Food Sci. Technol. Res., 5(3), 243-246, 1999

Note

Apoptosis-Inducing Effect of Fucoxanthin on Human Leukemia Cell Line HL-60

Masashi HOSOKAWA,[1] Satoshi WANEZAKI,[1] Kozue MIYAUCHI,[1] Hideyuki KURIHARA,[1] Hiroyuki KOHNO,[2] Jun KAWABATA,[3] Shizuo ODASHIMA[2] and Koretaro TAKAHASHI[1]*

[1] *Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*
[2] *Department Pathology, Kanazawa Medical University, Uchinada, Ishikawa 920-02, Japan*
[3] *Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan*

Received September 7, 1998; Accepted April 23, 1999

The apoptosis-inducing effect of fucoxanthin on human leukemic HL-60 cells was investigated. Fucoxanthin, which was obtained from the brown alga *Undaria pinnatifida*, inhibited the proliferation of HL-60 cells and induced DNA fragmentation on agarose gel electrophoresis, a typical characteristic feature of apoptotic cells. The results of sandwich ELISA using anti-biotin-antibody and anti-DNA-antibody also demonstrated DNA fragmentation in accordance with the increase in fucoxanthin concentration and incubation time. Since the level of DNA fragmentation did not increase after 24 h incubation, fucoxanthin appeared to have some effect on cell cycle. In contrast, β-carotene did not show an apoptosis-inducing effect on HL-60 cells. These results suggested that a carotenoid structure might be crucial for inducing apoptosis.

Keywords: fucoxanthin, antiproliferation, apoptosis, DNA fragmentation, HL-60 cell

Carotenoids appear to have beneficial effects on cancer chemoprevention (Astorg, 1997). It has been reported that β-carotene, astaxanthin, and canthaxanthin have chemopreventive effects on carcinogenesis (Krinsky, 1991; Tanaka *et al.*, 1995). Fucoxanthin, which is a major carotenoid in brown algae, is known to exhibit antitumor activity against human neuroblastoma GOTO cells (Okuzumi *et al.*, 1990). Inhibitory effects on mouse duodenum and on skin carcinogenesis have also been reported (Okuzumi *et al.*, 1993; Satomi *et al.*, 1996).

There is a wealth of information pertaining to apoptosis in anticancer research. Cells undergoing apoptosis are recognized and engulfed by macrophages without damage to neighboring cells (Compton, 1992). Therefore, apoptosis-inducing activities are expected to provide a novel means of chemoprevention and chemotherapy in the treatment of cancer. Canthaxanthin-induced apoptosis in human cancer cell lines has also been reported (Palozza *et al.*, 1998). But only limited information has been provided on the apoptosis-inducing activities of carotenoids pertaining to tumor cells.

We carried out a screening for antiproliferative activity in some seafood extracts, and fucoxanthin obtained from the brown alga, *Undaria pinnatifida*, was the active principle. Since fucoxanthin is available from edible seaweeds, its functions are of great interest. To the best of our knowledge, HL-60 cell apoptosis-inducing effect of fucoxanthin has not previously been reported. The present study demonstrates that fucoxanthin can induce apoptosis of HL-60 cells dose dependently.

* To whom correspondence should be addressed.
E-mail: kore@pop.hokudai.ac.jp

Materials and Methods

Materials HL-60 cells (ATCC CCL-240) were provided by the American Type Culture Collection (Rockville, CT). RPMI 1640 medium was obtained from GIBCO (New York) and fetal bovine serum (FBS) from Dainippon Pharmaceutical Co., Ltd. (Osaka). Camptothecin, β-carotene, all-*trans* retinoic acid (RA), ribonuclease A (RNase A), and proteinase K were purchased from Sigma Chemical Co. (St. Louis, MO). Sandwich ELISA kit used to estimate the levels of histone-associated DNA fragments was purchased from Boehringer Mannheim GmbH (Germany).

