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Marine algal fucoxanthin inhibits the metastatic potential of cancer cells

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ABSTRACT

Metastasis is major cause of malignant cancer-associated mortality. Fucoxanthin has effect on various pharmacological activities including anti-cancer activity. However, the inhibitory effect of fucoxanthin on cancer metastasis remains unclear. Here, we show that fucoxanthin isolated from brown alga *Saccharina japonica* has anti-metastatic activity. To check anti-metastatic properties of fucoxanthin, *in vitro* models including assays for invasion, migration, actin fiber organization and cancer cell–endothelial cell interaction were used. Fucoxanthin inhibited the expression and secretion of MMP-9 which plays a critical role in tumor invasion and migration, and also suppressed invasion of highly metastatic B16-F10 melanoma cells as evidenced by transwell invasion assay. In addition, fucoxanthin diminished the expressions of the cell surface glycoprotein CD44 and CXC chemokine receptor-4 (CXCR4) which play roles in migration, invasion and cancer–endothelial cell adhesion. Fucoxanthin markedly suppressed cell migration in wound healing assay and inhibited actin fiber formation. The adhesion of B16-F10 melanoma cells to the endothelial cells was significantly inhibited by fucoxanthin. Moreover, in experimental lung metastasis *in vivo* assay, fucoxanthin resulted in significant reduction of tumor nodules. Taken together, we demonstrate, for the first time, that fucoxanthin suppresses metastasis of highly metastatic B16-F10 melanoma cells *in vitro* and *in vivo*.

1. Introduction

Metastasis can be defined that cancer separated from a primary tumor site forms new tumors in distant organs elsewhere in the body. The cancer-metastatic process consists of complex steps [1,2]. That is, cancer cells invade into tissues around the initial lesion, enter into the microvasculature of the lymph and blood systems, move through the lymphatic system and the bloodstream to microvessels of distant tissues such as liver, lung, bone and brain, arrest in small blood vessels around new tissues, extravasate from the circulation into the surrounding tissues, and finally proliferate to form secondary tumors in new sites [1–4]. In addition, approximately 90% of human cancer deaths results from metastasis [2,3]. Thus, to prevent and treat cancer metastasis, many researchers are trying to understand metastasis-related molecular mechanisms [4].

The marine carotenoid fucoxanthin can be found in marine brown seaweeds, the macroalgae and diatoms [5]. Previously, our group has isolated fucoxanthin from edible brown alga *Saccharina japonica* [6]. Peng's review paper has shown that fucoxanthin has antioxidant, anti-inflammatory, anticancer, anti-obese, anti-diabetic, anti-angiogenic and antimalarial activities [5]. However, the inhibitory role of fucoxanthin on cancer metastasis remains unclear although it has several biological and biochemical activities. In this study, we show that fucoxanthin inhibited invasion, migration, arrangement of actin filament and adhesion of metastatic cancer cells to endothelial cells among critical steps for metastasis. In addition, fucoxanthin reduced the number of tumor nodules on the surface of lung in B16-F10 melanoma metastasis model.

2. Materials and methods

2.1. Isolation of fucoxanthin

Marine algal fucoxanthin was isolated from *S. japonica* as described previously [6]. The powder of the whole plant of

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S. japonica (1.0 kg) was refluxed with methanol (MeOH) for 3 h (4 × 5L). The total filtrate was then concentrated to dryness *in vacuo* at 40 °C in order to render the MeOH extract (180.15 g). This extract was suspended in distilled water and then successively partitioned with CH_2Cl_2, EtOAc, *n*-BuOH to yield CH_2Cl_2 (18.19 g), the EtOAc (0.29 g) and *n*-BuOH (6.64 g) fractions respectively, as well as the water residue (93.09 g). The CH_2Cl_2 fraction (46.0 g) was first chromatographed through a silica gel column, using a mixed solvent of *n*-hexane and EtOAc (*n*-hexane:EtOAc; 20:1∼0:1 gradient) to obtain 18 subfractions (F01-F18). Repeated chromatography of the fraction 10 over silica gel column yielded fucoxanthin (12 mg). The purity of fucoxanthin was checked by HPLC (⩾99%). Structure of fucoxanthin compound is given in Fig. 1A.

