Antitumor Effects of Fucoidan on Human Colon Cancer Cells via Activation of Akt Signaling

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Abstract

We identified a novel Akt signaling mechanism that mediates fucoidan-induced suppression of human colon cancer cell (HT29) proliferation and anticancer effects. Fucoidan treatment significantly inhibited growth, induced G1-phase-associated upregulation of p21WAF1 expression, and suppressed cyclin and cyclin-dependent kinase expression in HT29 colon cancer cells. Additionally, fucoidan treatment activated the Akt signaling pathway, which was inhibited by treatment with an Akt inhibitor. The inhibition of Akt activation reversed the fucoidan-induced decrease in cell proliferation, the induction of G1-phase-associated p21WAF1 expression, and the reduction in cell cycle regulatory protein expression. Intraperitoneal injection of fucoidan reduced tumor volume; this enhanced antitumor efficacy was associated with induction of apoptosis and decreased angiogenesis. These data suggest that the activation of Akt signaling is involved in the growth inhibition of colon cancer cells treated with fucoidan. Thus, fucoidan may serve as a potential therapeutic agent for colon cancer.

Key Words: Anticancer effect, Cell cycle arrest, Fucoidan, Human colorectal cancer cells

INTRODUCTION

Colorectal cancer has become an increasing global health concern. It is a leading cause of mortality in men and women, and affects more than one million people worldwide annually (Jemal *et al*., 2005). The features of the disease usually occur progressively over a protracted period owing to increased genomic instability, which leads to the upregulation of oncogenes and the downregulation of tumor suppressor genes (Samowitz and Slattery, 2002). Studies have shown that major intracellular signaling pathways are altered during tumorigenesis, leading to cell proliferation and survival (Fang *et al*., 2006).

The inhibition of proliferation and induction of apoptosis in tumor cells is a strategy used in antitumor therapy. The balance between proliferation and apoptosis signaling pathways controls tumor pathogenesis. A number of sulfated polysaccharides (SPs), which are natural products present in numerous brown seaweeds (Ahn *et al*., 2008), exert potent antitumor activity by inducing cell cycle arrest and apoptosis in several tumor cell lines (Croci *et al*., 2011; Coura *et al*., 2012). SP antitumor activity has been the subject of much attention in cur-

rent research. Fucoidans are a class of fucose-enriched sulfated polysaccharides and are found in the extracellular matrix of brown algae. Fucoidans have various biological activities, including antiviral, anticoagulant, anti-inflammatory, immuno-modulatory, anti-angiogenic, and anti-adhesive (Damonte *et al*., 2004; Cumashi *et al*., 2007). Furthermore, fucoidan induces antitumor effects in several tumor cell lines, both *in vitro* and *in vivo* (Itoh *et al*., 1993; Lee *et al*., 2012; Xue *et al*., 2012).

Akt signaling is activated by growth factors and other extracellular stimuli (Vivanco and Sawyers, 2002); it is involved in cell survival, apoptosis, transcription, and proliferation (Vivanco and Sawyers, 2002; Osaki *et al*., 2004; Song *et al*., 2005). The Akt signaling pathway is activated in several human cancers (Sarkar and Li, 2004). Numerous studies suggest that Akt inhibition is related to decreased cell growth and reduced cancer cell migration (Sarkar and Li, 2004), suggesting that Akt inhibition may be an attractive approach for preventing or treating human malignancies (Hill and Hemmings, 2002; Sarkar and Li, 2004). However, Akt activation has been implicated in cell death mediated by natural products in B16F10 murine melanoma and U373 glioblastoma cell lines (Jin *et al*.,

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2012). Thus, the roles of Akt activation and cell cycle regulation in the inhibition of cancer cell proliferation require further investigation. Our study suggests that Akt activation inhibits cell proliferation in fucoidan-treated HT29 colon cancer cells.

MATERIALS AND METHODS

Ethics statement

Experiments were performed on 8-week-old male Balb/C nude mice (Biogenomics, Seoul, Korea, http://www.orient.co.kr) that were maintained under a 12-h light/dark cycle, in accordance with the regulations of Soonchunhyang University, Seoul Hospital. All procedures were performed in accordance with the policies of the Institutional Animal Care and Use Committee of Soonchunhyang University, Seoul Hospital, Korea (IACUC2013-5).

Preparation of fucoidan

Fucoidan extract from the seaweed *Fucus vesiculosus* was obtained from Sigma (St. Louis, MO, USA). Fucoidan powder was dissolved in phosphate-buffered saline (PBS), filter-sterilized through a 0.45-μm pore filter (Sartorius Biotech GmbH, Gottingen, Germany), and stored as fucoidan extract (20 mg/mL) at 4°C until use.

Cell cultures

The human colon cancer HT29 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C with 5% CO_2 in a humidified incubator.

