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Original Article

Fucoidan induces G1 arrest of the cell cycle in EJ human bladder cancer cells through down-regulation of pRB phosphorylation

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ABSTRACT

Fucoidan, a sulfated polysaccharide found in marine algae and brown seaweeds, has been shown to inhibit the *in vitro* growth of human cancer cells. This study was conducted in cultured human bladder cancer EJ cells to elucidate the possible mechanisms by which fucoidan exerts its anti-proliferative activity, which until now has remained poorly understood. Fucoidan treatment of EJ cells resulted in dose-dependent inhibition of cell growth and induced apoptotic cell death. Flow cytometric analysis revealed that fucoidan led to G1 arrest in cell cycle progression. It was associated with down-regulation of cyclin D1, cyclin E, and cyclin-dependent-kinases (Cdks) in a concentration-dependent manner, without any change in Cdk inhibitors, such as p21 and p27. Furthermore, dephosphorylation of retinoblastoma protein (pRB) by this compound was associated with enhanced binding of pRB with the transcription factors E2F-1 and E2F-4. Overall, our results demonstrate that fucoidan possesses anticancer activity potential against bladder cancer cells by inhibiting pRB phosphorylation.

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Introduction

Bladder cancer is any of several types of malignancy arising from the epithelial lining of the urinary bladder. In the United States, bladder cancer is the fourth most common type of cancer in men and the ninth most common cancer in women, although the incidence of bladder cancer in Asia is much lower (Kakehi et al., 2010; Abdollah et al., 2013). Despite recent advances in surgical and chemotherapeutic procedures, long-term survival rates are poor, and the most common cause of mortality is recurrence with metastasis (Racioppi et al., 2012). Therefore, it is important to develop other effective strategies to improve the survival rate for bladder cancer patients.

Several epidemiological studies have revealed that dietary intake of marine algae and seaweeds are protective against the risk of various types of malignancies (Kim et al., 2011; Park and Pezzuto, 2013; Ahmed et al., 2014). Brown seaweeds are used as an important healthcare food and a pharmaceutical product in Asian countries. Fucoidan is a major sulfated polysaccharide found in brown seaweed; it has been well characterized and is known to have various biological functions, including antioxidant, anti-inflammatory, and anticancer effects (Li et al., 2008; Senni et al., 2011; Fitton, 2011; Wang et al., 2012; Senthilkumar et al., 2013; Thomas and Kim, 2014). We reported recently that fucoidan may offer substantial therapeutic potential for treatment of inflammatory and neurodegenerative diseases that are accompanied by microglial activation (Park et al., 2011a). In addition, fucoidan suppresses cancer cell proliferation and inhibits the growth of transplanted tumor xenografts by inducing apoptosis and/or by blocking abnormal cell cycle progression at the G1 or G2/M phase (Riou et al., 1996; Fukahori et al., 2008; Park et al., 2011b, 2013; Zhang et al., 2011, 2013; Hsu et al., 2013; Xue et al., 2013; Yang et al., 2013; Park et al., 2014; Banafa et al., 2013; Chen et al., 2014; Senthilkumar and Kim, 2014; Cho et al., 2014). However, this effect is selective for cancer cells, as normal cell lines are resistant to cell cycle arrest and apoptosis by fucoidan. Moreover, fucoidan also inhibits migration and invasion of highly metastatic cancer cells

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via down-regulation of matrix metalloproteinases and inhibition of the phosphoinositide 3-kinase/Akt and nuclear factor-kB signaling pathways (Saitoh et al., 2009; Lee et al., 2012; Wang et al., 2014; Senthilkumar and Kim, 2014). However, the molecular mechanisms of its anti-proliferative actions on human bladder carcinoma cell growth have not yet been examined. Thus, the purpose of this study was to investigate the effects of fucoidan on cell proliferation of the human bladder carcinoma cell line EJ and to explore the potential mechanisms of the effects. Our data indicate that fucoidan inhibited the growth of EJ cells in a concentration-dependent manner, arresting them in the G1 phase of their cell cycle and inducing apoptosis.

Materials and methods

Cell culture

EJ cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 2 mM of L-glutamine and penicillin/streptomycin. The cells were cultured in an incubator with 5% CO_2 at 37 °C. Fucoidan was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) as a stock solution at a 200 mg/ml concentration, and the stock solution was then diluted with the medium to the desired concentration prior to use.

MTT assay

Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay, which is based on the conversion of MTT to MTT-formazan by mitochondria. In brief, cells (2×10^4 cells/well) were seeded in 24-well plates and exposed to fucoidan for 48 h. After treatment, 5 mg/ml MTT solution was added, followed by 3 h incubation at 37 °C in the dark, and the media was then removed. The formazan precipitate was dissolved in dimethyl sulfoxide (Sigma-Aldrich), and absorbance of the formazan product was measured at a wavelength of 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA) (Lee et al., 2014). For the morphological study, cells were photographed directly using an inverted microscope (Carl Zeiss, Oberkochen, Germany).