Isolation of fucoxanthin Fucoxanthin (3'-acetoxy-5,6-epoxy-3,5'-dihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one) was isolated from the brown alga, *Undaria pinnatifida* as follows. Fresh *U. pinnatifida* purchased from a market was first soaked in two volumes (v/w) of methanol for two days. This extraction was repeated twice. The methanol solution was then filtered and evaporated to obtain the methanol extracts. Water and ethyl acetate were added to these extracts, then the ethyl acetate layer was obtained with a separatory funnel. The fucoxanthin fraction orange-colored was separated from the ethyl acetate-soluble fraction by means of preparative silica gel thin layer chromatography (TLC) developed with chloroform:methanol:water (65:25:4, v/v/v). Further purification was carried out with preparative silica gel TLC developed with *n*-hexane:acetone (6:4, v/v). Fucoxanthin thus obtained was characterized by MS and NMR analysis.

Characterization of Fucoxanthin VIS λ_{max} (acetone) 446, 465 nm; (hexane) 426, 448, 475 nm; FDMS *m/z*: 658 $(M)^+$; FDHRMS *m/z*: 658.4249 (calculated 658.4234 for $C_{42}H_{58}O_6$); ^1H NMR ($CDCl_3$) δ (Multiplicity, *J* in Hz): 7.13 (br d, ca 12), 6.73 (br dd, ca 12, ca 14), 6.65 (br d, ca 15), 6.62

(m), 6.58 (m), 6.55 (br dd, ca 12, ca 15), 6.39 (br d, ca 12), 6.33 (br d, ca 15), 6.25 (br d, ca 12), 6.11 (br d, ca 11), 6.03 (s), 5.36 (m), 3.80 (m), 3.63 (d, 18.3), 2.58 (d, 18.3), 2.31 (m), 2.28 (m), 2.02 (s), 1.98 (m), 1.97 (s), 1.97 (s), 1.92 (s), 1.79 (s), 1.76 (br dd, ca 11, ca 14), 1.49 (m), 1.49 (m), 1.39 (m), 1.36 (s), 1.33 (m), 1.33 (s), 1.20 (s), 1.05 (s), 1.01 (s), 0.94 (s); ^{13}C NMR ($CDCl_3$) d: 202.3, 197.8, 170.6, 145.0, 139.0, 138.0, 137.1, 136.6, 135.4, 134.6, 132.5, 132.5, 132.1, 129.4, 128.6, 125.7, 123.4, 117.6, 103.4, 72.7, 68.0, 67.1, 66.1, 64.3, 47.1, 45.5, 45.3, 41.7, 40.8, 35.8, 35.2, 32.1, 31.3, 29.2, 28.2, 25.1, 21.4, 21.2, 14.0, 12.9, 12.7, 11.8. The data of fucoxanthin obtained coincided with that in the literature (Haugan *et al.*, 1992; Palermo *et al.*, 1991).

Cell culture HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Fucoxanthin and RA were added to the culture medium as an ethanol solution, and β-carotene was added as a tetrahydrofuran solution. Camptothecin was added as a dimethyl sulfoxide solution. The final concentrations of ethanol, tetrahydrofuran, and dimethyl sulfoxide were below 0.1% (v/v).

Proliferation of HL-60 cells HL-60 cells (5×10^4 cells/ml or 1×10^5 cells/ml) were incubated in the culture medium containing fucoxanthin or β-carotene. Cell number was determined with a hematocytometer, and viability was determined with a dye exclusion test using trypan blue.

Agarose gel electrophoresis After HL-60 cells (1×10^5 cells/ml) were treated with fucoxanthin, β-carotene, RA, or camptothecin, cells were washed twice with PBS. The cell pellets were dissolved in 20 μl lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl salcosinate, pH 7.8), and lysate was incubated at 50°C for 30 min after the addition of 2 μl RNase A (10 mg/ml lysis buffer). A 2 μl of portion of proteinase K (10 mg/ml lysis buffer) was then added and the solution was further incubated at 50°C for 1 h. The lysate was transferred onto a 2% agarose gel, and electrophoresis was performed at 50 V. The DNA fragments were stained with ethidium bromide and visualized under a UV light.

