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Sulfated fucoidan FP08S2 inhibits lung cancer cell growth *in vivo* by disrupting angiogenesis via targeting VEGFR2/VEGF and blocking VEGFR2/Erk/VEGF signaling

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ABSTRACT

Fucoidan may inhibit angiogenesis. However, its functional target molecule and the underlying mechanism are still vague. In the present study, we showed that sulfated fucoidan FP08S2 from *Sargassum fusiforme* inhibited tube formation as well as migration and invasion of human microvascular endothelial cells (HMEC-1). In addition, FP08S2 was confirmed to disrupt VEGF-induced angiogenesis both *in vitro* and *in vivo*. Further study indicated that FP08S2 could bind to both VEGF and VEGFR2 to interfere with VEGF–VEGFR2 interaction. Moreover, VEGFR2/Erk/VEGF signaling pathway was blocked by FP08S2 in HMEC-1 cells. Importantly, FP08S2 impeded the growth and microvessel formation of A549 cancer cell xenograft in nude mice. These results suggested that FP08S2 presented remarkable anti-angiogenic activity via blocking VEGF signaling and could be a potential novel leading compound to inhibit lung cancer cell growth.

Introduction

The cancer ecosystem consists of blood vessels, fibroblastic stroma, immunocytes, inflammatory cells and cancer cells [1]. Blood vessels exert nutrient and catabolite exchange for the cancer cells and allow communication between the primary tumor and metastatic tumor [2,3]. Therefore, angiogenesis, the process of new blood-vessel growth, is crucial for cancer development and becomes a potential targeting approach for cancer therapy.

VEGF is discovered as an efficient inducer of angiogenesis, which influences on endothelial cell proliferation, migration, invasion and vascularization. Although a variety of angiogenic activators and inhibitors have been discovered [4], blockage of VEGF signal transduction represents one of the most efficient therapeutic strategies. VEGFR2, the subtype 2 receptor for VEGF, is implicated as vascular-endothelial-cell restricted and exerts a critical role on angiogenesis. Growth factors bind to the receptor to induce receptor phosphorylation and dimerization, leading to the activation of downstream signaling pathway [5–7]. Negative regulation of VEGFR2 is important for impairing angiogenesis [8,9], including tumor neovascularization. Hence, targeting VEGF and its receptor to disrupt angiogenesis might be an effective anti-tumor therapy strategy.

VEGF expression is regulated by a lot of transcription factors, including HIF-1, AP-1, AP-2, SP-1 and STAT3 [10–14]. HIF-1 (Hypoxia-inducible factor) is a major transcription factor to control the expression and secretion of VEGF. HIF-1 is identified as a heterodimeric trans-activator to regulate the expression of genes involved in angiogenesis, tumor growth and metastasis. HIF-1 is composed of α and β subunits, and HIF-1α is the limiting subunit to restrict transcription rate of VEGF gene under normoxic conditions [11,15].

Lung cancer continues to be the most common cause of cancer death. More than 1/4 of cancer deaths attribute to lung cancer [16]. As with other solid tumors, angiogenesis is recognized as a key step in the progression of lung cancers. Angiogenesis inhibitors like bevacizumab and some tyrosine kinase inhibitors are applied to lung cancer treatment [17–19]. Therefore, we postulate that the progression of lung cancer could be well-controlled by antiangiogenic treatment.

A lot of research revealed that fucoidan possessed anti-angiogenetic activity to inhibit tumor growth *in vitro* and *in vivo* [20–22]. In a previous report [23], we isolated a sulfated fucoidan FP08S2 from a brown alga *Sargassum fusiforme*. FP08S2 significantly inhibited tube formation and migration of HMEC-1 cells, but its target molecule and the precise mechanism underlying the

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anti-angiogenesis have not been illustrated well. In this study, we focused on one possible mechanism for the anti-angiogenetic effect of FP08S2. Our evidence suggested that FP08S2 could inhibit the growth of xenografted A549 lung tumor by anti-angiogenesis resulting from interaction of FP08S2 with VEGF and VEGFR2 as well as blockage of the VEGFR2/Erk/VEGF signaling pathway.

