Fucoxanthin Induces *GADD45A* Expression and G_1 Arrest with SAPK/JNK Activation in LNCap Human Prostate Cancer Cells

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Abstract. *Background/Aim: The antitumor effect of fucoxanthin, a marine carotenoid found in brown algae, was investigated on prostate cancer cells. Materials and Methods: LNCap prostate cancer cells were treated with fucoxanthin and the effects were evaluated in relation to cell proliferation, cell cycle, expression of growth arrest, DNA damage-inducible protein (GADD45) genes, and phosphorylation status of mitogen-activated protein kinases. Results: Fucoxanthin inhibited the growth of LNCap prostate cancer cells in a dose-dependent manner. Growth-inhibitory effects were accompanied by the induction of GADD45A expression and G_1 cell cycle arrest, but not apoptosis. Furthermore, fucoxanthin activated c-Jun N-terminal kinase (SAPK/JNK), while the inhibition of SAPK/JNK attenuated the induction of G_1 arrest and GADD45A expression by fucoxanthin. Conclusion: These results show that fucoxanthin induces G_1 cell cycle arrest in prostate cancer cells, and suggest that GADD45A and SAPK/JNK might be involved in these effects.*

Prostate cancer is one of the most common types of cancer in Western countries. It is well known that prostate tumor growth is stimulated by androgens, the controlled deprivation of which represents the base treatment for patients who cannot be treated by surgery or radiation therapy (1). However, after 1-3 years, most tumors re-grow independently of androgen and in a more aggressive manner. Recent findings indicate that the incidence of prostate cancer in Japan is increasing (2). Epidemiological research has shown that racial and ethnic backgrounds, and diet in particular, impact the risk for prostate cancer (3). In recent years, the chemoprevention of prostate cancer using dietary agents has been investigated (4, 5). Since prostate cancer typically occurs in elderly men, even a moderate effect on cancer using dietary agents could improve the quality of life of patients.

Carotenoids, such as lycopene, are promising agents for the prevention of prostate cancer. Some cohort and case -control studies have revealed that the consumption of lycopene is associated with lower risk of prostate cancer (5, 6). It was shown that lycopene has tumor-inhibitory activity in various cancer cell lines, including prostate cancer, with G_1 cell cycle arrest (7). It is well documented that carotenoids have general anticarcinogenic activity. Thus, it is of interest to analyze the preventive activity of carotenoids other than lycopene regarding prostate cancer progression. It was reported that fucoxanthin, a carotenoid mainly found in brown algae (Figure 1A), inhibited mouse duodenal tumor and skin tumor promotion, and inhibited the growth of cancer cells with G_1 cell cycle arrest and/or apoptosis (8-11). However, the precise mechanism of action of fucoxanthin remains unknown.

We previously reported that fucoxanthin induced expression of growth arrest and DNA damage-inducible protein alpha (GADD45A) and G_1 cell cycle arrest in human cancer cells HepG2 and DU145 (12). The GADD45 family play important roles in cell cycle progression, cell survival and apoptosis (13, 14). The GADD45 family of proteins consists of at least three members, A, B and G, the expression levels of which increase with genotoxic stress agents, terminal differentiation and growth arrest conditions (14-17). We have also shown that fucoxanthin activates mitogen-activated protein kinases (MAPKs), the activation of which differs depending on cell type (18). There are at least four subfamily members, comprising the extracellular signal-regulated kinases (ERK1/2), ERK5, p38 MAPK kinases, and the c-Jun *N*-terminal kinases (SAPK/JNK). MAPK family members have been implicated in playing roles in a variety of cellular processes, including cell growth, apoptosis and differentiation in response to environmental stimuli (19-21).

We have found that GADD45A and SAPK/JNK are partially involved in the action of fucoxanthin in androgen-independent DU145 prostate cancer cells (18). The purpose of the present study was to determine whether GADD45A and MAPK are involved in the action of fucoxanthin in the androgen-

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Key Words: Fucoxanthin, G_1 arrest, *GADD45*, SAPK/JNK, carotenoid, LNCap cells.

dependent prostate cancer cell line LNCap, the hormone dependency of which differs from the one of DU145 cells.

