Abstract. Fucoidan, a sulfated polysaccharide present in brown seaweed, has demonstrated anticancer activity in lung, breast, liver and colon cells. The insulin-like growth factor (IGF) signaling pathway regulates growth in HT-29 cells through the insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Ras/Raf/extracellular signal-regulated kinase (ERK) pathways. The aim of the present study was to investigate whether fucoidan downregulates the IGF-IR signaling pathway in HT-29 human colon cancer cells. Fucoidan treatment (0-1,000 µg/ml) was administered for 24 h in HT-29 cells. First, we investigated IRS-1/PI3K/AKT pathway-related protein expression levels following treatment with fucoidan (0-500 µg/ml) using western blot analysis. Fucoidan significantly inhibited the expression of IGF-IR, PTEN, PI3K and AKT as well as their phosphorylated forms (p-IRS-1, p-PI3K and p-AKT). Next, we investigated the effects of fucoidan on Ras/Raf/ERK pathway-related protein expression levels in HT-29 cells. Fucoidan significantly inhibited the expression of IGF-IR, Shc, Ras, SOS, Raf and MEK. HT-29 cells were then incubated in the presence of fucoidan (0 or 250 µg/ml), and IGF-1 (10 nM) was added for 0 to 60 min. Immunoprecipitation (IP) experiments showed that fucoidan inhibited IGF-IR phosphorylation of IGF-IR, PI3K, Shc (IP, IGF-IR), and phosphorylated IRS-1 and PI3K (IP, IRS-1) compared to the control group. Western blot analysis showed that fucoidan inhibited the expression of IGF-I-induced p-IGF-IR/IGF-IR and p-AKT/AKT, but not p-ERK/ERK. In conclusion, the inhibition of cell viability by fucoidan in HT-29 cells may be due to the downregulation of IGF-IR signaling through the main IRS-1/PI3K/AKT pathway. Fucoidan also partially impacted Ras/Raf signaling in the Ras/Raf/ERK pathway. Therefore, we suggest that fucoidan may be a suitable candidate chemopreventive agent in HT-29 colon cancer cells.

Introduction

Colon cancer is the most commonly diagnosed cancer and one of the leading causes of deaths in the United States and worldwide (1,2). It is the third most common cancer in men and the second most common cancer in women worldwide (2). Radiation treatment and chemotherapy are not therapeutically sufficient due to side effects and drug resistance (3). Several studies have shown close associations between colon cancer and dietary factors such as fruit, vegetables, and seaweeds containing a wide variety of phytochemicals. Some of these phytochemicals have been shown to protect cells from damage leading to cancer.

Fucoidan is a fucose-rich sulfated polysaccharide found in various species of brown seaweed (4-7). Fucoidan has been shown to exert various biological activities, including antioxidant, anti-inflammatory, anti-angiogenic, anti-coagulant, anti-bacterial, and anticancer effects (8-18). Studies have shown anticancer activities of fucoidan in lung, breast, liver, colon, prostate, and bladder cancer cells (19-23). Fucoidan has also been shown to induce tumor cell injury leading to growth arrest and tumor suppression via apoptosis and cell cycle arrest in various cancer cell types (24-29). Therefore, fucoidan shows promise as a new therapeutic compound for cancer treatment. Despite numerous studies showing the chemopreventive effects of fucoidan in several cancer models, its mechanism of action has not been fully elucidated.

The insulin-like growth factor (IGF) signaling system, consisting of ligands (IGF-I and IGF-II), growth factor receptors (IGF-IR and IGF-IIR), and IGF binding proteins (IGFBPs-1-6), regulates cell growth, proliferation, transformation, differentiation, migration and apoptosis (30,31). IGFs play a vital role in the growth of various cancer cells, including colon cancer cells (31,32). IGF-I and IGF-II mRNA levels are highly increased in colon cancer (33,34). Ligand binding to IGF-IR triggers two main downstream signaling pathways, the insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway and the Ras/Raf/extracellular signal-regulated kinase (ERK) pathway. The IRS-1/PI3K/AKT pathway is implicated in the transmission of cell survival signals, and the Ras/Raf/ERK pathway...
pathway is implicated in receptor-mediated mitogenesis and transformation (35,36).

In previous studies, fucoidan inhibited HT-29 cell proliferation by inducing apoptosis (23). In anti-colon cancer-related studies, the epidermal growth factor (EGF) pathway has been investigated, but no studies have investigated the IGF-I pathway (37). Therefore, in the present study, we examined whether fucoidan downregulates IGF-IR signaling in HT-29 cells.

