

Anti-Proliferation Activity of Fucoidan in MKN45 Gastric Cancer Cells and Downregulation of Phosphorylated ASK1, a Cell Cycle-Regulated Kinase

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ABSTRACT

Background Fucoidan is a high-molecular polysaccharide whose main constituent is sulfated fucose. We specifically focused on the anti-proliferation activity of fucoidan and examined the underlying mechanism in MKN45 gastric cancer cells.

Methods MKN45 cell proliferation was analyzed by BrdU assay and fucoidan cytotoxicity was examined by LDH and clonogenic assays. The Agilent Human microarray kit was used to identify upregulated and downregulated genes in response to fucoidan, and western blot analyses evaluated cell cycle proteins.

Results Fucoidan impeded the MKN45 cell cycle by approximately 50%, and inhibited cell proliferation. LDH assays showed no immediate cytotoxic effects of fucoidan at 24 h exposure, however longer time courses revealed cell growth inhibition at 4 days in a dose-dependent manner. Microarray analysis identified MAP3K5, or ASK1 (apoptosis signal-regulating kinase), which was upregulated by 1.38-fold upon fucoidan treatment. Fucoidan increased ASK1 protein levels, while reducing phosphorylated ASK1 levels. Reduction of ASK1 by siRNA decreased proliferation of MKN45 cells.

Conclusion Our findings show that fucoidan may suppress cellular proliferation and DNA synthesis in MKN45 cells by suppressing the ASK1-p38 signaling pathway through reduction of phosphorylated ASK1 levels.

Key words anti-proliferation activity; ASK1; cell cycle; fucoidan; MKN45

Fucoidan is a high-molecular polysaccharide only found in brown algae and its main constituent is sulfated fucose. The saccharide chain also includes galactose, man-

nose, xylose and uronic acid. Over the past decades, fucoidan has been extensively studied, owing to its numerous interesting biological activities, such as antioxidant,¹ immunomodulatory,² antiviral,³ antithrombotic⁴ and anticoagulant effects.⁵ In particular, we have focused on the anti-cancer activities of fucoidan.

Multiple studies have demonstrated antitumor activity for many polysaccharides. Fucoidans from *Eisenia bicyclis* and *Laminaria japonica* are effective against sarcoma 180.^{6,7} Fucoidan from *L. japonica* can inhibit hepatoma QGY7703 cell growth in logarithmic phase in vitro, thus restraining the growth of tumors.⁸ Fucoidans from *L. saccharina*, *L. digitata*, *Fucus serratus*, *F. distichus* and *F. vesiculosus* strongly blocked MDA-MB-231 breast carcinoma cell adhesion to platelets, an effect that might have critical implications in tumor metastasis.⁹

Among its various anti-cancer effects, we have focused on the antiproliferation activities of fucoidan. We hypothesized that fucoidan exerts its anti-cancer effects through alterations of gene expression. Thus, in this study, we performed mRNA microarray analysis to examine the effects of fucoidan on cellular gene expression. We selected MKN45 gastric cancer cells for analysis, as this common cancer cell line is relatively easy to culture and is often used for experimental analyses.^{10–12}

MATERIALS AND METHODS

Reagents

The human gastric adenocarcinoma cell line, MKN45 was obtained from the Riken BioResource Center (National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Tsukuba, Japan) in November 20, 2012. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). MKN45 gastric adenocarcinoma cells were obtained from a metastatic liver tumor in a 62-year-old Japanese female.

Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA, and the lactate dehydrogenase (LDH) assay kit were obtained from Wako (Tokyo, Japan). FBS was from Biowest (Nuaille, France). Rabbit polyclonal anti-ASK1 (H-300), anti-ERK1 (K-23), mouse monoclonal anti-phospho-ERK (E-4), and ASK1 siRNA

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Abbreviations: ASK-1, apoptosis signal-regulating kinase one; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle medium; IgG, immunoglobulin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MTT, methyl thiazolyl tetrazolium; RPMI, Roswell Park Memorial Institute

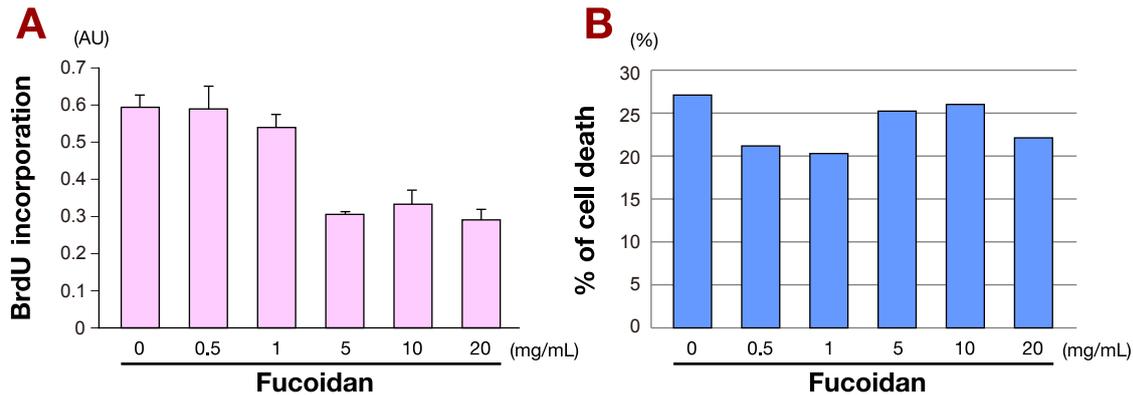


Fig. 1. Effect of fucoidan on MKN45 cells.

A: Fucoidan impedes proliferation of MKN45 cells. The rate of MKN45 cell proliferation inhibition in the presence of increasing amounts of fucoidan was examined using BrdU incorporation assay. Over 5 mg/mL of fucoidan resulted in decreased BrdU incorporation. Inhibition rate was approximately 50%.

B: Cytotoxic effects of fucoidan in MKN45 cells. Cytotoxic effects of increasing amounts of fucoidan in MKN45 cells were examined by LDH assay. Cells were cultured with fucoidan as indicated for 24 h. Increasing fucoidan concentrations did not affect the rate of cell death, even at high doses.

AU, arbitrary unit.

were purchased from Santa Cruz Biotech (Santa Cruz, CA). Rabbit polyclonal anti-phospho-ASK1 (Thr845), rabbit monoclonal anti-p38 MAPK (D13E1) and anti-phospho-p38 MAPK (Thr180/Tyr182) (D3F9) were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin (IgG), anti-mouse IgG, and the ECL plus detection kit were from GE Healthcare (Buckinghamshire, United Kingdom). Alexa Fluor 488-conjugated anti-rabbit IgG was from Invitrogen (Carlsbad, CA).

Preparation of fucoidan

High molecular weight fucoidan was obtained from Marine Products Kimuraya (Sakaiminato, Japan) as a sulfated polysaccharide, extracted from the brown seaweed *Cladosiphon okamuranus* (Okinawa Mozuku) as previously described.^{13, 14} The mean molecular weight was 4×10^5 and the active ingredient contained 14.2% sulfate (SO_4^{2-}). The material has been previously used in studies on biological activity and with respect to safety of human use.^{15–17}

Cell culture, cell growth and cell death assay

Cells were cultured in DMEM with 10% FBS. For fucoidan treatment, culture medium was replaced with fresh medium mixed with indicated concentrations of fucoidan, and cells were cultured until the assay. Cell growth assay was performed by counting cell numbers using a hemocytometer. Cytotoxic effects were evaluated by the LDH assay following the manufacturer's protocol. Briefly, extracellular LDH activity in the medium was

evaluated by an enzymatic reaction that results in formazan products, which were measured with a spectrophotometer (Tecan Sunrise Rainbow, Tecan Japan, Tokyo) at 600 nm.

siRNA transfection

Transfection of siRNA was performed with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol. Briefly, ASK1 siRNA (sc-20748; Santa Cruz Biotech) was mixed with Lipofectamine RNAiMAX reagent in serum-free DMEM. Suspended cells were added to the mixture and the cell mixture was plated and cultured for 24 h before experimental analysis.