Materials and Methods

Chemicals. Fucoxanthin (Figure 1A) was kindly provided by Dr. A. Nagao (National Food Research Institute, 2-1-12, Kannondai, Tsukuba, Ibaraki, 305-8642, Japan) and dissolved in dimethyl sulfoxide (DMSO). Kinase inhibitors PD98059 (an ERK1/2 MAPK inhibitor), SB203580 (a p38 MAPK inhibitor) and SP600125 (a SAPK/JNK inhibitor) were purchased from Tocris Cookson Inc. (Avonmouth, UK). All other chemicals were of biological grade.

Cell culture. LNCap human prostate cancer cells (kindly provided by Dr. T. Miki, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo, Kyoto, 602-8566, Japan), were cultured in RPMI supplemented with 10% heat-inactivated FBS. Cells were maintained in an incubator at 37˚C under a humidified atmosphere comprising 5% CO_2. Cell viability was determined by the trypan blue dye exclusion method.

Inhibition of MAPK pathways. Cells were pre-treated with 60 μM of PD98059 (an ERK1/2 MAPK inhibitor), 10 μM of SB203580 (a p38 MAPK inhibitor) or 10 μM of SP600125 (a SAPK/JNK inhibitor) for one hour before the treatment with fucoxanthin. Control cells were treated with vehicle (DMSO) alone.

Flow-cytometric analysis. Cells were plated at a density of 1×10^5 cells/10 ml of medium in 10 cm-diameter dishes and treated with fucoxanthin at 2.5, 3.8 and 4.5 μM one day later. Cells were then harvested, washed with phosphate buffered saline without Ca^{2+} and Mg^{2+} and resuspended in 1 ml of 0.1% Triton-X 100 solution. Resuspended cells were filtered through a 50 μm nylon mesh and nuclei were stained using 20 μl of 2.5 mg/ml propidium iodide and 10 μl of 100 mg/ml RNase A. The cell cycle distribution was analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the ModFit LT™ program.

Northern blot analysis. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Twenty micrograms of total RNA was electrophoresed and then transferred onto a nylon membrane. Northern blots were hybridized with a ^{32}P-labeled probe, which was labeled with [^{32}P]dCTP by the random primer labeling method. After hybridization, the membranes were washed and then analyzed with a STORM imager (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK). Reverse Transcription Polymerase Chain Reaction was performed to generate each cDNA probe for the *GADD45A* and *GADD45B* genes. The PCR primers used were as follows: for *GADD45A*, 5'-GAAGGATCCTGCCTTA AGTCAAC-3' and 5'-CCTCTTTCCATCTGCAAAGTCATC-3' (278 bp); for *GADD45B*, 5'-GGACCCAGACAGCGTGGTCCT CTG-3' and 5'-AGGAAGGGAAGACAGTGGAGGTCT-3' (223 bp) (GenBank accession No. NM_001924 and NM_015675, respectively). The expression levels of genes were normalized to the one of 36B4.

Western blot analysis. Treated cells were solubilized using cell lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml protease inhibitor mix (leupeptin, aprotinin and pepstatin A), 50 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, and 20 mM *para*-nitrophenyl phosphate. The cell lysate was sonicated for 30 s on ice and then centrifuged at 12,000 rpm (950 ×*g*) for 30 min at 4˚C. The protein content of samples was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Fifty micrograms of each whole-cell extract was separated by SDS-PAGE (10%) and then transferred onto a polyvinylidene difluoride membrane. Membranes were blocked overnight at 4˚C using 5% non-fat dry milk in TBST buffer containing 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20. Following washing with TBST, membranes were incubated for 1 h at room temperature with the primary antibody, washed, and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Tech., Inc., Beverly, MA, USA). Proteins were visualized using the enhanced chemiluminescence detection system (GE Healthcare UK Ltd.). The antibodies used were rabbit anti-p44/42 (ERK1/2) MAP kinase, -p38 MAP kinase, -SAPK/JNK, -phospho-p44/42 (ERK1/2) MAP kinase (Thr202/Tyr204), -phospho-p38 MAP kinase (Thr180/Tyr182), -phospho-SAPK/JNK (Thr183/Tyr185) (1:1000; Cell Signaling), and mouse anti-α-tubulin (1:20,000; Sigma, Saint Louis, MO, USA). For control, cells were treated with vehicle (DMSO) alone.

