Antitumor activity of fucoidan in anaplastic thyroid cancer via apoptosis and anti-angiogenesis

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Abstract. The present study demonstrated the effect of fucoidan, isolated from *Fucus vesiculosus*, on cell growth and apoptosis in anaplastic thyroid cancer cells. The cell viability was analyzed using a Cell Counting Kit-8 cell proliferation kit. Diamidino-2-phenylindole and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays were used to examine the apoptotic effect of fucoidan, which revealed the presence of apoptotic bodies and DNA fragmentation. Fucoidan inhibited the growth of FTC133 and TPC1 ATC cells in a dose-dependent manner. It also induced the apoptosis of FTC133 cells by promoting the expression levels of cleaved poly ADP-ribose polymerase and caspase-3. Significant decreases in the levels expression of hypoxia-inducible factor 1α and vascular endothelial growth factor were observed in the FTC133 cells following treatment of the cells with fucoidan. In addition, inhibition in tube formation and the migration of FTC133 cells were observed in the cells treated with fucoidan, compared with the cells in the control group. Therefore, fucoidan inhibited cell growth, induced apoptosis and suppressed angiogenesis in the thyroid cancer cells.

Introduction

In the US, thyroid cancer constitutes ~2% of malignancies, with cancer-associated mortality rates of >1,600 per year (1). Thyroid cancer accounts for 1% of cases of cancer in humans, and includes well-differentiated thyroid carcinoma of papillary and follicular types, which account for >95% of cases of thyroid cancer, and anaplastic thyroid carcinoma (ATC), which accounts for 1-5% of thyroid malignancies (2-4). ATC is a life threatening disease with a median survival rate of 6 months subsequent to diagnosis (2-5). Of the patients diagnosed with ATC, 90% have extraglandular spread at the time of diagnosis, with 75% of the patients developing distant metastases (6,7). Consequently, cases of ATC are staged as stage IV in the American Joint Commission on Cancer system (8).

Patients with ATC, as a life threatening type of tumor, have a median survival rate of ~6 months and a 1 year survival rate of <20% (9,10). It is more common in women, compared with men (11) and commonly affects the lungs (12). ATC is reported to induce significant site-specific morbidity and is associated with a poor prognosis. Patients with ATC present with an enlarged neck mass, and the symptoms of ATC include dysphagia, odynophagia, dyspnea, anxiety and vocal cord paralysis (13). If patients do not undergo treatment, including surgical resection and external beam radiation therapy, they develop uncontrolled local progression causing suffocation and mass bleeding, and eventually succumb to mortality (13,14). Despite tracheostomy, patients can suffer from obstruction of the wind pipe (15,16).

Fucus vesiculosus, commonly known as bladderwrack, is a brown edible seaweed, which has traditionally been used as an anti-obesity treatment, health supplement and for goiter treatment (17). It was the first source of iodine to be identified and has been used in the treatment of thyroid disorders (17). The brown color of the herb is due to the presence of fucoxanthin pigment (18). The compound. fucoidan, has been isolated from the extract of bladderwrack (19). The chemical structure of fucoidan is similar to that of heparin, which is a compound used as an anti-coagulant (20). In addition to fucoidan, bladderwrack also contains fucophlorethol and fucotriphlorethol A (20). The present study aimed to determine the anticancer effect of fucoidan in ATC cells by investigating apoptosis and anti-angiogenesis. The current study demonstrated that fucoidan inhibited the expression of vascular endothelial growth factor (VEGF) via suppression of HIF-1α.

Materials and methods

Extraction of fucoidan. F. vesiculosus was collected from the coast of the Baltic Sea and the identity of the specimens was confirmed as previously described (21,22). The fucoidan used in the present study was isolated from the extract of *F. vesiculosus* in 20:80 ethyl acetate:hexane using column

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chromatography and was characterized using ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and mass spectrometry.

