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Cancer Prevention by Natural Carotenoids

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Abstract Epidemiological investigations have shown that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits. Since 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 that coexist with β-carotene in vegetables and fruits also have anticarcinogenic activity. Some of them, such as α-carotene, showed higher potency than β-carotene in suppressing experimental carcinogenesis. Thus, we have carried out more extensive studies on cancer-preventive activities of natural carotenoids, which found that lycopene and lutein had potent anticarcinogenic activity. In the present study, the cancer-preventive activity of phytoene was also comfirmed biotechonologically when mammalian cells producing phytoene were resistant to H-*ras*-induced cell transformation. Further studies on various natural carotenoids besides β-carotene should be continued to obtain more information about the potential of natural carotenoids in the field of cancer prevention. J. Cell. Biochem. Suppl. 27:86–91. © 1998 Wiley-Liss, Inc.

Key words: cancer prevention; natural carotenoids; lycopene; lutein; phytoene; crtB gene

Cancer chemoprevention appears to be a promising method for cancer control. Among chemopreventive agents, constituents in foods are particularly important. We have extensively surveyed anticarcinogenic materials in foods; useful agents were found mainly in plant sources, flavonoids, terpenoids, and carotenoids being typical examples. Of the carotenoids, β-carotene has been studied extensively [1]. However, other types of carotenoids that co-exist with β-carotene in vegetables and fruits may also contribute to their anticarcinogenic activity. In fact, we found that carotenoids such as α-carotene showed higher potency than β-carotene in suppressing carcinogenesis in animal experiments [2]. Therefore, we have extended our cancer prevention studies to various additional natural carotenoids in vegetables and fruits. In the present study, we evaluated the anticarcinogenic activity of lycopene and lutein (Fig. 1).

Some natural carotenoids such as phytoene are unstable when purified, making it very difficult to examine their biological activities. In such cases, stable production of these carotenoids in target cells would help to more accurately evaluate their biological properties. To this end, we developed a new method of synthesizing phytoene in animal cells. Establishment of phytoene-producing mammalian cells was accomplished by the introduction of the crtB gene, which encodes phytoene synthase. These cells proved resistant to transformation imposed by transfection of the activated H-*ras* oncogene, thus confirming the preventive activity of phytoene against carcinogenesis.

MATERIALS AND METHODS

Chemicals

Lycopene (purity 93%) and lutein (purity 65%) were kindly supplied by Dr. Zohar Nir, LycoRed Natural Products Industries, Ltd., BeerSheva, Israel, and Dr. Frederick Khachik, US Department of Agriculture, respectively. These carotenoids were prepared as emulsions [2] to disperse into drinking water, or as a suspension in corn oil for intragastric administration.

Carcinogenesis Experiments

The spontaneous mouse liver carcinogenesis model and the two-stage mouse lung and skin carcinogenesis models were used as described

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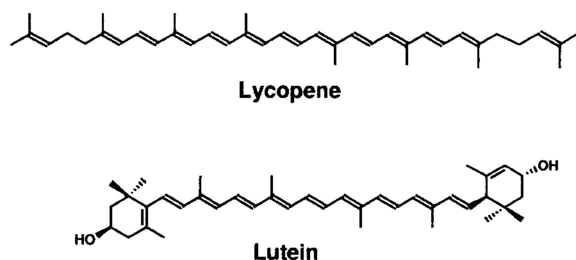

Fig. 1. Structure of lycopene and lutein.

previously to evaluate the chemopreventive effects of lycopene and lutein [1]. In the two-stage mouse skin carcinogenesis experiment with lutein, 12-*O*-teradecanoylphorbol-13-acetate (TPA, 1st-stage promoter) and mezerein (2nd-stage promoter) were used. The colonic aberrant crypt foci (ACF) model was also used, as follows: Female Sprague-Dawley rats, 7 weeks of age at the start of the experiment, were treated with *N*-methylnitrosourea (MNU, 4 mg, intrarectal instillations, 3 times in week 1). Intragastric administration of carotenoid samples, administered as oil suspension at a concentration of 0.24 mg in 0.2 ml corn oil, was continued during weeks 2 and 5. The control group received corn oil only. On week 6, the rats were sacrificed, the colon excised, cut open, lined with cold 0.9% NaCl solution on a flattened filter paper, and fixed in 4% neutral buffered formalin solution. The colons were stained with 0.2% methylene blue dye solution for 10 min, and the ACF were counted under a microscope.

Establishment of Phytoene-Producing Mammalian Cells and Analysis of Their Properties

CrtB gene, a phytoene synthase-encoding gene [3], was cloned from *Erwinia uredovora*. The mammalian expression plasmid, pCAcrtB (Fig. 2), used to transfect the crtB gene into mammalian cells, was constructed as follows. First the sequence around the initiation codon of the crtB gene on the plasmid pCRT-B was modified by PCR using primers to replace the original bacterial initiation codon TTG with the sequence CTCGAGCCACCATG. This sequence is a composite of the typical mammalian initiation codon, ATG, preceded by the Kozak consensus sequence and a Xhol recognition site. The Xhol linker, which harbors a cohesive end of the EcoRI site, was ligated to the EcoRI site at the 3'-end of this crtB gene, and the 969-base pair (bp) XhoI fragment was cloned into the XhoI 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). In the control vector, pCAGGS, a rabbit β-globin polyadenylation signal, is provided just downstream of the XhoI cloning site.

