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Inhibitory effects of fucoidan on activation of epidermal growth factor receptor and cell transformation in JB6 Cl41 cells

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Abstract

Algal fucoidan is a marine sulfated polysaccharide with a wide variety of biological activities including anti-thrombotic, anti-inflammatory, and anti-tumor activities. In this study, we tested the hypothesis that fucoidan may suppress neoplastic cell transformation by inhibiting the phosphorylation of epidermal growth factor receptor (EGFR) in mouse epidermal JB6 Cl41 cells. Our results provided the first evidence that fucoidan from *Laminaria guryanovae* exerted a potent inhibitory effect on EGF-induced phosphorylation of EGFR. Consistent with its inhibitory action on phosphorylation of EGFR, fucoidan clearly suppressed the phosphorylation of extracellular signal-regulated kinase or *c-jun* N-terminal kinases induced by EGF. Moreover, EGF-induced the *c-fos* and *c-jun* transcriptional activities were inhibited by fucoidan, resulting to suppressing of activator protein-1 (AP-1) activity and cell transformation induced by EGF. Taken together, these results indicate that fucoidan might exert chemopreventive effects through the inhibition of phosphorylation of the EGFR.

Keywords: Fucoidan; AP-1; Cell transformation; EGFR; c-fos; c-jun

1. Introduction

Brown seaweeds are known to produce different polysaccharides, namely alginates, laminarans and fucoidans (Painter, 1983; Percival, 1967). The latter polysaccharides usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars like xylose, galactose, mannose and glucuronic acid (Duarte et al., 2001; Percival, 1967). Several biological activities have been attributed to the fucoidans: anti-coagulant (Chevolot et al., 2001), anti-thrombotic (Mourao, 2004), anti-inflammatory (Cumashi et al., 2007), anti-tumoral (Itoh et al., 1993; Maruyama et al., 2006) (Teas et al., 1984), and anti-viral (Thompson and Dragar, 2004). Also, it was reported that fucoidan increases the level of nitric oxide (NO) production in quiescent macrophages, which was related with p38 kinase-dependent NF-kB activation (Nakamura et al., 2006). Although several studies on the biological activities of fucoidans have been performed, with particular focus on its anti-tumorigenic activity, it is unclear if fucoidan inhibits the neoplastic cell transformation and AP-1 transactivation activity induced by tumor promoter, such as EGF.

The epidermal growth factor receptor (EGFR), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell proliferation, differentiation, and transformation (Chen et al., 1987). The EGFR is an important target for cancer therapy (Yarden and Sliwkowski, 2001). Many carcinomas are promoted by EGFR activation, which can result from mutation of the receptor (Humphrey et al., 1990), its overexpression (Gorgoulis et al., 1992), or from EGFR stimulation through autocrine loops (Sizeland and Burgess, 1992).

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To elucidate the mechanism of the anti-tumorigenic effects of fucoidan, we studied the effects of fucoidan extracted from *Laminaria guryanovae* on the phosphorylation of EGFR and neoplastic cell transformation induced by EGF in mouse epidermal JB6 cells. Because the activation of EGFR has an important role in tumorigenesis, the results of this investigation may provide new insights in the mechanism of fucoidan in tumor suppression and the possibility for its application in tumor prevention and treatment.

2. Materials and methods

2.1. Reagents and antibodies

Eagle's minimal essential medium (MEM), L-glutamine, gentamicin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). EGF was purchased from Calbiochem–Novabiochem (San Diego, CA). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). The Dual-luciferase reporter assay system and CellTiter 96 non-radioactive cell proliferation assay kit were purchased from Promega (Madison, WI). Antibodies against phospho-MEK1/2, -ERK1/2, -p90RSK, -Elk1, -JNK, -*c-jun*, -EGFR, and total GAPDH were purchased from Cell Signaling Tech. Inc. (Danvers, MA).

2.2. Cell culture and transfection

JB6 Cl41 mouse epidermal cells were cultured in MEM supplemented with 5% FBS. Cells were transfected by a cationic liposome transfection method, Lipofectamine (Invirogen Co., Carlsbad, CA).

2.3. Polysaccharide extraction

L. guryanovae samples were collected in Troitsa Bay, Sea of Japan. The isolation and separation of water-soluble polysaccharides were carried out by the modified methods (Zvyagintseva et al., 1999). The fresh or deep-frozen seaweeds (3 kg) were initially treated with ethanol, acetone, and chloroform successively. A defatted seaweed *L. guryanovae* was stirred for 5 h in 0.1 M HCl for 5 h at room temperature, and the extracts were combined. The remaining seaweed was then extracted with water for 5 h at 60 °C. The extracts were concentrated to 1/5 of volume by ultrafiltration with the use of Millipore 3 kDa membrane, and polysaccharides were precipitated with four volumes of 96% ethanol. The precipitates were washed with 96% ethanol and acetone and air-dried.

