylinositol 3-kinase (PI3 K)/Akt and mitogen-activated protein kinases (MAPKs) pathways, and finally triggering the cellular responses, such as cell proliferation, cell survival and cell motility [2–5].

Overexpression and abnormal activation of EGFR plays a key role in the progression of lots of tumors by facilitating tumor cell proliferation, invasion and metastasis [6,7]. It has been confirmed that dysregulation of EGFR is associated with poor prognosis and low survival rate of patients suffering from different cancers, such as breast cancer [8], lung cancer [9], oral cancer [10], etc. Specifically, EGFR is overexpressed in more than 80% of the invasive squamous cell carcinoma of the head and neck (SCCHN) cases [11]. On the other hand, the upregulation of the EGFR ligands, like EGF, stimulates the phosphorylation of EGFR and multiple downstream protein kinases excessively to promote cancer cell metastasis [12]. Notably, EGFR is one of the most studied receptor tyrosine kinases (RTK) with regard to matrix metalloproteinases (MMPs) expression [13]. MMPs, a family of zinc dependent endopeptidases, are able to catalyze the destruction of extracellular matrix (ECM) to facilitate cancer metastasis [14]. Among human MMPs, MMP-2 and MMP-9 express abundantly in tumors and are closely

Abbreviations: SIP, *Sepiella maindroni* ink polysaccharide; SIP-SII, sulfated *Sepiella maindroni* ink polysaccharide; EGF, epidermal growth factor; MMPs, matrix metalloproteinases; EGFR, epidermal growth factor receptor; Erk1/2, extracellular signal-regulated kinase 1 and 2; JNK, the c-Jun N-terminal kinases; MAPKs, mitogen-activated protein kinases

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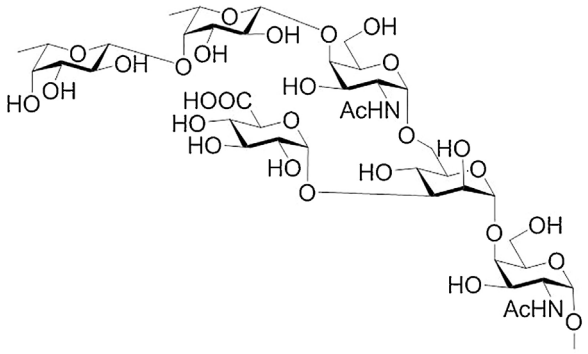

Fig. 1. Schematic structure of the repeating unit of SIP.

associated with the metastatic potential of the tumors, since they are the crucial enzymes participating in the degradation of type I and IV collagens and the ECM [15,16]. In cancer cells, the inhibition of EGFR will lead to the downregulation of MMP-2 and MMP-9 [17].

A number of EGFR inhibitors have been discovered, even clinically available, nevertheless the therapeutic efficacies are still not satisfactory [18]. Although great innovation has been devoted to discovering anti-cancer drugs, the cancer mortality is still high mostly because of tumor metastasis [19]. Development of anticancer polysaccharides drugs from natural resources has attracted great interest over the last decade [20]. *Spiella maindroni* ink polysaccharide, namely SIP, is isolated from the ink of cuttlefish *Sepiella maindroni*. SIP is a polysaccharide with a molecular weight about 1.13×10^4 Da and its repeating unit is composed of fucose, *N*-acetylgalactosamine and mannose in a molar ratio of 2:2:1, with a single branch of glucuronic acid at the C-3 position of mannose (Fig. 1.) [21]. After sulfation with the chlorosulfonic acid method, SIP-SII, a sulfated derivative of SIP was obtained, and it showed significant anti-metastasis activity both *in vitro* [22] and *in vivo* [23]. Furthermore, SIP-SII displayed marked antitumor activity as well [24]. After further research, SIP-SII was demonstrated to possess inhibitory effect on the expression and activation of EGFR (data not shown). Nonetheless, the effect of SIP-SII on EGF-induced metastasis remains uncertain. In the present study, we evaluated the effect of SIP-SII on the EGF-induced migration and invasion of human epidermoid carcinoma cell line (KB cell) which has been confirmed with overexpression of EGFR and EGF-induced enhancement of tyrosine kinase activity [25], and explored the underlying signaling molecular mechanisms.

