Revista Brasileira de Farmacognosia 25 (2015) 246–251

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

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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ARTICLE INFO

Article history:
Received 30 December 2014
Accepted 22 March 2015
Available online 25 April 2015

Keywords:
Fucoidan
EJ cells
G1 arrest
Apoptosis
pRB

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.

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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http://dx.doi.org/10.1016/j.bjp.2015.03.011

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

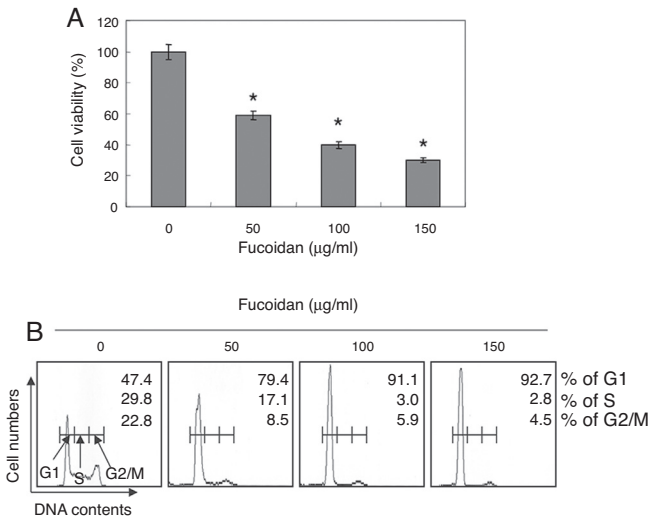

Fig. 1. Inhibition of cell viability and induction of G1 arrest by fucoidan treatment in human bladder cancer EJ cells. (A) Cells were treated with the indicated concentrations of fucoidan for 48 h. The cells were harvested, and the percentage of cell viability was calculated with an MTT assay. Data are presented as mean ± SD in triplicate. Significance was determined by the Student's *t*-test (*$p < 0.05$ vs. untreated control). (B) The cells were collected, fixed, and stained with PI for flow cytometry analysis. DNA content is represented on the *x*-axis, and the number of cells counted is represented on the *y*-axis. Each point represents the mean of two independent experiments.

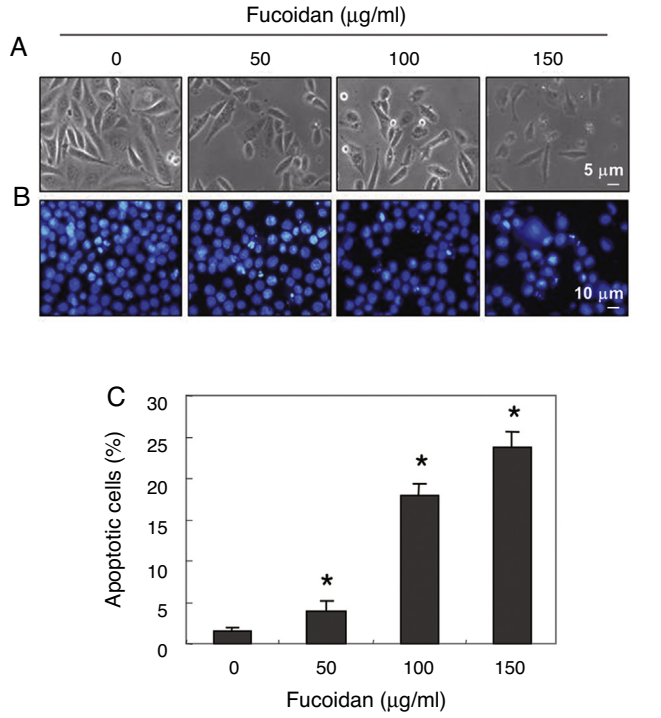

Fig. 2. Induction of apoptosis by fucoidan treatment in EJ cells. The cells were treated with various concentrations of fucoidan for 48 h. (A) The morphological changes of the cells were imaged using an inverted microscope (original magnification, 200×). (B) The cells were fixed and stained with DAPI solution. After 10 min incubation at room temperature, the stained nuclei were observed with a fluorescent microscope (original magnification, 400×). (C) The cells were harvested for determining the percentage of annexin-V positive/PI negative (apoptotic cells). The data are expressed as the mean ± SD of three independent experiments. The significance was determined by the Student's *t*-test (*$p < 0.05$ vs. untreated control).

