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Anti-neoplastic effect of halocynthiaxanthin, a metabolite of fucoxanthin

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We have reported that fucoxanthin, a natural carotenoid, inhibited the growth of human neuroblastoma GOTO cells. In the present study, we show that a metabolite of fucoxanthin, halocynthiaxanthin, which is isolated from sea squirt *Halocynthia roretzi*, has a more potent inhibitory effect. Halocynthiaxanthin (5 μg/ml) caused complete suppression of GOTO cell proliferation, whereas fucoxanthin reduced the growth rate by only 88.8% compared with the control, at day 2 after the drug treatment. Furthermore, halocynthiaxanthin also inhibited the growth of other human malignant tumor cells. Thus halocynthiaxanthin seems to be a promising anti-neoplastic agent.

Key words: Anti-neoplastic activity, halocynthiaxanthin, natural carotenoid.

Introduction

Epidemiological findings have focussed attention on the influence of diet on cancer incidence. The anti-tumor activity and cancer preventive activity of carotenoids present in vegetables and seaweeds[1] has been studied extensively.

It is well known that β-carotene and its metabolite vitamin A[2] have anti-cancer and cancer preventive activity.[3–7] We noticed that carotenoids other than β-carotene also effectively suppress neoplasms, e.g. α-carotene prepared from palm oil[8] and fucoxanthin isolated from brown algae[9] inhibited the proliferation of human neuroblastoma GOTO cells. In this respect they were more inhibitory than β-carotene.

In the present study, we extended our research to screen other effective anti-neoplastic carotenoids distributed in our daily foods. Here we report the anti-tumor activity of halocynthiaxanthin, a natural carotenoid isolated from sea squirt *Halocynthia roretzi*, which is eaten in Japan. Since halocynthiaxanthin is a metabolite of fucoxanthin, we compared the anti-proliferative activity of halocynthiaxanthin with that of fucoxanthin. We report here that halocynthiaxanthin shows more potent anti-cancer activity than fucoxanthin.

Materials and methods

Chemicals

Halocynthiaxanthin [(3*S*,5R,6*S*,3′R)-5,6-epoxy-3,3′-dihydroxy-7′,8′-didehydro-5,6,7,8-tetrahydro-β,β-caroten-8-one] (Figure 1) was isolated from sea squirt, *H. roretzi*[10] as follows. The carotenoids were extracted with acetone. After transfer to *n*-hexane:ether (1:1, v/v) by addition of water, the extracted solution was concentrated under reduced

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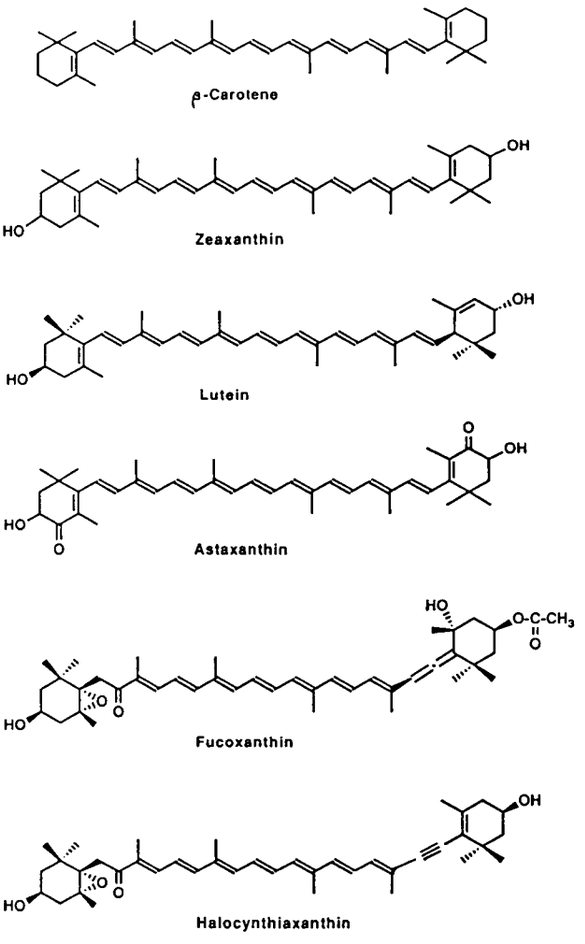

Figure 1. Structure of halocynthiaxanthin and other carotenoids.

pressure in nitrogen at −40°C. The crude carotenoids were separated by column chromatography on silica gel 60 (Merk) and the reddish band (halocynthiaxanthin fraction) was obtained by elution with stepwise increasing concentrations of acetone in *n*-hexane. The fraction containing halocynthiaxanthin was purified by preparative thin layer chromatography (P-TLC) on silica gel 60 G (Merck) using *n*-hexane:acetone (7:3, v/v). This carotenoid was finally submitted to purification by high performance liquid chromatography (HPLC) on a Shim-pack PREP-SIL column (Shimadzu Co.) with elution of acetone with a *n*-hexane gradient, monitoring the visible absorption at 450 nm.