**Materials and methods**

**Preparation of fucoidan.** Fucoidan purified from *Fucus vesiculosus* (cat. no. sc-255187) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Fucoidan was dissolved in RPMI-1640 medium (GenDEPOT, Inc., Barker, TX, USA) at concentrations of 0-1,000 µg/ml.

**Cell culture.** HT-29 human colon adenocarcinoma cells (cat. no. 30038) were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; GenDEPOT, Inc.) containing 50 µg/ml penicillin, 25 µg/ml amphotericin B, and 50 µg/ml streptomycin, in an incubator with 5% CO2 at 37°C.

**Cell proliferation assay.** Cell proliferation was estimated using a Cyto X cell viability assay kit (LPS Solution, Daejeon, Korea). Cells were seeded in 96-well plates at a density of 4x10^4 cells/well and allowed to attach for 24 h. Attached cells were treated with 62.5, 125, 250, 500 or 1,000 µg/ml of fucoidan in serum-free medium for 24 h. The cell proliferation assay solution was added and incubated for 1 h, and the absorbance of each well was measured at a wavelength of 450 nm using a FilterMax F5 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

**Immunoprecipitation and western blot analysis.** HT-29 cells were cultured with 0, 62.5, 125, 250, or 500 µg/ml of fucoidan for 24 h. Subsequently, cells were washed with phosphate-buffered saline (PBS) and lysed with extraction buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris, pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 µg/ml apro tinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM NaF, and 1 mM PMSF). The extracts were centrifuged at 9,750 x g for 10 min, and the supernatant was used for western blot analysis. For immunoprecipitation (IP), cells were incubated for 24 h with 0 or 250 µg/ml of fucoidan, and 10 nM of IGF-I (recombinant human IGF-I; Invitrogen Life Technologies, Frederick, MD, USA) was added. At 0, 5, 30 or 60 min after the addition of IGF-I, the cell lysates were centrifuged at 9,750 x g for 10 min. Supernatant (0.90 mg protein) were incubated with 3 µl of anti-IGF-IRβ or IRS-1 antibody overnight at 4°C. Protein A-agarose beads (GenDEPOT, Inc.) were added to the lysate-antibody mix, which was then incubated for 4 h at 4°C. The beads were washed 3 times with extraction buffer. The immunoprecipitates and total protein (40 µg) were electrophoresed using 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 1% bovine serum albumin (BSA; GenDEPOT, Inc.) in Tris-buffered saline-Tween-20 (TBS-T; 5 mM Tris-HCl, 20 mM sodium chloride pH 7.4, and 0.1% Tween-20) incubated with primary antibodies (1:1,000) in 1% BSA in TBS-T with gentle shaking overnight at 4°C. Membranes were washed twice for 15 min in TBS-T, and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000) for 2 h at room temperature and washed again. Immunoreactive bands were detected using an enhanced chemiluminescence substrate (Advansta, Inc., Menlo Park, CA, USA) and visualized using the GeneSys imaging system (SynGene Synoptics, Ltd., London, UK). The following primary antibodies from Santa Cruz Biotechnology, Inc., and Invitrogen were used: anti-p-IGF-IR (sc-10703, anti-rabbit), anti-IGF-IR (sc-390130, anti-mouse), anti-phospho-tyrosine (PY99; sc-7020, anti-mouse), anti-p-IRS-1 (sc-17200, anti-goat), anti-IRS-1 (sc-185, anti-mouse), anti-p-PTEN (sc-7985, anti-rabbit), anti-AKT (sc-8312, anti-rabbit), anti-p-P13K (PA5-17387, anti-mouse), anti-P13K (sc-374354, anti-mouse), anti-Ras (sc-520, anti-rabbit), anti-Raf (sc-227, anti-rabbit), anti-p-MEK (sc-81503, anti-mouse), anti-MEK (sc-81504, anti-mouse), anti-p-ERK (sc-7383, anti-mouse), anti-ERK (sc-292838, anti-rabbit), anti-SOS (sc-259, anti-rabbit), anti-Grb2 (sc-255, anti-rabbit), anti-Shc (sc-967, anti-mouse), anti-PTEN (sc-9794, anti-mouse), and anti-β-actin (sc-47778, anti-mouse). The secondary antibodies used were HRP-conjugated anti-mouse IgG (32430), anti-rabbit IgG (31460), and anti-goat IgG (31400) (all from Invitrogen Life Technologies).