Cell viability assay

Exponentially growing colon cancer cells were subconfluently incubated in 96-well plates with fucoidan for various lengths of time. Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay, which is based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium to formazan by mitochondrial dehydrogenase. Formazan was quantified by measuring the absorbance at 570 nm using a microplate reader (Tecan, Männedorf, Switzerland).

[3]H-thymidine incorporation

[3]H-thymidine incorporation experiments were conducted as previously described (Brett *et al.*, 1993). Cells were cultured in a well until they reached 50% confluence, after which they were washed twice in PBS and then maintained in serum-free DMEM with all supplements, and recorded. After 24 h of incubation, these cells were again washed twice in PBS and incubated under the same conditions. After the indicated incubation period, 1 μCi of [methyl-^{3}H] thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Uppsala, Sweden) was added to the cultures, and the incubation was continued for 1 h at 37°C. The cells were washed twice with PBS, fixed for 15 min in 10% trichloroacetic acid (TCA; Sigma) at 23°C, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23°C.

Aliquots were removed, and their radioactivity was determined using an LS 6500 Liquid Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). All values are expressed as the mean ± S.E. of triplicate experiments. The values were converted from the absolute counts to a percentage of the control to permit comparison between experiments.

Cell cycle analysis

Cells were harvested, fixed in 70% ethanol, and stored at -20°C. The cells were then washed twice with ice-cold PBS, and subsequently incubated with RNase and the DNA-intercalating dye propidium iodide (PI, Sigma). Cell cycle histograms were generated after analyzing the PI-stained cells by fluorescence-activated cell sorting (Beckman Coulter). At least 10^4 events were recorded for each sample. The samples were analyzed using CXP software (Beckman Coulter).

Western blot analysis

Protein expression of HT29 cells following treatment with fucoidan (100 μg/mL) and/or Akt 1/2 kinase inhibitor (10^{-6} mol/L; Akt inhibitor, Sigma) at indicated time-points was assessed using western blot analysis. Total protein was extracted using RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred to polyvinylidenefluoride membranes (PVDFs; Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies against vascular endothelial growth factor (VEGF), phospho-AKT, cyclin-dependent kinase 2 (CDK2), CDK4, Cyclin D, Cyclin E, p21, and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). After incubation of the membranes with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), bands were visualized using enhanced chemiluminescence reagents (Amersham Biosciences).

Tumor growth in mice

In vivo generation of tumors was accomplished by subcutaneous injection of 5×10^4 HT29 cells suspended in 50 μL PBS into mice. When the tumors reached a volume in the range of 8-10 mm^3, the mice were intraperitoneally injected with normal saline or fucoidan at doses of 5 or 10 mg/kg body weight every two days. After post-injection day 30, mice were sacrificed. Tumors of sacrificed mice were measured and histologically confirmed. Tumor size was measured in two perpendicular dimensions (a=length, b=width) with a Vernier caliper, and the volume (V; mm^3) was calculated using the formula V=a×b^2/2. The tumor specimens were fixed in 4% formaldehyde, embedded in paraffin, and cut into 4-μm thick sections for immunohistochemical analysis.

Immunohistochemistry

Immunofluorescent staining was performed using primary antibodies against CD31, VEGF, caspase-3, and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology), as well as secondary antibodies conjugated with Alexa-488 (Life Technologies, Carlsbad, CA, USA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Immunostained slides were imaged by confocal microscopy (Olympus, Tokyo, Japan).

Statistical analyses

All data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was assessed using the Student's *t* test, where *p*<0.05 was considered significant.

RESULTS

Fucoidan inhibited the proliferation of human colon cancer HT29 cells

To assess the effect of fucoidan on HT29 colon cancer cell viability, an MTT assay was performed to measure cell viability following the treatment of HT29 colon cancer cells with fucoidan (0-100 μg/mL) for 24 h (Fig. 1A). Fucoidan treatment decreased HT29 colon cancer cell viability in a dose-dependent manner, compared with control. In addition, the effects of fucoidan on HT29 colon cancer cell proliferation were investigated using [³H]-thymidine incorporation. Fig. 1B shows that HT29 colon cancer cell proliferation was significantly inhibited after treatment with fucoidan (0-100 μg/mL) for 24 h, compared with control (Fig. 1).