ELISA analysis DNA fragmentation was estimated by a biotin-labeled anti-histone-antibody and a peroxidase-conjugated anti-DNA-antibody in a sandwich ELISA kit. First, HL-60 cells (1×10^5 cells/ml) were treated with fucoxanthin or another agent. Then cells were washed with PBS and lysed with 200 μl of lysis buffer for 30 min at room temperature. The lysate was centrifuged at $200\times g$ for 10 min, and 20 μl of supernatant was transferred onto a streptavidin-coated microtiter plate. The presence of histone-associated DNA fragments in the cell lysates was determined using a sandwich ELISA kit. The level of DNA fragmentation of mono-, and oligo-nucleosomes in HL-60 cells treated with fucoxanthin was expressed as an enrichment factor, calculated by the following formula.

Enrichment factor=mU of the sample (HL-60 cells treated with fucoxanthin)/mU of the corresponding control (HL-60 cells without fucoxanthin treatment)
mU=absorbance [Indicated the wavelength at which the absorbance was measured] nm$\times10^{-3}$

Results and Discussion

Fucoxanthin obtained from fresh *U. pinnatifida* exhibited strong antiproliferative activity for HL-60 cells; its activity was higher than that of β-carotene (Fig. 1). At a concentration of 22.6 μM, fucoxanthin completely inhibited the proliferation. In HL-60 cells treated with fucoxanthin for 24 h, viable cell numbers estimated by trypan blue exclusion were decreased in a dose-dependent manner (Fig. 2). Viability was 46.0% and 17.3% against the control value (no addition of fucoxanthin) at 11.3 μM and 45.2 μM fucoxanthin, respectively. Okuzumi (1991) observed that the antiproliferative

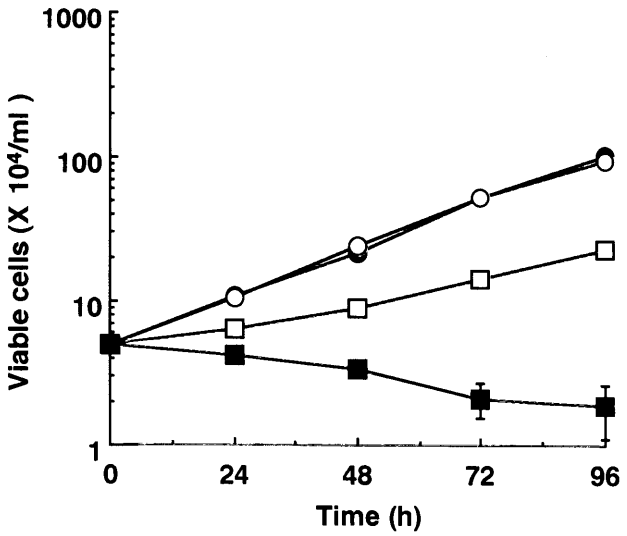

Fig. 1. Comparison of the inhibitory effects of fucoxanthin and β-carotene on viability of HL-60 cells. HL-60 cells (5×10^4 cells/ml) were incubated with 22.6 μM fucoxanthin or β-carotene. Results are shown as means±S.D. (n=3). ■, fucoxanthin; □, β-carotene; ●, ethanol; ○, tetrahydrofuran.

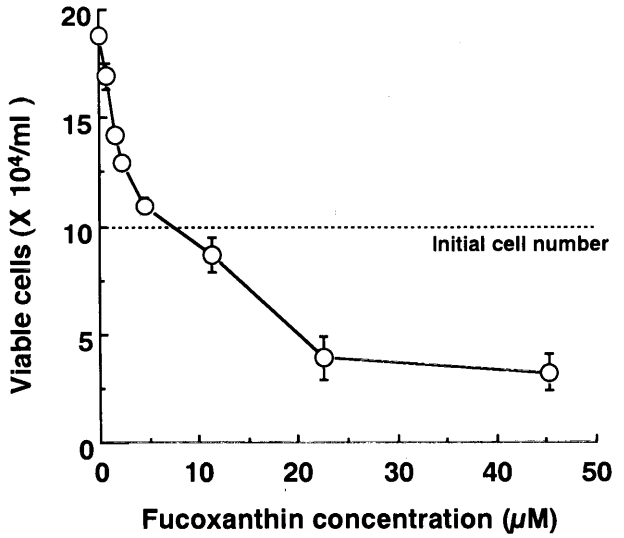

Fig. 2. Effect of fucoxanthin on viability of HL-60 cells. HL-60 cells (1×10^5 cells/ml) were incubated with fucoxanthin for 24 h. Percentage in viable cell number of cells treated with fucoxanthin relative to that of untreated cells was plotted. Results are shown as means±S.D. (n=3).