Cell lines and cell culture. Human FTC133 and TPC1 ATC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. The cell cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. A Cell Counting Kit-8 (CCK-8) cell proliferation kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to analyze the effect of fucoidan on cell proliferation. The cells were seeded into 96-well plates at a density of $2.5x10^5$ cells in each well. After 24 h, different concentrations of fucoidan (0, 1, 2, 4, 8, 10, 15 μM) were added to each well containing the cells. Following incubation for 48 h at 37˚C, 20 μl of CCK-8 solution (5 mg/ml) was added to each well and incubation was continued for another 4 h at 37˚C in an incubator with 5% CO_2. The proliferation of the cells was measured using a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Inc.) at 450 nm. The proliferation index was calculated from the resulting optical density values.

Western blot analysis. Following treatment of the cells with the aforementioned concentrations of fucoidan for 72 h, the cells were washed three times in ice-cold phosphate-buffered saline (PBS) and then lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40 and 5 mM cocktail. The concentration of proteins in the lysate was determined using a bicinchoninic acid assay. The proteins (2 μg) were resolved on a 10% polyacrylamide gel by electrophoresis. For the transfer of proteins onto a polyvinylidene difluoride membrane, a semi-dry method was used. The membrane was blocked in 5% non-fat dry milk overnight and then washed in Tris-buffered saline with Tween-20 (TBST). The membrane was then incubated overnight at 4˚C with anti-hypoxia inducible factor (HIF)-1α (1:50; cat. no. 100-449), anti-B cell lymphoma-2 (Bcl-2)-associated X protein (Bax; 1:50 ; cat. no. 100-56097), anti-Bcl-2 (1:50 ; cat. no. 100-2087), anti-cleaved caspase-3 (1:50 ; cat. no. 100-56113).) and anti-cleaved poly ADP-ribose polymerase (PARP; 1:50; cat. no. 100-56599; Cell Signaling Technology, Inc., Danvers, MA, USA). The incubation with antibodies was followed by washing with TBST. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. 610-103-121; GE Healthcare Life Sciences, Chalfont, UK) for 1 h at room temperature. Enhanced chemiluminescence (Pierce Biotechnology; Thermo Fisher Scientific, Inc.) was used for the antigen detection. β-actin was used as the control.

Assessment of apoptosis using 4,6-diamidino-2-phenylindole (DAPI) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays. Onto 18 mm cover slips, the cells in minimal essential medium (Gibco; Thermo Fisher Scientific, Inc.) were plated at a density of 3.0 10^6 and ~80% confluence for 24 h at 4˚C. For angiogenesis analysis, cells were treated with 100 μM $CoCl_2$ for 18 h to induce hypoxic conditions Treatment of the cells with 100 μg/ml fucoidan for 24 h was followed by fixing in 10% paraformaldehyde. The cells were then washed twice with PBS and stained with 2 μg/ml DAPI for 20 min at 37˚C. A fluorescent microscope was then used to analyze the nuclear fragmentation in the stained cells. A TUNEL kit (Chemicon, Temecula, CA, USA) was used for the TUNEL assay, which was performed according to the manufacturer's protocol.

Cell wounding assay. At 90% confluence, HUVECs were plated onto the 60 mm diameter culture dishes and then scratched with pipette tip. The cells were then subjected to PBS washing followed by incubation in endothelial cell growth medium MV2 (ECGM2) supplemented with 2% FBS along with recombinant human VEGF (50 ng/ml), thymidine (1 mM) and fucoidin (10 μM) obtained from PromoCell GmbH (Heidelberg, Germany). The cell cultures used as negative control were incubated with ECGM2 medium containing 2% FBS alone. After 16 h, HUVECs were rinsed in PBS followed by methanol fixation. The experiments were performed in triplicates and the data are presented as the mean of three experiments.

Tube formation assay. To each Matrigel-coated well (BD Biosciences, Franklin Lakes, NJ, USA), 0.2 ml of cell suspension comprising cells suspended in growth medium at a density of $2.5x10^5$ cells/ml, was added. The cells were incubated at 37˚C in an incubator with 5% CO_2 in the presence or absence of fucoidan for 12 h. The cells were also incubated at 4˚C in media containing 50 ng/ml VEGF and 1 mM thymidine along with fucoidan (10 μM) for 16 h. Subsequently, a phase contrast microscope was used to examine changes in the morphology of the cells.