assay (Bio-Rad Lab., Hercules, CA, USA), following the procedure described by the manufacturer. For immunoprecipitation, cell extracts were incubated with immunoprecipitating antibodies in extraction buffer for 1 h at 4 °C, and then the immunocomplexes were precipitated with protein A-Sepharose beads (Sigma-Aldrich). For Western blot assay, the total proteins and immunoprecipitated proteins were subjected to electrophoresis on sodium dodecyl sulphate (SDS)–polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) by electroblotting. Blots were subsequently blocked with 5% non-fat milk and probed with the desired antibodies for 1 h at 4 °C, incubated with diluted enzyme-linked secondary antibody at room temperature for a further 1 h, and then visualized by enhanced chemiluminescence (ECL, Amersham) according to recommended procedures. Eextracellular-regulated kinase (ERK) was used as an internal control. The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-labeled donkey antirabbit immunoglobulin and peroxidase-labeled sheep antimouse immunoglobulin were purchased from Amersham.

Statistical analysis

The data were expressed as means ± SD for triplicate experiments. Statistical analyses were performed using a Student's *t*-test. A *p* value <0.05 was considered statistically significant.

Results

Fucoidan inhibits cell viability and induces G1 arrest in EJ cells

To determine whether fucoidan influenced the cell viability of the EJ cells, the cells were cultured in the presence of fucoidan for 48 h, and the cell viability was determined with an MTT assay. As shown in Fig. 1A, fucoidan reduced the viability of the EJ cells in a concentration-dependent manner. The cell cycle phase distribution of exponentially growing EJ cells was next compared to cells treated with fucoidan. Compared with the untreated control, the fucoidan-treated cells accumulated in the G1 phase of the cell cycle in a concentration-dependent manner (Fig. 1B), and there was a decrease in the number of cells in the S and G2/M phases. Taken together, these results suggest that the growth inhibitory effect of fucoidan on EJ cells is the result of it blocking the G1 phase.

Fucoidan induces apoptosis in EJ cells

As fucoidan induced morphological changes, such as membrane blebbing and a reduction in cell volume under the same conditions (Fig. 2A), we assessed the effect of fucoidan treatment on the apoptotic characteristics of the EJ cells to determine whether apoptosis was involved in fucoidan-mediated antiproliferation[0]. Fig. 2B reveals that nucleic morphological changes of apoptosis as indicated by representative images of condensed and fragmented nuclei were clearly evident in fucoidan-treated EJ cells in contrast to the untreated cells. To quantify fucoidan-induced apoptosis, the EJ cells treated with fucoidan were stained with annexin V-FITC and analyzed by flow cytometry. As shown in Fig. 2C, fucoidan increased the annexin-V-FITC stained apoptotic population of cells, and these effects were dose dependent. These observations indicate that the inhibition of cell viability observed in response to fucoidan is also associated with the induction of apoptosis.