Statistical analysis. Data were analyzed using the Student's *t*-test. The significance level was set at $p<0.05$.

Results

Effect of fucoxanthin on cell growth and cell cycle distribution of prostate cancer cells. The growth of LNCap cells was inhibited by fucoxanthin in a dose-dependent manner (Figure 1B). The 50% cell growth inhibitory concentration on day 3 was approximately 2.5 μM. Flow cytometric analysis showed that G_1 cell cycle arrest was induced following treatment with fucoxanthin (Figure 1C). As shown in Figure 1C, the percentage of cells at 24 h in the G_1 phase was significantly greater in fucoxanthin- (2.5, 3.8 and 4.5 μM) treated LNCap cells (88.4±4.1%, 91.1±3.3%, and 89.5±1.6%, respectively), compared to the control (77.0±3.5%). Apoptosis was not observed under these conditions.

Fucoxanthin induced GADD45A gene expression. Expression of the *GADD45A* gene was enhanced by fucoxanthin in a dose-dependent manner (Figure 2A). However, the expression of *GADD45B* remained unchanged. At 24 h, 4.5 μM of fucoxanthin resulted in increased *GADD45A* mRNA levels by 9.3-fold in LNCap cells, although the effects varied. These results are similar to those previously observed in DU145 cells (18).

Effect of fucoxanthin on the phosphorylation of MAPKs. Since the MAPK family is involved in the regulation of genes induced by extracellular stimuli, the effect of fucoxanthin on MAPK activity was assessed. The level of phosphorylated SAPK/JNK increased with fucoxanthin treatment (Figure 3A). The induction was weak but distinct at 24 h. On the other hand, the phosphorylation of ERK1/2 MAPK in LNCap cells was reduced following fucoxanthin