Microarray expression analysis

Total RNA was collected from MKN45 cells using the RNeasy mini kit (Qiagen, Hilden, Germany). Microarray expression analyses were performed using the Agilent SurePrint G3 Human GE v2 8x60K Microarray (Design ID: 039494) (Agilent Technologies, Palo Alto, CA).

BrdU assay

BrdU assay was performed using the BrdU Cell Proliferation assay kit (Merck, Darmstadt, Germany) following the manufacturer's protocol. Briefly, cells in a 96-well plate were cultured in medium with BrdU for 12 h. BrdU incorporation was detected with anti-BrdU. Signals were measured by spectrophotometer analysis at 450/540 nm.

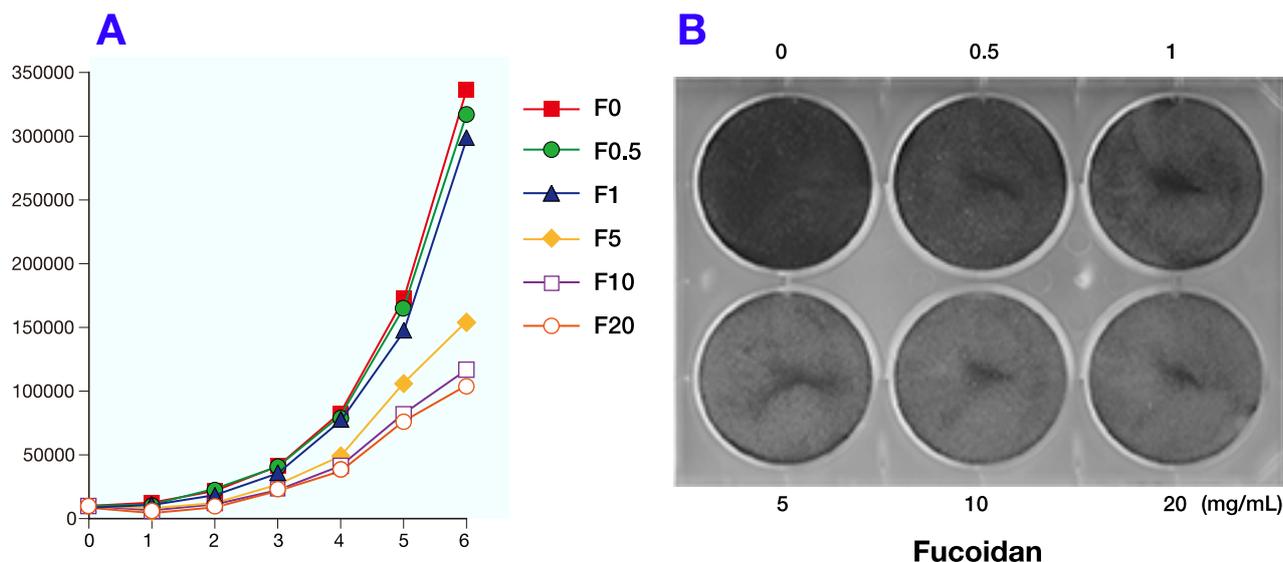


Fig. 2. Fucoidan inhibition of MKN45 cells.

A: Fucoidan inhibition of MKN45 cell growth. Clonogenic assays were performed by culturing MKN45 cells for 6 days in the presence of increasing amounts of fucoidan as indicated (F0-F20), and counting cells with a hemocytometer. Starting at day 4, the numbers of cells were clearly reduced with higher doses of fucoidan.

B: Fucoidan inhibits colony formation of MKN45 cells in a dose-dependent manner. Colony formation assay of MKN45 cells incubated with increasing amounts of fucoidan. Cell density was lower with increasing concentrations of fucoidan.