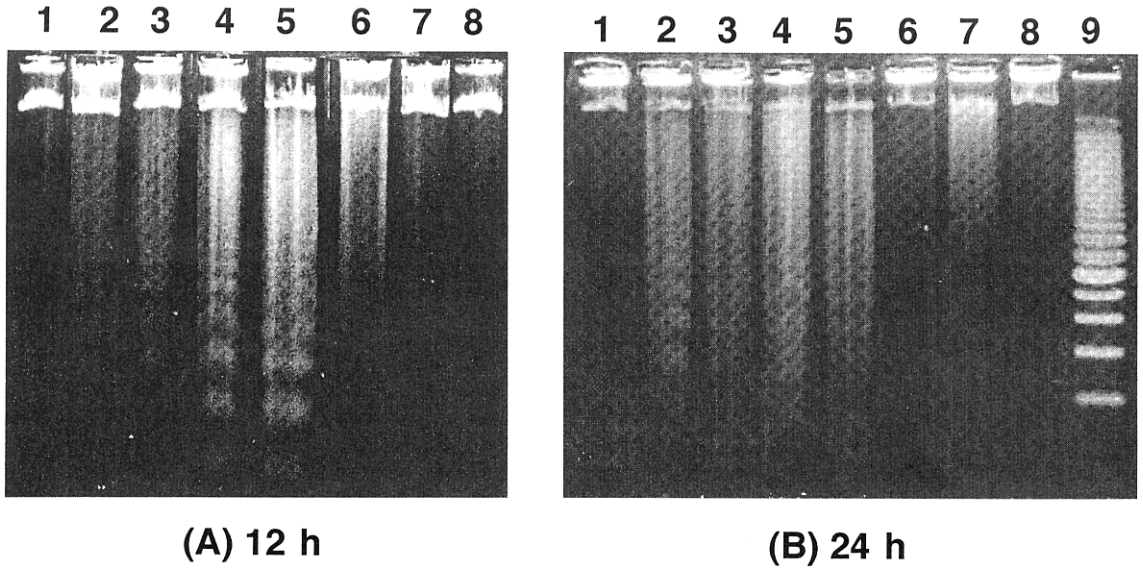

Fig. 3. Agarose gel electrophoresis of DNA in HL-60 cells treated with carotenoids and retinoic acid. HL-60 cells (1×10^5 cells/ml) were incubated for 12 h (A) and 24 h (B) with the following agents: lane 1: ethanol, lane 2: fucoxanthin 4.5 μM, lane 3: fucoxanthin 11.3 μM, lane 4: fucoxanthin 22.6 μM, lane 5: camptothecin 100 nM, lane 6: β-carotene 22.6 μM, lane 7: tetrahydrofuran, lane 8: retinoic acid 22.6 μM, lane 9: DNA size marker ($\times$180 bp.).

