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Implication of mitogen-activated protein kinase in the induction of G1 cell cycle arrest and *gadd45* expression by the carotenoid fucoxanthin in human cancer cells

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ABSTRACT

Background: The precise mechanism of the anti-tumor action of fucoxanthin has yet to be elucidated. We previously reported that *gadd45a* and *gadd45b* might play a role in the G1 arrest induced by fucoxanthin. In the present study, we show that several MAPKs modulate the induction of *gadd45* and G1 arrest.
Methods: HepG2 and DU145 cells were used. The cell cycle was analyzed using flow cytometry. Expression of *gadd45* was assayed by Northern blot and/or quantitative RT-PCR analyses. Activation of MAPK was assayed by Western blot analysis.
Results: Inhibition of p38 MAPK enhanced the induction of *gadd45a* expression and G1 arrest by fucoxanthin in HepG2 cells. Inhibition of ERK enhanced *gadd45b* expression but had no effect on the induction of G1 arrest by fucoxanthin in HepG2 cells. Inhibition of SAPK/JNK suppressed the induction of *gadd45a* expression and G1 arrest by fucoxanthin in DU145 cells.
These data suggest that *gadd45a* is closely related with the G1 arrest induced by fucoxanthin, and that the pattern of MAPK involvement in the induction of *gadd45a* and G1 arrest by fucoxanthin differs depending on the cell type.
General significance: The implication of GADD45 and MAPK involvement in the anti-tumor action of carotenoids is first described.

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1. Introduction

Carotenoids are a group of natural pigments comprising more than 700 members. Their effects on human health have been extensively investigated, with the anti-carcinogenic and anti-tumor activity of many carotenoids being well documented. However, intervention studies using β-carotene raised questions concerning the cancer prevention action of carotenoids [1]. Furthermore, research using various carotenoids other than β-carotene in efforts to delineate the precise mechanism of action of carotenoids have contributed to the consensus that the effects of carotenoids on human health require further verification. It has been suggested that carotenoids generally possess anti-oxidative activity. Additionally, carotenoids are thought to affect gene and protein expression events involved in the cell cycle, apoptosis, cell–cell communication, drug-metabolism, and other physiological phenomena [2–4]. Fucoxanthin is a carotenoid found in abundance in seaweeds such as brown algae. Recent attention has focused on the beneficial effects of fucoxanthin such as its anti-carcinogenic, anti-tumor and anti-obesity activities [5–7]. We and others have reported that fucoxanthin inhibited tumor cell growth by inducing cell cycle arrest at the G1 phase and/or apoptosis by modulating the expression of cell cycle and apoptosis-related genes [5,8,9].

We previously reported that GADD45A is partially implicated in fucoxanthin-induced G1 arrest in human cancer cells [8]. The GADD45 growth arrest and DNA-damage-inducible gene belongs to a group of genes comprising at least 3 members, A, B and G, the transcription levels of which increase under growth arrest conditions and following treatment with DNA-damaging agents [10]. GADD45 is known to regulate the cell cycle, apoptosis and DNA repair [10–16]. With regard to cell cycle progression, GADD45 was shown to be involved in G2/M and G1 arrest [11–14]. Although the regulation of GADD45 is not fully understood, mitogen-activated protein kinases (MAPKs) are thought to be involved in GADD45 induction [17–21]. It has also been suggested that GADD45 is an upstream factor in the activation of MAPKs [22–25]. The major members of the MAPK family comprise extracellular signal regulated kinase (ERK), p38 MAP kinase and c-Jun N-terminal kinase (SAPK/JNK), all of which are activated by phosphorylation at specific sites which in turn subsequently transmit signals by sequential phosphorylation events following activation. The MAPK family has been implicated to play a role in a variety of cellular processes including cell growth, apoptosis and differentiation in response to various stimuli [26–28].

In the present study, we investigated the role of MAPKs in the induction of G1 arrest and *gadd45* expression by fucoxanthin in cancer

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cells. We found that p38 MAPK and ERK1/2 MAPK are negatively involved in the induction of *gadd45a* and *gadd45b*, respectively, and that GADD45A is an important factor in the induction of G1 arrest by fucoxanthin in HepG2 cells. We also show that positive regulation by SAPK/JNK is involved in *gadd45a* induction and G1 arrest by fucoxanthin in DU145 cells. These results reveal that MAPK pathways are involved in fucoxanthin-induced *gadd45a* expression and G1 cell cycle arrest in cancer cells, and that the nature of the involvement is dependent on cell type. This is the first report that details a plausible mechanism to account for the inhibitory effect of the carotenoid fucoxanthin on the cell cycle and cell proliferation.

