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Antiangiogenic Activity of Brown Algae Fucoxanthin and Its Deacetylated Product, Fucoxanthinol

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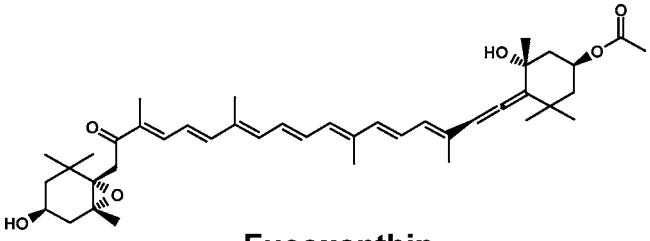
The antiangiogenic effects of fucoxanthin and a deacetylated product, fucoxanthinol, were examined. Fucoxanthin significantly suppressed HUVEC proliferation and tube formation at more than 10 μM, but it had no significant effect on HUVEC chemotaxis. The formation of blood vessel-like structures from CD31-positive cells was evaluated using embryonic stem cell-derived embryoid bodies. Fucoxanthin effectively suppressed the development of these structures at 10−20 μM, suggesting that it could suppress differentiation of endothelial progenitor cells into endothelial cells involving new blood vessel formation. Fucoxanthin and fucoxanthinol suppressed microvessel outgrowth in an ex vivo angiogenesis assay using a rat aortic ring, in a dose-dependent manner. These results imply that fucoxanthin having antiangiogenic activity might be useful in preventing angiogenesis-related diseases.

KEYWORDS: Angiogenesis; aortic ring; endothelial cells; fucoxanthin; fucoxanthinol

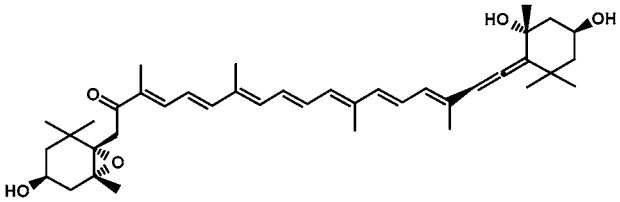
INTRODUCTION

Angiogenesis, the process of new blood vessel growth, is involved in many physiological and pathological situations. Antiangiogenic therapy has become established as a strategy for cancer prevention, and numerous efforts have been conducted on angiogenesis inhibitors, because the aggravation of some pathogenesis, such as cancer, atherosclerosis, and diabetic retinopathy, is known to depend on the angiogenic phenotype (*1*, *2*). Many food compounds have been believed to be beneficial for human health due to their anticarcinogenic activity. Therefore, the antiangiogenic activity of food components has received increased attention as potent inhibitors of tumor growth (*3*). We previously revealed that some natural products, such as vitamin B_6 (*4*), algal polysaccharides (*5*, *6*), and nasunin (*7*), suppress angiogenesis.

Fucoxanthin (**Figure 1**) is a major carotenoid of edible brown algae (*8*). There have recently been several reports that fucoxanthin had beneficial effects on chemoprevention of cancer (*9*, *10*) and induced apoptosis in several cancer cell lines (*11*−*17*). We have found that fucoxanthin reduced the viability of prostate cancer cells by inducing apoptosis more remarkably than the other carotenoids present in foodstuffs (*12*). Several studies have shown that fucoxanthin decreases the expression level of Bcl-2, an antiapoptotic protein (*15*, *16*). The formation of fucoxanthinol from fucoxanthin has been demonstrated in human intestinal Caco-2 cells and in mice fed with fucoxanthin, and amarouciaxanthin A is generated from fucoxanthinol in the liver after intestinal absorption of fucoxanthinol (*18*, *19*). Fucoxanthinol has a more potent antiproliferative effect on prostate cancer cells than does fucoxanthin (*19*). However, it remained unknown whether fucoxanthin and its metabolites affected angiogenesis. In the present study, we investigated whether fucoxanthin and fucoxanthinol, its deacetylated form, could suppress angiogenesis.



Fucoxanthin



Fucoxanthinol

Figure 1. Chemical structures of fucoxanthin and fucoxanthinol.

