Pure Appl. Chem., Vol. 71, No. 12, pp. 2273–2278, 1999.
Printed in Great Britain.

Cancer prevention by carotenoids*

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Abstract: Various natural carotenoids have been proven to have anticarcinogenic activity. Epidemiological investigations have shown that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits. As β-carotene is present in abundance in these vegetables and fruits, it has been investigated extensively as a possible cancer preventive agent. However, various carotenoids which coexist with β-carotene in vegetables and fruits also have anticarcinogenic activity, and some of these, such as α-carotene, lutein and lycopene, show a higher potency than β-carotene in suppressing experimental carcinogenesis. Thus, we have carried out more extensive studies on cancer preventive activities of natural carotenoids in foods. For example, we found that β-cryptoxanthin showed antitumor initiating activity, as well as antitumor promoting activity. It is of interest that not only carotenoids distributed in vegetables and fruits, but also animal carotenoids, such as astaxanthin, are promising as cancer preventive agents. In the present study, the cancer preventive potential of phytoene was also confirmed. The establishment of NIH3T3 cells that produce phytoene by introducing the *crtB* gene provides evidence that resistance against transformation, imposed by transfection of activated H-*ras* oncogene, was acquired by phytoene production. Analysis of the action mechanism of these natural carotenoids is now in progress, and some interesting results have already been obtained; for example, various carotenoids were suggested to stimulate the expression of RB gene, an antioncogene.

INTRODUCTION

Various factors in foods, such as antioxidative vitamins and carotenoids, phenolic compounds, terpenoids, steroids, indoles and fibers, have been considered to be responsible for the reduction of life style-related diseases, including cancer. Among them, carotenoids have been studied widely and have been proven to show diverse beneficial effects on human health. Initially, carotenoids were suggested to serve as precursors of vitamin A as the active compound. In this context, β-carotene has been studied most extensively, as β-carotene has the highest provitamin A activity among carotenoids. However, Peto *et al*. [1] suggested that β-carotene could have a protective effect against cancer without converting to vitamin A. Therefore, various carotenoids other than β-carotene may also be able to suppress

*Lecture presented at the 12th International Symposium on Carotenoids, Cairns, Australia, 18–23 July 1999, pp. 2205–2302.
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carcinogenesis. Of more than 600 carotenoids identified to date, about 40 carotenoids are found in our daily foods. Thus, we decided to evaluate the biological activities of these carotenoids, and found that some showed more potent activity than β-carotene in suppressing the process of carcinogenesis. It is of interest that not only plant carotenoids, but also animal carotenoids, are promising as cancer preventive agents.

Some natural carotenoids, such as phytoene, are unstable when they are purified, and it is thus very difficult to examine their biological activities. In such cases, stable production of these carotenoids in target cells may be helpful for more accurate evaluation of their biological properties. In this context, we tried to develop a new method for the synthesis of phytoene in animal cells. Establishment of mammalian cells producing phytoene was performed by the introduction of the *crtB* gene, which encodes phytoene synthase. These cells were proven to acquire resistance against transformation, imposed by transfection of activated H-*ras* oncogene.

ANTICARCINOGENIC ACTIVITY OF NATURAL CAROTENOIDS

Among the carotenoids distributed in our daily foods, α-carotene, lutein, zeaxanthin and lycopene, as well as β-carotene, are now being investigated by international collaboration as promising candidates for cancer prevention, as these carotenoids are commonly found in vegetables and fruits, and are also detectable in human plasma.

α-Carotene is found in vegetables, such as carrots and pumpkin. Initially, we found that α-carotene induced G1-arrest in the cell cycle [2]. As various agents which induce G1-arrest have been proven to have cancer preventive activity, we evaluated the anticarcinogenic activity of α-carotene. α-Carotene showed a higher activity than β-carotene in suppressing tumorigenesis in skin, lung, liver and colon [3,4].