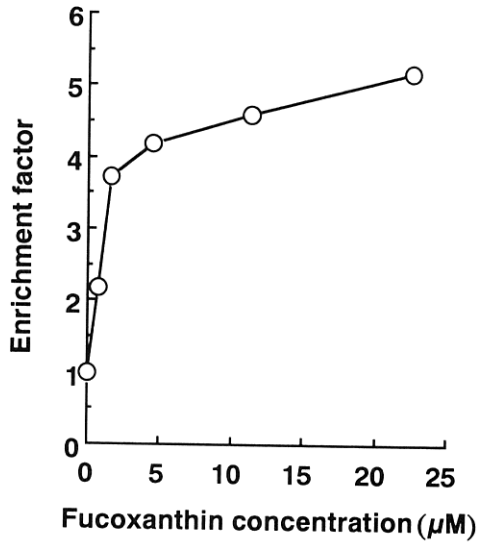

Fig. 4. Effect of fucoxanthin on DNA fragmentation in HL-60 cells. HL-60 cells (1×10^5 cells/ml) were incubated with fucoxanthin for 24 h. DNA fragmentation was analyzed by sandwich ELISA with biotin labeled anti-histone antibody and peroxidase conjugated anti-DNA antibody. The level of DNA fragmentation was expressed as an enrichment factor defined as absorbance (405 nm) of cells treated with fucoxanthin relative to that of untreated cells.

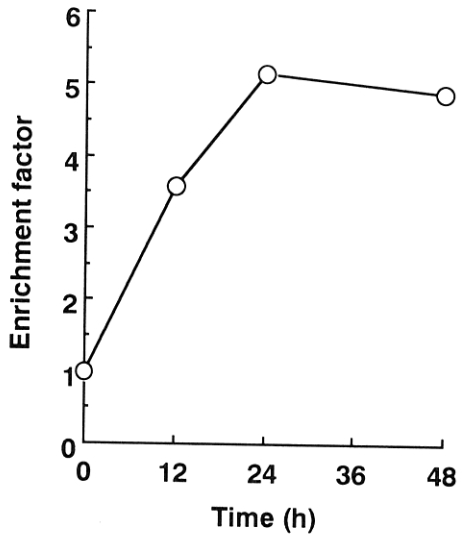

Fig. 5. Time dependency of DNA fragmentation in HL-60 cells treated with fucoxanthin. Cells (1×10^5 cells/ml) were incubated with 11.3 μM fucoxanthin. DNA fragmentation was analyzed in the same manner as in Fig. 4.

activity of fucoxanthin against tumor cell lines is much higher than that of normal cells. Thus, fucoxanthin has an antiproliferative effect on tumor cell lines.

Since cell proliferation is key to the promotion and progression stages of carcinogenesis, we then investigated the apoptosis-inducing activity of fucoxanthin. As shown in Fig. 3, a DNA ladder, which is a characteristic feature of apoptotic cells (Wyllie, 1980), was clearly observed in HL-60 cells treated with 22.6 μM fucoxanthin for 12 h. Similar results have been obtained with camptothecin, which is known as a strong apotosis-inducing agent (Solary *et al.*, 1993). After 24 h incubation, the DNA ladder became much clearer, even though we decreased the concentration of fucoxanthin to 11.3 μM or to 4.5 μM. It is assumed that the apoptosis-inducing activity of fucoxanthin is almost comparable to that of cisplatin or VP16, which are typical reagents that induce apoptosis in HL-60 cells, when treated at the concentration of 2–4 μM for 24 h (Jing *et al.*, 1994). In contrast, DNA fragmentation was not observed in HL-60 cells treated with 22.6 μM β-carotene or RA (Fig. 3). Thus, apoptosis induction toward HL-60 cells might differ among carotenoids. In fact, just recently it was reported that canthaxanthin can induce apoptosis in human cancer cell lines (Palozza *et al.*, 1998). Therefore, the apoptosis-inducing activity of carotenoids may

vary depending on the cell line employed.

Enrichments of mono- and oligo-nucleosomes in HL-60 cells were estimated by sandwich ELISA after treatment with fucoxanthin. The fragmented DNA content, designated as an enrichment factor, increased with the concentration of fucoxanthin in the medium. The enrichment factor went over 5 by treatment of 22.6 μM fucoxanthin for 24 h as illustrated in Fig. 4. Since viable cell numbers were also decreased in a dose dependent manner (Fig. 2), it was suggested that fucoxanthin inhibited HL-60 cell proliferation by inducing apoptosis. The enrichment factor increased in a time-dependent manner and reached a plateau after incubation for 24 h (Fig. 5). Some apoptosis-inducing agents are known to arrest a specific cell phase (Bino *et al.*, 1991). Since doubling time of HL-60 cells is approximately 24 h, fucoxanthin may affect cell cycle. However, viable cell numbers were decreased gradually until 96 h after fucoxanthin addition. These observations suggest that other mechanisms besides apoptosis may be involved in the antiproliferation of fucoxanthin on HL-60 cells.