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MATERIALS AND METHODS

Materials. Fucoxanthin was extracted and refined from brown algae (*Undaria pinnatifida*), and fucoxanthinol was prepared from fucoxanthin as described previously (*18*−*20*). Briefly, the acetone extract from the brown algae was applied to a silica gel (Keisel gel 60, Merck, Darmstadt, Germany) column and was eluted by stepwise elution with a hexane:ethyl acetate mixture (10:0−4:6, v/v). Fucoxanthin was recovered in the hexane:ethyl acetate fraction (5:5−4:6, v/v). The fucoxanthin-rich fraction was further subjected to a flash column chromatography on a LiChroprep RP-18 (40−63 μm, 11 × 240 mm, Merck) with acetonitrile/methanol/water (75:15:10) containing 0.1% ammonium acetate to isolate pure fucoxanthin. Fucoxanthinol was prepared from fucoxanthin by enzymatic hydrolysis. Approximately 5 μmol of fucoxanthin and 100 mg of taurocholate were together dissolved in a small volume of dichloromethane/methanol (2:1), and the solvent was dried under a stream of nitrogen gas in a centrifuge tube. Next, 10 units of cholesterol esterase in 10 mL of potassium phosphate buffer (0.1 M, pH 7.0) was added to the residue and dissolved. After incubation at 37 °C for 2 h, 4 volumes of dichloromethane/methanol (2:1, v/v) were added to the reaction mixture, and fucoxanthinol was extracted into the dichloromethane phase. The extracted fucoxanthinol was then purified by preparative HPLC on a TSK-gel ODS 80Ts column, 10 × 250 mm (Tosoh), with acetonitrile/methanol/water (67.5:13.5:19, v/v) containing 0.1% (w/v) ammonium acetate as a mobile phase.

Human recombinant vascular endothelial growth factor (VEGF) was obtained from R&D Systems (MN). WST-1 reagent was from Dojindo Laboratories (Kumamoto, Japan). Other reagents were special grades commercially available.

Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in the medium, HuMedia EG 2 (Kurabo Industries), which was a modified MCDB 131 medium containing 2% fetal bovine serum (FBS), 10 ng/mL recombinant human epidermal growth factor (EGF), 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, 5 ng/mL recombinant human basic fibroblast growth factor (bFGF), and 10 μg/mL heparin, at 37 °C in humidified 5% CO_2. Subcultures were obtained by treating the HUVEC cultures with a 0.025% trypsin−0.01% EDTA solution. HUVECs at passages three to five were used in this experiment.

HUVEC Proliferation Assay. A cell suspension (15 000 cell/mL) was plated onto 96-well culture plates (100 μL/well). After 24 h, the medium was replaced with fresh HuMedia EG2 containing fucoxanthin (0−100 μM). Fucoxanthin dissolved in tetrahydrofuran (THF) was added to the culture medium. The final concentration of the vehicle (THF) was 0.1%, and the control culture received the vehicle alone. After 72 h, 10 μL of WST-1 reagent was added into each well of a 96-well plate and incubated for 4 h at 37 °C. The absorbance at 450 nm was measured using a micro-plate spectrophotometer.

HUVEC Tube Formation Assay. Tube formation assay was performed using BD Matrigel (Becton, Dickinson and Co., Tokyo, Japan). Briefly, solid gels were prepared according to the manufacturer's manual on a 96-well tissue culture plate. HUVECs (1×10^5 cells/mL) in HuMedia EG-2 medium containing 0−25 μM of fucoxanthin was seeded 100 μL per well onto the surface of the solid gel, BD Matrigel. After 12 h in culture, tube formation was observed under an inverted light microscope at 40× magnification. Microscopic fields were photographed with a COOLPICKS 950 digital camera (Nikon). The total length of tube structures in each photograph was measured using Adobe Photoshop software.

HUVEC Chemotaxis Assay. This assay was carried out by a modified Boyden chamber assay (*21*). The microporous membrane (8 μm) of 24-well cell culture inserts (BD Biosciences, MA) were coated with 0.1% gelatin. HUVECs were detached with cell dissociation buffer (Invitrogen Corp., Carlsbad, CA), collected by centrifugation, resuspended in Medium 199 (Invitrogen) with 0.1% bovine serum albumin (BSA), and seeded in triplicate in the chamber (1.0×10^5 cells/400 μL). The well was filled with 400 μL of Medium 199 containing 0.1% BSA and 10 ng/mL of VEGF with or without fucoxanthin. The assembled chamber was cultured for 6 h. Nonmigrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol and stained with Diff-Quik stain (Sysmex, Kobe, Japan). Migrated cells were counted in five fields of each membrane under the microscope at 200× magnification, and the average number of a field was calculated.