2. Materials and methods

2.1. Chemicals

Fucoxanthin (Fig. 1A) was kindly provided from Dr. A. Nagao (National Food Research Institute, Japan) and dissolved in DMSO. The kinase inhibitors PD98059 (an ERK1/2 MAPK inhibitor), SB203580 (a p38 MAPK inhibitor) and SP600125 (a SAPK/JNK inhibitor) were purchased from Merck Biosciences (Darmstadt, Germany) or Tocris Cookson Inc. (Avonmouth, UK). All other chemicals were of biological grade.

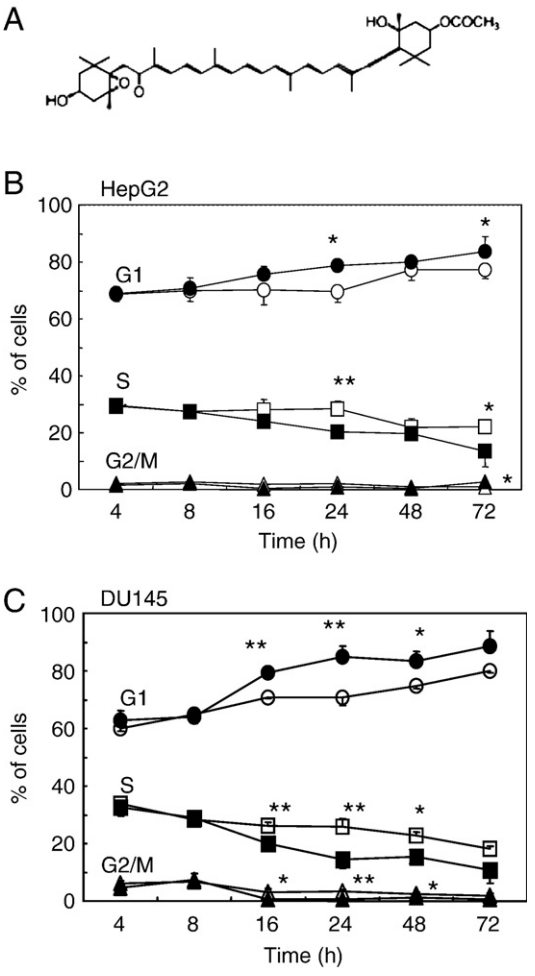

Fig. 1. Effects of fucoxanthin on cell cycle progression. (A) Chemical structure of fucoxanthin. HepG2 (B) and DU145 (C) cells were treated with fucoxanthin (5.5 μM and 5.2 μM, respectively) for the indicated times and then subjected to flow-cytometric analysis. ● and ○ G1 phase cells; ■ and □ S phase cells; ▲ and △ G2/M phase cells. Black and open symbols show the data of fucoxanthin-treated and control cells, respectively. Data are expressed as mean ± SD ($n=3$).*$p<0.05$ and **$p<0.01$, compared to the control.

2.2. Cell culture and cell proliferation assay

Human hepatocellular carcinoma HepG2 and prostate cancer DU145 cell lines were cultured in Dulbecco's modified Eagle's medium and RPMI, respectively, supplemented with 10% heat-inactivated FBS. Cells were maintained in a 37 °C incubator under a humidified atmosphere comprising 5% CO_2. Cells were seeded into 3.5 cm-diameter dishes at a density of 2×10^4 cells/2 ml of medium. After 1 day, cells were treated with fucoxanthin or 0.5% vehicle alone. On days 1, 3 and 5, cell viability was determined using the trypan blue dye exclusion assay and the number of viable cells was counted.

2.3. Flow-cytometric analysis

Cells were plated at a density of 1×10^5 cells/10 ml of medium in 10 cm-diameter dishes and fucoxanthin was added 1 day later. Cells were harvested at various time points, washed with PBS (−) 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 was analyzed using a FACS Calibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the ModFit LT™ program.

2.4. Northern blot and quantitative RT-PCR analyses

Cells were harvested at various time points and total RNA was extracted 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. RT-PCR was performed to generate each cDNA probe for the *gadd45a* and *gadd45b* genes (GenBank Accession Nos. NM_001924 and NM_015675, respectively). The expression levels of each gene were normalized to that of *36b4*. In an effort to estimate the expression of *gadd45b*, quantitative RT-PCR using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) was also performed according to the manufacturer's protocol since the basal expression level of *gadd45b* in HepG2 cells is very low. Pre-designed gene-specific primer and TaqMan probes were supplied from the manufacturer. The expression level of *gadd45b* was normalized to that of *gapdh*.

2.5. Western blot analysis

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% SDS, 1 mM PMSF, 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 for 30 min at 4 °C. The protein content of the 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 PVDF 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 primary antibody, washed, and then incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:1000, Cell Signaling Tech., Inc., Beverly, MA, USA). Proteins were visualized using the ECL detection system (Amersham, Piscataway, NJ, USA). 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).

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