2.2. Cell culture

Highly metastatic murine B16-F10 melanoma cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 200 mg/L L-glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA) and U/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich) in a humidified incubator with 37 °C, 5% CO_2 prior to experimentation. Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA), and were cultured in sterile endothelial cell medium (ECM, ScienCell Research Laboratories) and were maintained at 37 °C in a humidified 5% CO_2 incubator. Passages 5–8 of HUVECs were used in monolayer cell adhesion assay.

2.3. Cell viability assay

The cytotoxicity caused by fucoxanthin was examined with MTT assay. In brief, B16-F10 cells were cultured in 24-well plates with or without fucoxanthin (indicated concentrations) for 24 h. After Griess reaction, MTT solution (2.0 mg/mL) was added to each well of cells. At 4 h after incubation at 37 °C in a CO_2 cell culture incubator, the supernatants were removed, and formazan crystals formed in viable cells were measured at 540 nm with a microplate reader. The percentage of living cells was calculated against untreated cells.

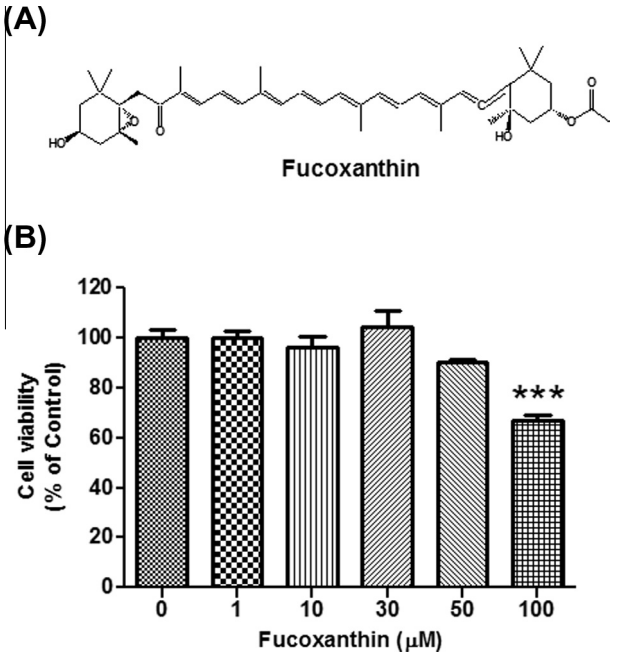

Fig. 1. The effect of fucoxanthin on viability of B16-F10 melanoma cells. (A) Chemical structure of fucoxanthin isolated from *S. japonica*. (B) B16-F10 cells were treated with the indicated concentrations of fucoxanthin. At 24 h after the treatment, cell viability was estimated using an XTT assay. Data represent the mean ± SD of 3 independent measurements. ***$P < 0.001$ vs. the untreated control.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from each cell using the TRIzol method (Invitrogen, CA). First-strand cDNA was synthesized using oligo-dT primer and AccuPower® RT-PreMix (Bioneer Co., Daejeon, Korea). The cDNA was amplified by PCR with the following primers: MMP-9, 5′-AGTCCGGCAGACAATCCTTGCA-3′ (sense) and 5′-ATCCACGCGAATGACGCTCTGG-3′ (antisense); CD44s, 5′-GTATCTCCCGGACTGAGGCA-3′ (sense) and 5′-CGGTCCATCGAAGGAATTGG-3′ (antisense); CXCR4, 5′-AGCCTGTGGATGGTGGTGTTTC-3′ (sense) and 5′-CCTTGCTTGATGACTCCCAAAAG-3′ (antisense); CXCR6, 5′-TACGATGGGCACTACGAGGGAG-3′ (sense) and 5′-GCAAAGAAACCAACAGGGAGACCAC-3′ (antisense); GAPDH, 5′-GGAGCCAAAAGGGTCATCAT-3′ (sense) and 5′-GTGATGGCATGGACTGTGGT-3′ (antisense). Amplified products were analyzed in 1.0% agarose gels under UV light, and the images were captured with GelDoc-It TS Imaging System (UVP, Upland, CA, USA).