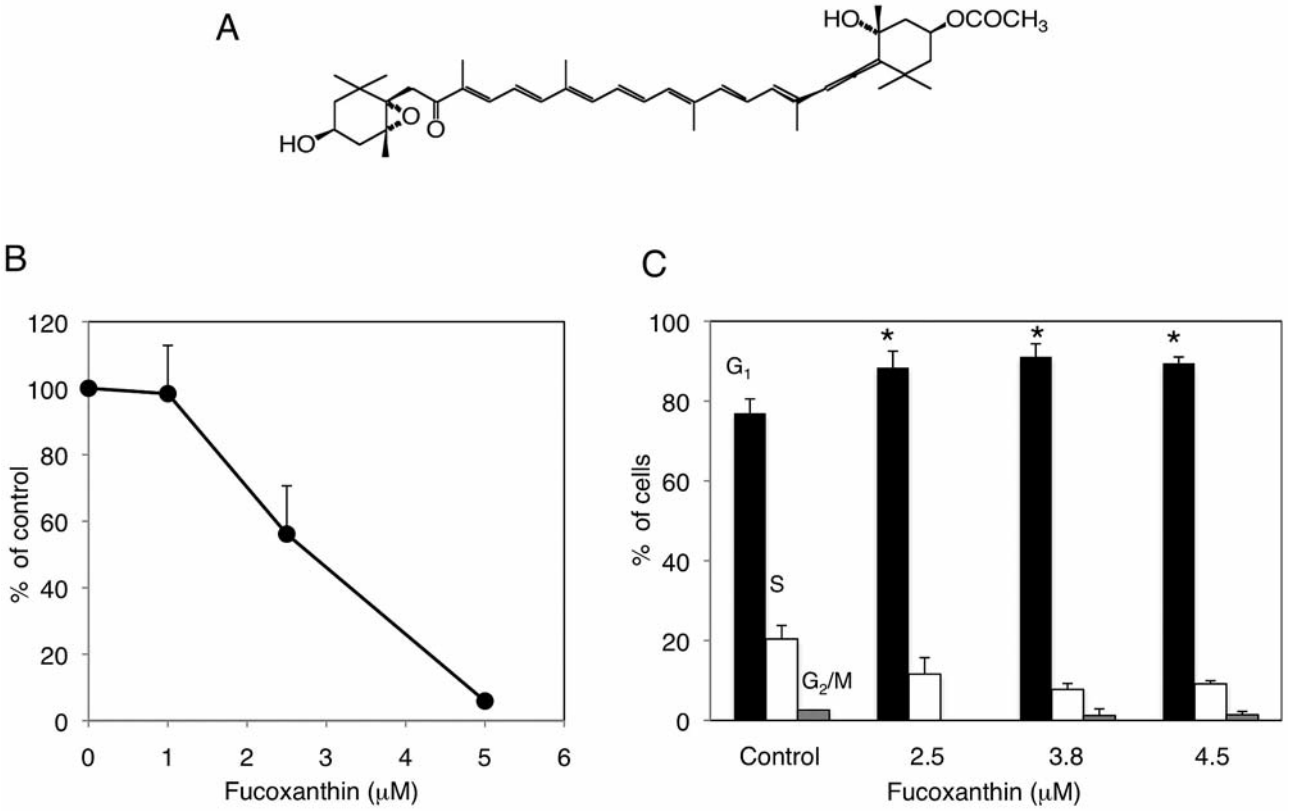

Figure 1. *Fucoxanthin inhibited cell growth and induced cell cycle arrest at the G_1 phase. A: Chemical structure of fucoxanthin. B: Cells were treated with the indicated concentrations of fucoxanthin for three days and the viable cell number was counted. C: Cells were treated with the indicated concentrations of fucoxanthin for 24 h and subjected to flow-cytometric analysis. Data are expressed as the mean±SD (n=2-3). *p<0.05 compared to control.*

treatment for 24 h (Figure 3B); the phosphorylation of p38 MAPK was unaffected (Figure 3C). We previously found that SAPK/JNK, and not ERK1/2 or p38 MAPK, was activated by fucoxanthin in DU145 cells (18). The current results show that fucoxanthin modulates different MAPK pathways in prostate cancer cells depending on the cell type.

Effect of inhibition of MAPK pathways on induction of GADD45A by fucoxanthin. In an effort to ascertain the role of MAPKs in the induction of *GADD45A* by fucoxanthin, the effect of inhibiting MAPK pathways on *GADD45A* expression was assessed. MAPKs are involved in the functionality of the GADD45 family (14, 22-26). MAPK pathways were inhibited by pre-treatment of cells with each MAPK inhibitor for 1 h. Fucoxanthin was then added for another 24 h and the effect on *GADD45A* expression in cells was then determined. Inhibition of the SAPK/JNK pathway using SP600125 reduced *GADD45A* induction by fucoxanthin in LNCap cells (Figure 4; 0.72-fold compared with no pre-treatment of cells). On the other hand, inhibition of the ERK1/2 pathway stimulated the induction of *GADD45A* by 2.2-fold compared with no pre-treatment of cells (Figure 4). Surprisingly, inhibition of the p38 MAPK pathway enhanced *GADD45A* induction in LNCap cells (Figure 4; 3.0-fold compared with no pre-treatment of cells), although p38 MAPK was not activated by fucoxanthin in the cells (Figure 3).

