Cytotechnology (2005) 47:117–126
DOI: 10.1007/s10616-005-3761-8


# Enzyme-digested fucoidan extracts derived from seaweed *Mozuku* of *Cladosiphon novae-caledoniae kylin* inhibit invasion and angiogenesis of tumor cells

Jun Ye[1], Yuping Li[1], Kiichiro Teruya[1], Yoshinori Katakura[1], Akira Ichikawa[2], Hiroshi Eto[3], Mutsutaka Hosoi[4], Masako Hosoi[5], Shinji Nishimoto[6] and Sanetaka Shirahata[1,*]
*[1]Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; [2]Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan; [3]Daiichi Sangyo Co. Ltd., Osaka 530-0037, Japan; [4]Hosoi Dermatological Clinic, Fukuoka 812-0054, Japan; [5]Department of Clinical Medicine, Faculty of Medical Sciences, Kyushu University, 812-8582, Japan; [6]Nishimoto Daini Clinic, Wakayama, 640-8156, Japan; *Author for correspondence (e-mail: sirahata@grt.Kyushu-u.ac.jp; phone: +81-92-642-3045; fax: +81-92-642-3052)*

Received 3 February 2005; accepted in revised form 4 March 2005

*Key words:* Angiogenesis, Antitumor activity, Fucoidan, Invasion, Matrix metalloproteinase, VEGF

**Abstract**

Fucoidan is a uniquely-structured sulfated polysaccharide found in the cell walls of several types of brown seaweed that has recently, especially as enzyme-digested fucoidan extract, attracted a lot attention due to its anti-tumor potential. In this study, we evaluated the effects of enzyme-digested fucoidan extracts prepared from seaweed *Mozuku* of *Cladosiphon novae-caledoniae kylin* on *in vitro* invasion and angiogenesis abilities of human tumor cells. First, we evaluated the effect of the fucoidan extracts on oxidative stress of tumor cells, and demonstrated that intracellular $H_2O_2$ level and released $H_2O_2$ from tumor cells were both greatly repressed upon the treatment with the fucoidan extracts, suggesting that fucoidan extracts ameliorate oxidative stress of tumor cells. Next, we tested for the effects of fucoidan extracts on invasion ability of human fibrosarcoma HT1080 cells, showing that fucoidan extracts significantly inhibit their invasion, possibly via suppressing matrix metalloproteinases (MMPs) MMP-2/9 activities. Further, we investigated the effects of the fucoidan extracts on angiogenesis of human uterine carcinoma HeLa cells, and found that fucoidan extracts suppressed expression and secretion of an angiogenesis factor vascular endothelial growth factor (VEGF), resulting in suppressed vascular tubules formation of tumor cells. The results taken together clarified that enzyme-digested fucoidan extracts from *Cladosiphon novae-caledoniae kylin* possess inhibitory effects on invasion and angiogenesis of tumor cells. These effects might, at least partially, be elicited by the antioxidative potential of enzyme digested fucoidan extracts.

## Introduction

Metastasis is a major problem in cancer treatment/therapy; there being several sequential steps in cancer cell metastasis. Cancer cells migrate from the primary cancerous site to other parts of the body via the bloodstream or lymph system, absorb to extracellular matrix (ECM) and degrade its surrounding proteins. Matrix metalloproteinases (MMPs) are key enzymes involved in tumor

invasion, where MMPs degrade ECM proteins such as collagen, proteoglycan, elastin, laminin, and fibronectin (Johnson et al. 1998). In human, MMP-2 (gelatinase A/$M_r$ 72,000 type IV collagenase) and MMP-9 (gelatinase B/$M_r$ 92,000 type IV collagenase) are thought to be key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Westermarck and Kahari 1999). Both MMP-2 and MMP-9 are abundantly expressed in various malignant tumors (Johnsen et al. 1998) and contribute to invasion and metastasis (Liabakk et al. 1996).

Tumor angiogenesis, the formation of new blood capillaries by vascular endothelial cells from existing vessels, is an important mechanism for supplying nutrients to tumor cells that are distant from existing blood vessels. Tumor angiogenesis is thought to be controlled by angiogenic factors including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). VEGF is a highly conserved dimeric heparin-binding glycoprotein and has a pivotal role in the regulation of normal and abnormal angiogenesis (Ferrara 1993). At present, reactive oxygen species (ROS) are thought to be involved in the regulation of angiogenesis, suggesting that ROS also regulate the expression of VEGF, and that ROS-scavengers is ideal anti-angiogenesis agents.

Fucoidan is a uniquely-structured sulfated polysaccharide found in the cell walls of several types of brown seaweed. Recently, fucoidan has attracted a lot of clinical attention due to its strong anti-tumor potential, which has been intensively investigated. Fucoidan suppresses the growth of tumor cells *in vivo* and activates the immune system against tumors (Usui et al. 1980; Yamamoto et al. 1984; Noda et al. 1990; Itoh et al. 1993; Zhuang et al. 1995; Maruyama et al. 2003). Sulfation of fucoidan enhanced its antitumor activity (Yamamoto et al. 1984). Koyanagi et al. (2003) reported that fucoidan inhibited tube formation following migration of human umbilical vein endothelial cells (HUVEC) and its chemical oversulfation enhances the inhibitory potency. They suggested that fucoidan inhibited the binding of VEGF to the VEGF receptor. Recently it has been reported that a low molecular weight fucoidan (MW. ca. 4 kDa) prepared by radical degradation promotes basic fibroblast growth factor-induced tube formation of endothelial cells (Chabut et al. 2003, 2004). Here we present the first evidence that enzyme-digested fucoidan extract derived from *Mozuku* of *Cladosiphon novae-caledoniae kylin*, which originates in the Kingdom of Tonga, scavenges intracellular ROS and suppresses the invasion and angiogenesis abilities of tumor cells.