Fucoxanthin is rich in the edible seaweeds e.g. *Undaria pinnatifida* and *Hizikia fusiformis*. They may be beneficial, for designing of primary health care foods. Further studies, especially *in vivo*, should be carried out to confirm the apoptosis-inducing effect of fucoxanthin.

Acknowledgement The authors are grateful to Mr. Kenji Watanabe (GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University) for the MS analysis of fucoxanthin.

References

Astorg, P. (1997). Food carotenoids and cancer prevention: An overview of current research. *Trends Food Sci. Technol.*, **8**, 406-413.

Bino, G.D., Skierski, J.S. and Darzynkiewicz, Z. (1991). The concentration-dependent diversity of effects of DNA topoisomerase I and II inhibitors on the cell cycle of HL-60 cells. *Exp. Cell Res.*, **195**, 485-491.

Compton, M.M. (1992). A biochemical hallmark of apoptosis: Internucleosomal degradation of the genome. *Cancer Metastasis Rev.*, **11**, 105-119.

Haugan, J.A., Englert, G., Glinz, E. and Jensen, S.L. (1992). Algal carotenoids. 48. Structural assignments of geometrical isomers of fucoxanthin. *Acta Chem. Scand.*, **46**, 389-395.

Jing, Y., Ohizumi, H., Kawazoe, N., Hashimoto, S., Masuda, Y., Nakajo, N., Yoshida, T., Kuroiwa, Y. and Nakaya, K. (1994). Selective inhibitory effect of bufalin on growth of human tumor cells *in vitro*: Association with the induction of apoptosis in leukemia HL-60 cells. *Jpn. J. Cancer Res.*, **85**, 645-651.

Krinsky, N.I. (1991). Effect of carotenoids in cellular and animal systems. *Am. J. Clin. Nutr.*, **53** (Suppl.), 238S-246S.

Okuzumi, J. (1991). Antiproliferative and antitumor promoting activities of fucoxanthin, a natural carotenoid. *J. Kyoto Pref. Univ. Med.*, **100**, 551-566.

Okuzumi, J., Nishino, H., Murokoshi, M., Iwashima, A., Tanaka, Y., Yamane, T., Fujita, Y. and Takahashi, T. (1990). Inhibitory effects of fucoxanthin, a natural carotenoid, on N-*myc* expression and cell cycle progression in human malignant tumor cells. *Cancer Lett.*, **55**, 75-81.

Okuzumi, J., Takahashi, T., Yamane, T., Kitao, Y., Inagake, M., Ohya, K., Nishino, H. and Tanaka, Y. (1993). Inhibitory effects of fucoxanthin, a natural carotenoid, on *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine-induced mouse duodenal carcinogenesis. *Cancer Lett.*, **68**, 159-168.

Palermo, J.A., Gros, E.G. and Seldes, A.M. (1991). Carotenoids from three red algae of the corallinaceae. *Phytochemistry*, **30**, 2983-2986.

Palozza, P., Maggiano, N., Calviello, G., Lanza, P., Piccioni, E., Ranelletti, F.O. and Bartoli, G.M. (1998). Canthaxanthin induces apoptosis in human cancer cell lines. *Carcinogenesis*, **19**, 373-376.

Satomi, Y., Tokuda, H., Fujii, H., Shimidzu, N., Tanaka, Y. and Nishino, H. (1996). Anti-tumor-promoting activity of fucoxanthin, a natural carorenoid. *J. Kyoto Pref. Univ. Med.*, **105**, 739-743.

Solary, B.E., Bertrand, R., Kohn, K.W. and Pommier, Y. (1993). Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by DNA topoisomerase I and II inhibitors. *Blood*, **81**, 1359-1368.

Tanaka, T., Kawamori, T., Ohnishi, M., Makita, H., Mori, M., Satoh, K. and Hara, A. (1995). Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis*, **16**, 2957-2963.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284**, 555-556.