

Potential chemoprevention effect of dietary fucoxanthin on urinary bladder cancer EJ-1 cell line

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Abstract. Bladder cancer is a common but serious malignancy. It is widely accepted that chemoprevention may be an effective way to decrease the rate of recurrence and morbidity. We first determined antigrowth and apoptosis-induction activity of fucoxanthin from dietary *Laminaria japonica* against EJ-1 human bladder cancers. Fucoxanthin significantly reduced the cell viability in a dose- and time-dependent manner. The induction of apoptosis in EJ-1 cells was characterized by morphological changes, DNA ladder, and increased percentage of hypodiploid cells, activating caspase-3 activity. The ratio of apoptotic cells reached >93% after treatment for 72 h with 20 μ M fucoxanthin. The findings obtained indicate that fucoxanthin may act as a chemopreventive and/or chemotherapeutic carotenoid in bladder cancer cells by modulating cell viability.

Introduction

Bladder cancer is not only a significant public health problem responsible for >130,000 deaths annually worldwide but also the most expensive cancer to survey and treat because of the need for frequent interval cystourethroscopy, urine cytology and radiological evaluations (1,2). Bladder carcinogenesis is considered to be linked to diets with high animal fat intake (3-6). In contrast, a number of studies have suggested that high consumption of fruits and vegetables decreases the risk of bladder cancer (6-8). Although the anti-angiogenic activity of food components has received increased attention as a strategy

for bladder cancer prevention, there are too few or conflicting studies to formulate accurate guidelines, such as vitamins C and E and selenium, studies demonstrating benefits are balanced by studies demonstrating no benefits (9-15). Other natural products, such as soy, green tea and isothiocyanates, have been suggested by some studies to be protective and by others to be tumor-promoting (16-20).

Fucoxanthin is a major carotenoid of edible brown algae (21). There is a wealth of information pertaining to fucoxanthin-induced apoptosis in cancer cell lines (22-28). Cells undergoing apoptosis are recognized and engulfed by macrophages without damage to neighboring cells (29). Therefore, apoptosis-inducing activities are expected to provide a novel means of chemoprevention and chemotherapy in the treatment of cancer. However, little information is available on the potential of fucoxanthin to prevent bladder cancer. In the present study, we obtained clear evidence of apoptosis induction and antiproliferative effects of fucoxanthin on EJ-1 human bladder cancer cells.

Materials and methods

Materials. Propidium iodide and WST-8 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hoechst 33258 was purchased from Calbiochem-Novabiochem Co. (San Diego, CA, USA). Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was purchased from CSL Limited (Parkville, Australia). 2',7'-Dichlorodihydro fluorescein (H₂DCFDA) was purchased from Molecular Probes Inc. (Eugene, Oregon, USA). An Apoptotic DNA ladder extraction kit was purchased from Bio-Vision Research Products (USA). An apoptosis caspase-3 colorimetric assay kit was purchased from MBL (Nagoya, Japan).

Preparation of fucoxanthin. Fucoxanthin was extracted and refined from brown algae (*Laminaria japonica*) as described previously (30). Briefly, 80% ethanol extract from brown algae was applied to a silica gel column (400 mm, 50 mm i.d.) and 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-

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rich fraction was further subjected to flash column chromatography on a LiChroprep RP-18 (40–63 μm , 11x24 mm, Merck) with acetonitrile/methanol/water (75:15:10) containing 0.1% ammonium acetate to isolate pure fucoxanthin. The purity of the fucoxanthin used in this study was >97% as determined by HPLC. The fucoxanthin was dissolved in ethanol and stored at -80°C .

Cell proliferation assay. EJ-1 cancer cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University. The cells were cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum, 4 mM l-glutamine, and antibiotics (100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged twice a week. To evaluate the effect of the fucoxanthin on the proliferation and viability of EJ-1 cancer cells, the cells were seeded at a density of 5×10^3 cells per well containing 100 μl of culture medium in 96-well plates, and the medium was replaced with fresh medium supplemented with the fucoxanthin of different concentrations after cultivation for 24 h and further cultivated for the indicated period. The fucoxanthin dissolved in ethanol was added to the culture medium at a final concentration of 22.5, 20, 12.5 and 6.25 μM , and the final concentration of ethanol in the culture medium was 0.5% (v/v). The control culture received only ethanol at a concentration of 0.5% (v/v) (vehicle alone). After further incubation, 10 μl of WST-8 reagent was added to each well of a 96-well plate and incubated for 4 h at 37°C . The number of viable cells was monitored with absorbance at 450 nm. The wells without the fucoxanthin and the free cells (culture medium alone) were used as blanks.