Western blot

Cells were lysed by sonication in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 4 °C. Protein concentrations were determined with a protein assay rapid kit (Wako, Tokyo). Cell lysates (10 µg of protein) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with primary antibodies at 1:1000 dilution with TBS-Tween 20 buffer, followed by incubation with HRP-conjugated secondary antibody at 1:5000 dilution. The membranes were processed using the ECL detection kit and images were obtained using a LAS-4000 image analyzer (Fujifilm, Tokyo).

Immunofluorescence staining

Cells were plated on coverslips and fixed with 3.7% formaldehyde in PBS for 30 min, followed by permeabilization in 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated with anti-phospho-p38 antibody (1:200 in 0.1% BSA/PBS) for 1 h, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG for 1 h. The coverslips were mounted with mounting medium containing DAPI (ProLong Gold Antifade Mounting with DAPI; Invitrogen), and fluorescence images were obtained with confocal microscopy (Leica TCS-SP2, Wetzlar, Germany).

RESULTS

Fucoidan impedes the cell cycle

We first examined the effects of fucoidan on proliferation of MKN45 cells using the BrdU incorporation assay. Low doses of fucoidan had a minimal impact on MKN45 cell proliferation, while high doses of fucoidan, over 5 mg/mL, dramatically decreased BrdU incorporation. The rate of MKN45 cell proliferation inhibition by fucoidan was approximately 50% (Fig. 1A).

Cytotoxicity of fucoidan by LDH assay

The cytotoxic effects of treatment of MKN45 cells with increasing amounts of fucoidan for 24 h were examined by LDH assay (Fig. 1B). The results showed that 24 h fucoidan treatment did not induce cell death, even at high doses.

Clonogenic assay reveals fucoidan cytotoxic effects

To gain fuller insight into the potential cytotoxic effects of fucoidan, we performed clonogenic assays and extended the incubation time to 6 days (Fig. 2A). Starting at day 4, the numbers of cells were clearly reduced with larger doses of fucoidan. Colony formation assays showed similar results (Fig. 2B). We observed lower cell density of MKN45 cells cultured with high doses of fucoidan, and this reduction occurred in a dose-dependent manner.

Table 1. The differentially expressed G0 categories and gene lists in fucoidan treated MKN45 cells

Upregulated	Downregulated
Human insulin signaling (7) <i>SORBS1</i> <i>FLOT2</i> <i>PSCD3</i> <i>MAP4K1</i> <i>MAP3K5</i> <i>FOS</i> <i>EGR1</i>	Irinotecan pathway (<i>Homo sapiens</i>) (2) <i>CES1</i> <i>ABCC2</i>
GPCRs, class a rhodopsin-like (5) <i>HTR6</i> <i>OR3A3</i> <i>P2RY4</i> <i>ADRA2A</i> <i>OR5V1</i>	Blood clotting cascade (2) <i>SERPINE1</i> <i>F10</i> Fatty acid omega oxidation (2) <i>CYP1A2</i> <i>CYP1A1</i>
T-cell receptor signaling pathway (5) <i>MAP4K1</i> <i>RASGRP2</i> <i>FOS</i> <i>CABIN1</i> <i>CARD11</i>	Focal adhesion (9) <i>TNC</i> <i>EGFR</i> <i>CAV1 (A_24_P12626)</i> <i>CAV3</i> <i>CAV1 (A_23_P134454)</i> <i>RAPGEF1</i> <i>COMP</i> <i>MAPK8</i> <i>LAMC2</i>
Monoamine GPCRs (2) <i>HTR6</i> <i>ADRA2A</i>	Complement activation, classical pathway (3) <i>CFH</i>
MAPK signaling pathway (5) <i>HSPA8</i> <i>MAP4K1</i> <i>MAP3K5</i> <i>FOS</i> <i>MAPT</i>	<i>SERPINE1</i> <i>F10</i>

Genes that showed significant ($P < 0.05$) changes in upregulated or downregulated expression in MKN45 cells in the presence of fucoidan.