2.5. Gelatin zymography

The cells were grown in serum-free conditioned medium with the indicated concentrations of fucoxanthin for 24 h. The amount of secreted protein in the conditioned media was estimated by cell numbers. Conditioned media were prepared in a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue without boiling. The samples were loaded in an acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin, and then electrophoresized at 4 °C. The gels were washed twice in 0.25% Triton X-100 for 30 min at room temperature and were incubated at 37 °C in the incubation buffer [50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 3 mM NaN_3 and 1 μM $ZnCl_2$]. At 18 h after incubation, the gels were stained for 30 min in 0.5% (w/v) Coomassie blue R-250 solubilized in 30% methanol and 10% acetic acid, and then incubated in a destaining solution with 30% methanol and 10% acetic acid. The stained gel was photographed.

2.6. Transwell invasion assay

For invasion assay, the upper sides of the filters were coated with 100 μL of 0.2% collagen-fragmented gelatin. Serum free DMEM with or without fucoxanthin (indicated concentrations) was added to the lower compartment of the chamber. The chambers were incubated at 37 °C for 24 h in a 5% CO_2 atmosphere. After incubation, the filter inserts were removed from the chamber well, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, stained with hematoxylin and eosin, and mounted on microscope slides. The cells located on the underside of the filter were counted for invaded cells.

2.7. Wound healing assay

The confluent B16-F10 cell monolayer in 6-well plate was wounded by manually scraping the cells with a cell scraper. And then, the cells were treated with fucoxanthin. After 24 h, cell migration into the wound surface was captured by microscopy with a Nikon DS-Fi1 camera (Nikon, Tokyo, Japan).

2.8. Actin staining and immunofluorescence microscope

B16-F10 cells, which were seeded on 12 mm π-sterilized coverslips in 24-well tissue culture plates, were treated with or without fucoxanthin (indicated concentrations) for 24 h. After washed with PBS, the cells were fixed in 3.7% formalin for 10 min. The cells were washed with PBS, and then permeabilized with 0.1% Triton X-100 in PBS. For actin staining, the cells were washed with PBS, and then incubated with PBS including 1uint of Texas Red®-X phalloidin (Invitrogen) for 30 min at RT. The cells were washed with PBS again, and were then analyzed using a fluorescence microscope.

2.9. Monolayer cell adhesion assay

HUVECs were confluently cultured in 12 well plate and stimulated with or without 10 ng/mL TNF-α (R&D systems, Minneapolis, MN, USA) for 8 h. B16-F10 cells treated with or without fucoxanthin (indicated concentrations) for 24 h were added on a monolayer culture of TNF-α-unstimulated or stimulated HUVECs, incubated for 20 min at 37 °C with rotation at 40 rpm in shaking incubator, and washed to exclude non-specific cell to cell interaction. The attached cells were visualized using a microscope.

2.10. Experimental lung metastasis assay

One day before injection of B16-F10 melanoma cells, 5 of C57BL/6 mice (6-week-old male, OrientBio Co., Osan, Korea) were intraperitoneally injected with and without 100 μl of fucoxanthin (1 mg/mL) dissolved in ethanol/corn oil (1:9 v/v) solution. B16-F10 melanoma cells (2×10^5) suspended in 100 μl PBS were injected into the tail vein of fucoxanthin-untreated and pretreated mice. On injecting B16-F10 cells, 5 mice of group pretreated with fucoxanthin were directly injected with 100 μl fucoxanthin. Continuously, 5 mice pretreated with fucoxanthin were injected with fucoxanthin once every other day for 6 days. At 21 days after injection with B16-F10 cells, the mice were euthanized, and lungs from each mouse were isolated. Metastatic foci on lung surfaces were counted. The significance of difference between two groups (each $n = 5$) was presented by Student's t test. Data were considered significant at $P < 0.05$. The isolated lung tissues were fixed in phosphate-buffered 10% formaldehyde, embedded in paraffin, and cut into 4 μm section. The sections were then stained with hematoxylin and eosin.

2.11. Statistical analysis

The values from the proliferation, invasion and cell adhesion assays were calculated by the fold differences compared to the control and were expressed as mean ± SD. The differences between the mean values for the 2 groups were determined by one-way analysis of variance with a post hoc Dunnet's comparison. The minimum significance level was set at a P value of 0.05 for all the analyses. All the experiments were performed at least 3 times, independently.