Morphological study of EJ-1 apoptotic cells. The cells were seeded in six-well plates at a density of 2×10^5 cells/ml containing 2 ml of the medium and treated with fucoxanthin as described above for the WST-8 assay. Morphological changes were observed after exposure to 20 μM fucoxanthin for 72 h using a phase-contrast microscope. Chromatin condensation and nuclei fragmentation were observed using a fluorescence microscope after staining with Hoechst 33258 (5 $\mu\text{g/ml}$).

Agarose gel electrophoresis. The cells were seeded in a six-well plate at a density of 2×10^5 cells/ml and treated with fucoxanthin as described above for the WST-8 assay. After exposure to 20 μM fucoxanthin for 72 h, the treated cells were harvested using trypsin and washed twice with PBS, and the DNA of apoptotic cells was extracted according to the method of the Apoptotic DNA ladder extraction kit. The DNA ladder was detected by agarose gel electrophoresis.

Quantitative analysis of EJ-1 apoptotic cells. PI staining was used for quantitative analysis of apoptosis. Briefly, the cells were seeded in six-well plates at a density of 2×10^5 cells/ml and treated with fucoxanthin as described above for the WST-8 assay. After exposure to 20 μM fucoxanthin for the indicated period, a total of 1×10^6 cells were harvested using trypsin, washed twice with PBS, fixed with cold 70% ethanol in PBS for 1 h at 4°C , re-suspended in 3 ml PBS for 5 min, filtered with a 400 mesh sieve, stained with 1 ml 100 $\mu\text{g/ml}$ of PI at 4°C for 30 min in the dark, and subjected to flow cyto-

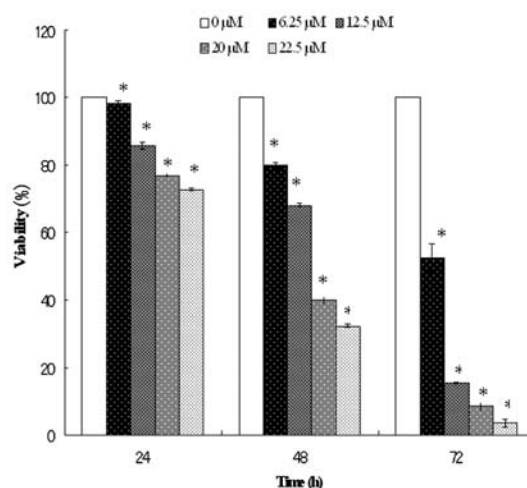


Figure 1. Fucoxanthin inhibits the proliferation of EJ-1 cancer cells. EJ-1 cells in 96-well plates were treated with fucoxanthin at the concentration indicated. Cell proliferation was measured using WST-8 assay at the indicated times. The number of viable cells is proportional to the absorbance. Data are means \pm SD for three independent experiments. The values marked with an asterisk are significantly different from control values, $P < 0.05$.

metry. The percentage of apoptotic cells was estimated using a BD FACS can flow cytometer and the data were obtained and analyzed with CellQuest.

Caspase activity assay. The cells were seeded in a six-well plate at a density of 2×10^5 cells/ml and treated with fucoxanthin as described above for the WST-8 assay. After exposure to 20 μM fucoxanthin for the indicated period, the cells were harvested using trypsin and washed twice with PBS. The caspase-3 activity was then determined using Caspase activity assay kits (MBL, Nagoya, Japan).

Intracellular reactive oxygen species assay. The level of intracellular reactive oxygen species (ROS) was determined by the change in fluorescence resulting from the oxidation of the fluorescent probe H_2DCFDA . Briefly, 5×10^5 cells/well were exposed to fucoxanthin at the indicated concentration for 24 h. After incubation, cells were washed once with MEM medium. Cells were incubated with 50 μM of the fluorescent probe H_2DCFDA for 1 h at 37°C . The degree of fluorescence, corresponding to intracellular ROS, was determined using Fluoroscan Ascent FL (Type 374, Labsystems, Finland) (excitation 485 nm; emission 538 nm).