Fucoidan induced G1-phase cell cycle arrest

Cell cycle distribution and DNA content were analyzed in fucoidan-treated HT29 colon cancer cells by flow cytometry. Fucoidan treatment (100 μg/mL) induced a higher population of cells in G0/G1 phase compared with that of control cells, and a concomitant reduction was observed in the population of cells in the S-phases relative to that in control cells (Fig. 2). Next, the effect of fucoidan on cell cycle regulatory proteins, which are functional in the G1-phase, was investigated using immunoblot assay in HT29 colon cancer cells. Fig. 3A shows

that fucoidan treatment significantly decreased the expression of cyclin D1, cyclin E, CDK2, and CDK4 in HT29 colon cancer cells in a dose-dependent manner. p21WAF1 has been identified as a negative regulator of the G1- to S-phase transition checkpoint (Harper *et al.*, 1993; Sherr, 1994, 1996). We examined the effect of fucoidan on p21WAF1 expression in HT29 colon cancer cells. Fig. 3C shows that 24-h fucoidan treatment significantly induced p21WAF1 expression in a dose-depen-
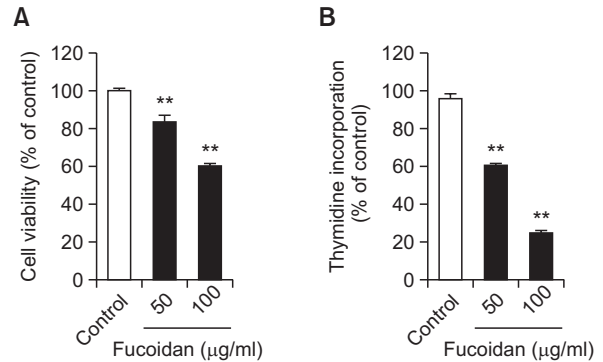

Fig. 1. Fucoidan-mediated inhibition of cell viability and cell proliferation in HT29 colon cancer cells. (A) HT29 colon cancer cells were treated with fucoidan (0-100 μg/mL) for 24 h. Cell viability was measured using a modified MTT assay. (B) HT29 colon cancer cells were incubated for 24 h with or without fucoidan as indicated and labeled with [methyl-³H] thymidine at 1 μCi/mL during the last 12 h of the incubation period. Values are expressed as the mean ± SEM of three independent experiments with triplicate dishes. **p<0.01 vs. control.
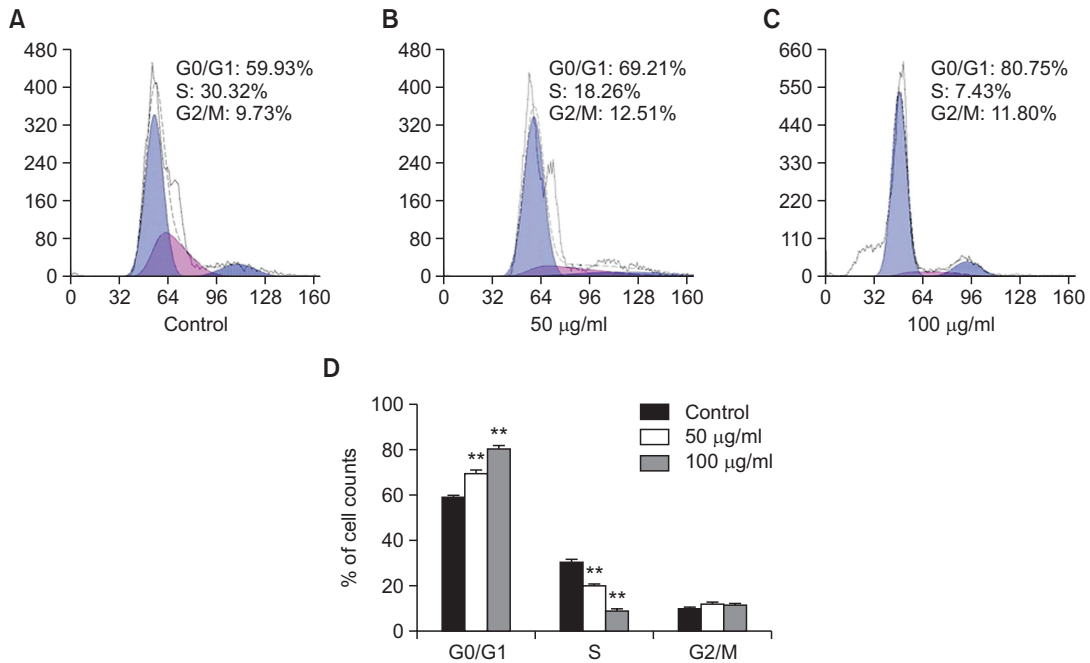