Materials and methods

Reagents

FP08S2 was prepared in this lab as described previously [23] and dissolved in PBS for research. Matrigel with growth factors and reduced growth factors was purchased from BD Biosciences (New Jersey, USA). Fetal bovine serum was obtained from Sijiqing Co., Ltd. (Hangzhou, China). EGF and VEGF proteins were from Prime Gene (Shanghai, China). VEGFR2 was purchased from Sino Biological (Beijing, China). Inhibitors used included BIBF1120 (Selleck Chemicals, Houston, USA), PD98059 (Sigma Aldrich, Missouri, USA), and 2-Methoxyestradiol (MedChem Express, New Jersey, USA).

Cell culture

Human microvascular endothelial cells (HMEC-1) [24] were cultured in MCDB131 (GIBCO, USA) medium, supplemented with 2 mM L-glutamine, 10 ng/mL EGF, 15% FBS and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin, Invitrogen). A549 lung cancer cells were cultured in RIPM 1640 medium containing 10% FBS and antibiotics. All cells were cultured in a humidified atmosphere of 5% CO_2 in air.

Chick embryo chorioallantoic membrane assay

The chick embryo chorioallantoic membrane (CAM) assay was carried out as described previously [25]. Fertilized chick eggs were incubated at 37 °C with adequate humidity. On the seventh day, the eggshell was cracked and peeled away from the region over the airspace. 0.5 cm × 0.5 cm filter papers soaked with saline containing FP08S2 (0, 50, 100, 150 μg/egg) were covered on the CAMs. The eggs were sealed and incubated for another 48 h. 10 eggs were used for each group and nonviable eggs were abandoned during this period. The CAMs were photographed with a microscope (Olympus BX51) after fixation (methanol:acetone = 1:1).

Tube formation assay

A 96-well plate was coated with cold Matrigel 50 μL/well and incubated at 37 °C to solidify the Matrigel. HMEC-1 cells (5×10^4 cells/well) with different doses (0, 4.21, 8.42, 16.84 μM) of FP08S2 were seeded onto the Matrigel and incubated at 37 °C for 12 h. For VEGF-induced tube formation assay in serum-free medium, the cells were seeded on the growth factor reduced Matrigel in the presence or absence of VEGF (100 ng/mL) and FP08S2 (8.42 μM) and incubated for 12 h. The capillary networks were photographed by a microscope (Olympus IX51).

Migration and invasion assays

Migration and invasion of HMEC-1 cells were evaluated by a Transwell assay using a 24-well, 8-μm-pore size Transwell plate (Costar, Cambridge, MA). For migration assay, HMEC-1 cells (1.5×10^5 cells/well) in 100 μL of serum-free medium containing different doses of FP08S2 (0, 4.21, 8.42, 16.84 μM) were seeded in the upper chamber. And the lower chamber contained the same medium with 15% FBS. After 8 h incubation, the migrated cells were stained by 0.1% crystal violet and the upper surface of the membrane was cleansed with a cotton swab to remove non-migrated cells. Migrated cells were photographed by a microscope (Olympus BX51). For invasion assay, Matrigel was thawed and diluted to 5 mg/mL in serum-free cold medium, 80 μL of the diluted Matrigel was added into the upper chamber. After incubation at 37 °C for 5 h, the gelled Matrigel was washed gently with warm serum-free medium. The followed steps were the same as migration assay. After 24 h incubation, invasive cells were also photographed after fixing and staining.

Immunofluorescence staining

HMEC-1 cells treated with or without FP08S2 were incubated for F-actin staining. Cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100 and blocked with 5% BSA in PBS for 1 h at room temperature. Then cells were probed with Rhodamine-labeled Phalloidin for 1 h and counterstained with DAPI for 30 min. Photomicrographs were analyzed under a fluorescence microscope (Olympus FV 1000).