## Materials and methods

### *Preparation of fucoidan and reagents*

The abalone glycosidase-digested fucoidan extract prepared from seaweed *Mozuku* of *Cladosiphon novae-caledoniae kylin* from the Kingdom of Tonga, commercially available as a product named 'Power fucoidan', was donated for the study by the Daiichi Sangyo Corporation (Osaka, Japan). An undiluted solution (pH 3.7) was neutralized to pH 7.0 with NaOH. The precipitants were removed by centrifugation at $2200 \times g$ for 15 min. The supernatants were then sterilized with a 0.2 $\mu$m pore filter (Millipore, MA, USA), and stored as 'fucoidan extract (43.5 mg/ml)' at 4 °C. 2′,7′-Dichlorodihydro-fluorescin diacetate ($H_2$DCFDA) was purchased from Molecular probes (Eugene, OR, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was purchased from Wako (Osaka, Japan). Matrigel and type I collagen were obtained from Funakoshi (Tokyo, Japan). The human VEGF immunoassay kit was obtained from R&D system (MN, USA). Angiogenesis tubule staining kit (for staining CD31) was purchased from TCS Cellworks (Buchingham, UK).

### *Cell culture and treatment*

The human fibrosarcoma cell line HT1080, human uterine carcinoma cell line HeLa and human normal fibroblast TIG-1 were cultured in Minimum Eagle's medium (MEM: Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, France), 2 mM L-glutamine and 10 mM HEPES (10% FBS/MEM). HUVEC were cultured in EBM-2 medium (Cambrex, MD, USA). The fucoidan extract (final conc.: 10–20%) was mixed with 10×MEM, and diluted with

MilliQ water. Treatments with the fucoidan extract were performed as described below.

*Cell viability assay*

After HT1080 cells ($5\times10^5$ cells/well) were seeded to 24-well plates and cultured in 10% FBS/MEM in the 5% $CO_2$ atmosphere at 37 °C for 24 h, the culture medium was replaced with 10% FBS/MEM containing the fucoidan extracts, and the cells were cultured for another 24 h. The culture medium was then removed, 500 $\mu$l of WST-1 reagent added to the wells, and the plates incubated at 37 °C for 2 h. A 100 $\mu$l of reaction solution was moved to a new 96-well microplate, and the absorbance at 450 nm was measured using a microplate reader (Tecan, Maennedorf, Switzerland).

*Flow cytometric analysis of intracellular $H_2O_2$*

$H_2$DCFDA is a cell-permeable indicator for reactive oxygen species that is non-fluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. HT1080 cells were pre-treated with MEM containing the fucoidan extracts for 24 h, and incubated with 5 $\mu$M of $H_2$DCFDA for 30 min at 37 °C. Fluorescent intensity of 2′,7′-dicholorofluorescein (DCF) was measured by a flow cytometer (EPICS XL system II, Beckman Coulter, Miami, FL, USA).

*Measurement of $H_2O_2$ release*

$H_2O_2$ release from HeLa cells into the culture medium was assayed according to the method described by Ruch et al. (1983). This fluorometric method is based on the conversion of homovanillic acid to its fluorescent dimer in the presence of $H_2O_2$ and horseradish peroxidase. After HT1080 cells were challenged with the fucoidan extracts for 24 h, the cells were first washed with PBS and incubated with 800 $\mu$l of reaction buffer (100 $\mu$M homovanillic acid, 5 units $ml^{-1}$ horseradish peroxidase type IV and 1 mM HEPES in Hanks' balanced salt solution without phenol red pH 7.4). The reaction buffer alone was treated in the same way and used as a control. This solution was collected after 30 min incubation; pH was then adjusted to 10.0 with 0.1 M glycine–NaOH buffer. Fluorescence was measured by using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 321 nm and 421 nm, respectively. The concentration of $H_2O_2$ in the solution was estimated by a standard calibration curve prepared by using a series of 0$\sim$10 mM $H_2O_2$. The exact concentrations of $H_2O_2$ in solutions used to plot standard curves were determined spectrophotometrically at 240 nm using an extinction coefficient of 43.6 $M^{-1}$ $cm^{-1}$.

*Matrigel invasion assay*

Each well of 24-well microplates was coated with 700 $\mu$l of serum-free MEM containing 10 $\mu$g of fibronectin. Upper parts of the 8 $\mu$m chambers (Kurabo, Osaka, Japan) were then coated with 20 $\mu$l matrigel, and lower parts with 10 $\mu$l collagen. The chambers were then inserted into the 24-well microplate. HT1080 cells were pretreated with the fucoidan extract for 24 h, inoculated onto the chamber ($1\times10^5$ cells per well) and incubated for 12 h at 37 °C. The cells that invaded into the lower surface of collagen were fixed and stained with Diff-Quik (Sysmex, Hyogo, Japan), and three random fields counted under a light microscope.

*Zymography assay*

HT1080 cells were cultured in 10% FBS/MEM overnight and subsequently treated with 10% FBS/MEM containing the fucoidan extract for 24 h. The culture medium was replaced with 2 ml of fresh serum-free MEM, and cells cultured for another 24 h. The supernatant was collected and concentrated by ultracentrifugation (Millipore). A 5 $\mu$l of concentrated culture medium was mixed with 5 $\mu$l of 2 × sample buffer (0.25 M Tris–HCl, pH 6.0, 8.5% glycerol, 4% SDS, 0.01% bromophenol blue), and then electrophoresed on a gelatin-containing gel (7.5% polyacrylamide and 2 mg/ml gelatin). After electrophoresis, the gel was washed three times for 10 min in 2.5% Triton X-100, and incubated with incubation buffer (50 mM Tris–HCl, pH 7.6, 10 mM $CaCl_2$, 50 mM NaCl, 0.05% Brij35) overnight at 37 °C. After

incubation, the gel was stained by CBB solution (0.25% Coomassie blue R250, 40% methanol and 10% acetic acid) for 1 h at room temperature, and decolorized with 40% methanol and 10% acetic acid.