Fig. 2. Fucoidan-induced G1-phase cell cycle arrest in HT29 colon cancer cells. Cells were treated with 0 (A), 50 (B), and 100 (C) μg/mL fucoidan for 24 h. Flow cytometric analysis was performed to determine the cell cycle distribution in HT29 colon cancer cells treated with fucoidan. (D) Standard quantification of each phase (G0/G1, S, and G2/M) presented as the percent of cell counts. The example shown is representative of four independent experiments. Values are expressed as the mean ± SEM. **p<0.01 vs. control.
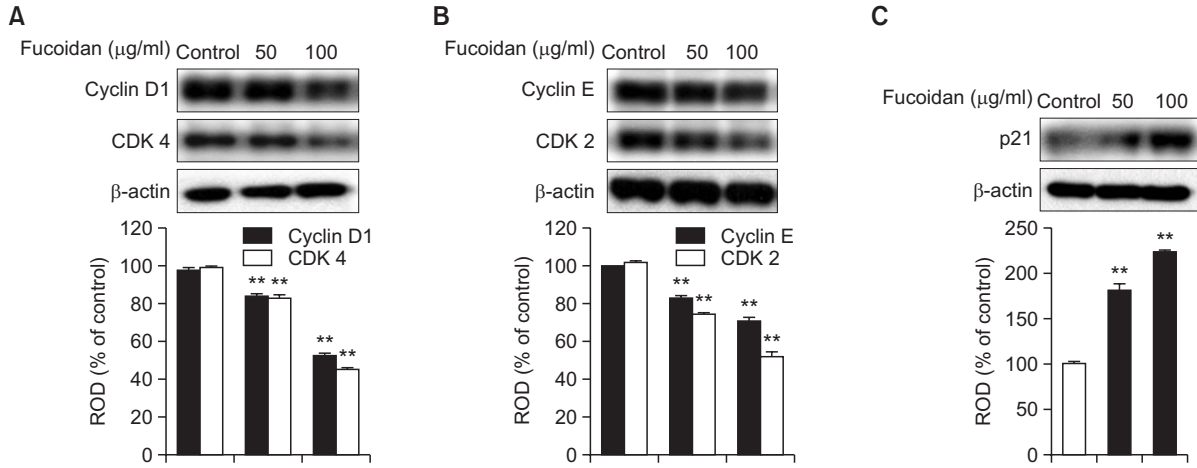
Fig. 3. Fucoidan-induced cell cycle arrest was correlated with the upregulation of p21WAF1 in HT29 colon cancer cells. (A and C) HT29 colon cancer cells were treated with fucoidan (0-100 μg/ml) for 24 h. Western blot analysis was performed using cyclin D1, cyclin E, CDK2, CDK4, and p21WAF1 antibodies. The lower panel depicts the mean ± SEM of three independent experiments for each condition, as determined from densitometry relative to β-actin. **$p < 0.01$ vs. control.
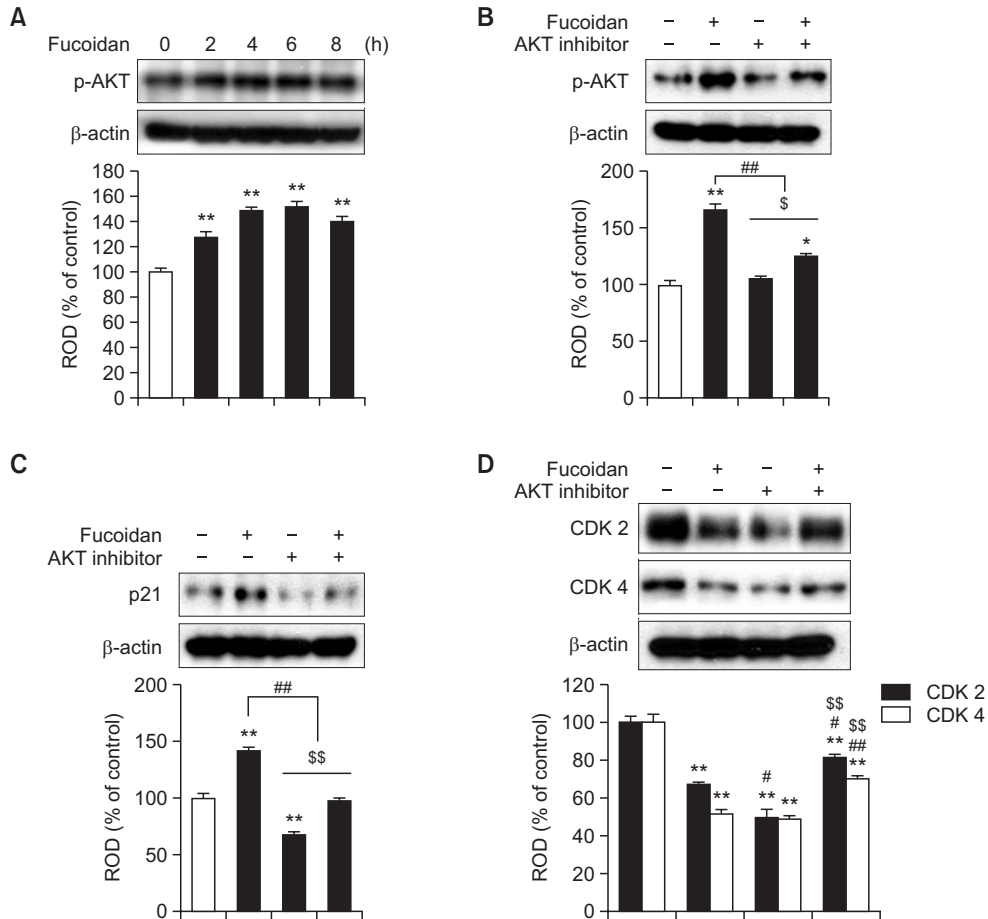