In vivo *Matrigel plug assay*

C57/BL6 mice were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All *in vivo* studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were injected subcutaneously with 0.5 mL growth factor reduced Matrigel supplemented with 150 ng recombinant Human VEGF protein. Control mice were injected with Matrigel only. FP08S2 (0, 0.5 and 10 mg/kg) was administrated via tail vein injection every day for 14 days. The Matrigel plugs were removed and photographed after the mice were sacrificed.

Surface plasmon resonance (SPR) analysis

SPR measurements were performed on a BIACORE T200 (GE Healthcare, Stockholm, Sweden). Proteins were immobilized on a CM5 sensor chip by the amine coupling method. For interaction measurements, different concentrations of FP08S2 or VEGF were injected into the chips. In competitive inhibition assays, constant concentration of VEGF (29.67 nM) was incubated with increasing concentrations of FP08S2 for 30 min before injecting to the chip with immobilized VEGFR2. All procedures were conducted in HBS-EP (0.15 M NaCl, 0.01 M HEPES, 3 mM EDTA and 0.005% surfactant P20, pH 7.4) running buffer. Kinetic parameters were achieved using 1:1 binding model by BIACORE T200 Evaluation Software Version 1.0.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNAs of cultured cells were extracted with TRIzol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 2 μg total RNA using M-MLV reverse transcriptase (Takara Biotechnology, Dalian, China). Real-time PCR was performed with SYBR green Premix Ex Taq II kit (Takara Biotechnology, Dalian, China) using an Applied Biosystems ViiA™ 7 Fast Real-time PCR system as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 3 s, and annealing and extension at 57 °C for 30 s. Each sample was run in triplicate and the C_T value was evaluated for the target transcripts, where C_T represents the threshold cycle for each target transcript. The expression of the genes was normalized to that of the housekeeping gene 18S rRNA using the $2^{-\Delta\Delta CT}$ method. qPCR primers were shown in Supplementary Table S1.

Western blotting

HMEC-1 cells treated with different conditions were lysed with lysis buffer. Protein samples were separated by electrophoresis on SDS–PAGE gels and transferred to a PVDF membrane. Blots were incubated with primary antibodies at 4 °C, overnight. After incubation with secondary antibodies, enhanced chemiluminescence (ECL) reagent was used for signal detection. Antibodies used were listed in Supplementary Table S2. The data were quantified and normalized using ImageJ software.

Tumor xenograft model

A549 cancer cells (5×10^6 cells/mouse) were subcutaneously injected into BALB/cA nu/nu mice (6–8 weeks old). Xenografted animals were administrated with saline or different doses of FP08S2 (0.5 and 10 mg/kg) via tail vein injection every other day when tumors were palpable. Tumor volume (V) was calculated: $V = (\text{length} \times \text{width}^2)/2$. On the 27th day after treatment, mice were sacrificed and tumors were weighed and photographed.

Immunohistochemistry

Tumors were harvested and excised, fixed in 4% neutral paraformaldehyde, then embedded in paraffin and sectioned for the immunohistochemical analysis. Endothelial cells were identified by immunostaining with a CD31 antibody (Abcam). Cell proliferation was determined by staining with Ki67 antibody (Abcam). VEGF expression was shown by staining with VEGF antibody (Santa Cruz). To evaluate the protein expression, semiquantitative image analysis on the sections was conducted by Image Pro Plus software.

Statistical analysis

All data are expressed as the means ± S.D. P-value of the difference between groups was measured using Student's t-test for comparison or one-way ANOVA for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

FP08S2 inhibited angiogenesis in vivo *and* in vitro *as well as migration and invasion of HMEC-1 cells*

As discussed in the previous study [23], FP08S2 was associated with a significant decreased level of angiogenesis. To validate the antiangiogenic effects of FP08S2, we conducted chick chorioallantoic membrane (CAM) assay and tube formation assay. In the CAM assay, FP08S2 displayed potent antiangiogenic effects. FP08S2 led to a decreased rate of microvessel density in a dose-dependent manner (Fig. 1A). In tube formation assay (Fig. 1B), FP08S2 (4.21, 8.42,