Inhibition of the SAPK/JNK pathway attenuated G_1 arrest induced by fucoxanthin. The effect of MAPK inhibition on the induction of G_1 arrest by fucoxanthin was investigated since the effect on *GADD45A* expression following co-treatment with MAPK inhibitor and fucoxanthin varied. It is supposed that the MAPK pathway could be involved in the G_1 arrest induced by fucoxanthin since we found that GADD45A was partially involved in the induction of G_1 arrest by fucoxanthin (12). The cell population in the G_1 phase decreased when cells were pre-treated with SP600125, as a SAPK/JNK inhibitor, following fucoxanthin treatment compared with LNCap cells treated with fucoxanthin alone (Figure 5). This is consistent with the observed reduction of *GADD45A* expression following co-treatment with fucoxanthin and SP600125 (Figure 4). The percentage of

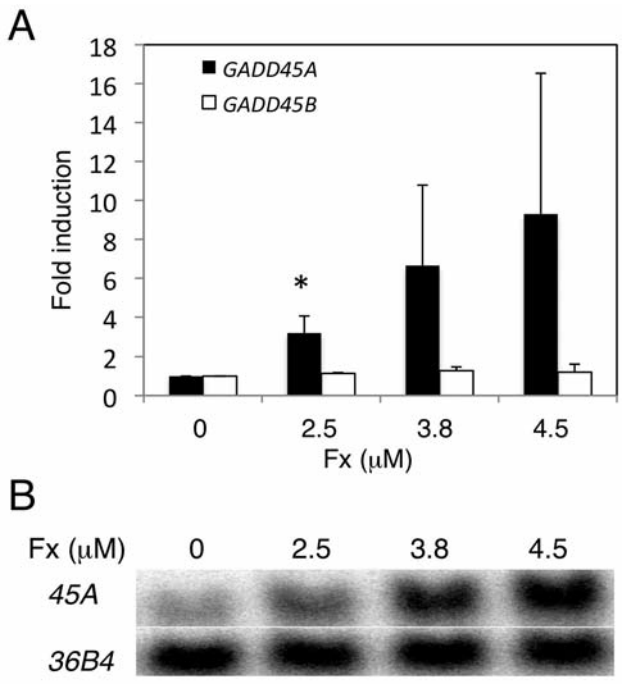

Figure 2. *Effect of fucoxanthin (Fx) on GADD45A and GADD45B gene expression. A: Cells were treated with the indicated concentrations of fucoxanthin for 24 h. B: Representative data for GADD45A expression as determined by northern blot analysis. 45A, GADD45A. Data are expressed as the mean±SD (n=3). *p<0.05 compared to control.*