Fig. 4. Fucoidan-induced activation of the Akt pathway and Akt inhibitor-mediated reversal of p21WAF1 and CDK expression. (A) HT29 colon cancer cells were treated with fucoidan (100 μg/mL) for various times (0-8 h). The activation of Akt was analyzed by western blot analysis using a phospho-Akt antibody. (B) HT29 colon cancer cells were pretreated for 4 h with an Akt inhibitor (10^{-6} M) and then incubated with or without fucoidan for 6 h. The activation of Akt was analyzed by western blot analysis using a phospho-Akt antibody. (C and D) HT29 colon cancer cells were pretreated for 4 h with an Akt inhibitor (10^{-6} M) and then incubated with or without fucoidan for 24 h. Western blot analysis was performed with antibodies specific for p21WAF1, CDK2, and CDK4. The lower panel depicts the mean ± SEM of three independent experiments for each condition, as determined from densitometry relative to β-actin. *$p < 0.05$ and **$p < 0.01$ vs. control, #$p < 0.05$ and ##$p < 0.01$ vs. fucoidan only group, \$$p < 0.05$ and \$$p < 0.01$ vs. Akt inhibitor only group.
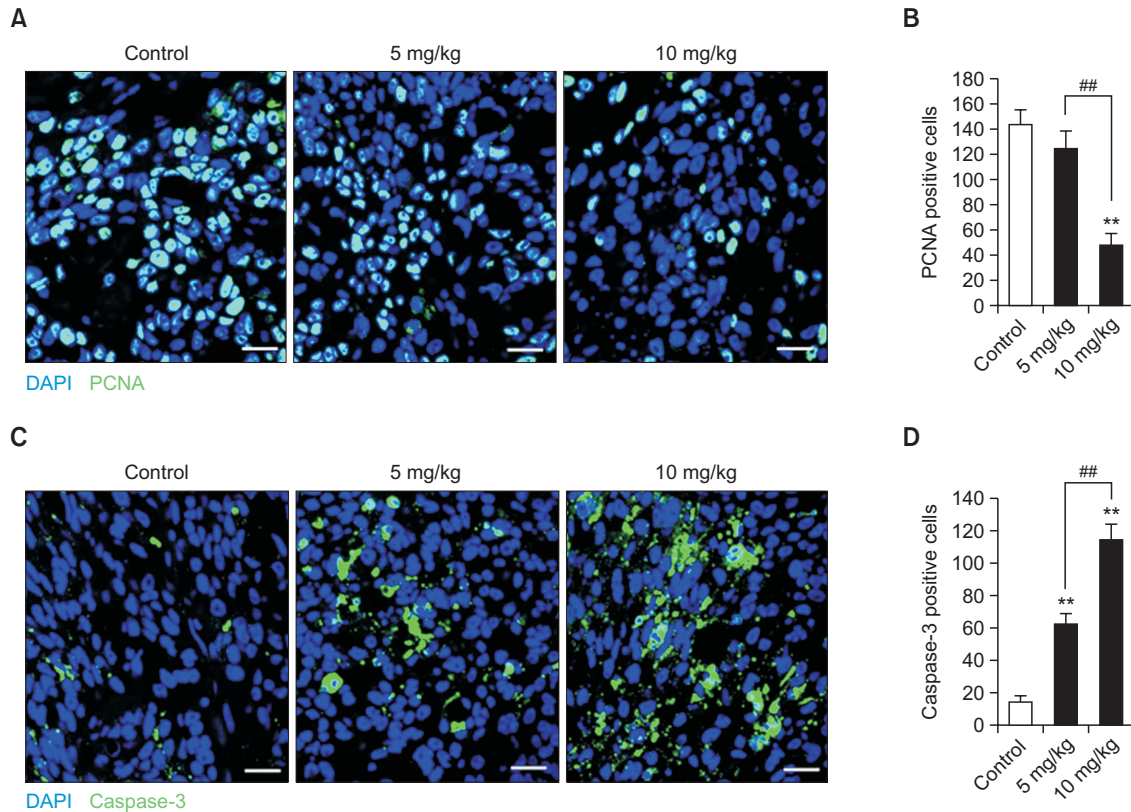
Fig. 5. Fucoidan induced apoptosis *in vivo*. (A) At 30 days after tumor injection, tumor tissue samples were analyzed by immunofluorescence staining to determine proliferation of tumor cells. Proliferation of tumor cells was assessed by staining with anti-proliferating cell nuclear antigen (PCNA) antibodies (green) (scale bar=50 μm). (B) Quantification of tumor proliferation is represented as PCNA-positive cells. (C) At 30 days after tumor injection, tumor tissue samples were analyzed by immunofluorescence staining to determine the survival of tumor cells. Apoptosis of tumor cells was assessed by staining with anti-caspase-3 antibodies (green) (scale bar=50 μm). (D) Quantification of tumor apoptosis is represented as caspase-3-positive cells. Values are expressed as the mean ± SEM (n=9). **$p<0.01$ vs. control. ##$p<0.01$ vs. 5 mg/kg injection group.