**Statistical analyses.** The results are presented as means ± standard deviation of three independent experiments. The difference in protein expression levels was determined by quantifying the density of the bands using ImageJ (NIH). Significant differences among multiple mean values were assessed using one-way or two-way analysis of variance followed by Bonferroni’s multiple comparison test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**Fucoidan induces cell death in HT-29 cells.** To investigate the effects of fucoidan on cell viability, HT-29 cells were incubated with 0-1,000 µg/ml fucoidan for 24 h. Data are presented as means ± standard deviation (SD) of three independent experiments. Data were analyzed using one-way analysis of variance. *P*<0.05 vs. control.
with various concentrations (0-1,000 µg/ml) of fucoidan for 24 h. Fucoidan treatment significantly decreased the viability of HT-29 cells in a concentration-dependent manner (Fig. 1). After treatment with 125, 250, 500 and 1,000 µg/ml fucoidan, cell viability was significantly decreased to 91.7±2.3, 86.3±5.3, 61.5±4.4 and 44.1±8.3%, respectively.

**Fucoidan reduces the levels of IRS-1/PI3K/AKT pathway-related proteins in HT-29 cells.** IGFs signaling is known to affect cell survival, and IGF-I mRNA is increased in colon cancer cells (33,34). Therefore, we investigated whether the IGF-IR signaling pathway is involved in fucoidan-induced cell death. First, we investigated IRS-1/PI3K/AKT pathway-related protein expression levels, one of the two main downstream IGF-IR signaling pathways. Fucoidan treatment significantly decreased the expression of IGF-IR, phospho-tyrosine and PTEN in HT-29 cells in a concentration-dependent manner (Fig. 2). Fucoidan treatment also significantly decreased the ratios of p-IRS-1/IRS-1, p-PI3K/PI3K and p-AKT/AKT expression.

**Fucoidan reduces the levels of Ras/Raf/ERK pathway-related proteins in HT-29 cells.** Next, we investigated Ras/Raf/ERK pathway-related protein expression levels, the other main IGF-IR downstream signaling pathway. Fucoidan treatment significantly decreased the expression of IGF-IR, phospho-tyrosine, Shc, Ras, SOS, Grb2, and Raf in HT-29 cells in a concentration-dependent manner (Fig. 3). Fucoidan treatment also significantly decreased the expression of p-MEK/MEK, but not p-ERK/ERK.

**Fucoidan reduces IGF-I-induced IGF-IR activation of the IGF-IR signaling pathway.** We examined whether fucoidan downregulates IGF-I-induced tyrosine phosphorylation of IGF-IR. HT-29 cells were treated for 24 h with 0 or 250 µg/ml fucoidan, and IGF-IR was stimulated with 10 nM IGF-I for 0, 5, 30, or 60 min. Total cell lysates were prepared and immunoprecipitated using an IGF-IR β antibody. The immune complexes were used in western blot analysis with an anti-phospho-tyrosine antibody (PY99). IGF-I induced tyrosine phosphorylation of IGF-IR at 5 min, and tyrosine phosphorylation levels persisted at 60 min in control cells. Cells treated with fucoidan exhibited significantly inhibited IGF-IR β phosphorylation up to 30 min after IGF-I stimulation (Fig. 4).

To investigate the association of the Shc and p85 subunits of PI3K with IGF-IR, we performed IP of cell lysates with an IGF-IR β antibody and subsequent western blot analysis with p85 and Shc antibodies. IGF-I stimulated the association of the p85 regulatory subunit of PI3K with IGF-IR. Expression of the p85 regulatory subunit of PI3K in the control group

---

Figure 2. Fucoidan treatment reduces the levels of proteins in the IRS-1/PI3K/AKT pathway in HT-29 cells. HT-29 cells were incubated with various concentrations of fucoidan (0-500 µg/ml) for 24 h. The protein expression levels of IGF-IR, IRS-1, PTEN, PI3K, and AKT were examined by western blot analysis. Data were analyzed using one-way analysis of variance. *P<0.05 vs. control. IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; IGF, insulin-like growth factor. (Values at the left of the western blot indicate the molecular weights of the proteins in kDa).
was induced within 5 min, but fucoidan treatment delayed its expression by up to 30 min. IGF-I also stimulated the association of the Shc subunit with IGF-IR. The expression of Shc in the control cells was induced within 1 min, but in cells treated with fucoidan, expression was induced at 5 min.