3. Results and discussions

Metastasis is fundamental property of malignant cancer cells, and occurs through a series of sequential steps including invasion, intravasation, survival and translocation in the circulation system, extravasation and survival in new organ. Furthermore, metastasis causes about 90% of human cancer-associated deaths [2,3,7]. Thus, a number of researchers are investigating molecular mechanisms related to cancer metastasis, and searching drugs which can suppress metastatic potential of cancer. It has been reported that fucoxanthin isolated from edible brown algae which is used as a popular health food induces apoptosis of various cancer cells [6,8]. However, anti-metastatic effect of fucoxanthin remains

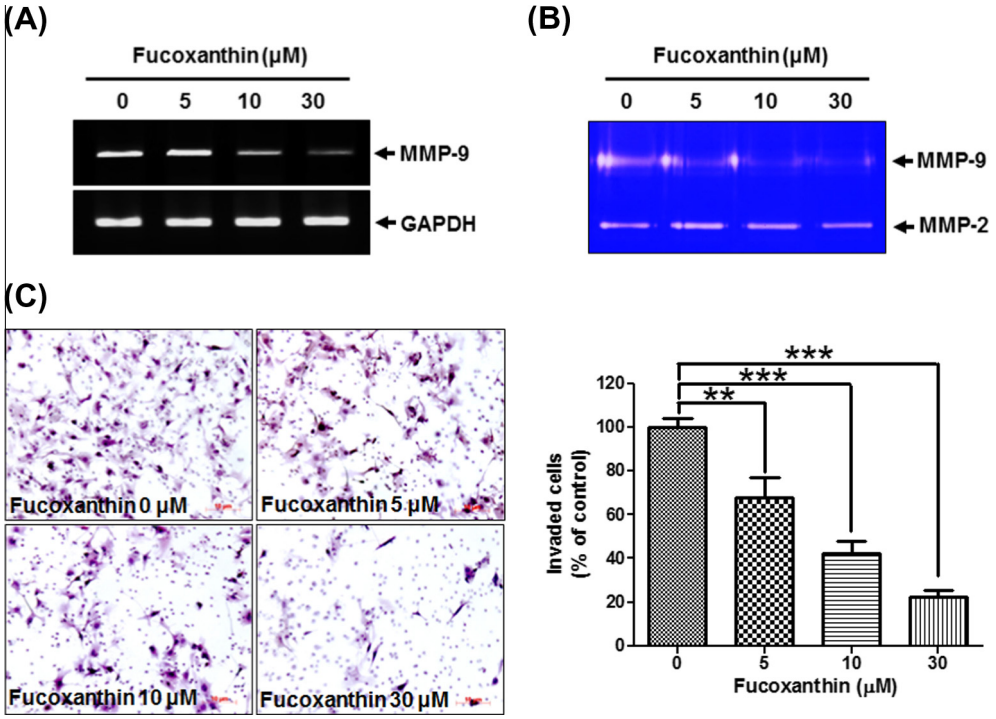

Fig. 2. The effect of fucoxanthin on the expression and activity of MMP-9 and invasion of B16-F10 melanoma cells. B16-F10 cells were treated with the indicated concentrations of fucoxanthin in serum free-conditioned medium for 24 h. The levels of MMP-9 mRNA and the activities of MMP-9 were determined by (A) RT-PCR and (B) gelatin zymography. (C) B16-F10 cells were treated with the indicated concentrations of fucoxanthin for 24 h and invasion assay was performed. The invaded cells were stained with hematoxylin and eosin, and counted. Data represent the mean ± SD of 3 independent measurements. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the untreated control.