dent manner, compared to control.

Fucoidan induced activation of Akt, and an Akt inhibitor reversed fucoidan-mediated effects on p21WAF1 and CDK expression

Previous studies have suggested that mitogen-activated protein kinase ([MAPK:c-Jun N-terminal kinases 1/2 (JNK1/2), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38]) and Akt signaling play important roles in cancer cell proliferation (Sarkar and Li, 2004). Here, we wanted to identify the signaling pathway responsible for the inhibitory effect of fucoidan on HT29 colon cancer cell proliferation. Western blot experiments indicated that Akt activation was induced upon fucoidan treatment for 1-6 h (Fig. 4A). In addition, Akt activation was inhibited by an Akt inhibitor (10^{-6} M) (Fig. 4B). These unexpected results suggest that fucoidan inhibits cell proliferation via Akt activation in HT29 colon cancer cells. Next, to determine if fucoidan-mediated Akt activation was associated with the cell cycle regulatory proteins p21WAF1 and CDKs, HT29 colon cancer cells were pretreated with Akt inhibitor (10^{-6} M) or untreated, followed by addition of 100 μg/mL fucoidan. Fig. 4C shows that the fucoidan-mediated increase in p21WAF1 expression was reduced to the same level as in the control after pretreatment with the Akt inhibitor. In addition, the fucoidan-

induced decrease in CDK2 and CDK4 protein levels was also reversed by treatment with the Akt inhibitor for 24 h (Fig. 4D).

Fucoidan reduced proliferation and induced apoptosis in tumor tissue

To further confirm the ability of fucoidan to induce apoptosis *in vivo*, immunohistochemical staining was performed on tissue sections of tumors excised from mice that were treated with various doses of fucoidan 30 days after HT29 colon cancer cell implantation. As illustrated in Fig. 5A, B, fucoidan treatment significantly decreased the number of PCNA-positive cells and increased the number of caspase-3-positive apoptotic cells (Fig. 5C, D). These results suggest that fucoidan has a strong antitumor effect in this colon cancer model and is a potent apoptosis-inducing agent *in vivo*.