*Semi-quantitative RT-PCR*

Total RNA was isolated using a GenElute Mammalian Total RNA isolation kit (Sigma, St. Louis, MO) following the manufacturer's protocol. Primer sequences for amplifying GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse), for MMP-2: 5′-TTCTATGGCTGCCCCAAGGAGAGCTGCAAC-3′ (forward) and 5′-CAGCTCAGCAGCCTAGCCAGTCGGATTTGA-3′ (reverse), and for VEGF: 5′-GGGCCTCCGAAACCATGAAC-3′ (forward) and 5′-CTGGTTCCCGAAACCCTGAG-3′ (reverse). PCR conditions were 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min for 30 cycles for GAPDH, 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min for 30 cycles for MMP-2, and 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min for 35 cycles for VEGF.

*Measurement of VEGF secreted into the culture medium*

HeLa cells ($5\times10^5$ cells/well) were seeded in 24-well plates with 10% FBS/MEM overnight. The medium was then replaced with serum-free MEM containing the fucoidan extract and cultured for another 24 h. Culture medium was replaced with fresh serum-free MEM and cells were cultured for another 24 h. Supernatants were collected to measure the amount of VEGF secretion using a procedure following the manufacturer's protocol.

*Tubles formation assay*

HeLa cells ($1\times10^6$ cells) were seeded in a 100 mm dish with 10% FBS/MEM overnight. The medium was replaced with serum-free MEM or MEM containing the fucoidan extract, the cells were cultured for 24 h, and then further cultured in serum-free MEM for 72 h. The conditioned medium was collected and filtered with a 0.2 $\mu$m filter. Tubules formation assay was performed with a co-culture system. HUVEC were mixed with TIG-1 cells in a ratio of 1:40, seeded in 24-well microplates, and cultured in EBM-2 medium overnight. The medium was removed and a mixture of tumor cell conditioned medium and EMB-2 at 2:1 was added. The medium was changed every 2 days. Formed tubules were detected with HUVEC-specific markers CD31 (PECAM-1). Briefly, at day 11, medium was completely removed, and the co-culture plate was fixed for 30 min with a 70% ethanol solution. After incubation with PBS containing 1% BSA, the co-culture plate was incubated with a mouse anti-human CD31 antibody for 60 min, followed by another 60 min incubation with a secondary goat anti-mouse IgG antibody conjugated with alkaline phosphatase. After washing the plate, BCIP/NBT substrate was added until tubules developed a dark purple color. Recorded images were analyzed by software for angiogenesis quantification (AngioSys 1.0, TCS, Cellworks, UK). Twelve random fields per well were taken for tubule formation assessment.

## Results

*Preparation of the fucoidan extract and its properties*

Extract containing high molecular weight fucoidan was purified to 85% from seaweed of *Cladosiphon novae-caledoniae kylin*, digested with an abalone glycosidase, and used as the fucoidan extract in this study. Molecular composition of the fucoidan extract was evaluated by size exclusion chromatography using a TSK-gel G3000$PW_{XL}$ (TOSOH, Tokyo, Japan); the fucoidan extract was demonstrated to consist of a digested small molecular weight (MW) fraction (72%, MW: <500) and non-digestive fractions [less than 28%, peak MW: 800 kDa (data not shown)]. Sugar composition was further evaluated by a spectrofluorometer (LC-9A, Shimadzu, Kyoto, Japan) equipped with a TAK-gel Sugar AXG column (TOSHO); showing that sugars in the fucoidan extract consisted of mostly fucose (73%), xylose (12%) and mannose (7%) (data not shown). The ratio of sulfation determined by flask burning and ion chromatography method was 14.5%. Taking these results together, we considered that the fucoidan extract

used here consists mostly of a mixture of digested fucoidan and other substances, as well as non-digested fucoidan.

*Effect of the fucoidan extract on tumor cell viability*

To examine the effect of the fucoidan extract on the viability of HT1080 and HeLa cells, cells were treated with different concentrations of the fucoidan extract (1–80%) for 2 days and cell proliferation was evaluated by WST-1 assay. As shown in Figure 1, no significant effect was observed on the viability of HT1080 cells when exposed to less than 20% of the fucoidan extract. Further, the fucoidan extract did not affect viability of HeLa cells until exposure to 20% of the fucoidan extract (data not shown). To exclude the toxic effect of the fucoidan extract on proliferation of tumor cells, less than 20% of the fucoidan extract was used throughout the experiments.

*Fucoidan extract reduced the accumulation and release of $H_2O_2$ in tumor cells*

We next investigated the intracellular $H_2O_2$-scavenging ability of the fucoidan extract in HT1080 cells. $H_2$DCFDA was used to measure intracellular $H_2O_2$ production in HT1080 cells. HT1080 cells were pre-treated with MEM containing the fucoidan extract for 24 h, then incubated with 5 $\mu$M $H_2$DCFDA for 30 min at 37 °C. As shown in Figure 2a, fucoidan extract was shown to scavenge intracellular $H_2O_2$ in HT1080 cells. Fucoidan extract also scavenged intracellular $H_2O_2$ in HeLa cells (data not shown). Furthermore, the release of $H_2O_2$ into culture medium from HeLa cells was also significantly reduced upon the treatment with fucoidan extract (Figure 2b). These results indicate that fucoidan extract relieves the oxidative stress induced by ROS, and reduces oxidative stress of cells and their surroundings.