Differentiation of Embryoid Bodies and Treatment with Fucoxanthin. Mouse embryonic stem (ES) cells (129SV) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). ES cells passages 15 were used throughout this study. The experimental method was modified according to Wartenberg et al. (*22*) and Wobus et al. (*23*). To differentiate ES cells and to form an embryoid body (EB), ES cells were suspended in αMEM medium containing 10% FBS, 100 μM 2-mercaptoethanol, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10 ng/mL of VEGF. EBs were formed by the hanging drop method. Hanging drops containing 2.5×10^3 cells in 50 μL of the medium were maintained for 3 days on the lids of bacterial grade Petri dishes filled with phosphate-buffered saline. The EBs formed were transferred onto the cover glass coated type I collagen in a 12-well culture plate and maintained with the medium for 5−7 days to develop vascular structures. To evaluate the antiangiogenic effect on EBs, fucoxanthin was added to the medium after EBs were transferred onto the collagen-coated plate. The EBs were treated with fucoxanthin from day 4 to day 8, then fixed and stained with PE-labeled anti-CD31 (PECAM-1) monoclonal antibody (BD Biosciences), following the observation by a LSM 410 confocal laser scanning microscopy (Zeiss).

Assessment of Angiogenesis in EBs. Antibody staining was performed by the slightly modified method of Wartenberg et al. (*22*). PE-labeled anti-CD31 monoclonal antibody was used at a concentration of 2.5 μg/mL. EBs were fixed in ice-cold methanol/acetone (7:3) for 30 min at −20 °C and washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Blocking against nonspecific binding was performed for 60 min with 10% fat-free milk powder dissolved in PBS. Subsequently, EBs were incubated for 2 h at room temperature with anti-CD31 monoclonal antibody dissolved in PBS with 10% fat-free milk powder. EBs were then washed three times with PBS. Angiogenesis (blood vessel-like structures) was evaluated by scoring under the observation by confocal laser scanning microscopy. EBs with blood vessel-like structures formed with CD31 positive cells were counted as positive, and the percentage of positive EBs against the total EBs used was calculated as an indicator of neovascularization.

Ex Vivo Angiogenesis Assay. Six-weeks-old male Wistar rats (Clea Japan, Inc., Tokyo, Japan) were housed two to a metal cage in a room with controlled temperature (24 ± 1 °C) and a 12-h light:dark cycle (lights on, 08:00−20:00 h). They had free access to diets and deionized water. The rats were maintained according to the Guide for the Care and Use of Laboratory Animals established by Okayama Prefectural University. The ex vivo angiogenesis assay was performed according to slightly modified methods as described previously (*24*, *25*). Briefly, a male Wistar rat (body weight ≈ 200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. A thoracic aorta was removed and washed with RPMI 1640 medium to avoid contamination with blood. It was then turned inside out and cut into short segments of about 1−1.5 mm. Collagen gel (gel matrix solution) was made with 8 volumes of porcine tendon collagen solution (3 mg/mL) (Cellmatrix Ia, Nitta Gelatin Co., Osaka, Japan), 1 volume of 10 × Eagle's MEM (Gibco, NY), and 1 volume of reconstitution buffer (80 mM NaOH and 200 mM HEPES). These solutions were mixed gently at 4 °C. Each aortic segment was placed in the center of a well on a 6-well culture plate and covered with 0.5 mL of gel matrix solution reconstituted as described. The solution was allowed to gel at 37 °C for 20 min, and then overlaid with 2 mL of RPMI 1640 medium containing 1% of ITS+ (Becton Dickinson Labware, MA). Sample solution or vehicle was then added. Incubation was carried out for 10 days in a fully humidified system of 5% CO_2 in the air at 37 °C. The medium was changed on day 7 of the culture. An estimation of the length of the capillary was performed under phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate mean point of capillary. Microscopic fields were photographed with a COOLPICKS 950 digital camera (Nikon). The length of the capillary was measured using Adobe Photoshop software.