Fucoidan inhibited colon tumor growth *in vivo*

The prominent inhibitory effect of fucoidan on HT29 colon cancer cell proliferation *in vitro* suggested that it might suppress tumor growth *in vivo*. To verify this hypothesis, we subcutaneously inoculated male nude mice with HT29 colon cancer cells. After 30 days, visible tumors developed at the injection sites (8-10 mm^3 in size). The tumor-bearing mice were intraperitoneally injected with 5 and 10 mg/kg body weight
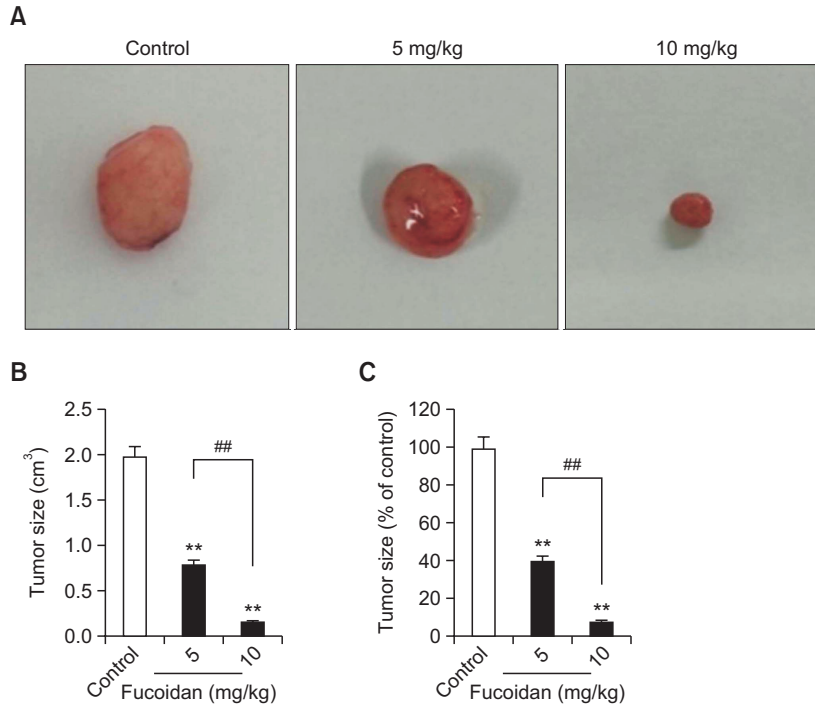
Fig. 6. Antitumor effects of fucoidan on colon cancer *in vivo*. (A) Mice were euthanized 30 days after treatment with fucoidan. Representative images illustrating the different size of tumor tissue injected with fucoidan (5 and 10 mg/kg) after 30 days. (B) The bar graph shows the quantitative analysis of tumor size 30 days after treatment. (C) Standard quantification of tumor size presented as the percent of control. Values are expressed as the mean ± SEM (n=9). **$p<0.01$ vs. control, ##$p<0.01$ vs. 5 mg/kg injection group.
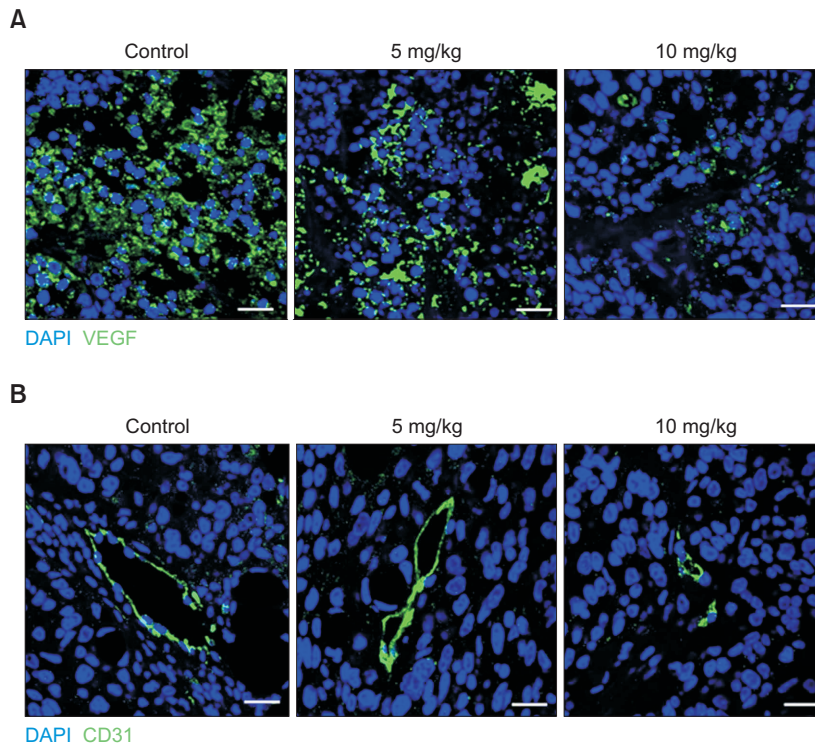

Fig. 7. Fucoidan inhibited tumor angiogenesis *in vivo*. (A) At 30 days after tumor injection, tumor tissue samples were analyzed by immunofluorescence staining to determine VEGF secretion in response to treatment with fucoidan. VEGF secretion was assessed by staining with anti-VEGF antibodies (green) (scale bar=50 μm).(B) At 30 days after tumor injection, tumor tissue samples were analyzed by immunofluorescence staining to determine vessel formation in response to treatment with fucoidan. Vessel formation in tumor tissues was assessed by staining with anti-CD31 antibodies (green) (scale bar=50 μm).

fucoidan or 0.1 mL normal saline alone (as a control), and seven animals were used in each treatment. This process was repeated every 2 days for 10 administrations. As shown in Fig. 6A, B, fucoidan treatment reduced the tumor volume in mice. Tumor size decreased by 39% ± 2.64% and 7.51% ± 1.17% after injection of 5 and 10 mg/kg fucoidan, respectively (Fig. 6C).

Fucoidan inhibited angiogenesis in tumor tissue

To investigate the effect of fucoidan on angiogenesis *in vivo*, we analyzed tumors by immunohistochemical staining to measure the expression of VEGF. Immunoreactive VEGF in the tumors decreased after fucoidan treatment (Fig. 7A). In addition, we evaluated CD31 expression in tumors. Fig. 7B shows that CD31 expression was reduced in the tumors of mice treated with fucoidan.