Migration assay. To determine the migration potential of the fucoidan-treated cells, Transwell cell culture inserts were used. The cells ($3x10^5$) in 200 ml of growth medium were seeded into the upper chambers of the Transwell inserts. To the lower chamber, 750 ml of medium was added, which contained 20% FBS as a chemoattractant. In the control wells, medium was added to the upper and the lower chambers. The non-migrated cells in the upper chamber were removed by swabbing following 24 h of incubation at 4˚C. The inserts were observed under an inverted fluorescent microscope subsequent to fixing and staining. The number of cells in five randomly selected fields of view were counted in triplicate.

Statistical analysis. One-way analysis of variance and Student's *t*-test were used for statistical analysis. The data are presented as the mean ± standard deviation. $P \leq 0.05$ was considered to indicate a statistically significant difference. SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA) was used for statistical calculations.

Results

Inhibition of ATC cell growth by fucoidan. Treatment of the FTC133 and TPC1 ATC cell lines with different doses

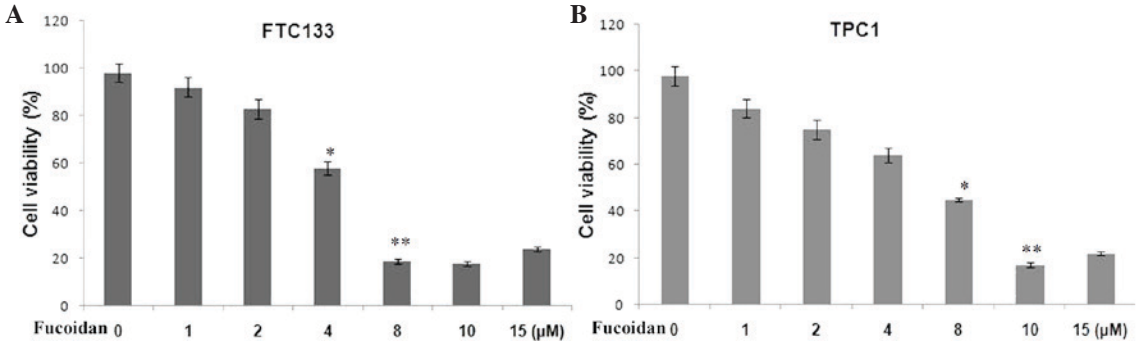

Figure 1. Cytotoxic effects of fucoidan on (A) FTC133 and (B) TPC1 ATC cells. Cyotoxic effects were measured using a CCK-8 cell proliferation kit. Following incubation for 24 h, the ATC cells were treated with varying concentrations of fucoidan. Following incubation for 48 h, the cells were treated with CCK-8 solution. Results are expressed as the percentage of cell proliferation relative to the control and data are presented as the mean ± standard deviation. *P<0.05; **P<0.01 compared with the control. ATC, anaplastic thyroid carcinoma; CCK, Cell Counting Kit-8.