**Fucoidan reduces IGF-I-induced IRS-1 activation in the IGF-IR signaling pathway.** We examined whether fucoidan downregulates IGF-I-induced tyrosine phosphorylation of IRS-1. HT-29 cells were treated for 24 h with 0 or 250 µg/ml fucoidan, and IGF-IR was stimulated with 10 nM IGF-I for 0, 5, 30, or 60 min. Total cell lysates were prepared and immunoprecipitated using an IRS-1 antibody. The immune complexes were used in western blot analysis with anti-phospho-tyrosine antibody. IGF-I induced tyrosine phosphorylation of IRS-1 at 5 min, and tyrosine phosphorylation levels persisted for 30 min in control cells. Treatment with fucoidan significantly inhibited the phosphorylation of IRS-1 for up to 60 min after IGF-I stimulation (Fig. 5).

To investigate the association of the p85 subunit of PI3K with IRS-1, we performed IP of cell lysates with an IRS-1 antibody and subsequent western blot analysis with a p85 antibody. IGF-I stimulated the association of the p85 regulatory subunit of PI3K and IRS-1 with IRS-1. Expression of the p85 regulatory subunit of PI3K and IRS-1 in control cells were stimulated within 5 min, but fucoidan treatment delayed expression for up to 60 min.

**Fucoidan reduces IGF-I-induced activation of AKT in HT-29 cells.** AKT and ERK1/2 are known to play vital roles in cell survival and are activated by IGF-I (33,37,38). HT-29 cells were treated for 24 h with 0 or 250 µg/ml fucoidan, and IGF-IR was stimulated with 10 nM IGF-I for 0, 5, 30, or 60 min. We investigated whether fucoidan inhibits IGF-I-induced phosphorylation of IGF-IR, AKT, and ERK, a downstream target of
the IGF-IR signaling pathway. Levels of p-IGF-IR/IGF-IR and p-AKT/AKT increased in control cells in a time-dependent manner with IGF-I stimulation (Fig. 6). Fucoidan treatment delayed p-IGF-IR/IGF-IR and p-AKT/AKT expression for up to 60 min, lower than the control cells level. In addition, in the control group, p-ERK/ERK increased with IGF-I stimulation without changing total ERK levels. There were no differences in p-ERK/ERK1/2 expression in the fucoidan treatment group.

Discussion

Colon cancer is one of the most common cancers in both men and women and is prevalent worldwide (1,2). Globally, the incidence of colon cancer is increasing, seemingly due to changes in dietary habits and preferences. Dietary habits can affect the development of colon cancer, and food ingredients may serve as chemotherapeutic agents (22,24,38-43). Many studies have investigated potential colon cancer treatments. Some studies have suggested marine natural products with pharmacological activities as a method for treating colon cancer (44). Among these marine natural products, seaweeds have been reported to contain various useful compounds such as laminarin, fucoidan, dactyline, and meroditerpenoids with various effects in cancer cells (37-44). Numerous studies have investigated the effects of seaweeds on cell death pathways including apoptosis. The inhibition of apoptosis in colon cancer cells promotes tumor growth and tumor progression and imparts tolerance to cytotoxic anticancer drugs (7,22,23,27,40-43).

Studies related to colon cancer have found that laminarin inhibits cancer cells via Fas and IGF-IR signaling through the intrinsic apoptotic and ErbB pathways, and induces Fas-mediated apoptosis by regulating Fas and Fas-associated protein with death domain (FADD) protein levels (34,45-47). Fucoidan suppressed growth, decreased metastasis, inhibited angiogenesis, and induced apoptosis through activated caspases, resulting in the induction of apoptosis through both death receptor-mediated and mitochondria-mediated apoptotic pathways (22,38,40-43). Dactyline also induced G1-S cell cycle arrest and apoptosis in tumor cells (44).

In previous studies, fucoidan induced apoptosis through the apoptotic pathway and cytotoxicity, and inhibited migration and proliferation in HT-29 colon cancer cells (22,24,28,40,42,43). Fucoidan exerted an anticancer effect by downregulating the PI3K-AKT-mTOR signaling pathway (40). In the present study, we investigated the downregulation of IGF signaling pathways by fucoidan in HT-29 cells.

The present study provided the first evidence that fucoidan reduces IGF-IR protein expression and IGF-IR-mediated signaling through the IRS-1/PI3K/AKT pathway. We examined cell proliferation using a cell viability assay kit with various concentrations of fucoidan (0-1,000 µg/ml). The results indicated that fucoidan inhibited cell proliferation in a dose-dependent manner in HT-29 cells (Fig. 1).