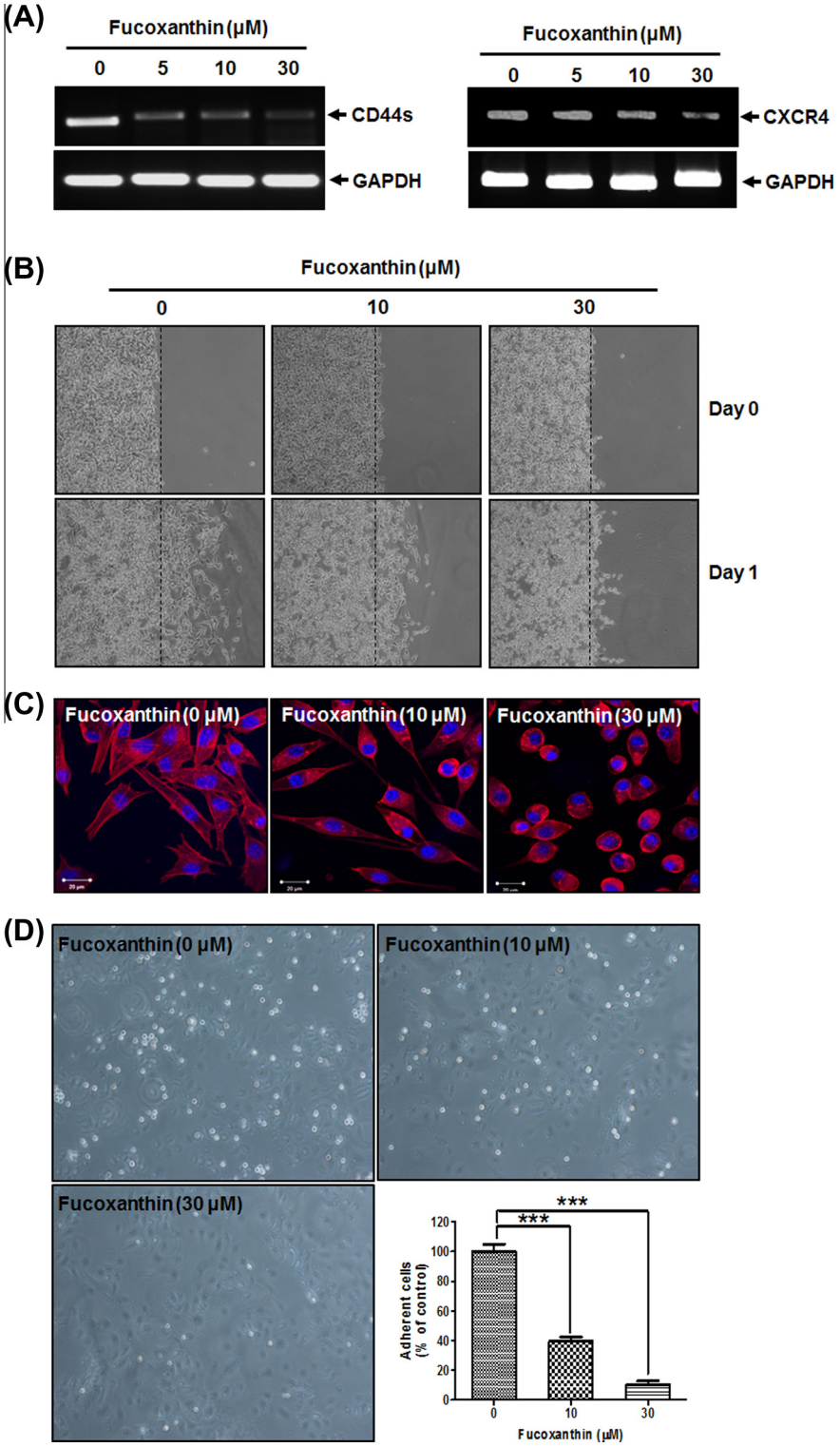

Fig. 3. The effect of fucoxanthin on cell motility, actin arrangement and interaction of cancer cell–endothelial cell through the regulation of CD44s and CXCR4 expression. (A) Total RNAs were isolated from B16-F10 cells treated with the indicated concentrations of fucoxanthin for 24 h. The expressions of CD44s and CXCR4 mRNA were analyzed by RT-PCR. (B) The confluent B16-F10 cell monolayer was wounded by a cell scraper and the cells were treated with the indicated concentrations of fucoxanthin for 24 h. The cells migrated on wound area were photographed. (C) B16-F10 cells treated with the indicated concentrations of fucoxanthin for 24 h were fixed, and stained with Texas Red-X phalloidin. The Texas Red-X phalloidin stained cells were captured by fluorescence microscopy with a camera. (D) B16-F10 cells treated with the indicated concentrations of fucoxanthin for 24 h were added on TNF-α-stimulated HUVECs, and was incubated for 20 min at 37 °C with rotation at 40 rpm in a shaking incubator. The attached cells were then visualized using a microscope. Data represent the mean ± SD of 3 independent measurements. $^{***}P < 0.001$ vs. the untreated control.