Statistical analysis. Values are expressed as means \pm SD. The significance of the difference from the respective controls for each experiment test condition was assayed using Student's t-test analysis, with $P < 0.05$ or 0.01 considered significant.

Results

Antiproliferative effects of fucoxanthin on urinary bladder cancer EJ-1 cells. To test the effect of fucoxanthin on the proliferation of EJ-1 cancer cells, the cells were treated with different concentrations of fucoxanthin and different exposure time. The growth inhibition of EJ-1 cells was determined by

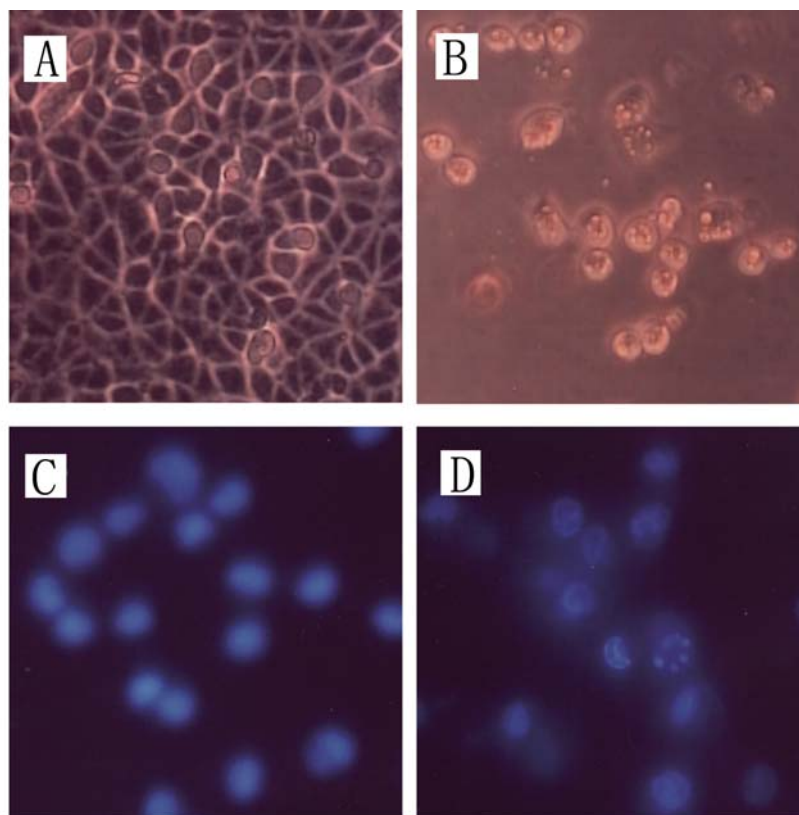


Figure 2. Apoptosis induced in EJ-1 cancer cells by fucoxanthin. Morphological changes observed by phase-contrast microscopy (A and B), by fluorescence microscopy with Hoechst 33258 staining (C and D). EJ-1 cells were incubated for 72 h in the presence of ethanol alone (A and C) and in the presence of 20 μ M fucoxanthin (B and D). Phase-contrast images and fluorescence images of the same fields are shown. Magnification x400.