Lutein was isolated from spinach *Spinacia oleracea*. Astaxanthin and zeaxanthin were kindly donated from Hoffmann-La Roche. β-Carotene was purchased from Sigma. [6-^{3}H]Thymidine (9.99×10^{11} Bq/mmol), [5-^{3}H]-uridine (9.99×10^{11} Bq/mmol), L-[4,5-^{3}H]leucine (2.63×10^{12} Bq/mmol) and deoxycytidine 5′-[α-^{32}P]triphosphate (1.11×10^{14} Bq/mmol) were purchased from Amersham International.

Cell culture

GOTO (neuroblastoma), HGC-27 (gastric cancer), COLO 320DM (colon cancer) and PANC-1 (pancreatic cancer) cells were maintained in Dulbecco's modified Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum. HeLa (cervical cancer) cells were maintained in Eagle's MEM supplemented with 10% calf serum.

Northern blot analysis

Total cellular RNA was isolated by the guanidium/cesium chloride method. The RNA samples were electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde and then transfered to nitrocellulose membranes (Schuller & Schleicher Co.). The membranes were hybridized with appropriate nick-translated [^{32}P]DNA probe and washed. Relative intensities of bands on autoradiograms were quantified by scanning densitometer (Shimadzu dual wavelength flying spot scanner CS-9000). Both N-*myc* and *β-actin* probes were purchased from Oncor Inc.

Measurements of DNA, RNA and protein syntheses

Cells were labelled for 1 h with [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine, and then washed with phosphate buffered saline and treated with 5% trichloroacetic acid. The acid-insoluble fraction was dissolved in 2% sodium dodecyl sulfate. The radioactivity in the aliquot was counted.

Results

Anti-tumor activity of various kinds of carotenoids distributed in food materials

As shown in Table 1, various carotenoids distributed in our daily foods proved to have

Table 1. Effect of various carotenoids in foods on the growth of GOTO cells

Carotenoid	Inhibition (%)
β-Carotene	8.0
Zeaxanthin	9.4
Lutein	10.4
Astaxanthin	39.0
Halocynthiaxanthin	100

GOTO cells (4×10^4 cells in 2 ml medium) were inoculated into 35 mm diameter Petri dishes. After 1 day, the carotenoid sample at a concentration of 10 μg/ml was added into the medium. Culture was continued for 3 days and then the number of viable cells was counted. Data are mean values of duplicate experiments, each of which consisted of three cultures for each carotenoid, and are expressed as percentage of growth inhibition (the average number of viable cells in the control culture was 3.74×10^5 cells/dish).

anti-tumor activity. Among them, halocynthiaxanthin showed a relatively strong anti-proliferative activity.

Comparison of the inhibitory effects of halocynthiaxanthin and fucoxanthin on the proliferation of GOTO cells

Since halocynthiaxanthin is a metabolite of fucoxanthin, which was previously found to have anti-tumor activity,[9] we compared the effects of halocynthiaxanthin and fucoxanthin on the proliferation of tumor cells. As shown in Figure 2, halocynthiaxanthin showed higher activity than fucoxanthin. At a concentration of 5 μg/ml, halocynthiaxanthin completely inhibited the proliferation of GOTO cells, while fucoxanthin reduced the growth rate by 88.8% compared with the control. ID_{50} was calculated to be about 2.5 μg/ml for halocynthiaxanthin and 7.5 μg/ml for fucoxanthin.

Time course of anti-proliferative effect of halocynthiaxanthin

At a concentration as low as 1 μg/ml, halocynthiaxanthin definitely suppressed the growth of GOTO cells. However, at this concentration, GOTO cells did not lose the ability of growth until 6 days after treatment with halocynthiaxanthin. At a concentration of 2 μg/ml, halocynthiaxanthin caused the loss of the ability of proliferation later than 4 days after the treatment. At a concentration of 5 μg/ml, GOTO cells were completely suppressed by halocynthiaxanthin and no cell proliferation was observed during the whole period of the experiment (Figure 3).