Microarray analyses of fucoidan-treated cells

To identify potential genes involved in mediating the cellular effects of fucoidan, total RNA was collected from MKN45 cells and microarray expression analyses were performed using the Agilent Human microarray kit. A total of 20278 sequences were examined at DNA Chip Research (Kanagawa, Japan), and 496 genes were found to be upregulated and 774 genes were downregulated by fucoidan. Table 1 lists the genes in MKN45 cells with significant changes in expression in response to fucoidan ($P < 0.05$). Among these genes, we observed that MAP3K5, also known as ASK1 (apoptosis signal-regulating kinase), was upregulated by 1.38-fold in response to fucoidan. Microarray analyses accession number: GSE58774.

Fucoidan downregulates cell cycle promoting proteins

From the results of the BrdU incorporation assay and mRNA microarray analysis, we hypothesized that fucoidan affected the expressions of cell cycle-related genes. Western blot analysis revealed that treatment of MKN45 cells with over 5 mg/mL of fucoidan resulted in decreased levels of phosphorylated ASK1 and phosphorylated p38 MAPK. In contrast, the expression of ASK1 was slightly increased in a dose-dependent manner. The expressions of p38 MAPK, ERK, and phosphorylated ERK were unchanged (Fig. 3A).

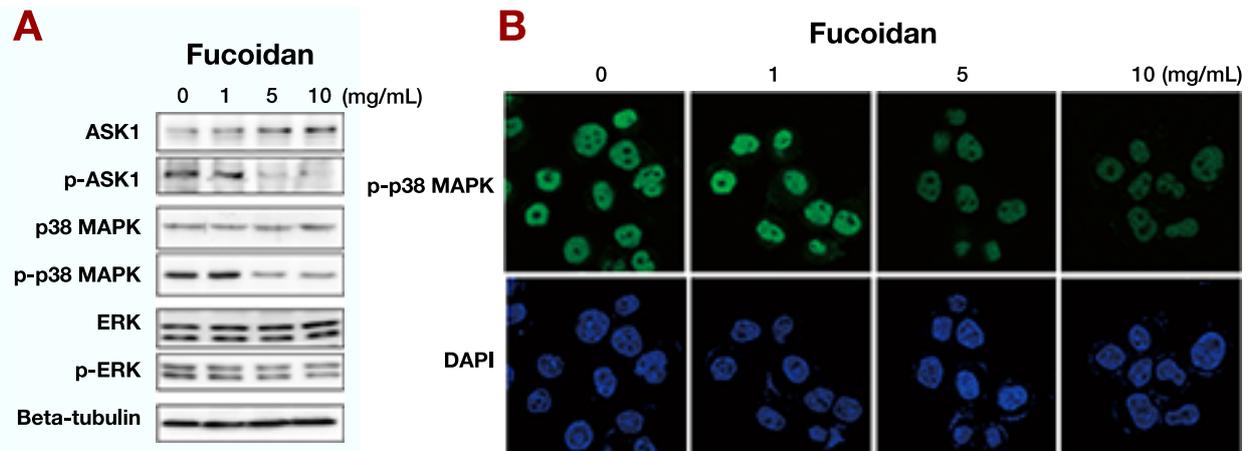


Fig. 3. Effects of fucoidan on phosphorylation.

A: Fucoidan downregulates cell-cycle promoting proteins. Western blot analysis revealed that over 5 mg/mL of fucoidan decreased the levels of phosphorylated ASK1 and phosphorylated p38 MAPK. However, the expression of ASK1 was slightly increased in a dose-dependent manner. The expressions of p38 MAPK, ERK, and phosphorylated ERK were not changed.

B: Fucoidan reduces phosphorylated p38 in the nucleus. MKN45 cells were incubated with fucoidan as indicated and analyzed by immunofluorescence for phosphorylated p38. Treatment with fucoidan at 5 and 10 mg/mL clearly reduced the levels of phosphorylated p38 protein in the nucleus of MKN45 cells, which was consistent with the western blot results.