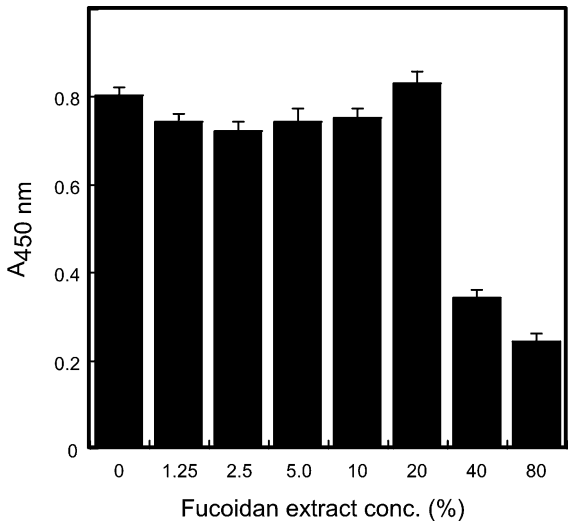


*Figure 1.* Effect of fucoidan extract on HT1080 cell viability. To examine the effect of the fucoidan extract on the viability of HT1080 cells, cells were treated with different concentrations of fucoidan extract (11.0 mg/ml, 100%) for 2 days and cell proliferation was evaluated by WST-1 assay.

*Fucoidan extract inhibits invasion of HT1080 cells*

MMP-2 and -9 are secreted from tumor cells and are involved in tumor cell invasion. First, we performed RT-PCR analysis to investigate the effect of fucoidan extract on the transcription of MMP-2. Figure 3a shows that transcription of MMP-2 in HT1080 cells slightly decreased upon treatment with fucoidan extract. Next, we performed zymography assay to detect the levels of MMP-2 and -9 in culture medium. As shown in Figure 3b, activities of both enzymes secreted from HT1080 decreased upon treatment with fucoidan extract. Finally, we investigated the invasion ability of HT1080 cells treated with fucoidan extract by matrigel penetrating assay. As shown in Figure 3c, the number of cells penetrating the matrigel also decreased upon treatment with fucoidan extract. Taken together, all these results indicate that fucoidan extract attenuates invasion ability of tumor cells.

*Fucoidan extract inhibits expression and secretion of VEGF*

VEGF and $H_2O_2$ are known angiogenic factors. As shown in Figure 2, fucoidan extract decreased the accumulation and release of $H_2O_2$, which might lead to a reduction of the angiogenic ability of tumor cells. Thus, we next investigated the effect of fucoidan extract on the expression of VEGF. As shown in Figure 4a, VEGF expression in HeLa cells greatly repressed upon treatment with fucoidan

extract. In accordance with this result, the secretion of VEGF was also inhibited upon treatment with fucoidan extract (Figure 4b). All these results demonstrate that fucoidan extract reduces the angiogenic ability of tumor cells by decreasing the release of $H_2O_2$ and inhibiting secretion of VEGF.

*Fucoidan extract inhibits tumor angiogenesis*

Next, we investigated the effect of fucoidan extract on tumor angiogenesis by using a co-culture system. The results showed that conditioned medium of HeLa cells (HeLa CM) significantly augmented the formation of the vascular tubules in all tested aspects as compared to no cell conditioned medium (control). In contrast, the conditioned medium of HeLa cells treated with fucoidan extract (fucoidan ex.–HeLa) significantly suppressed all parameters of tubules formation assay (Figure 5). These results indicate that the fucoidan extract inhibits angiogenesis of tumor cells.

## Discussion

Fucoidan is reported to have anti-tumor activity (Soeda et al. 1994; Haroun-Bouhedja et al. 2002). However, the mechanism by which of fucoidan inhibits the invasion/angiogenesis of tumor cells has not been clearly elucidated. In this study we used fucoidan extract derived from the seaweed *Cladosiphon novae-caledoniae kylin* and digested with abalone glycosidase. The fucoidan extract was found to consist mostly of fucose (73%) by sugar composition analysis, and digested small MW fraction (72%, MW: <500) by size-exclusion chromatography; therefore, fucoidan extract consists mostly of digested fucoidan. In this study, we tried to evaluate the functionalities of this novel fucoidan derivative on the invasion/angiogenesis abilities of tumor cells.

Tumor cells carry a high level of oxidative stress in comparison to normal cells (Szatrowski and Nathan 1991), along with elevated levels of MMP-2 and 9 (Johnsen et al. 1998), which strongly suggest a correlation between oxidative stress and MMP-2/9 expression in tumor cells (Belkhiri et al. 1997; Gurjar et al. 2001; Grote et al. 2003; Kolev et al. 2003); and further, that alleviating intracellular oxidative stress can result in the downregulation of MMP-2/9. In fact, several fucoidan derivatives have shown *in vitro* or *in vivo* antioxidative potential (Xue et al. 1998, 2001; Ruperez et al. 2002; Zhang et al. 2003). We demonstrated