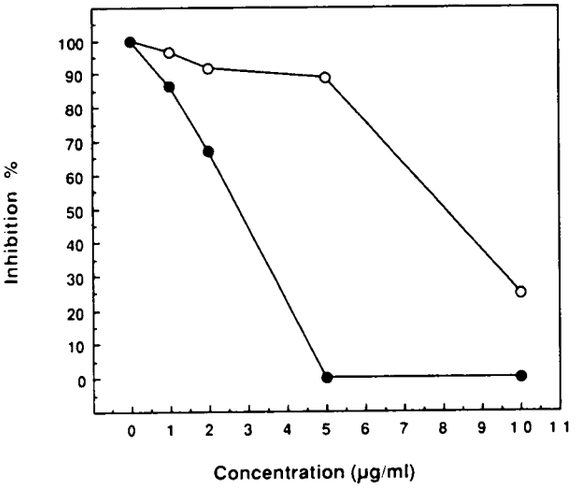

Figure 2. Effect of halocynthiaxanthin and fucoxanthin on the proliferation of GOTO cells. GOTO cells (4×10^4 cells in 2 ml medium) were inoculated into 35 mm diameter dishes. After 2 days, halocynthiaxanthin (●) or fucoxanthin (○) at a concentration of 1, 2, 5 and 10 μg/ml was added into medium. Culture was continued for 2 days and then the number of viable cells was counted. Data are mean values of duplicate experiments.

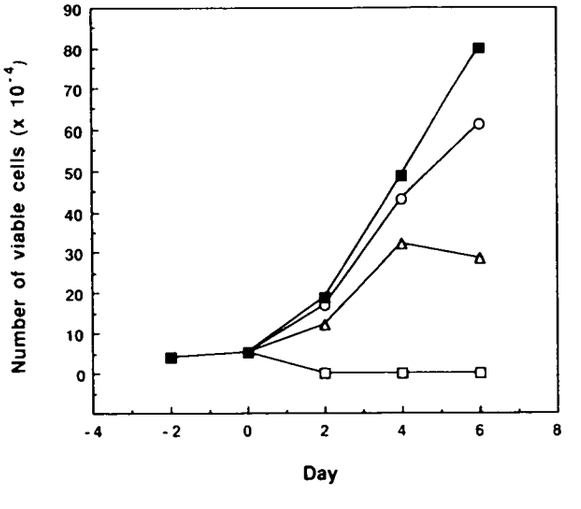

Figure 3. Effect of halocynthiaxanthin on the growth of GOTO cells. GOTO cells were treated with halocynthiaxanthin (1, 2 and 5 μg/ml), and the number of viable cells was counted at 2, 4 and 6 days after the administration. Data are mean values of duplicate experiments. ■, Control; ○, halocynthiaxanthin (1 μg/ml); △, halocynthiaxanthin (2 μg/ml); □, halocynthiaxanthin (5 μg/ml).

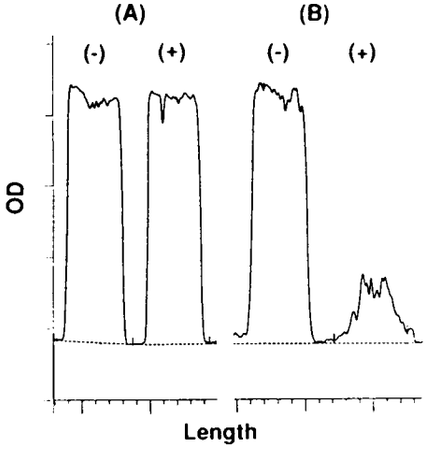

Figure 4. Effect of halocynthiaxanthin on N-*myc* gene expression in GOTO cells. GOTO cells were treated with (A) halocynthiaxanthin (3.3 μg/ml) or (B) fucoxanthin (10 μg/ml) for 24 h. Total RNA was isolated and Northern blot analysis was carried out using ^{32}P-labelled probes of N-*myc*. The autoradiogram was scanned by a densitometer. (−), Control; (+), treated with carotenoids.

Effect of halocynthiaxanthin on N-*myc* gene expression in GOTO cells

The N-*myc* gene, a possible marker of malignancy in neuroblastoma, is amplified in GOTO cells and its expression is suppressed by some anti-neoplastic carotenoids, e.g. fucoxanthin and α-carotene.[8,9] Thus, it is of interest to examine the effect of halocynthiaxanthin on N-*myc* gene expression. As shown in Fig. 4(A), the level of N-*myc* mRNA was scarcely affected by the treatment with halocynthiaxanthin (3.3 μg/ml) for 24 h. Under the same experimental conditions, fucoxanthin (10 μg/ml; at this concentration fucoxanthin showed about the same anti-tumor activity as that of 3.3 μg/ml halocynthiaxanthin, as shown in Figure 2) caused strong suppression of N-*myc* gene expression. The expression of the *β-actin* gene was little affected by treatment with either halocynthiaxanthin or fucoxanthin (data not shown).