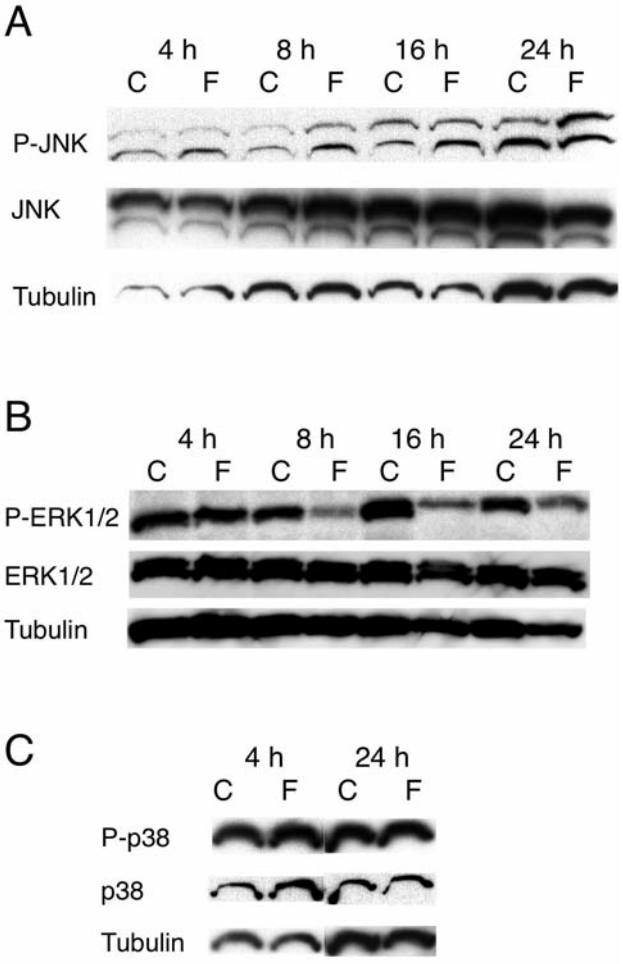

Figure 3. *Effect of fucoxanthin on the phosphorylation of MAPKs. Cells were treated with fucoxanthin (3.8 μM) for the indicated times and proteins were then extracted. Representative data are shown. C, Control; F, fucoxanthin-treated; P-JNK, P-ERK1/2, and P-p38 represent phosphorylated forms of SAPK/JNK, ERK1/2, and p38 MAPKs, respectively.*

cells in the G_1 phase following treatment with fucoxanthin and SB203580 (a p38 MAPK inhibitor) did not increase compared when to cells treated with fucoxanthin alone (Figure 5). Although this is consistent with the observation that p38 MAPK was not affected following the treatment of LNCap cells with fucoxanthin (Figure 3C), it is inconsistent with the observed induction of *GADD45A* expression following co-treatment with fucoxanthin and SB203580 (Figure 4). Co-treatment with fucoxanthin and PD98059 (an ERK1/2 inhibitor) tended to reduce the LNCap cell population in the G_1 phase compared to cells treated with fucoxanthin alone (Figure 5). Although this is consistent with the observation that the active form of ERK1/2 MAPK was reduced following the treatment of LNCap cells with fucoxanthin (Figure 3B), it is inconsistent with the observed induction of *GADD45A* expression following co-treatment with fucoxanthin and PD98059 (Figure 4).

Discussion

Many epidemiological and experimental studies have shown that carotenoids possess antitumor and anticarcinogenic activities. Among these carotenoids, lycopene has been regarded as a promising agent for the prevention of prostate cancer (4, 6). However, recent clinical trials have shown conflicting results (5). Thus, we investigated the efficacy of another carotenoid, fucoxanthin, regarding its potential use in prostate cancer prevention. Fucoxanthin is mainly found in brown algae, and the consumption of fucoxanthin is thought to be higher in Asian countries, including Japan. Previous studies have demonstrated that fucoxanthin inhibited growth of various cancer cell lines and induced cell cycle arrest at the G_1 phase and/or apoptosis (8-10). In the present study, fucoxanthin inhibited the growth of androgen-dependent LNCap prostate cancer cells. Fucoxanthin induced G_1 cell cycle arrest but not apoptosis in these cells. Furthermore, fucoxanthin induced *GADD45A* expression. The GADD45 family of proteins is represented by GADD45A, GADD45B and GADD45G, at least, and is

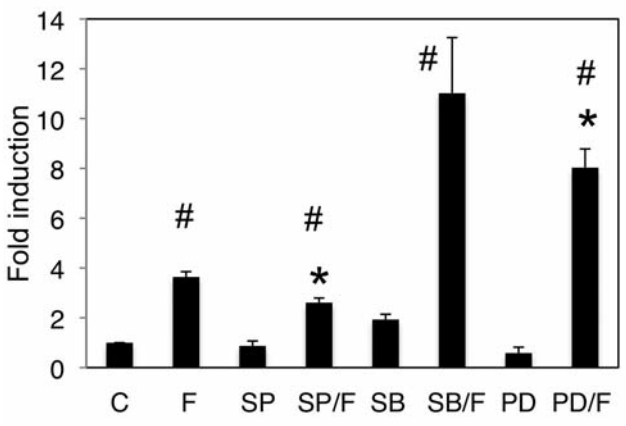

Figure 4. *Effect of MAPK inhibitors on induction of GADD45A by fucoxanthin. Cells were pre-treated with each MAPK inhibitor (10 μM of SP600125, 10 μM of SB203580, or 60 μM of PD98059) for 1 h and then treated with fucoxanthin (3.8 μM) for 24 h. C, Control; F, fucoxanthin-treated; SP, SP600125; SB, SB203580; PD, PD98059; SP/F, SB/F and PD/F, SP600125, SB203580, or PD98059 in combination with fucoxanthin, respectively. Data are expressed as the mean±SD (n=2-3). *p<0.05 compared to fucoxanthin alone. #p<0.05 compared to control.*