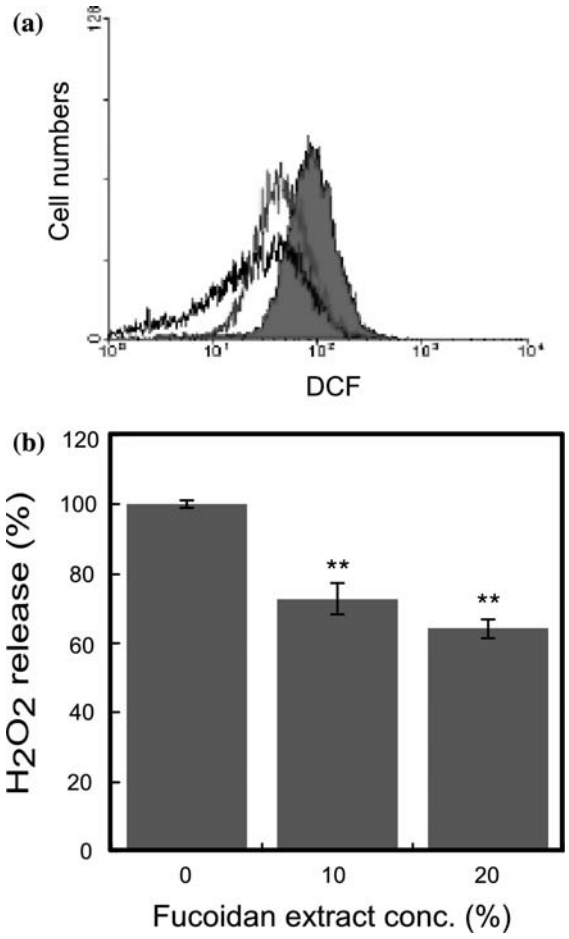


*Figure 2.* Fucoidan extract reduced the accumulation and release of $H_2O_2$ in tumor cells. (a) $H_2$DCFDA was used to measure intracellular $H_2O_2$ production in HT1080 cells. HT1080 cells were pre-treated with 10% FBS/MEM containing fucoidan extract for 24 h, then incubated with 5 $\mu$M $H_2$DCFDA for 30 min at 37 °C. The fluorescent intensity of DCF was measured by using a flow cytometer. The horizontal axis shows the fluorescence intensity of DCF, which indicates the intracellular oxidative status of HT1080 cells. The vertical axis shows cell numbers. (b) $H_2O_2$ release from HeLa cells into the culture medium was assayed according to the method described by Ruch et al. (1983). This fluorometric method is based on the conversion of homovanillic acid to its fluorescent dimer in the presence of $H_2O_2$ and horseradish peroxidase. Fluorescence intensity was measured by a fluorescence spectrophotometer at excitation and emission wavelengths of 321 nm and 421 nm, respectively. Data are expressed as the mean ± SD of three independent experiments. Results were statistically significant (**$p < 0.01$; $n = 3$) using the Student's *t*-test.

that the fucoidan extract derived form *Cladosiphon novae-caledoniae kylin* effectively reduced both intracellular and released $H_2O_2$ of HT1080 cells, which might lead to the suppression of MMP-2/9 expression and subsequent inhibition of the invasive ability. We have not clarified the mechanisms for the reduction of ROS by fucoidan extract, but we can speculate that fucoidan extract stimulates antioxidant enzymes such as superoxide dismutase and glutathione peroxidase.

Release of $H_2O_2$ is thought to be one of the triggers in the angiogenic process of cancer cells (Szatrowski and Nathan 1991). During tumor growth, $H_2O_2$ is reported to play a pivotal role in ethanol-induced *in vitro* angiogenesis (Qian et al. 2003). Although tumor angiogenesis is thought to directly correlate with VEGF production from tumors (Berkman et al. 1993; Guidi et al. 1995; Wizigmann-Voos et al. 1995; Mattern et al. 1996; Suzuki et al. 1996; Balsari et al. 1999), $H_2O_2$ also causes significant induction of VEGF expression in many cell lines (Brauchle et al. 1996; Chua et al. 1998; Cho et al. 2001; Masuda et al. 2002; Zhu et al. 2002). Thus, scavenging ROS and reduction of the ROS stress of tumor cells and their surroundings should lead to the inhibition of angiogenesis of tumor cells via suppressing the release of $H_2O_2$ and donwnregulating VEGF expression.

Several antioxidants inhibit tumor angiogenesis. N-acetyl cysteine (NAC) inhibits tumor angiogenesis via suppressing the production of VEGF (Albini et al. 2001) and by promoting angiostatin production, a potent endothelial apoptotic factor (Agarwal et al. 2004). Vitamin E has been shown to suppress tubules formation induced by exogenous $H_2O_2$ (Tang and Meydani 2001), and to inhibit melanoma angiogenesis through suppressing the expression of VEGF (Malafa et al. 2002). In the present study, a decreased intracellular ROS level and $H_2O_2$ release upon treatment with enzyme-digested fucoidan extract derived from *Cladosiphon novae-caledoniae kylin* was responsible for a reduction of MMP-2/9 activities and a decrease in VEGF expression; and further, the subsequent inhibition of invasion, and the suppression of tubules formation in tumor cells. The active substances in fucoidan extract remain to be clarified. Low molecular weight and sulfate con-