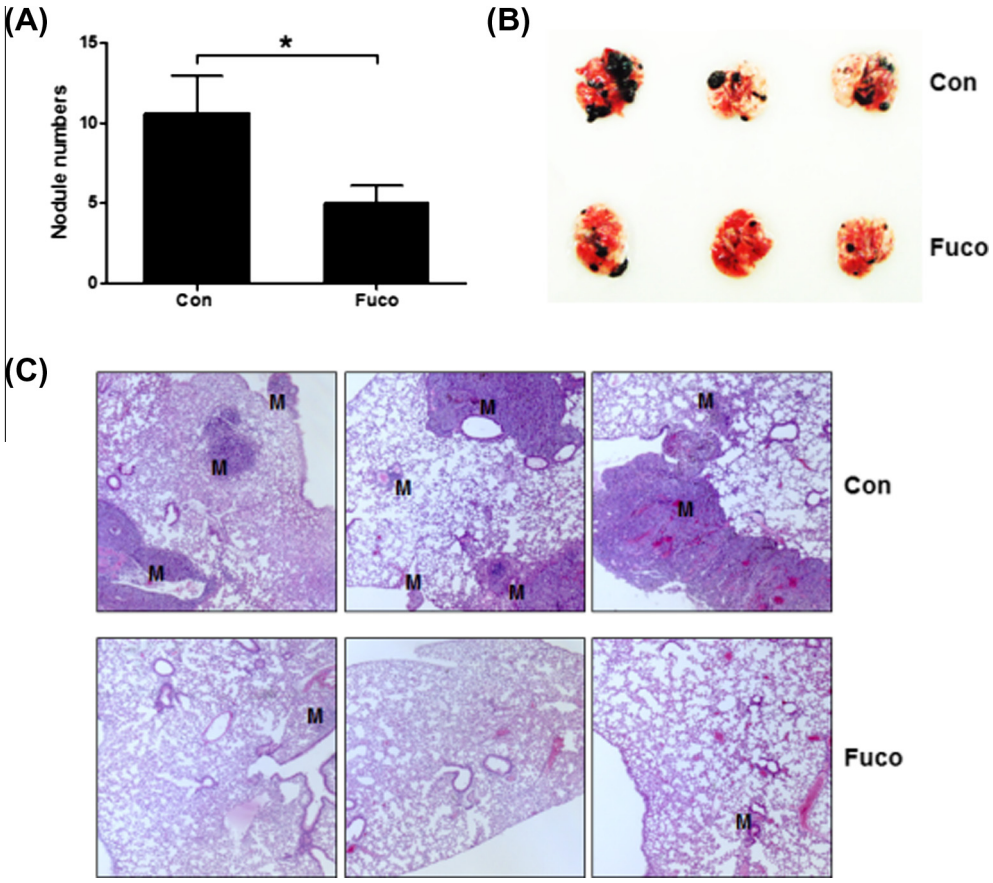

Fig. 4. The effect of fucoxanthin on experimental lung metastasis with B16-F10 melanoma cells. As described in Section 2, to perform experimental lung metastasis assay, B16-F10 melanoma cells were injected into tail veins of C57BL/6 mice with or without intraperitoneal injection of fucoxanthin. The mice were continuously injected with fucoxanthin once every other day for 6 days. At 21 days after injection with B16-F10 cells, mice were sacrificed. Metastatic nodules on lung surface were counted (A), and photographed (B). $^{*}P < 0.05$ vs. the untreated control. (C) Lung tissue sections were analyzed histopathologically after H & E staining. Metastatic foci in lung tissue sections are indicated by M.

unclear although it has anti-tumor activities in various cancer cells. Here, we checked whether fucoxanthin can affect invasion, migration, arrangement of actin filament and adhesion of cancer cells to endothelial cells. First of all, we initially checked whether fucoxanthin has effect on the viability of B16-F10 cells using MTT assay. As shown in Fig. 1B, fucoxanthin did not have significant cytotoxic activity up to 30 μM, thus we used fucoxanthin levels up to 30 μM for subsequent experiments.