Effect of fucoidan on the expression of cell cycle checkpoint regulators

To further investigate the molecular mechanisms underlying fucoidan-induced G1 arrest, the EJ cells were treated with fucoidan,

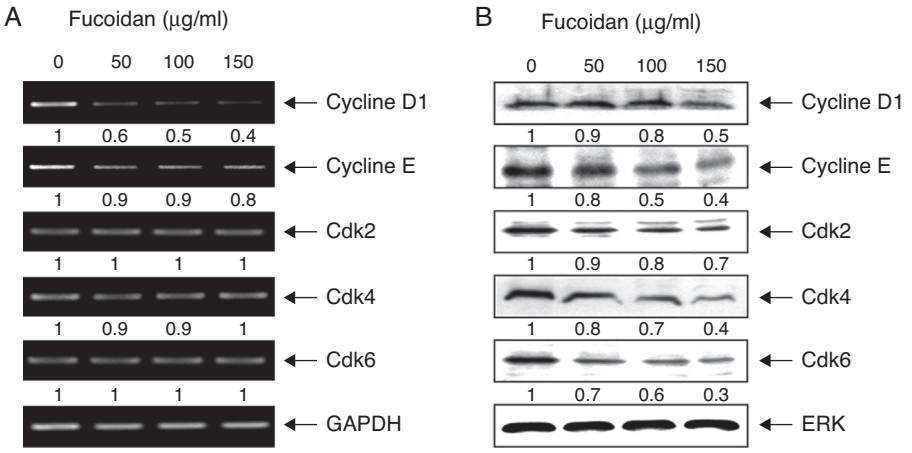

Fig. 3. Down-regulation of G1-associated cyclins and Cdks by fucoidan treatment in EJ cells. (A) After treatment with various concentrations of fucoidan for 48 h, the cells were collected, and total RNA was isolated and reverse-transcribed. The resulting cDNAs were then subjected to PCR with the indicated primers, and the reaction products were separated in 1.0% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) The cell lysates were separated and equal amounts of total cell lysates were subjected to SDS–polyacrylamide gels, transferred, and probed with the indicated antibodies. ERK was used as an internal control. The relative ratios of expression in the results of the Western blotting were presented at the bottom of each of the results as relative values of the GAPDH and ERK expression, respectively.

and then RT-PCR and Western blot analysis were performed. As illustrated in Fig. 3, fucoidan suppressed the expression of biomarkers, such as cyclin D1, cyclin E, cyclin-dependent-kinase (Cdk) 2, Cdk4, and Cdk6, in the transition of the G1 to the S phase in a concentration-dependent manner. However, fucoidan did not significantly affect the levels of Cdk inhibitors, such as p21 and p27 (Fig. 4).

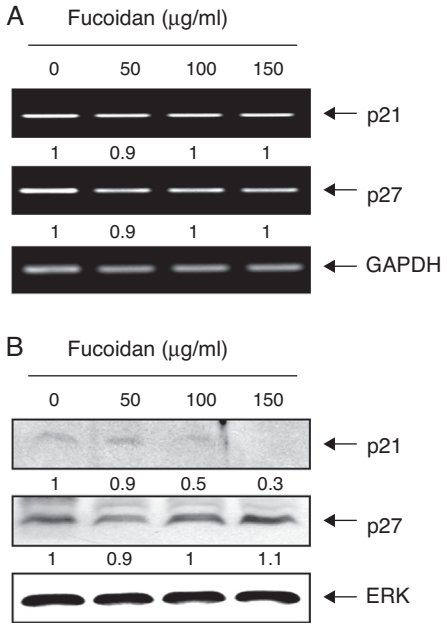

Fig. 4. Effects of fucoidan on the levels of Cdk inhibitors in EJ cells. (A) The cells were treated with different concentrations of fucoidan for 48 h. Total RNA was isolated and reverse-transcribed, and the resulting cDNAs were subjected to PCR. The reaction products were subjected to electrophoresis in 1.0% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) After incubation with fucoidan under the same conditions, the cell lysates were separated, and equal amounts of total cell lysates were subjected to SDS–polyacrylamide gels, transferred, and probed with antibodies against p21 and p27. ERK was used as an internal control. The relative ratios of expression in the results of the Western blotting were presented at the bottom of each of the results as relative values of the GAPDH and ERK expression, respectively.