IGF signaling plays a vital role in promoting normal cell proliferation, tumorigenesis, and cancer cell proliferation. IGF-I is also known to inhibit cell death and promote growth.
in various cancer cells, including colon cancer cells. IGF-IR expression is increased in colon cancer compared to normal mucosal tissues (48). In the present study, we investigated whether the anticancer effects of fucoidan involve changes in the IGF-IR signaling pathway. The IGF-IR-related pathway can be divided into two main downstream signaling pathways: The IRS-1/Pi3K/AKT and Ras/Raf/ERK pathways (35,36). In the IRS-1/Pi3K/AKT pathway, the protein expression of pathway members, including IGF-IR, PTEN, Pi3K, and AKT, decreased in a concentration-dependent manner upon fucoidan treatment (Fig. 2). The protein expression of Ras/Raf/ERK pathway members, including IGF-IR, She, Ras, SOS, Grab2, Raf, and MEK, also decreased in a concentration-dependent manner (Fig. 3).

The present study suggests that fucoidan inhibits phosphorylation of the β-subunit of IGF-IR by downregulating the α-subunit, directly interfering with the binding of IGF-I to IGF-IR. In immunoprecipitation assays and western blot analysis, fucoidan reduced IGF-I-induced tyrosine phosphorylation of IGF-IR and IRS-1, leading to reduced interaction between Pi3K p85 and IGF-IRβ, and the subsequent activation of Pi3K/AKT but not ERK1/2 (Figs. 4-6). AKT plays a vital role in Pi3K-mediated suppression of cell proliferation as a downstream target of the Pi3K/AKT pathway (28,40,49). We found that fucoidan inhibited IGF-I-induced activation of AKT, which may have been due to reduced IGF-IR levels and the subsequent reduction in IGF-IR activation. The moderate reduction in AKT protein levels may also have contributed to the reduced p-AKT levels (Fig. 6). However, fucoidan did not affect IGF-I-induced activation of p-ERK/ERK levels. Fucoidan was also partially associated with the Ras/Raf pathway in the Ras/Raf/ERK pathway.

Recent data indicate that fucoidan triggers G1 phase arrest and apoptosis in HCT116 colon cancer cells through a p53-independent pathway. In particular, it has been suggested that fucoidan is able to enhance p21 expression at transcriptional level in a p53-independent manner (50). IGF-I signaling is strongly associated with cell growth and has a significant role in ribosome biogenesis by regulating the expression of proteins directly involved in the processing of ribosomal subunits and p53-mediated regulation of cell cycle progression (51). In particular, IGF-I activates the murine double minute 2 (MDM2) protein which is a component crucial regulator of cell proliferation and apoptosis and acts in association to some ribosomal proteins by inhibiting the p53 tumor suppressor (51). It has been demonstrated that fucoidan is able to synergize with standard anticancer agents as 5-FU and reduce its toxicity (52,53). Recent data showed that in HCT116 colon cancer cells 5-FU is able to induce perturbation of ribosome biogenesis led to nucleolar stress (52). This condition activates p53 independent pathway involving ribosomal proteins and MDM2 leading to p21-mediated cell cycle arrest and apoptosis. In previous study, fucoidan were induced G1 phase arrest and apoptosis in p53 mutated colon cancer cell line, HT-29 (23). In present study, we examined whether HT-29 cells affect the IGF-IR signaling pathway involved in cell proliferation during fucoidan. In this result, an interesting possibility may be that fucoidan through the IGF-I signaling could activate ribosomal protein/MDM2/ p21 induced cell death. The research on this is expected to be further progressed.

Based on these findings, we conclude that fucoidan inhibits cell proliferation and induces apoptosis by inhibiting the IGF-I-induced IGF-IR signaling pathway, including the IRS-1/Pi3K/AKT pathway, in HT-29 cells. These results suggest that downregulation of IGF-IR/IRS-1/Pi3K/AKT signaling may be one of the mechanisms by which fucoidan impacts HT-29 cells. These results suggest that the anti-proliferative effect of fucoidan may be mediated through downregulation of the IGF-I/IGF-IR/IRS-1/Pi3K/AKT signaling pathway. Therefore, fucoidan may be useful as a chemopreventive agent in colon cancer cells.

Acknowledgements

This study was supported by Pukyong National University (grant no. C-D-2016-0267), Busan, Republic of Korea.

References