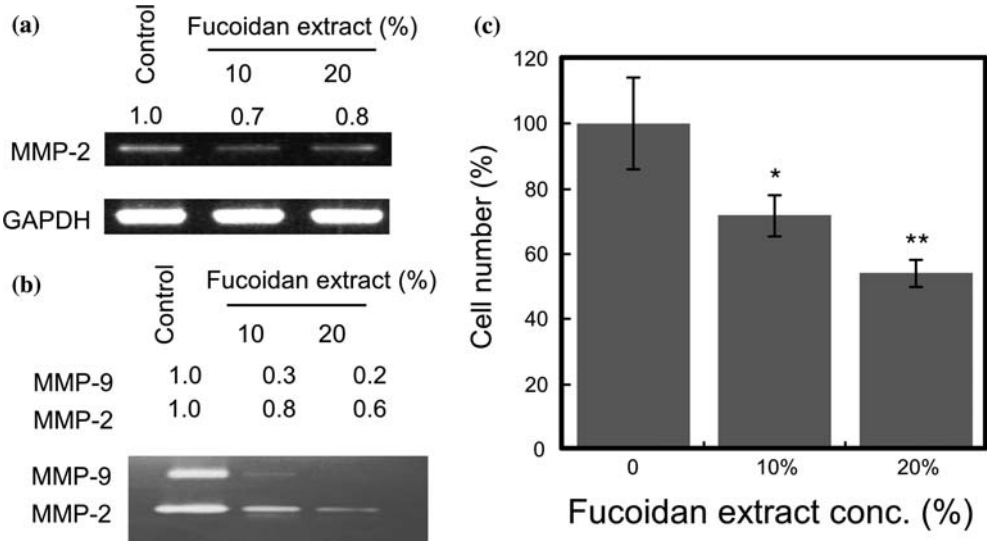


*Figure 3*. Fucoidan extract suppresses invasion of HT1080 cells. (a) After HT1080 cells were pre-treated with 10% FBS/MEM containing fucoidan extract for 24 h, total RNAs were prepared and RT-PCR analyses for MMP-2 and GAPDH performed. Numerical RT-PCR data analyzed by Image Gauge 4.0 software (Fuji Film Co. Ltd., Tokyo) are shown at the top of the photo. (b) After HT1080 cells were pre-treated with 10% FBS/MEM containing fucoidan extract for 24 h, the medium was replaced with 2 ml of serum-free MEM and cells were cultured for another 24 h. The supernatant was concentrated and applied to zymography assay. Numerical zymography data analyzed by Image Gauge 4.0 software are shown at the top of the zymography photo. (c) After HT1080 cells were pre-treated with 10% FBS/MEM containing fucoidan extract for 24 h, $1 \times 10^5$ cells per well were seeded into the chamber, then incubated for 12 h at 37 °C. Cells invading into the lower surface of the collagen were fixed and stained with Diff-Quick. Invaded cells were counted in three random fields under a light microscope. Data are expressed as the mean ± SD of three independent experiments. Results were statically significant (*$p < 0.05$; **$p < 0.01$, $n = 3$) using the Student's *t*-test.

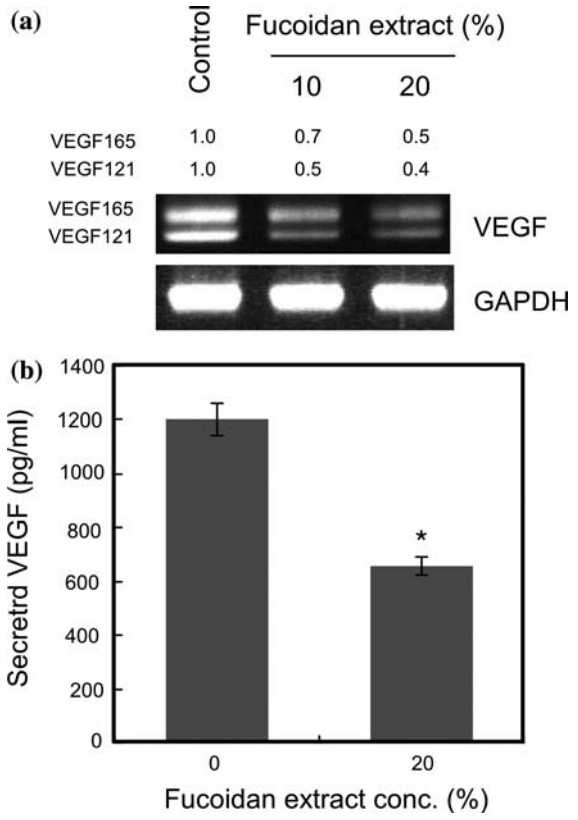


*Figure 4*. Fucoidan extract inhibits expression and secretion of VEGF in HeLa cells. (a) After HeLa cells were cultured in 10% FBS/MEM for 24 h, the culture medium was replaced with serum-free MEM containing fucoidan extract and cells were cultured for another 24 h. After total RNA was prepared, RT-PCR for VEGF was performed. Numerical RT-PCR data analyzed by Image Gauge 4.0 software are shown at the top of the photo. (b) After the supernatant of HeLa cells treated as above were replaced with fresh serum-free MEM and cultured for another 24 h, supernatants were collected to measure VEGF secretion. The procedure of VEGF measurement is described in Materials and methods. Data are expressed as the mean ± SD of three independent experiments. Results were statistically significant ($*p < 0.05$; $n = 3$) using the Student's *t*-test.

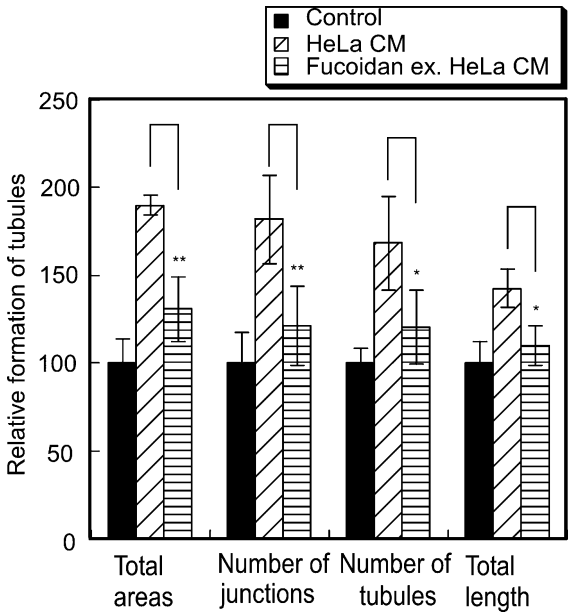


*Figure 5*. Fucoidan inhibits vascular tubules formation. The HUVEC/TIG-1 co-culture system was challenged with a mixture of EBM-2 and non-cell treated MEM (control), HeLa cell conditioned MEM (HeLa CM) and conditioned MEM of HeLa cells treated with fucoidan extract (Fucoidan ex. HeLa CM) in a ratio of 1:2, respectively. At day 11, tubules formation was detected with a tubule staining kit and then visualized under a phase-contrast microscope. Photographs were analyzed using software for angiogenesis quantification analysis. Twelve random fields per well were assessed for tubule formation. Data are expressed as the mean ± SD of three independent experiments. Results were statistically significant ($*p < 0.05$; $**p < 0.01$; $n = 3$) using the Student's *t*-test.