Fucoidan down-regulates pRB phosphorylation and increases the binding of pRB and E2Fs

As D-type cyclins and cyclin E-stimulated Cdk activity converges in hyperphosphorylation of the retinoblastoma protein (pRB), the effect of fucoidan on the phosphorylation status of pRB was investigated by Western blot analysis. Fucoidan caused a remarkable decrease in total levels of pRB expression and changed from a hyperphosphorylated form to a hypophosphorylated form (Fig. 5A). Furthermore, co-immunoprecipitation analysis indicated that there appeared to be no association between pRB and E2Fs, such as E2F-1 and E2F-4, in the untreated control cells[0]. However, there was a strong increase in the binding of pRB and E2Fs in the fucoidan-treated EJ cells (Fig. 5B), suggesting that fucoidan inhibits the release of E2Fs protein from pRB.

Discussion

Although fucoidan exerts various pharmacological activities, such as anti-inflammatory, antioxidant, and anticancer effects (Riou et al., 1996; Li et al., 2008; Fukahori et al., 2008; Saitoh et al., 2009; Fitton, 2011; Senni et al., 2011; Park et al., 2011b, 2013; Zhang et al., 2011, 2013; Wang et al., 2012; Lee et al., 2012; Liu et al., 2012; Hsu et al., 2013; Xue et al., 2013; Yang et al., 2013; Senthilkumar et al., 2013; Park et al., 2014; Banafa et al., 2013; Chen et al., 2014; Senthilkumar and Kim, 2014; Wang et al., 2014), the anti-cancer activity of fucoidan in human bladder cancer cells has rarely been reported. Therefore, we elucidated the ability of fucoidan to inhibit the growth of human bladder cancer EJ cells. In this study, we found that fucoidan shows considerable potential to inhibit the proliferation of EJ cells via cell cycle arrest in the G1 phase and apoptosis induction.

Cancer cells exhibit deregulation of cell cycle and apoptosis and activation of signal transduction, resulting in abnormal proliferation. Cyclins and Cdks play an important role in the regulation of the cell cycle through the formation of cyclin/Cdk complexes. Alteration in the formation of these complexes could lead to increased or decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis (Sperka et al., 2012; Canavese et al., 2012). In general, D-type cyclins/Cdk4/6 and cyclin E/Cdk2 complexes are crucial factors in the transition of the G1 to the S phase cell cycle, and these factors are negatively regulated by Cdk

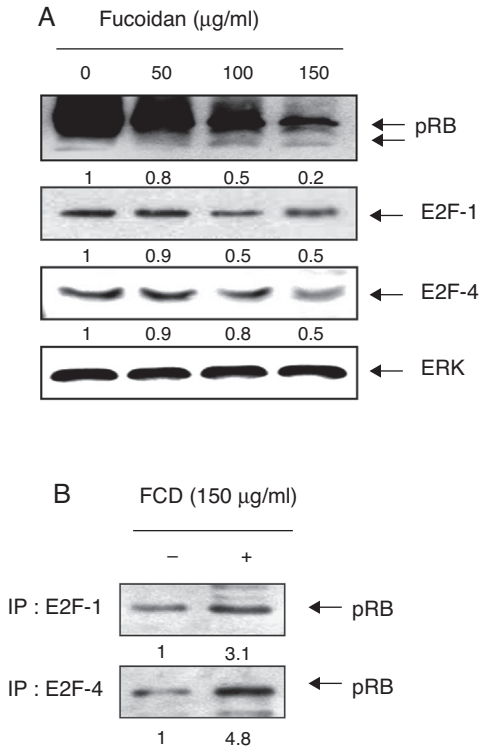

Fig. 5. Induction of hypophosphorylation of pRB and enhanced association of pRB and E2Fs by fucoidan treatment in EJ cells. (A) After treatment with various concentrations of fucoidan for 48 h, the total cell lysates were prepared and separated by electrophoresis on an 8% or 10% SDS–polyacrylamide gel. Western blotting was then performed using anti-pRB, anti-E2F-1, and anti-E2F-4 antibodies. ERK was used as an internal control. (B) The cells were incubated with or without 150 μg/ml of fucoidan for 48 h, and then equal amounts of proteins (0.5 mg of protein) were immunoprecipitated with anti-E2F-1 or anti-E2F-4 antibody. Immunocomplexes were separated by 8% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-pRB antibody. Proteins were detected by ECL detection. The relative ratios of expression in the results of the Western blotting were presented at the bottom of each of the results as relative values of the ERK expression or untreated control, respectively.