Statistical Analysis. Values are presented as means ± SEM. Data

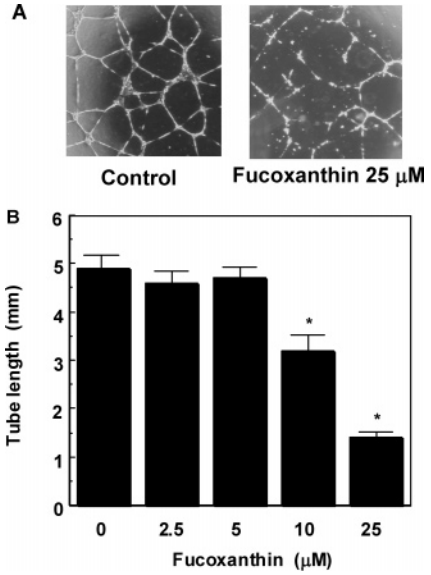

Figure 2. Effect of fucoxanthin on HUVEC tube formation on reconstituted basement membrane gel. (**A**) Cells were plated on reconstituted gel and observed 12 h later. (**B**) Capillary length was measured, and values are means ± SEM ($n = 3$). The values marked with an asterisk are significantly different from control values, $p < 0.05$.

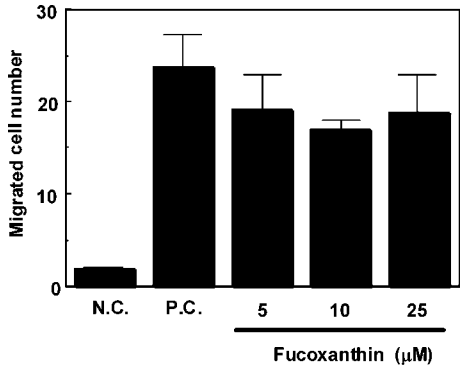

Figure 3. Effect of fucoxanthin on HUVEC chemotaxis. HUVECs migrated after 6 h incubation to lower surface of the filter were counted in five 200× fields of a filter. Means of a field of three filters ± SEM ($n = 3$) are shown. N. C., medium without VEGF and fucoxanthin (negative control). P. C., VEGF containing medium without fucoxanthin (positive control). No significant inhibitory effect on HUVEC chemotaxis was observed.

were analyzed by one-way analysis of variance with Dunnett's test to identify significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Fucoxanthin on HUVEC Functions. The effect of fucoxanthin on HUVEC tube formation as an in vitro angiogenesis model was examined. HUVECs on reconstituted basement membrane migrated, attached to each other, and formed tube structures (**Figure 2A**). Fucoxanthin significantly reduced the tube length of HUVEC at more than 10 μM (**Figure 2B**).

To elucidate the antiangiogenic mechanism, we examined the effect of fucoxanthin on HUVEC migration on gelation-coated Boyden chambers (**Figure 3**). VEGF strongly stimulated HUVEC migration, but fucoxanthin had no effect on HUVEC migration in the presence of VEGF. Fucoxanthin applied to HUVEC proliferation assay, because many angiogenesis inhibitors suppress endothelial cell proliferation. Fucoxanthin inhibited HUVEC proliferation in a dose-dependent manner, and significant differences are found between control and fucoxanthin treated HUVECs (10−100 μM) (**Figure 4**). Taken together, antiangiogenic activity of fucoxanthin would be due to inhibiting endothelial cell tube formation and proliferation but not migration.

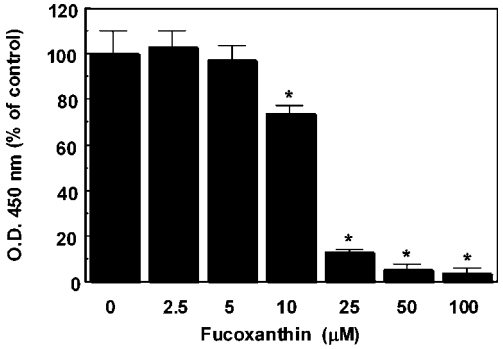

Figure 4. Effect of fucoxanthin on HUVEC proliferation. Values are means ± SEM ($n = 3$). The values marked with an asterisk are significantly different from control values, $p < 0.05$.