We also performed fluorescence immunostaining of MKN45 cells with phosphorylated p38 antibody and found that 5 and 10 mg/mL fucoidan treatment clearly reduced phosphorylated p38 protein levels in the nucleus (Fig. 3B), which was consistent with the western blot results. These results suggested the possibility that fucoidan inhibited proliferation of MKN45 cells by suppressing the ASK1-p38 signaling pathway.

ASK1 knockdown reduces proliferation of MKN45 cells

To examine the effect of ASK1 suppression on MKN45 cell proliferation, ASK1 siRNA was transfected into MKN45 cells. Western blot analyses confirmed that ASK1 siRNA reduced ASK1 protein levels compared with control cells (Fig. 4A). BrdU assay revealed that genetic inhibition of *ASK1* repressed MKN45 cell proliferation (Fig. 4B).

DISCUSSION

Recent studies have shown that marine bioactive compounds can either modulate signaling pathways leading to cell cycle regulation or directly alter cell cycle regula-

tory molecules in cancer therapy. The anti-proliferative activity of fucoidan may be a result of its interference in cell cycle progression.

Previous studies demonstrated that fucoidan caused a cell cycle arrest at the G0/G1 phase in hepatocarcinoma cell lines¹⁸ and suppressed cell proliferation and arrested the cell cycle in hepatocellular carcinoma cell lines.¹⁹ Our BrdU results showed that cell proliferation was suppressed at a dose of fucoidan of over 5 mg/mL in 24 h, which impeded DNA synthesis and cell proliferation during S phase in MKN45 cells. This result demonstrated that fucoidan induced cell cycle arrest in MKN45 cells.

We explored the cytotoxic effects of fucoidan in MKN45 cells by LDH assay. After 24h of incubation, fucoidan-treated MKN45 cells did not exhibit any elevation in LDH release compared with the control group, even at high doses (Fig. 1B). This result was consistent with previous studies.^{18, 19} However, other studies demonstrated cytotoxicity of fucoidan in different cancer cells.^{19, 20}

These discrepancies in cytotoxicity among fucoidans are likely due to variations in fucoidan polymer structure and function, as fucoidans originate from various species, anatomical regions, and under different growing conditions of brown seaweeds. Fucoidans are also subject to specific extraction and purification procedures. Furthermore, fucoidans may also exhibit cell-type-specific effects, with different functions in different cancer cell lines. Additionally, even though cell proliferation was repressed by fucoidan in previous reports, the repression was not evident at 24 h by MTT assay.¹⁹ We also did not observe cytotoxic effects of fucoidan at 24 h. We speculated that a longer time course may better reveal fucoidan effects under long exposure conditions, and subsequently extended the incubation time to 6 days. Under longer treatment conditions, we observed both a marked decrease in cell number and reduction of cell density in a dose-dependent manner.

We performed mRNA microarray analysis to identify potential gene targets of fucoidan in mediating its cell cycle effects. MAP3K5 or ASK1 that was identified as a MAPK kinase kinase, was upregulated 1.38-fold in response to fucoidan. ASK1 is involved in regulating the cell cycle and is phosphorylated at the beginning of S phase.²¹ This suggested that phosphorylation might facilitate ASK1's function in the spindle-kinetochore interaction, which presumably occurs during the S phase after the duplication of kinetochores. This also implies that decreasing phosphorylated ASK1 would impair the cell cycle during S phase.

In the MAPK pathway, p38 MAPK functions as a downstream kinase of ASK1. p38 MAPK was reported

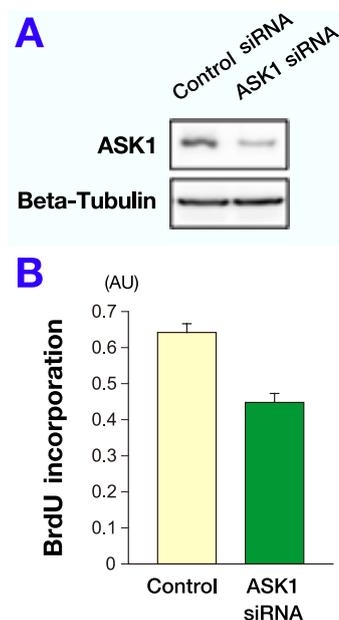


Fig. 4. ASK1 knockdown decreases proliferation of MKN45 cells.