tent of fucoidan are reported to be responsible for its antioxidative abilities (Xue et al. 2001, 2004; Quanbin et al. 2003). On the other hand, oversulfation of fucoidan could enhance its anti-tumor invasion (Soeda et al. 1994) and angiogenic activities (Koyanagi et al. 2003). We observed that the non-digested high molecular weight of the fucoidan extract did not scavenge intracellular ROS of HT1080 cells, and did not enhance the expression of MMP-2 mRNA (data not shown). Thus, the active substances in enzyme-digested fucoidan extract derived from seaweed *Mozuku* are thought to be of low molecular weight and a sulfated fraction. However, other possibility also considered is that active substances of low molecular weight such as an antioxidative carotenoid, fucoxanthin (Okuzumi et al. 1990, 1993; Hoyoku 1995) may also be released by the enzyme-digestion of fucoidan extract. Further investigation regarding the active substances in enzyme-digested fucoidan extract needs to be undertaken.

## References

Agarwal A., Munoz-Najar U., Klueh U., Shih S.C. and Claffey K.P. 2004. N-acetyl-cysteine promotes angiostatin production and vascular collapse in an orthotopic model of breast cancer. Am. J. Pathol. 164: 1683–1696.

Albini A., Morini M., D'Agostini F., Ferrari N., Campelli F., Arena G., Noonan D.M., Pesce C. and De Flora S. 2001. Inhibition of angiogenesis-driven Kaposi's sarcoma tumor growth in nude mice by oral N-acetylcysteine. Cancer Res. 61: 8171–8178.

Balsari A., Maier J.A., Colnaghi M.I. and Menard S. 1999. Correlation between tumor vascularity, vascular endothelial growth factor production by tumor cells, serum vascular endothelial growth factor levels, and serum angiogenic activity in patients with breast carcinoma. Lab. Invest. 79: 897–902.

Belkhiri A., Richards C., Whaley M., McQueen S.A. and Orr F.W. 1997. Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal $H_2O_2$ exposure. Lab. Invest. 77: 533–539.

Berkman R.A., Merrill M.J., Reinhold W.C., Monacci W.T., Saxena A., Clark, W.C., Robertson J.T., Ali I.U. and Oldfield E.H. 1993. Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J. Clin. Invest. 91: 153–159.

Brauchle M., Funk J.O., Kind P. and Werner S. 1996. Ultraviolet B and $H_2O_2$ are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. J Biol Chem 271: 21793–21797.

Chabut D., Fischer A.-M., Colliec-Jouault S., Laurendeau I., Matou S., Le Bonniec B. and Helley D. 2003. Low molecular weight fucoidan and heparin enhance the basic fibroblast growth factor-induced tube formation of endothelial cells through heparin sulfate-dependent a6 overexpression. Mol. Pharmcol. 64: 696–702.

Chabut D., Fischer A.M., Helley D. and Colliec S. 2004. Low molecular weight fucoidan promotes FGF-2-induced vascular tube formation by human endothelial cells, with decreased PAI-1 release and ICAM-1 downregulation. Thrombosis Res. 113: 93–95.

Cho M., Hunt T.K. and Hussain M.Z. 2001. Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. Am. J. Physiol. Heart Circ. Physiol. 280: H2357–2363.

Chua C.C., Hamdy R.C. and Chua B.H. 1998. Upregulation of vascular endothelial growth factor by H2O2 in rat heart endothelial cells. Free Radic. Biol. Med. 25: 891–897.

Ferrara N. 1993. Vascular endothelial growth factor. Trends Cardiovasc. Med. 3: 244–250.

Grote K., Flach I., Luchtefeld M., Akin E., Holland S.M., Drexler H. and Schieffer B. 2003. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. Circ. Res. 92: e80–86.

Guidi A.J., Abu-Jawdeh G., Berse B., Jackman R.W., Tognazzi K., Dvorak H.F. and Brown L.F. 1995. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J. Natl. Cancer Inst. 87: 1237–1245.

Gurjar M.V., Deleon J., Sharma R.V. and Bhalla R.C. 2001. Role of reactive oxygen species in IL-1 beta-stimulated sustained ERK activation and MMP-9 induction. Am. J. Physiol. Heart Circ. Physiol. 281: H2568–2574.

Haroun-Bouhedja F., Lindenmeyer F., Lu H., Soria C., Jozefonvicz J. and Boisson-Vidal C. 2002. *In vitro* effects of fucans on DA-MB231 tumor cell adhesion and invasion. Anticancer Res. 22: 2285–2292.

Hoyoku H. 1995. Cancer chemoprevention by natural carotenoids and their related compounds. J. Cell. Biochem. Suppl. 22: 231–235.

Itoh H., Noda H., Amano H., Zhuang C., Mizuno T. and Ito H. 1993. Antitumor activity and immunological properties of marine algal polysaccharides, especially Fucoidan, prepared from *Sargassum thunbergii* of Pheophyceae. Anticancer Res. 13(6A): 2045–2052.

Johnsen M., Lund L.R., Romer J., Almholt K. and Dano K. 1998. Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. Curr. Opin. Cell Biol. 10: 667–6671.

Johnson L.L., Dyer R. and Hupe D.J. 1998. Matrix metalloproteinases. Curr. Opin. Chem. Biol. 2: 466–471.

Kolev K., Skopal J., Simon L., Csonka E., Machovich R. and Nagy Z. 2003. Matrix metalloproteinase-9 expression in post-hypoxic human brain capillary endothelial cells: $H_2O_2$ as a trigger and NF-kappaB as a signal transducer. Thromb. Haemost. 90: 528–537.

Koyanagi S., Tanigawa N., Nakagawa H., Soeda S. and Shimeno H. 2003. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochem. Phamacol. 65: 173–179.