For cancer invasion, matrix metalloproteinases (MMPs) are well known to help cell migration through the basement membranes to reach the circulation system, and the remigration of tumors at metastatic sites by degrading extracellular matrix (ECM) and basement membranes for intra and extravasation [7,9]. Among MMPs, MMP-2 and MMP-9, which are expressed in various cancer cells, degrade type IV collagen, a major constituent of the basement membrane during cancer invasion [7]. We investigated whether fucoxanthin regulates the expression and secretion of MMP-9. As determined by RT-PCR and zymography, fucoxanthin markedly decreased expression levels of MMP-9 mRNA and the secretion levels of MMP-9 protein in a dose-dependent manner, but not MMP-2 (Fig. 2A and B). Furthermore, a transwell assay was applied to investigate whether fucoxanthin represses the invasion ability of highly metastatic B16-F10 cells. As shown in Fig. 2C, fucoxanthin resulted in a significant decrease in the number of invaded B16-F10 cells. These results suggest that fucoxanthin has inhibitory effect on invasiveness of cancer cells by suppressing expression of gelatinolytic enzyme MMP-9.

The cell surface glycoprotein CD44 and CXCR4 among CXC chemokine receptors (CXCR)s are known to play principal roles in cancer metastasis, and also often highly expressed in various cancer including melanoma cells [10–12]. It has been reported that CD44 has effects on cytoskeleton reorganization, migration of cancer cells [13]. Previous reports have shown that the chemokine stromal cell-derived factor-1 (SDF-1)/CXCR4 axis enhances the migration of tumor cells into the metastatic sites in variety of cancers [14,15], and also induces MMP-9 expression for tumor invasion and metastasis [16]. In the metastatic B16-F10 melanoma cells expressing CD44 and CXCR4, we checked whether fucoxanthin can inhibit expression of CD44 and CXCR4. Interestingly, levels of CD44 and CXCR4 mRNA expression were reduced by fucoxanthin in a dose-dependent manner as evidenced by RT-PCR (Fig. 3A). Thus, we further checked inhibitory effect of fucoxanthin on migration of melanoma cells using wound healing assay. As shown Fig. 3B, fucoxanthin clearly suppressed motility of melanoma cells compared to the marked increase of cell migration on wound area in fucoxanthin-untreated control. In our previous data, due to inhibitory effect of fucoxanthin on cell motility, we also investigated whether fucoxanthin affects actin organization of melanoma cells. As shown in Fig. 3C, fucoxanthin resulted in the remarkable reduction of stress fiber and lamellipodia formation for cell migration. It is known that CD44 expressed on cancer cells binds to P-, L- and E-selectins on tumor-associated immune and endothelial cells activated by cytokines, which is associated with extravasation of malignant cancer cells for metastasis [17,18].

Furthermore, CXCR4 expression induces adhesion of melanoma cells to lung microvascular endothelial cells for the pulmonary metastatic potential [19]. Since our data showed that fucoxanthin decreased CD44 and CXCR4 expression in B16-F10 melanoma cells, next, we examined whether fucoxanthin inhibits cell adhesion of B16-F10 cells to endothelial cells stimulated by inflammatory cytokine TNF-α. As shown in Fig. 3D, fucoxanthin significantly reduced number of adherent B16-F10 cells to endothelial cells treated with TNF-α compared to fucoxanthin-untreated B16-F10 cells.

On the basis of our findings from *in vitro* metastasis models, we further have experiment about *in vivo* lung metastasis assay. Fucoxanthin significantly inhibited lung metastasis of B16-F10 melanoma cells injected into tail veins of C57BL/6 mice compared to fucoxanthin-untreated group, indicating that fucoxanthin reduced metastatic foci of lung surface in experimental lung metastasis model (Fig. 4A and B). Microscopic analysis of lung tissue sections also showed that metastatic nodule number was clearly decreased by fucoxanthin (Fig. 4C).

In conclusion, we demonstrate, for the first time, that fucoxanthin suppresses metastatic potential through inhibition of MMP-9-mediated tumor invasion, formation of stress fiber for cell migration and interaction of cancer cell–endothealial cell in multiple steps for tumor metastasis via down-regulation of MMP-9, CD44 and CXCR4 expression, and might be value in preventing cancer metastasis.

Authorship

T.W.C., H.J.C. and K.T.H. designed research; T.W.C., H.J.C. and J.Y.L. performed research; T.W.C., H.J.C., H.S.J., C.H.K., M.J., C.W.H., S.Y.K., J.S.C and K.T.H. analyzed data; J.S.C contributed new reagent; T.W.C., H.J.C. and K.T.H. wrote paper.

Acknowledgments

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