Flow cytometry analysis

Exponentially growing cells were compared to cells treated with various concentrations of fucoidan for 48 h. After treatment with fucoidan, the cells were collected and fixed in 70% ethanol at 4 °C for 30 min, and the DNA content of cells was stained with propidium iodide (PI) using a DNA staining kit (CycleTEST PLUS Kit, Becton Dickinson, San Jose, CA, USA) in accordance with the manufacturer's instructions. The cells were then subjected to a FACScan flow cytometer (Becton Dickinson) with the percentages of cells in different phases of the cell cycle calculated from DNA histograms. Cells with sub-G1 DNA content were considered apoptotic cells (Kim et al., 2014).

Morphological observation of nuclear change

After culture with various concentrations of fucoidan, cells were washed twice with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. The fixed cells were washed twice with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) solution for 10 min at room temperature. The cells were washed two more times with PBS. Coverslips were mounted on glass slides and analyzed by fluorescence microscopy using a Zeiss Axiophot microscope (Carl Zeiss).

Table 1
Oligonucleotides used in RT-PCR.

Gene name		Sequence
Cyclin D1	Sense	5′-TGG-ATG-CTG-GAG-GTC-TGC-GAG-GAA-3′
	Antisense	5′-GGC-TTC-GAT-CTG-CTC-CTG-GCA-GGC-3′
Cyclin E	Sense	5′-AGT-TCT-CGG-CTC-GCT-CCA-GGA-AGA-3′
	Antisense	5′-TCT-TGT-GTC-GCC-ATA-TAC-CGG-TCA-3′
Cdk2	Sense	5′-GCT TTC TGC CAT TCT CAT CG-3′
	Antisense	5′-GTC CCC AGA GTC CGA AAG AT-3′
Cdk4	Sense	5′-ACG-GGT-GTA-AGT-GCC-ATC-TG-3′
	Antisense	5′-TGG-TGT-CGG-TGC-CTA-TGG-GA-3′
Cdk6	Sense	5′-CGA-ATG-CGT-GGC-GGA-GAT-C-3′
	Antisense	5′-CCA-CTG-AGG-TTA-GAG-CCA-TC-3′
p21	Sense	5′-CTC AGA GGA GGC GCC ATG-3′
	Antisense	5′-GGG CGG ATT AGG GCT TCC-3′
p27	Sense	5′-AAG-CAC-TGC-CGG-GAT-ATG-GA-3′
	Antisense	5′-AAC-CCA-GCC-TGA-TTG-TCT-GAC-3′
GAPDH	Sense	5′-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3′
	Antisense	5′-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3′

Annexin-V staining

To analyze apoptosis, the cells were treated with the indicated concentrations of fucoidan for 48 h and resuspended in an annexin-V binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Aliquots of the cells were incubated with annexin-V fluorescein isothiocyanate (FITC, R&D Systems, Minneapolis, MN, USA), mixed, and incubated for 15 min at room temperature in the dark. PI at a concentration of 5 μg/ml was added to distinguish necrotic cells. The apoptotic cells (Annexin V^+/PI^- cells) were measured by a fluorescence-activated cell sorter analysis in a FACScan flow cytometer (Park and Han, 2014).

RNA extraction and reverse transcription-polymerase chain reaction (PCR)

Total RNA was isolated using an RNeasy minikit (Qiagen, La Jolla, CA, USA) and primed with random hexamers to synthesize complementary DNA using AMV reverse transcpriptase (Amersham Corp., Arlington Heights, IL, USA) following the manufacturer's instructions. PCR was performed in a Mastercycler (Eppendorf, Hamburg, Germany) with the indicated primers, which were purchased from Bioneer (Seoul, Republic of Korea), in Table 1. The conditions for the PCR reactions were 1× (94 °C for 3 min), 35X (94 °C for 45 s; 58 °C for 45 s; and 72 °C for 1 min), and 1× (72 °C for 10 min). The amplification products obtained by PCR were electrophoretically separated on a 1.0% agarose gel and visualized by ethidium bromide (EtBr, Sigma-Aldrich) staining (Gong et al., 2014). In a parallel experiment, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Total protein extraction, immunoprecipitation, and Western blot analysis

For isolation of total protein fractions, the cells were harvested and washed once with ice-cold PBS, and lysed with cell lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 0.5 g/ml leupeptin, 1% Na_3CO_4, 1 mM phenylmethane-sulfonyl fluoride], and protein concentrations were quantified using the Bio-Rad protein

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