Lutein is the dihydroxy form of α-carotene, and is distributed among a variety of vegetables, such as kale, spinach and winter squash, and fruits, such as mango, papaya, peaches, prunes and oranges. An epidemiological study in the Pacific Islands indicated that people with a high intake of all three compounds (β-carotene, α-carotene and lutein) had the lowest risk of lung cancer [5]. Thus, the effect of lutein on lung carcinogenesis was examined. Lutein showed antitumour promoting activity in a two-stage carcinogenesis experiment in the lung of ddY mice, initiated with 4-nitroquinoline-1-oxide (4NQO) and promoted with glycerol. 4NQO (10 mg/kg body weight), dissolved in a mixture of olive oil and cholesterol (20:1), was given by a single subcutaneous injection on the first experimental day. Glycerol (10% in drinking water) was given as a tumor promoter from experimental week 5 to week 30 continuously. Lutein, 0.2 mg in 0.2 mL of a mixture of olive oil and Tween 80 (49:1), was given by oral intubation three times a week during the tumor promotion stage (25 weeks). Treatment with lutein showed a decreased tendency for lung tumor formation: the control group developed 3.1 tumors per mouse, whereas the lutein-treated group had 2.2 tumors per mouse. Lutein also inhibited the development of aberrant crypt foci in Sprague–Dawley (SD) rat colon induced by *N*-methyl-nitrosourea (MNU) [4].

Zeaxanthin is the dihydroxy form of β-carotene, and is distributed in various vegetables. Recently, some features of zeaxanthin were elucidated. For example, it was found that spontaneous liver carcinogenesis in C3H/He male mice was suppressed by treatment with zeaxanthin (at a concentration of 0.005%, mixed as an emulsion in drinking water), as shown in Table 1.

Table 1 Effect of zeaxanthin on tumorigenesis in mouse liver

Group	Number of mice	Tumor-bearing mice (%)	Average number of tumors per mouse
Control	14	35.7	1.75
+ Zeaxanthin	12	8.3	0.08

C3H/He male mice were used. Zeaxanthin, 0.005% in drinking water, was given during the whole period of the experiment (40 weeks).

Lycopene occurs in our diet predominantly in tomato products. Recently, the exceptionally high singlet oxygen quenching ability of lycopene was found [6,7]. An epidemiological study in elderly Americans indicated that a high tomato intake was associated with a 50% reduction of mortality from cancers at all sites [8]. A case-control study in Italy showed the potential protection of a high consumption of lycopene in the form of tomatoes against cancers of the digestive tract [9]. An inverse association between a high intake of tomato products and prostate cancer risk was also reported [10]. The anticarcinogenic activity of lycopene was found in animal models in the mammary gland, liver, lung, skin and colon [4,11]. A study in mice with a high rate of spontaneous mammary tumors showed that the intake of lycopene delayed and reduced tumor growth. Spontaneous liver carcinogenesis in C3H/He male mice was also suppressed. Treatment for 40 weeks with lycopene (at a concentration of 0.005%, mixed as an emulsion in drinking water) resulted in a significant decrease of liver tumor formation: the control group developed 7.7 tumors per mouse, whereas the lycopene-treated group had 0.92 tumors per mouse ($P < 0.005$). Lycopene showed antitumor promoting activity in a two-stage carcinogenesis experiment in the lung of ddY mice, initiated with 4NQO and promoted with glycerol. Lycopene, 0.2 mg in 0.2 ml of a mixture of olive oil and Tween 80 (49:1), was given by oral intubation three times a week during the tumor promotion stage (25 weeks). Treatment with lycopene resulted in a significant decrease of lung tumor formation: the control group developed 3.1 tumors per mouse, whereas the lycopene-treated group had 1.4 tumors per mouse ($P < 0.05$). The antitumor promoting activity of lycopene was confirmed by another two-stage carcinogenesis experiment: lycopene showed antitumor promoting activity in a two-stage carcinogenesis experiment in the skin of ICR mice. From 1 week after initiation by 100 μg of 7,12-dimethylbenz[a]anthracene (DMBA), 1.0 μg (= 1.6 nmol) of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was applied twice a week for 20 weeks. Lycopene (160 nmol, molar ratio to TPA = 1:100) was applied with each TPA application. At week 20 of promotion, the average number of tumors per mouse in the control group was 8.5, whereas the lycopene-treated group had 2.1 tumors per mouse ($P < 0.05$). Lycopene also inhibited the development of aberrant crypt foci in SD rat colon induced by MNU (three intrarectal administrations of 4 mg in week 1). Lycopene (0.12 mg, suspended in 0.2 ml of corn oil, intragastric gavage daily) or vehicle as control were administered during weeks 2 and 5. The mean number of colonic aberrant crypt foci in the control group at week 5 was 69, whereas the lycopene-treated group had 34 ($P < 0.05$).