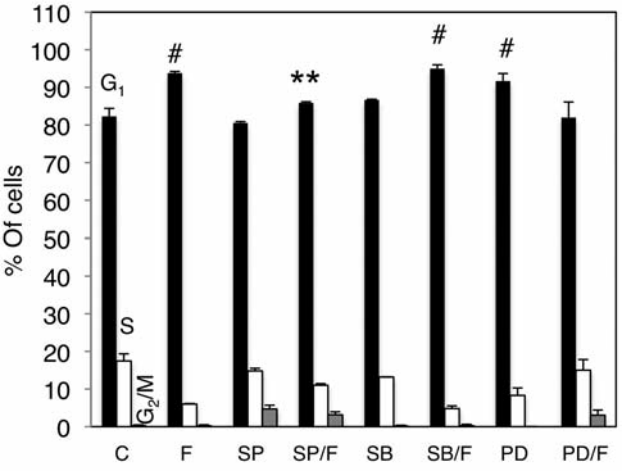

Figure 5. *Effect of MAPK inhibitors on induction of G_1 arrest by fucoxanthin. Cells were pre-treated with each MAPK inhibitor (10 μM of SP600125, 10 μM of SB203580, or 60 μM of PD98059) for 1 h and then treated with fucoxanthin (3.8 μM) for 24 h. C, Control; F, fucoxanthin-treated; SP, SP600125; SB, SB203580; PD, PD98059; SP/F, SB/F and PD/F, SP600125, SB203580, or PD98059 in combination with fucoxanthin, respectively. Data are expressed as the mean±SD (n=2-3). **p<0.01, compared to fucoxanthin alone. #p<0.05 compared to control.*

known to regulate the cell cycle, apoptosis and DNA repair (13-17). GADD45 is known to be involved in G_1 and G_2/M arrest (15, 16). The data suggest that GADD45A may be implicated in the G_1 arrest induced by fucoxanthin in LNCap cells. Our former studies showed that fucoxanthin induced

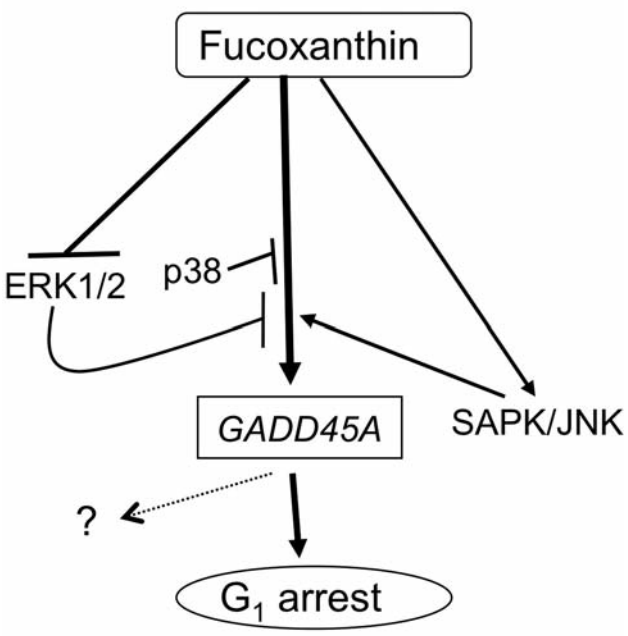

Figure 6. *Proposed signaling pathway involved in the induction of GADD45A and G_1 arrest by fucoxanthin in LNCap cells. Fucoxanthin induces GADD45A, which leads to G_1 arrest in prostate cancer LNCap cells. Fucoxanthin activates SAPK/JNK, which positively regulates the induction of GADD45A by fucoxanthin in cells. Positive regulation by the SAPK/JNK pathway seems to be involved in fucoxanthin-induced GADD45A expression and G_1 arrest in prostate cancer cells. Negative regulation by the ERK1/2 MAPK pathway is likely to be involved in fucoxanthin-induced GADD45A expression, but not in the G_1 arrest, in LNCap cells.*