Effect of halocynthiaxanthin on DNA, RNA and protein synthesis

As shown in Table 2, halocynthiaxanthin (3.3 μg/ml) had little or no inhibitory effect on the incorporation of [^{3}H]thymidine into DNA and of [^{3}H]uridine into RNA. By contrast, the incorporation of L-[^{3}H]leucine into cellular proteins was suppressed by 69.1% compared with the control by treatment with halocynthiaxanthin.

Table 2. Effect of halocynthiaxanthin on DNA, RNA and protein synthesis

	Inhibition (%)
[^{3}H]Thymidine incorporation	0
[^{3}H]Uridine incorporation	12.5
L-[^{3}H]Leucine incorporation	30.9

GOTO cells were treated with halocynthiaxanthin (3.3 μg/ml) for 5 h and then labelled with [^{3}H]thymidine (1.35 × 10^4 Bq/culture), [^{3}H]uridine (1.35 × 10^4 Bq/culture) or L-[^{3}H]leucine (3.7 × 10^4 Bq/culture). After 1 h, radioactivity in the acid-insoluble fraction was measured. Data are mean values of duplicate experiments and are expressed as percent of inhibition.

Effect of halocynthiaxanthin on the proliferation of various kinds of human malignant tumor cells

As shown in Table 3, halocynthiaxanthin also reduced the growth of other human malignant tumor cells, i.e. HGC-27 (gastric cancer), COLO 320DM (colon cancer), PANC-1 (pancreatic cancer) and HeLa (cervical cancer) cells by 0.1, 10.7, 8.0 and 6.5% compared with the control, respectively, at day 2 after drug treatment (5 μg/ml).

Discussion

In the present study, halocynthiaxanthin, a metabolite of fucoxanthin, was shown to inhibit the proliferation of GOTO cells in a dose- and time-dependent manner, and to be several times more inhibitory than fucoxanthin.

Since N-*myc* expression of GOTO cells was inhibited by various anti-tumor carotenoids, including fucoxanthin, N-*myc* has been thought to play an important role in the mechanism of the anti-proliferative action of carotenoids. However, halocynthiaxanthin showed little effect on N-*myc* gene expression. Therefore, it is necessary to re-evaluate the role of N-*myc* suppression by anti-tumor carotenoids in their mechanism of action.

DNA and RNA syntheses in the early stage after treatment with halocynthiaxanthin were also little affected. Thus, the mechanism of anti-tumor action of halocynthiaxanthin is not clear yet and should be investigated more extensively. In a preliminary

Table 3. Effect of halocynthiaxanthin on the proliferation of various tumor cells

Concentration of halocynthiaxanthin (μg/ml)	Number of viable cells ($\times 10^{-4}$ cells/dish)			
	HGC-27	COLO 320DM	PANC-1	HeLa
0 (control)	6.6	27.1	31.0	59.0
1	5.1	18.1	24.5	40.0
2	3.9	13.6	22.0	25.5
5	0.1	10.7	8.0	6.5

Various kinds of human malignant tumor cells were treated with halocynthiaxanthin. The number of viable cells was counted after 2 days. Data are mean values of duplicate experiments.

experiment, we found that the release rate of membrane-bound Ca^{2+} was decreased by halocynthiaxanthin. Therefore, it seems to be possible that modulation of the structure and function of cellular membranes may be involved in the mechanism of anti-tumor action of halocynthiaxanthin. In the present study, the incorporation of L-[^{3}H]leucine into the acid-insoluble fraction was found to be decreased by treatment with halocynthiaxanthin. This inhibition may be explained by the functional retardation in amino acid transport across cell membranes modified with halocynthiaxanthin. Thus, analysis of halocynthiaxanthin-induced alteration in the membrane transport activity for nutrients is now in progress.

Some of natural carotenoids have been demonstrated to have not only anti-tumor activity, but also anti-tumor-promoting activity.[9] In a preliminary study, we found that halocynthiaxanthin was also effective as an anti-tumor promoter, e.g. it inhibited tumor promoter-enhanced phospholipid metabolism. Tumor promoter-induced expression of Epstein–Barr virus early antigen in Raji cells was also inhibited by halocynthiaxanthin. Thus, halocynthiaxanthin may be a possible anti-tumor promoting agent *in vivo*.

From these results, halocynthiaxanthin seems to be promising as a cancer control agent and worthy of being investigated more extensively.

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