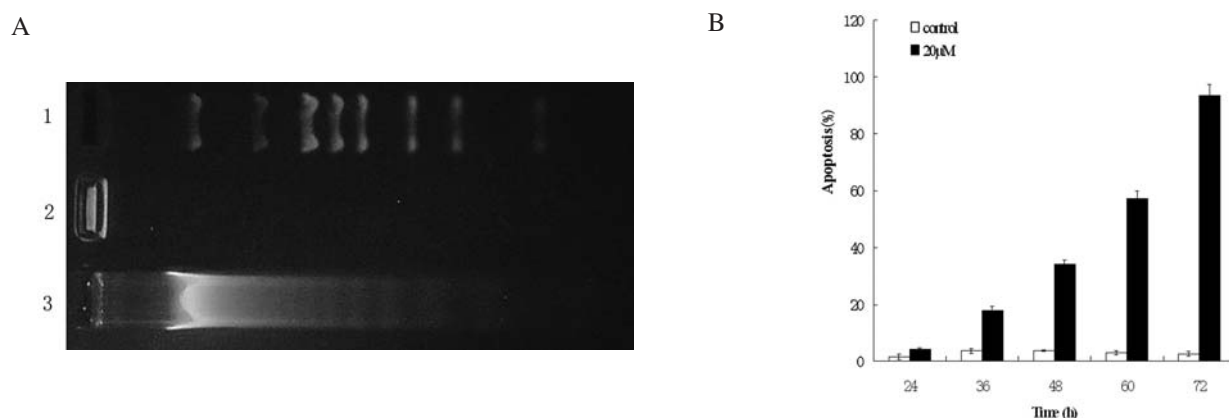


Figure 3. (A) Agarose gel electrophoresis of DNA extracted from EJ-1 cells treated with fucoxanthin. EJ-1 cells were incubated for 72 h in the presence of ethanol alone (lane 2) or 20 μ M fucoxanthin (lane 3). The DNA extracted from the cells and the DNA marker (λ -HindIII; lane 1) were subjected to agarose gel electrophoresis. (B) Quantitative analysis of apoptotic cells using flow cytometry in EJ-1 cells treated with ethanol alone and 20 μ M fucoxanthin.

using WST-8 assay. The amount of the yellow formazan dye generated by activity of dehydrogenase in cells is directly proportional to the number of living cells in a culture medium. The results indicated that fucoxanthin isolated from *Laminaria japonica* exhibited remarkable antiproliferative effects on EJ-1 cancer cells. The viability of EJ-1 cells treated with fucoxanthin decreased in a dose- and time-dependent manner from 24 to 72 h incubation (Fig. 1). After 72 h of incubation with 6.25 μ M (22.5 μ M) fucoxanthin, the number of viable cells decreased to 52.62% (3.56%) compared to the control.

Fucoxanthin induces apoptosis in EJ-1 cancer cells. To examine whether fucoxanthin inhibits the proliferation of EJ-1 cells by inducing apoptosis, we used Hoechst 33258 staining to observe the morphological changes in EJ-1 cells induced by fucoxanthin. Control cells exhibited round nuclei with well distributed chromatin, whereas typical apoptotic morphology characterized by condensed chromatin, nuclear fragmentation, and appearance of apoptotic bodies was demonstrated after 72 h exposure to 20 μ M fucoxanthin (Fig. 2). Furthermore, a DNA ladder, which is a characteristic feature of apoptotic cells,

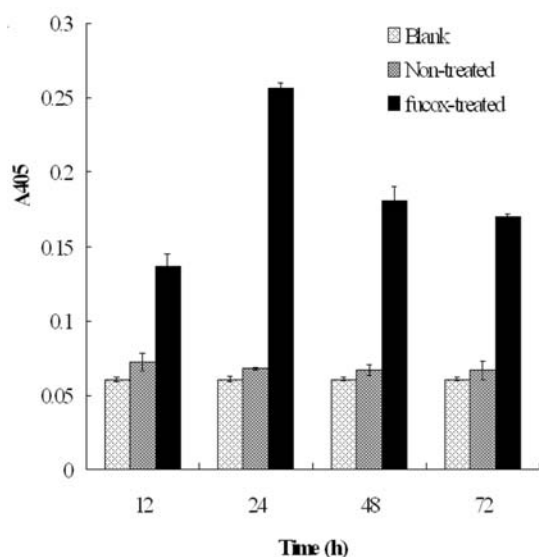


Figure 4. Caspase-3 activity in EJ-1 cells treated with 20 μ M fucoxanthin in a time-dependent manner.

was observed in EJ-1 cells treated with 20 μ M fucoxanthin for 72 h (Fig. 3A). To further characterize the apoptosis process quantitatively, the ratio of apoptotic cells was evaluated by FACS. The ratio of apoptotic cells increased significantly in a time-dependent manner compared to the control group (Fig. 3B). After 72 h incubation with 20 μ M fucoxanthin, the ratio of apoptotic cells exceeded 93% compared to the control.

Intracellular ROS levels. We investigated whether exposing EJ-1 cells to fucoxanthin could induce the generation of hydrogen peroxide in the cells. When EJ-1 cells were exposed to fucoxanthin for 24 h and treated with H₂DCFDA, the relative fluorescence intensity of the cells was 10.3 \pm 8.4 when treated with vehicle alone, 9.7 \pm 4.6 when treated with 10 μ M fucoxanthin, and 10.2 \pm 3.4% when treated with 20 μ M fucoxanthin, with no significant difference among the treatments.