2.4. Anion exchange chromatography

A solution of polysaccharide in 0.1 M NaCl (2.3 g in 50 ml) was applied onto a DEAE-cellulose column (Cl^- form, 3 × 21 cm) equilibrated with 0.1 M NaCl. The column was then successively eluted with 0.1, 0.5, 1, and 2 M NaCl solutions, each time until the disappearance in elute of positive reaction for carbohydrates by the phenol–sulfuric acid method (Dubois et al., 1956). The correspondent polysaccharides fractions were concentrated by ultrafiltration (1 kDa cutoff), dialyzed, and lyophilized. Fucoidan was dissolved in sterile water to test the biological activity in JB6 Cl41 cells.

2.5. Analytical procedures

Neutral carbohydrates were quantified by the phenol–sulfuric acid method (Dubois et al., 1956); reducing carbohydrates were determined according to Nelson method (Nelson, 1944). Monosaccharide composition was determined by HPLC with a LC-5001 carbohydrate analyzer (a Durrum DA-X8-11 column (385 × 3.2 mm) (Biotronic), bicinhoniate assay, and a C-R2 AX integrating system (Shimadzu) after hydrolysis by 2 M TFA (6 h, 100 °C).

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To estimate cell cytotoxicity, JB6 Cl41 cells were seeded (1×10^4) in 96-well plates in 100 μl of 5% FBS-MEM at 37 °C in a 5% CO_2 incubator. After culturing for 24 h, the various concentrations of fucoidan were treated and incubated for additional 24 h at 37 °C in a 5% CO_2 incubator. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (15 μl) were added to each well, and cells were then incubated for 4 h at 37 °C in a 5% CO_2. Absorbance was measured at 570 nm.

2.7. Anchorage-independent cell transformation assay (soft agar assay)

EGF-induced cell transformation was investigated in JB6 Cl41 cells. In brief, cells (8×10^3/ml) were exposed to EGF (1 ng/ml) with or without fucoidan in 1 ml of 0.3% basal medium Eagle (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. The cultures were maintained at 37 °C, in a 5% CO_2 incubator for 10 days, and the cell colonies were scored using a microscope and the Image-Pro PLUS computer software program (Media Cybernetics, Silver Spring, MD) as described by Colburn et al. (1981). The effects of the fucoidan on cell transformation of JB6 C141 cells are presented as an inhibition of cell transformation compared with EGF-stimulated cells in soft agar.

2.8. Reporter gene assays

The reporter gene assay for firefly luciferase activity was performed using lysates from transfected cells. In addition, the reporter gene vector phRL-SV40 (Promega) was co-transfected into each cell line and the *Renilla* luciferase activity generated by this vector was used to normalize the results for transfection efficiency. Cell lysates were prepared by first washing the transfected JB6 Cl41 cells once in phosphate buffered saline (PBS) at 37 °C. After removing the PBS completely, passive lysis buffer (PLB, Promega) were added, and then cells were incubated for 1 h with gentle shaking. The supernatant fraction was used for the measurement of firefly and *Renilla* luciferase activities. Cell lysates (20 μl each) were mixed with 100 μl of luciferase assay II reagent (Promega) and firefly luciferase light emission was measured by TriStar LB 941 (Berthold Tech. GmbH and Co. KG, Germany). Subsequently, 100 μl of *Renilla* luciferase substrate (Promega) was added in order to normalize the firefly luciferase data. *c-Fos* promoter luciferase (pGL3-Fos) and *c-jun* promoter luciferase (JC6GL3) constructs were kindly provided by Dr. Ron Prywes (Columbia University, NY). The AP-1 luciferase reporter plasmid (−73/+63 collagenase–luciferase) was kindly provided by Dr. Dong Zigang (Hormel Institute, University of Minnesota, MN).

2.9. Immunoblotting

The proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes. The membranes were blocked, hybridized with the appropriate primary antibody overnight at 4 °C. Protein bands were visualized by the chemiluminescence detection kit (ECL of Amersham Biosciences Corp., Piscataway, NJ) after hybridization with the horseradish peroxidase (HRP)-conjugated secondary antibody from rabbit or mouse.

3. Results

3.1. Purification and cell viability of fucoidan from L. gurjanovae

The brown seaweed *L. gurjanovae* was collected in the coast of island Big Shantar (Okhotsk sea). Polysaccharides were isolated from seaweed by a combination of methods