2. Material and methods

2.1. Materials

SIP-SII was prepared as described previously [21,22]. RPMI 1640 was obtained from HyClone, USA. Fetal bovine serum was obtained from TBD (Tianjin, China). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was acquired from Sigma-Aldrich (St Louis, MO, USA). Transwell chamber was obtained from Corning Incorporated (NY, US). Matrigel was bought from BD Biosciences (MA, USA). Total protein extraction kit was purchased from BestBio (Shanghai, China). The BCA protein assay kit was bought from Beyotime (Shanghai, China). Anti-phospho-EGFR, anti-phospho-Akt (Ser473), anti-phospho-ERK, anti-MMP-2 and anti-MMP-9 antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Anti-phospho-p38 and anti-phospho-JNK antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-GAPDH and secondary antibodies (anti-rabbit or anti-mouse) were purchased from ZSGB-BIO (Beijing, China). Immobilon™ Western chemiluminescent HRP substrate was produced by Millipore Corporation (Billerica, MA, USA). Other chemicals and reagents were of analytical grade.

2.2. Cell culture

Human epidermoid carcinoma cell line (KB cells), a gift from the Department of Pharmacology of Shandong University, was grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/mL–100 μg/mL) at 37 °C in a 5% CO_2 humidified incubator. The cells were subcultured every 2–3 days using 0.02% EDTA–0.25% trypsin to harvest the cells.

2.3. MTT assay

The MTT assay was performed as previously described with slight modification [26]. Briefly, KB cells were seeded in 96-well plates and allowed to attach overnight. The attached cells were treated with SIP-SII at different concentrations (0, 4, 20, 100, 500 μg/mL) in the absence or presence of EGF (100 ng/mL) for 24 h, 48 h or 72 h. After incubation, 20 μL of MTT (5 mg/mL) was added to the medium and the cells were further incubated at 37 °C for 4 h. After centrifugation, the precipitate, namely formazan blue, was dissolved in 150 μL of DMSO. Light absorption of the DMSO solution was measured at 570 nm on a microplate reader (Bio-Rad 680, Hercules, CA, USA).

2.4. Wound healing assay

The wound healing assay was performed as previously described with slight modification [27]. Briefly, KB cells were seeded in 6-well plates and cultured until 90%-95% confluency. The monolayer culture was scratched by a sterile 200 μL pipette tip to generate an experimental wound, and then rinsed twice with serum-free RPMI 1640 to remove the non-adherent cells. The cells were exposed to various drug treatments at 37 °C for different time. Images were captured at indicated time points using an inverted microscope at 10 × (Olympus IX51, Japan) or 4 × (Olympus IX71, Japan) magnification. The distance of cell migration was calculated using the IPP software. Each value was derived from nine fields randomly selected along the wound scratch. The migration rate was calculated using the equation:

$$Migration\ \ rate = \frac{(\text{migrated distance at indicatedtime-initialdistance})}{\text{initial distance}} \times 100\%$$

2.5. Transwell invasion assay

The transwell invasion assay was performed as previously described with slight modification [27]. Briefly, the stimulation activity of EGF on the invasion of KB cells was evaluated using transwell chambers (8 μm). The upper surface of the chamber was coated with 50 μL of matrigel (1:8 dilution in serum-free RPMI 1640) and then incubated at 37 °C for 1 h. The liquid was removed and the chamber was rinsed with 50 μL of serum-free RPMI 1640 twice. The lower surface of the chamber was coated with 0.2 μg/μL of fibronectin and air dried before use. KB cells (5×10^4) in 50 μL of serum-free RPMI 1640 were seeded in the chambers and exposed to different drug treatments. 600 μL of RPMI 1640 with 10% FBS was added to the lower well. After incubation at 37 °C for 24 h, the cells on the upper surface of chamber were removed with cotton swabs and the invasive cells on the other side of the chamber were fixed with fixing solution (methanol: glacial acetic acid at 3:1 ratio). The fixed cells were stained with 0.1% crystal violet and counted under an inverted microscope (Olympus IX71, Japan) at 20 × magnification from at least 5 random fields in each condition. The relative cell invasion was calculated using the equation:

$$Relatinve\ cell\ invasion = \frac{\text{number of invaded cells in treated sample}}{\text{number ofinvaded cells in control}} \times 100\%$$