Effect of Fucoxanthin on Angiogenesis in Embryonic Stem Cell-Derived Embryoid Bodies. To clarify whether fucoxanthin affects differentiation of endothelial progenitor cells into endothelial cells, its effect on angiogenesis in embryonic stem cell-derived embryoid bodies (EBs) was investigated by evaluating the formation of blood vessel-like structure by CD31-positive cells. **Figure 5** shows the representative result of vessel-like structure formation in EBs. Fucoxanthin effectively suppressed development of blood vessel-like structures in EBs (93.8% for nontreated control, $n = 32$; 73.9% for 10 μM fucoxanthin, $n = 16$; 43.8% for 20 μM fucoxanthin, $n = 46$). This result was consistent with the observation on HUVEC assays.

Ex Vivo Angiogenesis Assay. The effects of fucoxanthin and fucoxanthinol, which is a deacetylated product of fucoxanthin, on angiogenesis were examined in a rat aortic ring angiogenesis model. This method is widely used as a useful one to evaluate antiangiogenic agents in a complex system in which endothelial cells, fibroblasts, pericytes, and smooth muscle cells interact (*26*−*28*). Fibroblastic fusiform cells migrated from the ends of the aortic rings after 2−3 days, and then they spread in the collagen gel. Microvessels appeared from the ends of aortic rings after 5−6 days and elongated (**Figure 6**). Fucoxanthin and fucoxanthinol strongly suppressed outgrowth of microvessels in a dose-dependent manner (**Figure 7**).

The present study revealed the antiangiogenic activity of brown algae fucoxanthin. We demonstrated that fucoxanthin exerted the antiangiogenic activity through suppressing HUVEC tube formation and endothelial cell proliferation, but it did not affect migration. The mechanisms by which antiangiogenic compounds suppress endothelial cell proliferation are very complex and not fully understood yet. It has been reported that antiangiogenic compounds affect receptor phosphorylation, protein kinase C, tyrosine kinase, and phosphoinositide 3-kinase (*29*, *30*). Cell cycle and matrix metalloproteinases are also their targets (*31*, *32*). Das et al. (*14*) reported that fucoxanthin induces cell cycle arrest in colon cancer cells during the G0/G1 phase, which is mediated through the up-regulation of p21WAF1/Cip1. In the present study, fucoxanthin inhibited endothelial cell proliferation but not migration by VEGF. VEGF is one of the most important players that regulate vessel formation (*33*). VEGF receptor-2 is the predominant receptor in angiogenic signaling and regulates cell migration (*34*). However, fucoxanthin might not affect the signal transduction by VEGF receptor-2, because it had no effect on VEGF-induced HUVEC

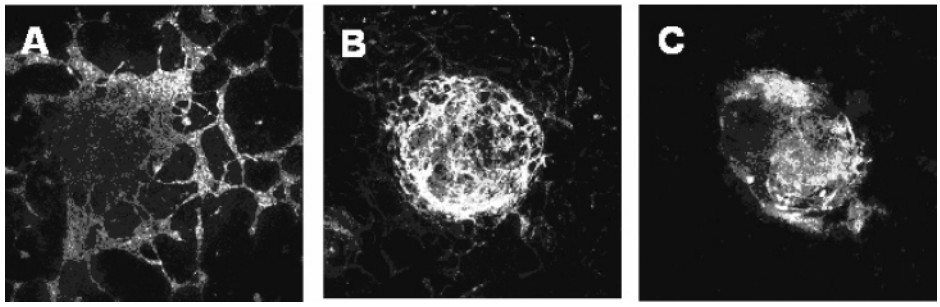

Figure 5. Representative results of the suppressive effect of fucoxanthin on vessel-like structure formation in EBs. EB at day 8 was stained with PE-labeled anti-CD31 antibody. Blood vessel-like structures were observed (**A**). EB subcultured on collagen gel in the presence of 10 μM (**B**) and 20 μM (**C**) fucoxanthin at day 8.