Liabakk N.B., Talbot I., Smith R.A.Wilkinson K. and Balkwill F. 1996. Matrix metalloprotease 2 (MMP-2) and matrix metalloprotease 9 (MMP-9) type IV collagenases in colorectal cancer. Cancer Res. 56: 190–196.

Malafa M.P., Fokum F.D., Smith L. and Louis A. 2002. Inhibition of angiogenesis and promotion of melanoma dormancy by vitamin E succinate. Ann. Surg. Oncol. 9: 1023–1032.

Maruyama H., Tamauchi H., Hashimoto M. and Nakano T. 2003. Antitumor activity and immune response of Mekabu Fucoidan extracted from Sporophyll of *Undaria pinnatifida*. In Vivo 17: 245–249.

Masuda M., Suzui M., Lim J.T., Deguchi A., Soh J.W. and Weinstein I.B. 2002. Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. J. Exp. Ther. Oncol. 2: 350–359.

Mattern J., Koomagi R. and Volm M. 1996. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumor cell proliferation in human epidermoid lung carcinoma. Br. J. Cancer 73: 931–934.

Noda H., Amano H., Arashima K. and Nisizawa K. 1990. Antitumor activity of marine algae. Hydrobiologica 204–205: 577–584.

Okuzumi J., Nishino H., Murakoshi M., Iwashima A., Tanaka Y., Yamane T., Fujita Y. and Takahashi T. 1990. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. Cancer Lett. 55: 75–81.

Okuzumi J., Takahashi T., Yamane T., Kitao Y., Inagake M., Ohya K., Nishino H. and Tanaka Y. 1993. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-ethyl-N′-nitro-N-nitrosoguanidine-induced mouse duodenal carcinogenesis. Cancer Lett. 68: 159–168.

Qian Y., Luo J., Leonard S.S., Harris G.K., Millecchia L., Flynn D.C. and Shi X. 2003. Hydrogen peroxide formation and actin filament reorganization by Cdc42 are essential for

ethanol-induced *in vitro* angiogenesis. J. Biol. Chem. 278: 16189–16197.

Quanbin Z., Pengzhan Y., Zhien L., Hong Z., Zuhong X. and Pengcheng L. 2003. Antioxidant activities of sulfated polysaccharide fractions from *Porphyra haitanesis*. J. Appl. Phycol. 15: 305–310.

Ruch W., Cooper P.H. and Baggiolini M. 1983. Assay of $H_2O_2$ production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. J. Immunol. Methods 63: 347–357.

Ruperez P., Ahrazem O. and Leal J.A. 2002. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. J. Agric. Food Chem. 50: 840–845.

Soeda S., Ishida S., Shimeno H. and Nagamatsu A. 1994. Inhibitory effect of oversulfated fucoidan on invasion through reconstituted basement membrane by murine Lewis lung carcinoma. Jpn. J. Cancer Res. 85: 1144–1150.

Suzuki K., Hayashi N., Miyamoto Y., Yamamoto M., Ohkawa K., Ito Y., Sasaki Y., Yamaguchi Y., Nakase H., Noda K., Enomoto N., Arai K., Yamada Y., Yoshihara H., Tujimura T., Kawano K., Yoshikawa K. and Kamada T. 1996. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. Cancer Res. 56: 3004–3009.

Szatrowski T.P. and Nathan C.F. 1991. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 51: 794–798.

Tang F.Y. and Meydani M. 2001. Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells through suppression of IL-8 production. Nutr. Cancer 41: 119–125.

Usui T., Asari K. and Mizuno T. 1980. Isolation of highly purified "Fucoidan" from *Eisenia bicyclios* and its anticoagulant and antitumor activities. Agric. Biol. Chem. 44: 1965–1966.

Westermarck J. and Kahari V.M. 1999. Regulation of matrix metalloproteinase expression in tumor invasion. Faseb. J. 13: 781–792.

Wizigmann-Voos S., Breier G., Risau W. and Plate K.H. 1995. Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res. 55: 1358–1364.

Xue C.-H., Fang Y., Lin H., Chen L., Li Z.-J., Deng D. and Lu C.-X. 2001. Chemical characters and antioxidative properties of sulfated polysaccharides from *Laminaria japonica*. J. Appl. Phycol. 13: 67–70.

Xue C., Yu G., Hirata T., Terao J. and Lin H. 1998. Antioxidative activities of several marine polysaccharides evaluated in a phosphatidylcholine-liposomal suspension and organic solvents. Biosci. Biotechnol. Biochem. 62: 206–209.

Xue Z., Chang-Hu X., Zhao-Jie L., Yue-Piao C., Hong-Ying L and Hong-Tao Q. 2004. Antioxidant and hepatoprotective activities of low molecular weight sulfated polysaccharide from *Laminaria japonica*. J. Appl. Phycol. 16: 111–115.

Yamamoto I., Takahashi M., Suzuki T., Seino H. and Mori H. 1984. Antitumor effect of seaweeds. IV. Enhancement of antitumor activity by sulfation of a crude Fucoidan from *Sargassum kjellmanianum*. Jpn. J. Exp. Med. 54: 143–151.

Zhang Q., Li N., Zhou G., Lu X., Xu Z. and Li Z. 2003. *In vivo* antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodephyta) in aging mice. Pharmacol. Res. 48: 151–155.

Zhu J.W., Yu B.M., Ji Y.B., Zheng M.H. and Li D.H. 2002. Upregulation of vascular endothelial growth factor by hydrogen peroxide in human colon cancer. World J. Gastroenterol. 8: 153–157.

Zhuang C., Itoh H., Mizuno T. and Ito H. 1995. Antitumor active Fucoidan from the brown seaweed, Umitoranoo (*Aargassum thunbergii*). Biosci. Biotech. Biochem. 59: 563–567.