GADD45A expression and G_1 arrest in androgen-independent DU145 prostate cancer cells, and that the introduction of siRNA against *GADD45A* partially attenuated the induction of this G_1 arrest (12). Therefore, the data suggest that GADD45A might be involved in the G_1 arrest induced by fucoxanthin in prostate cancer cells.

MAPKs are thought to be involved in GADD45A activation (14, 22-26). Fucoxanthin activated SAPK/JNK, but had no affect on p38 MAPK in LNCap cells. The inhibition of SAPK/JNK using a specific inhibitor attenuated *GADD45A* expression and the induction of G_1 arrest by fucoxanthin in LNCap cells. These results are similar to those previously observed in DU145 cells (18). Since SAPK/JNK is thought to be an upstream activator of *GADD45* expression (23, 25, 26), these results are consistent with the former findings. Although the mechanism underlying the activation of GADD45 by SAPK/JNK is unknown, there are several reports suggesting that MAPKs, including SAPK/JNK, induce GADD45 in a p53 -dependent or -independent manner (23, 25, 27, 28). It is supposed that fucoxanthin induces *GADD45A via* SAPK/JNK in a p53-independent manner, since similar results were obtained in LNCap (wild-type p53)

and DU145 (mutated p53) cells. It is reported that MAPK, including SAPK/JNK, activates the *GADD45A* promoter *via* octamer-binding protein 1 (OCT1) and CAAT motifs (23). Thus, the induction of *GADD45A* following SAPK/JNK activation by fucoxanthin might involve activation of its promoter in these prostate cancer cells. Interestingly, inhibition of the p38 MAPK pathway enhanced *GADD45A* expression in LNCap cells, regardless of the absence of an effect on p38 MAPK by fucoxanthin. This observation was similar to the one observed in DU145 cells. The present results combined with the former data may suggest that negative regulation of p38 MAPK is involved in the expression of *GADD45A* in these prostate cancer cells under basal conditions. These results suggest that each MAPK plays a different role in the induction of *GADD45A* and the G_1 arrest by fucoxanthin in prostate cancer cells. On the other hand, fucoxanthin reduced the phosphorylation of ERK1/2 MAPK in LNCap cells. Inhibition of the ERK1/2 MAPK pathway attenuated G_1 arrest regardless of *GADD45A* induction in LNCap cells. These results differed from those observed in DU145 cells where ERK1/2 MAPK was not affected by fucoxanthin, and may suggest that GADD45A is not the only player involved in G_1 arrest in LNCap cells.

In conclusion, and combined with our former data, it has been shown that fucoxanthin inhibits the proliferation of prostate cancer cells regardless of cell hormone dependency. The growth inhibitory effect of fucoxanthin is due to the induction of G_1 cell cycle arrest, which occurs partly through a GADD45A-dependent pathway. It is suggested that fucoxanthin-induced *GADD45A* expression and G_1 arrest are positively regulated by SAPK/JNK in both DU145 and LNCap prostate cancer cells. The proposed pathway in LNCap cells is illustrated in Figure 6. Our future studies will focus on elucidating the precise mechanism by which fucoxanthin activates MAPKs. The results of this study suggest that fucoxanthin may be a promising agent for use in prostate cancer prevention.

Conflict of Interest

No potential conflicts were disclosed.

Acknowledgements

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Received December 1, 2011
Revised January 9, 2012
Accepted January 10, 2012