In addition to the carotenoids mentioned above, β-cryptoxanthin seems to be a promising carotenoid, as it showed the strongest inhibitory activity in the *in vitro* screening test: β-crytoxanthin suppressed TPA-induced expression of the early antigen of Epstein–Barr virus in Raji cells with the highest potency among the carotenoids tested [12]. TPA-enhanced ^{32}Pi-incorporation into phospholipids of cultured cells was also inhibited by β-cryptoxanthin. β-Cryptoxanthin is distributed in our daily food, such as oranges, and is one of the major carotenoids which is detectable in human blood. Thus, it seems worthwhile to investigate this compound more precisely. In this context, we further examined the anticarcinogenic activity *in vivo*.

β-Cryptoxanthin showed antitumor promoting activity in a two-stage carcinogenesis experiment in the skin of ICR mice, initiated with DMBA and promoted with TPA. β-Cryptoxanthin (160 nmol, molar ratio to TPA = 1:100) was applied 1 h before each TPA application. At week 20 of promotion, the percentage of tumor-bearing mice in the control group was 64%, whereas the percentage of tumor-bearing mice in the group treated with β-cryptoxanthin was 29%. The average number of tumors per mouse in the control group was 2.7, whereas the β-cryptoxanthin-treated group had 1.6 tumors per mouse ($P < 0.05$).

β-Cryptoxanthin also showed antitumor initiating activity in a two-stage carcinogenesis experiment in the skin of SENCAR mice, initiated with peroxynitrite (390 nmol, once) and promoted with TPA. β-Cryptoxanthin (0.0025% in drinking water) was administered from 1 week before to 1 week after the initiation by peroxynitrite. At week 20 of promotion, the average number of tumors per mouse in the control group was 6.3, whereas the β-cryptoxanthin-treated group had 2.5 tumors per mouse (Table 2).

Furthermore, β-cryptoxanthin inhibited the development of aberrant crypt foci in SD rat colon induced by MNU (three intrarectal administrations of 4 mg in week 1). β-Cryptoxanthin (0.048 mg, 0.24 mg or 1.2 mg suspended in 0.2 ml of corn oil, intragastric gavage daily) or vehicle as control were administered during weeks 2 and 5. The mean number of colonic aberrant crypt foci in the control group at week 5 was

Table 2 Effect of β-cryptoxanthin on tumorigenesis in mouse skin

Group	Number of mice	Tumor-bearing mice (%)	Average number of tumors per mouse
Control	15	100	6.3
+β-Cryptoxanthin	15	93	2.5

SENCAR mice were used. tumor initiator: peroxynitrite (390 nmol, once). Tumor promoter: TPA (1.7 nmol/painting, twice a week). β-Cryptoxanthin (0.002% drinking water) was given from 1 week before to 1 week after the initiation.

42, whereas those in the groups treated with β-cryptoxanthin at doses of 0.048 mg, 0.24 mg or 1.2 mg were 26, 25 and 25, respectively. Based on these data, an additional study on β-cryptoxanthin was carried out. Four groups of F344 rats ($n = 25$ each) received an intrarectal dose of 2 mg MNU, three times a week for 5 weeks, and were fed a diet supplemented with or without β-cryptoxanthin (0.0025%). The colon cancer incidence at week 30 was significantly lower in the β-cryptoxanthin diet group (68%) than in the control group (96%) ($P < 0.05$). The tumor multiplicity was also lower in the β-cryptoxanthin-treated group (1.4 tumors per rat) than in the control group (1.7 tumors per rat), but not statistically significant.