A: Western blot analysis confirmed that transfection with ASK1 siRNA reduced the expression level of ASK1 protein compared with control cells. Beta-tubulin was used as an internal control.

B: To examine the effect of ASK1 suppression on proliferation of MKN45 cells, ASK1 siRNA was transfected into MKN45 cells and BrdU assay was performed. Genetic inhibition of ASK1 repressed the proliferative activity of MKN45 cells.

AU, arbitrary unit.

to promote cancer cell growth and survival.²² In a study investigating the effect of fucoidan on lung carcinoma, fucoidan treatment resulted in decreased phospho-p38 levels,²³ which demonstrated anti-cancer effect due to fucoidan.

Another MAPK, ERKs are known to promote cell survival, differentiation, cell proliferation²⁴ and cell death.²⁵ A previous study showed fucoidan clearly decreased the phosphorylation of ERK in HS-Sultan cells.²⁶

In evaluating the results of the mRNA microarray analysis, we therefore focused on genes involved in cell cycle regulation to examine potential mediators of fucoidan. Based on the BrdU incorporation results, we hypothesized that the protein levels of ASK1 and its related proteins would be altered by fucoidan, and we performed western blot analysis of ASK1, p38 MAPK, and ERK steady state and phosphorylated forms to determine the effects of fucoidan.

Treatment with over 5 mg/mL fucoidan effectively decreased phosphorylated ASK1 and phosphorylated p38 MAPK levels, while ERK expression remained unchanged. The western blot results furthered the BrdU incorporation findings, suggesting that fucoidan caused reduction of phosphorylated ASK1 expression, arresting the cell cycle in S phase and eventually impairing DNA synthesis. This results in damaging proliferation of cancer cells.

Consistent with the microarray analysis, fucoidan increased ASK1 protein levels. Increased ASK1 protein could possibly result from a reduction of phosphorylated ASK1 by fucoidan. As a consequence of reduced phosphorylated ASK1, the mRNA levels were compensatory amplified, resulting in increased expression of ASK1.

A previous study demonstrated elevation of ASK1 in human gastric cancer and showed that overexpression of ASK1 promoted gastric cancer cell proliferation.²⁷ Another report found that ASK1 inhibition prevents gastric cancer cell growth in vitro and in vivo.²⁸ This mechanism involved cyclin D, a known regulator of the G1 to S phase transition.²⁹ However, this mechanism seemed specific to gastric cancer cells, as ASK1 knockdown in other cell lines did not inhibit proliferation.²⁸ The previous study supports the results of the current study, demonstrating that genetic inhibition of ASK1 repressed MKN45 cell proliferation.

To elucidate the mechanisms underlying the anti-cancer activities of fucoidan, determining its binding partners and targets would help clarify its activities. One study reported that fucoidan acts as a polysaccharide ligand of P-selectin, with nanomolar affinity.³⁰ P-selectin is an adhesion molecule expressed at the surface of en-

dothelial cells and platelets on activation. P-selectin promotes platelet, endothelium, and leukocyte interactions, and was reported to cause cardiovascular events.³¹

Fucoidan exhibited the highest affinity for P-selectin³² and repressed its function in platelet aggregation.³¹ This implied that fucoidan targets the surface of endothelial cells, with potential consequences on downstream signal transduction pathways. Our findings show that fucoidan induces alterations of gene expression, which may possibly involve its function at the cell surface.

In conclusion, our study indicated that fucoidan may suppress cellular proliferation and DNA synthesis in MKN45 cells by suppressing the ASK1-p38 signaling pathway through a reduction in phosphorylated ASK1 levels. Our findings strongly suggest fucoidan as a potential therapeutic agent for cancer therapies. Further investigations would provide new approaches to the development of effective chemotherapy regimens.

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The authors declare no conflict of interest.

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