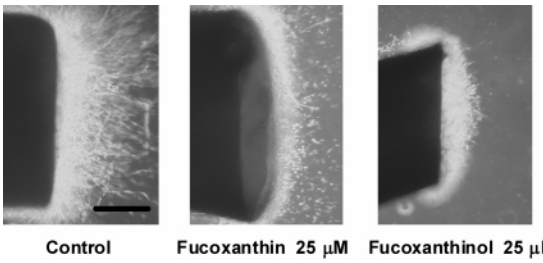

Figure 6. Representative results of the inhibitory effect of fucoxanthin (25 μM) or fucoxanthinol (25 μM) on ex vivo angiogenesis using a rat aortic ring. Bar equals 500 μm.

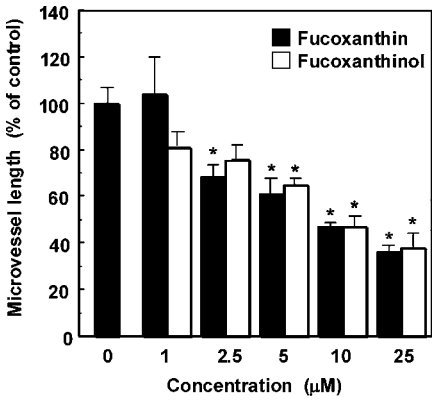

Figure 7. Effect of fucoxanthin (black bar) and fucoxanthinol (white bar) on ex vivo angiogenesis using a rat aortic ring. Microvessel length was measured on day 7 of culture. Values are means ± SEM (n = 5–12). The values marked with an asterisk are significantly different from control values, $p < 0.05$.

migration. The antioxidant activity of fucoxanthin might be involved in the antiangiogenic effect, because reactive oxygen species (ROS) stimulate angiogenesis (*35*, *36*). The molecular mechanism of the antiangiogenic effect of fucoxanthin should be addressed in a future study.

Recent evidence shows that endothelial progenitor cells (EPCs) in blood circulation also participate in neovascularization (*37*). EPCs are thought to be important targets to prevent new blood vessel formation (*38*). Therefore, the effect of fucoxanthin on EPC differentiation should be addressed. ES cells in EBs have been reported to differentiate into endothelial cells and form vascular-like structures (*22*, *39*). This EB model is recognized as an excellent model to examine the effects of antiangiogenic agents on neovascularization (*22*, *40*, *41*). In the present study, fucoxanthin at ≥10 μM suppressed blood vessel development in EBs. These results are consistent with their suppressive effects in ex vivo and in vitro angiogenesis assays.

Several studies indicated that fucoxanthinol, a deacetylated product of fucoxanthin, has a more potent antiproliferative effect via apoptosis induction in cancer cells than does fucoxanthin (*19*, *42*). Our result shows that fucoxanthinol also strongly suppressed outgrowth of microvessels in a dose-dependent manner. We previously reported that fucoxanthinol appears in plasma when mice ingest fucoxanthin and showed that the concentration of fucoxanthinol had reached approximately 30 μM in plasma after high dose oral administration (*19*). Thus, dietary fucoxanthin appears to serve as a bioactive component to suppress angiogenesis in vivo.

Various types of brown algae, such as hijiki (*Sargassum fusiforme*), kombu (*Laminaria japonica*), and wakame (*Undaria pinnatifida*), are staples in the diet of East Asians. The average intake of edible algae in Japanese is estimated to be 5.5 g/day. Based on this value, the average intake of fucoxanthin is calculated to be about 0.6 μmol/day. Thus, fucoxanthin in one meal served with brown algae would reach 10-fold the average intake. It has been reported on the basis of animal experiments that the consumption of seaweeds suppresses carcinogenesis (*43*, *44*). Fucoxanthin and its metabolites may contribute to suppression of cancer by dietary brown algae. Several studies have shown the anti-inflammatory and anti-obesity effect of fucoxanthin (*45*, *46*). Because it has been shown that antiangiogenic agents have an anti-obesity effect (*47*, *48*), fucoxanthin exerts its anti-obesity effect at least in part through its antiangiogenic activity as shown here. Consequently, dietary fucoxanthin seems to be a useful food component for human health.

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