inhibitors, including p21 and p27 (Dobashi et al., 2003; Paternot et al., 2010). In the present study, the results from RT-PCR and immunoblotting analysis clearly demonstrated that the levels of cyclin D1 and cyclin E expression were markedly inhibited by fucoidan treatment at both transcriptional and translational levels (Fig. 3). In addition, fucoidan-induced G1 arrest was correlated with the down-regulation of Cdks, such as Cdk2, Cdk4, and Cdk6. Although, a recent study indicated that fucoidan down-regulated cyclin E, Cdk2, Cdk4 resulting in G0/G1 arrest through binding of Cdk inhibitor p21 to Cdk2 and Cdk4 in cells treated with fucoidan in human bladder cancer 5637 cells (Cho et al., 2014), the expression of Cdk inhibitors such as p21 and p27 was remained unchanged in fucoidan-treated EJ cells (Fig. 4).

On the other hand, pRB as a tumor suppressor is also important for cell cycle progression during the G1 to S phase transition. Dephosphorylation of pRB inhibits cell cycle progression by interacting with transcription factors of the E2F family, whereas phosphorylation of pRB results in induction of cell cycle progression through the breaking of pRB/E2F complexes (Dobashi et al., 2003; Paternot et al., 2010). Therefore, if the levels of either protein are decreased or the association between their respective bindings partners is diminished, a concomitant decrease in the degree of pRB phosphorylation would be expected. In conjunction with down-regulation of total levels of pRB, the data showed that treatment with fucoidan inhibited the phosphorylation of pRB and enhanced the association of pRB and E2Fs (Fig. 5). The role of Cdk inhibitors has attracted attention in the induction of G1 phase arrest, which is related to the restriction of the proliferation of cancer cells via fucoidan treatment. However, according to Boo et al. (2012), restriction of E2F expression plays an important role in inducing G1 cell cycle arrest in PC-3 prostatic cancer cells, along with the increased expression of Cdk inhibitor p21. Moreover, a recent study conducted in our lab revealed that phosphorylation inhibition of pRB was simultaneously involved in addition to the expression increase of Cdk inhibitor p21 in T24 bladder cancer cells' G1 arrest brought about by fucoidan (Park et al., 2014). Hence, even if cancer cells' G1 arrest resulting from fucoidan treatment differs according to types of cancer cells, it can at least be said that expression increase of Cdk inhibitors such as p21 and phosphorylation inhibition play an important factors in G1 arrest.

Conclusions

In conclusion, the results presented here demonstrate that: (i) reduced survival of EJ cells after exposure to fucoidan is associated with G1 phase cell cycle arrest and apoptosis induction; (ii) fucoidan inhibits cell cycle progression in the G1 phase by decreasing G1-related cyclins and Cdks; (iii) treatment with fucoidan results in increased binding of pRB to E2Fs. These novel phenomena have not been previously described in bladder cancer cells and suggest that fucoidan and related compounds may have significant potential in the development of cancer treatments.

Authors' contributions

HYP and IWC contributed in running the laboratory work, analysis of the data and drafted the paper. GYK, BWK and WJK contributed to critical reading of the manuscript. WJK and YHC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Blue-Bio Industry Regional Innovation Center (RIC08-06-07) at Dongeui University as a RIC program under Ministry of Trade, Industry & Energy and Busan city and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. NRF-2014R1A2A1A09006983).

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