Analysis of the action mechanism of β-cryptoxanthin reveals that it stimulates the expression of the RB gene, an antioncogene.

Astaxanthin, which is found in shrimp, crab and salmon, has been proven to suppress spontaneous liver carcinogenesis in C3H/He male mice. The mean number of liver tumors was significantly decreased by astaxanthin treatment as compared with that in the control group: the control group developed 0.87 tumors per mouse, whereas the astaxanthin-treated group had 0.27 tumors per mouse ($P < 0.05$) (Table 3).

Table 3 Effect of astaxanthin on tumorigenesis in mouse liver

Group	Number of mice	Tumor-bearing mice (%)	Average number of tumors per mouse
Control	15	53	0.87
+Astaxanthin	15	27	0.27

C3H/He male mice were used. Astaxanthin (0.2 mg suspended in 0.2 mL of corn oil, intragastric gavage, three times per week) was given for 40 weeks.

ESTABLISHMENT OF PHYTOENE PRODUCING MAMMALIAN CELLS, AND ANALYSIS OF THEIR PROPERTIES

Phytoene, which is detectable in human blood, has been proven to suppress tumorigenesis in skin. It was suggested that the antioxidative activity of phytoene may play an important role in its action mechanism. In order to confirm the mechanism, a more precise study is needed. However, phytoene becomes unstable when it is purified, and thus it is very difficult to examine its biological activity. Therefore, the stable production of these carotenoids in target cells, which may be helpful for the evaluation of their biological properties, was attempted. As the phytoene synthase encoding gene, *crtB*, has already been cloned from *Erwinia uredovora* [13], we used it for the expression of the enzyme in animal cells. Mammalian expression plasmids, pCAcrtB, to transfer the *crtB* gene to mammalian cells, were constructed as follows. First, the sequence around the initiation codon of the *crtB* gene on the plasmid pCRT-B was modified by polymerase chain reaction (PCR) using the primers to replace the original bacterial initiation codon TTG with CTCGAGCCACCATG, which is a composite of the typical mammalian initiation codon ATG preceded by the Kozak consensus sequence and a *Xho*1 recognition site. The *Xho*1 linker which harbors a cohesive end for the *Eco*R1 site was ligated to the *Eco*R1 site at the 3′-end of the *crtB* gene, and the 969-base pair (bp) *Xho*1 fragment was cloned into the *Xho*1 site of the expression vector pCAGGS. The resulting plasmid pCAcrtB drives the *crtB* gene by the CAG promoter (modified chicken β-actin promoter coupled with cytomegalovirus immediate early enhancer).

Plasmids were transfected either by electroporation or lipofection. For the gene transfer to NIH3T3 cells, which were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 4 mM L-glutamine, 80 U/mL penicillin, 80 mg/mL streptomycin and 10% calf serum (CS), the parameter for electroporation using a Gene Pulser (BioRad) was set at 1500 V/25 mF with a DNA concentration of 12.5–62.5 μg/mL. Lipofection was carried out using Lipofectamine (Gibco BRL) according to the protocol supplied by the manufacturer.

For Northern blot analysis, 20 μg of total RNA was loaded onto a 1.2% formaldehyde agarose gel, electrophoresed and transferred to a nitrocellulose filter (Nitroplus). The 969-bp *Xho*1 fragment of the *crtB* gene as mentioned above was labeled with [^{32}P]dCTP by the random primer labeling method and used as a probe to hybridize the target RNA on the filter.

NIH3T3 cells transfected with pCAcrtB showed the expression of a 1.5 kilobase mRNA from the *crtB* gene as a major transcript. Those transcripts were not present in the cells transfected with the vector alone.