DISCUSSION

Recent reports have chronicled the ability of natural products to inhibit cancer growth (Sarkar and Li, 2004; Boo *et al*., 2011; Lee *et al*., 2012). Previous studies have demonstrated the potential of fucoidan, a complex sulfated polysaccharide, as a chemo-preventive or anticancer agent (Itoh *et al*., 1993; Aisa *et al*., 2005; Xue *et al*., 2012). Mechanistic studies have suggested that fucoidan inhibits the growth of tumor cells via the downregulation of signaling pathways such as the ERK, JNK, MAPK, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Itoh *et al*., 1993; Aisa *et al*., 2005; Xue *et al*., 2012). In addition, several studies have documented the antitumor effects of fucoidan, which is associated with cell cycle regulation and apoptosis (Aisa *et al*., 2005; Xue *et al*., 2012). However, the molecular mechanisms of fucoidan-induced Akt inhibition of cell growth and cell cycle regulation have not previously been investigated. In the present study, we examined the mechanisms involved in fucoidan-mediated inhibition of cell growth through activation of the Akt signaling pathway.

First, we investigated the effects of 0-100 μg/mL fucoidan on HT29 colon cancer cells by using MTT viability and thymidine-incorporation assays. Fucoidan treatment resulted in decreased cell viability and proliferation. Consistent with the results of the present study, previous studies indicated that fucoidan inhibits the growth of various types of cancer cells (Itoh *et al*., 1993; Aisa *et al*., 2005; Xue *et al*., 2012). Moreover, in the present study, fucoidan treatment of HT29 colon cancer cells induced G1-phase cell cycle arrest via increased p21WAF1 expression and decreased levels of cyclinD1/CDK4 and cyclin E/CDK2 expression. These results suggest that p21WAF1 is involved in the inhibition of cell growth and G1-phase cell-cycle arrest and that G1-phase cell cycle arrest is induced by fucoidan (Xue *et al*., 2013), thus implying that our study is the first systematic investigation to demonstrate the involvement of the p21WAF1-cyclin-CDK machinery in fucoidan-stimulated G1-phase cell cycle arrest.

The Akt signaling pathway regulates the development and progression of various tumors (Hill and Hemmings, 2002; Vivanco and Sawyers, 2002; Song *et al*., 2005). Several studies have demonstrated the inhibition of cell growth in response to Akt signaling (Hill and Hemmings, 2002; Vivanco and Sawyers, 2002; Osaki *et al*., 2004; Song *et al*., 2005). In fact, previous

studies have shown that Akt activation is decreased in growth-retarded tumor cells (Hill and Hemmings, 2002; Vivanco and Sawyers, 2002; Osaki *et al*., 2004; Song *et al*., 2005). However, the results of our study showed that fucoidan treatment stimulated Akt activation in colon cancer cells. In addition, pre-treatment of HT29 colon cancer cells with an Akt inhibitor reversed the fucoidan-induced G1-phase arrest associated with p21WAF1 expression. Subsequently, Akt inhibitor treatment reversed the decreased CDK levels in fucoidan-treated cells. Previous studies supported the notion that Akt inhibition transmitted cell death signals in fucoidan-treated cells (Boo *et al*., 2011). However, recent reports have shown that Akt activation is involved in the regulation of magnolol-induced growth inhibition in human glioblastoma U373 cells (Chen and Lee, 2013). The results of the present study demonstrate that Akt inhibition reverses the fucoidan-mediated inhibition of cell growth via G1-phase arrest associated with p21WAF1 in HT29 colon cancer cells.

Furthermore, fucoidan potently suppressed proliferation and exhibited anti-angiogenic activity against colon cancer *in vivo*. Subcutaneous injection of fucoidan in mice bearing colon cancer cells resulted in decreased proliferation; however, fucoidan treatment increased the number of caspase-3-positive cells. In addition, we observed downregulation of VEGF expression *in vivo*. Suppression of VEGF expression induces a decrease in capillary formation (Koyanagi *et al*., 2003). Found that over-sulfated fucoidan clearly suppresses the neovascularization observed when Sarcoma 180 cells are implanted in mice. VEGF can promote tumor cell metastasis by triggering cell migration and invasion in an autocrine manner. In our studies, fucoidan suppressed the mitogenic and chemotactic activity of vascular VEGF by preventing VEGF from binding with its cell surface receptor. Fucoidan treatment also resulted in less capillary formation, which was at least partially relevant to the downregulation of VEGF expression. Therefore, further studies are required to provide further evidence.

In conclusion, fucoidan inhibited the growth and angiogenesis of colon cancer *in vitro* and *in vivo*. The molecular mechanism of this action involved Akt signaling in fucoidan-treated colon cancer cells. First, fucoidan induced the inhibition of cell growth via p21WAF1-mediated G1-phase cell cycle arrest. Second, fucoidan inhibited tumor growth and angiogenesis of colon cancer *in vivo*. The results of the present study provide insights into the novel therapeutic effects of fucoidan for the prevention and treatment of colon cancer.

ACKNOWLEDGMENTS

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