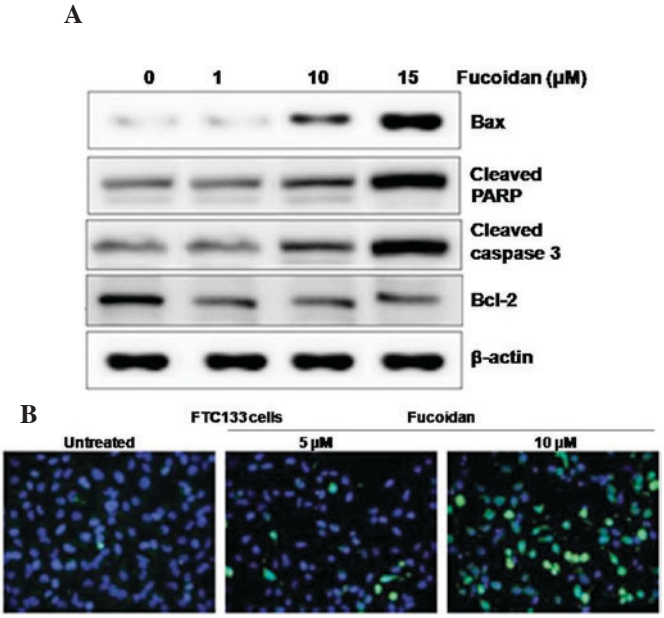

Figure 2. Effect of fucoidan on the apoptosis of FTC133 anaplastic thyroid cancer cells. (A) Expression levels of PARP, cleaved caspase-3, Bax and Bcl-2 were determined using western blot analysis in cells treated with fucoidan at the indicated doses for 48 h. (B) Induction of apoptosis by fucoidan was analyzed using terminal deoxynucleotidyl transferasemediated dUTP nick-end labeling and 4,6-diamidino-2-phenylindole staining. Following staining, images of the cells were captured (magnification, x200). PARP, poly ADP-ribose polymerase; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

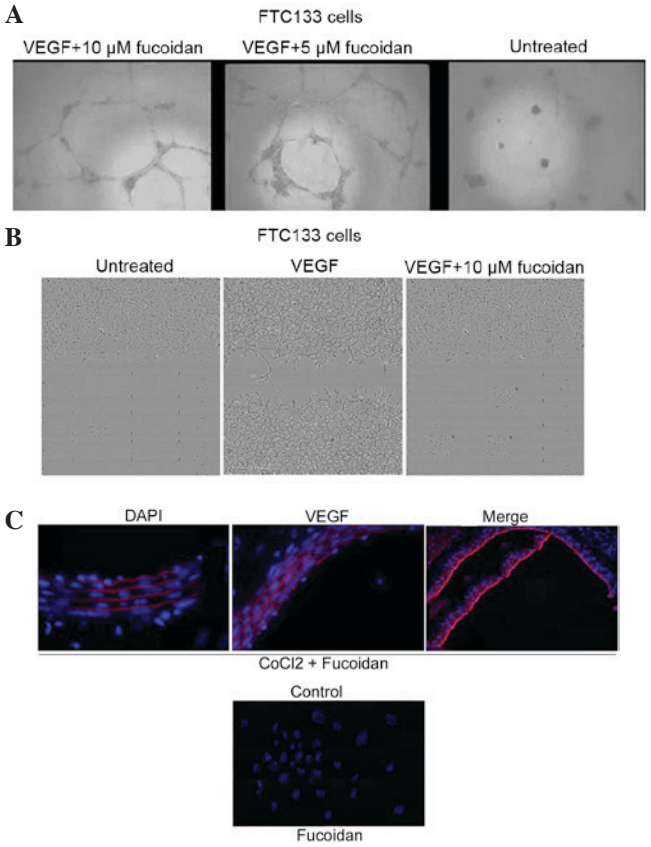

Figure 3. Effects of fucoidan on angiogenesis in human thyroid cells. (A) Cells were plated on Matrigel (200 μl/well) and treated with various concentrations of fucoidan. After 12 h, capillary tube formation was assessed under a phase-contrast microscope and images were captured (magnification, x400). (B) Effects of fucoidan on migration. Following wounding, the cells were washed with phosphate-buffered saline and incubated in ECGM2 containing 2% FBS and 50 ng/ml recombinant human VEGF, 1 mM thymidine and/or fucoidan. ECGM2 medium with 2% FBS was used as a negative control. Results are representative of at least three independent experiments performed in triplicate. Magnification, x350. (C) Immunocytochemistry of VEGF in hypoxia-induced cells (100 μM $CoCl_2$). Magnification, vx350. VEGF, vascular endothelial factor; FBS, fetal bovine serum; ECGM2, endothelial cell growth medium 2; DAPI, 4,6-diamidino-2-phenylindole.

of fucoidan (1-15 μM) led to inhibition of cell growth 36 h following treatment. The inhibition of cell growth by fucoidan was observed to be concentration-dependent (Fig. 1). For the FTC133 and TPC1 cell lines, the half maximal inhibitory concentrations for growth inhibition were 8 and 10 μM, respectively.