Fucoxanthin induces activation of caspase-3. Activation of caspase cascades has been shown to occur in apoptosis (31). To clarify the apoptotic mechanism induced by fucoxanthin, the caspase-3 activity was detected after EJ-1 cells were treated with 20 μ M fucoxanthin for 12, 24, 48 and 72 h (Fig. 4). According to the calculated formula of caspase-3 activity as described in the Apocyto caspase-3 colorimetric assay kit, the calculated results indicated that the activity of caspase-3 was 2.6-fold that of the control after 12 h incubation with fucoxanthin. The maximal caspase-3 activity reached 6.1 times the activity of the control after treatment with fucoxanthin for 24 h, and then began to decrease to 3.9-fold of the control at late apoptosis.

Discussion

In the current study, we demonstrated for the first time that fucoxanthin remarkably reduces the viability and induces

apoptosis in the human urinary bladder cancer EJ-1 cell line based on cell viability assay, morphological observations, DNA ladder, FACS analysis, and assay of caspase-3 activity.

The induction of apoptosis is now considered to be an attractive strategy for cancer therapy (32,33). The caspases play a central role in the extrinsic and intrinsic pathways of apoptosis (34). One of the apoptosis-inducing pathways is known to be mediated through the release of cytochrome c from mitochondria to cytosol, followed by the activation of caspase-9 and -3. The activated caspase-3 is capable of cleaving many cellular substrates, including inhibitor of caspase-activated DNase (ICAD), Poly ADP-Ribose Polymerase (PARP; a DNA repair enzyme), and lamin. Once the ICAD is cleaved, CAD enters the nucleus and breaks chromatin into DNA fragments. Subsequent disassembly of the cell structure eventually leads to cell death (35,36). In the present study, activation of caspase-3 was identified in fucoxanthin-induced apoptosis of EJ-1 cells.

In addition, time-dependent DNA fragmentation was observed. Following EJ-1 cell incubation with 20 μ M fucoxanthin for 24 and 48 h, no DNA ladder was observed. However, a clear DNA ladder appeared after 60-72 h incubation. Meanwhile, the ratio of apoptotic cells determined by FACS quantitative analysis also increased in a time-dependent manner. These effects were correspondingly reflected in the reduction of cell viability. Internucleosomal degradation of DNA due to the activation of endogenous endonuclease occurs during apoptosis. These results indicated for the first time that fucoxanthin reduces the viability of human urinary bladder cancer cells by inducing apoptosis. A similar induction of apoptosis in prostate cancer cells has been reported for fucoxanthin, β -carotene, and cantaxanthin (37-39). Since fucoxanthinol exhibits stronger growth inhibition than fucoxanthin, the antiproliferative effect of fucoxanthin may be attributable to the action of its metabolites (40). In the present study, no increase of intracellular ROS was found in EJ-1 cells treated with fucoxanthin. Fucoxanthin was stable under cell culture conditions for 24 h incubation with the medium only (data not shown). These results suggest that neither the possible pro-oxidant action of fucoxanthin nor their oxidation products are involved in inducing apoptosis of EJ-1 cells.

Fucoxanthin has a unique structure including an unusual allenic bond and 5, 6-monoepoxide in its molecule. The structure of carotenoids may be important in reducing growth and in inducing apoptosis in cancer cells. Fucoxanthin may also regulate the redox signals, and then facilitate the progression of apoptosis through Bcl-2 protein suppression and caspase-dependent and -independent pathways (23). To our knowledge, fucoxanthin is the only dietary phytochemical reported to induce apoptosis in EJ-1 cells. *Scutellariae radix* was considered a promising cancer-preventing agent that inhibited the growth of EJ-1 cells by 50% at 64 μ g/ml after 96 h (41). Thus, dietary fucoxanthin would be a more effective agent than *Scutellariae radix* against some types of urinary bladder cancer EJ-1 cell lines. In conclusion, fucoxanthin decreased cell viability and induced apoptosis in the human urinary bladder cancer EJ-1 cell line. Our findings indicate the possibility of chemopreventive or chemotherapeutic effects of fucoxanthin on urinary bladder cancer.

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