For analysis of phytoene by high performance liquid chromatography (HPLC), the lipid fraction including phytoene was extracted from cells (10^7–10^8). The sample was subjected to HPLC (column: 3.9 × 300 mm, Nova-pakHR, 6 m C18, Waters) at a flow rate of 1 mL/min. To detect phytoene, UV absorbance of the eluate at 286 nm was measured by a UV detector (JASCO875).

Phytoene was detected as a major peak in the HPLC profile in NIH3T3 cells transfected with pCAcrtB, but not in control cells. Phytoene was identified by UV and field desorption mass spectra.

As lipid peroxidation is considered to play a critical role in tumorigenesis, and it was suggested that the antioxidative activity of phytoene may play an important role in its mechanism of anticarcinogenic action, the level of phospholipid peroxidation induced by oxidative stress in cells transfected with pCAcrtB or with vector alone was compared.

Oxidative stress was imposed by culturing the cells in a Fe^{3+}/adenosine 5′-diphosphate (ADP) containing medium (374 mM iron(III) chloride, 10 mM ADP dissolved in DMEM) for 4 h. The cells were then washed three times with a Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS(−)), harvested by scraping, washed once with PBS(−), suspended in 1 mL of PBS(−) and freeze–thawed once. The lipid fraction was extracted from the cell suspension twice with 6 ml of chloroform–-methanol (2:1). The chloroform layer was collected and dried with sodium sulfate. The sample was evaporated, and its residue was dissolved in a small volume of HPLC solvent (2-propanol–n-hexane–methanol–H_2O = 7:5:1:1) and then subjected to chemiluminescence-HPLC (CL-HPLC). The lipid was separated with the column (Finepack SIL NH2-5, 250 mm × 4.6 mm i.d., JASCO) by eluting with the HPLC solvent (see above) at a flow rate of 1 mL/min at 35 °C. Post-column chemiluminescent reaction was carried out in a mixture of 10 mg/mL cytochrome *c* and 2 mg/mL luminol in a borate buffer (pH 10.0) at a flow rate of 1.1 mL/min. To detect lipids, UV absorbance of the eluate at 210 nm was measured by a UV-8011 detector (TOSOH), and chemiluminescence was detected with a CLD-110 detector (Tohoku Electric Ind.).

The phospholipid hydroperoxidation level in the cells transfected with pCAcrtB, and confirmed to produce phytoene by HPLC, was lower than that in the cells transfected with vector alone. Thus, the antioxidative activity of phytoene in animal cells was confirmed.

It is of interest to test the effect of the endogenous synthesis of phytoene on the malignant transformation process which is newly triggered in noncancerous cells. Thus, the study was carried out on NIH3T3 cells producing phytoene for its possible resistance against oncogenic insult imposed by transection of the activated H-*ras* oncogene. Plasmids with activated H-*ras* gene were transfected to NIH3T3 cells with or without phytoene production, and the rate of transformation focus formation in 100 mm diameter dishes was compared. It was proven that the rate of transformation focus formation induced by the transfection of activated H-*ras* oncogene was lower in the phytoene producing cells than in controls cells (Table 4).

This type of experimental method may be applied to the evaluation of the anticarcinogenic activity of other phytochemicals, as the cloning of genes for the synthesis of various kinds of substances in vegetables and fruits has already been accomplished. It is particularly useful for the evaluation of the biological activity of unstable phytochemicals, such as phytoene and other carotenoids.

Table 4 Suppression of transformation focus formation induced by activated H-*ras* gene in phytoene producing NIH3T3 cells

	Number of transformed foci	
Oncogene	Control	+*crtB*
ras-1 (pNCO102)	47	22
ras-2 (pNCO602)	80	15

ACKNOWLEDGEMENTS

This work was supported in part by grants from BRAIN, the Ministry of Health and Welfare, the Ministry of Education, Science, Sports and Culture, SRF, and the Plant Science Research Foundation. The study was carried out in collaboration with Dr. Zohar Nir, Lyco-Red Natural Products Industries, Ltd., Israel, and Dr. Frederick Khachik, University of Maryland.

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