Effects of fucoidan on apoptotic cell death in FTC133 ATC cells. The results of the western blot analysis revealed that the expression levels of Bax, cleaved PARP and caspase-3 were increased, and the expression of Bcl-2 was decreased in the FTC133 cells treated with fucoidan for 36 h, compared with the control cells (Fig. 2A). DAPI staining showed the appearance of DNA fragmentation and perinuclear apoptotic bodies in the FTC133 cells treated with 8 μM of fucoidan (Fig. 2B). Thus, fucoidan led to apoptosis of the FTC133 cells. The

fucoidan-induced apoptosis was indicated by DNA strand breakage, which were also observed in the results of the TUNEL analysis.

Effects of fucoidan on angiogenesis. The cells were treated with $CoCl_2$ (100 μM) for 18 h to induce hypoxia-like conditions, and were then treated with different doses of fucoidan. Under the hypoxic conditions, the expression of HIF-1α was promoted, however, fucoidan treatment (10 μM) reduced the hypoxia-induced expression of HIF-1α. The expression of VEGF was also enhanced under the hypoxic conditions and this hypoxia-induced expression of VEGF was decreased by treatment with fucoidan. The results from the tube formation assay clearly revealed that the formation of vessel-like structures was suppressed following treatment with fucoidan (Fig. 3A). Incubation of the wounded cells in media containing 50 ng/ml VEGF and 1 mM thymidine in the presence of fucoidan (10 μM) for 16 h showed failure of wound healing capacity (Fig. 3B). Investigation of the effect of fucoidan on the expression of VEGF revealed a reduction in the expression of VEGF by fucoidan in hypoxia (Fig. 3C). Thus, tube formation and the migration of cells were inhibited by fucoidan, suggesting that fucoidan has a potent anti-angiogenic effect.

Discussion

Fucus vseiculosus has been used in the treatment of thyroid disorders (17) and fucoidan has been isolated from its extract (19). Fucoidan is structurally similar to heparin, which is used as an anti-coagulant agent. In the present study, the effect of fucoidan on the growth of ATC cells was investigated. A previous study investigated the anticancer effects of plants or their components (23). Of the drugs used in cancer therapy, ~70% are natural products or their derivatives (24). The results of the present study revealed that fucoidan inhibited the growth of tumor cells and induced apoptosis of the cells. Fucoidan also suppressed angiogenesis by decreasing the expression levels of HIF-1α and VEGF. Apoptosis is vital in cancer treatment by removing infected cells through programmed cell death (25). Caspase-3, a member of the caspase family, is important for inducing apoptosis. It is involved in the proteolytic cleavage of various key proteins, including PARP, a protein repairing DNA and maintaining genomic DNA integrity (26,27). The results from the present study demonstrated that fucoidan promoted the expression levels of cleaved caspase-3 and PARP, and induced apoptosis of the thyroid cells. TUNEL and DAPI staining were used to investigate the apoptotic effects of fucoidan. Anti-apoptotic regulators, including Bcl-2, and pro-apoptotic regulators, including Bax, are responsible for the maintenance of cell homeostasis. The results of the present study showed that fucoidan enhanced the expression of Bax and decreased the expression of Bcl-2 in the FTC133 human thyroid cells. Angiogenic factors, including VEGF, are responsible for inducing angiogenesis (28,29). In the present study, the expression levels of HIF-1α and VEGF were inhibited under $CoCl_2$-induced hypoxic conditions in the FTC133 cells. This anti-angiogenic effect of fucoidan was supported by the inhibition of cell migration and tube formation observed, indicating that fucoidan inhibited angiogenesis through VEGF, in addition to targeting the endothelial cells directly. Thus fucoidan offers significant potential as an anticancer agent. In conclusion, the present study demonstrated the anticancer effects of fucoidan in ATC cells, which involved the induction of apoptosis and anti-angiogenesis by inhibiting the expression of VEGF via the suppression of HIF-1α.

These findings suggested that fucoidan may be a potential candidate for cancer therapy against ATC.

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