Plasmids were transfected either by electroporation or lipofection. For gene transfer to NIH3T3 cells, cultured in Dulbeccoo's modified minimum essential medium (DMEM) supplemented with 4 mM L-gultamine, 80 U/ml penicillin, 80 mg/ml streptomycin, and 10% calf serum (CS), the parameter for electroporation using a Gene Pulser (BioRad, Richmond, CA) was set at 1,500 V/25mF, and a DNA concentration of 12.5–62.5 μg/ml was used. Lipofection was carried out using Lipofectamine (GIBCO BRL, Gaithersburg, MD) according to the protocol supplied by the manufacturer. pCAcrtB or pCAGGS were contransfected with the plasmid pKOneo (kindly provided by Dr. Douglas Hanahan, University of California, San Francisco), which harbors a neomycin resistance encoding gene.

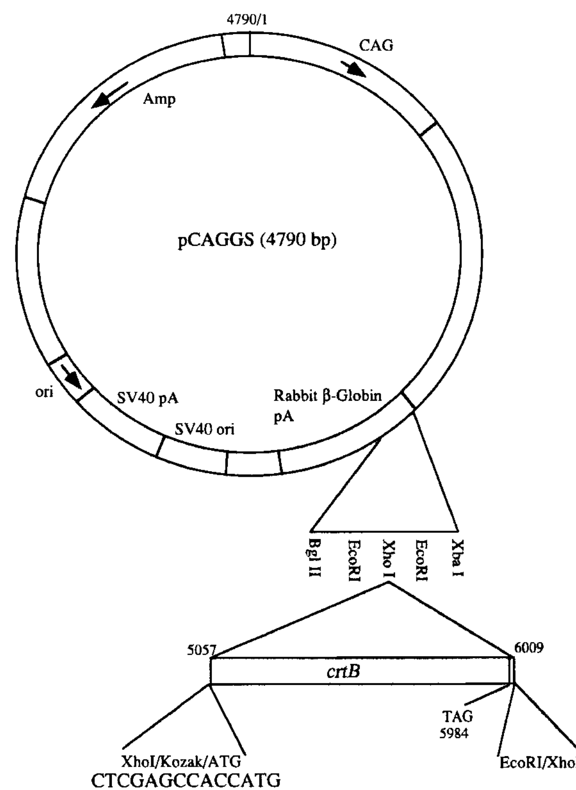

Fig. 2. Construction of pCAcrtB.

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*I fragment of the crtB gene 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.

To analyze phytoene by HPLC, a lipid fraction including phytoene was extracted from the NIH3T3 cells (10^7–10^8) by the standard 2-phase partition method. The extracted sample was subjected to HPLC (column: 3.9 by 300 mm, Nova-pakHR,6μm C18, Waters, Rochester, MN) at a flow rate of 1 ml/min (acetonitrill:methanol: 2-propanol = 90:6:4). To detect phytoene, UV absorbance of the eluate at 286 nm was measured using a UV detector (JASCO875, Jasco, Inc., Easton, MD).

The lipid hydroperoxidation level in the NIH3T3 cells was measured as an index of oxidative stress-induced cellular damage. 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 hours. The cells were then washed three times in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS(-)), harvested by scraping, washed once with PBS(-), resuspended 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 from the column (Finepack SIL NH2–5,250 mm X 4.6 mm i.d. JASCO) by eluting with an HPLC solvent (see above) at a flow rate of 1 ml/min at 35°C. A post-column chemiluminescent reaction was carried out using a mixture of 10 mg/ml cytochrome c and 2 mg/ml luminal in 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.).

NIH3T3 cells producing phytoene were then evaluated for resistance to transformation by activated H-*ras* oncogene. Plasmids containing the activated H-*ras* genes pNCO102 and pNCO602 were transfected into NIH3T3 cells that either produced or did not produce phytoene, and the rate of formation of transformed foci in 100 mm dishes was compared.

RESULTS

Anti-Carcinogenic Activity of Lycopene

Lycopene was shown to suppress (88% reduction in incidence [$P < 0.05$], 88% reduction in multiplicity [$P < 0.01$]) spontaneous liver carcinogenesis in C3H/He male mice (Table I). It also showed anti-tumor promoting activity in a two-stage carcinogenesis experiment in the lungs of ddY mice, initiated with 4-nitro-quinoline-1-oxide (4NQO) and promoted with glycerol (Table I).

The anti-tumor promoting activity of lycopene was confirmed in the ICR mouse skin model in which tumors were initiated with dimethlybenz[*a*]anthracene and promoted with TPA (Fig. 3) (76% reduction at week 18 of promotion, $P < 0.05$). Lycopene also inhibited (38% reduction) the development of aberrant crypt foci in the colon of Spraque Dawley (SD) rats induced by MNU (Table II).

TABLE I. Effect of Lycopene on Tumorigenesis in Mouse Liver and Lung

Group	Number of mice	Tumor-bearing mice (%)	Average number of tumors per mouse
Liver carcinogenesis[a]			
Control	17	88.2	7.65
+Lycopene	13	38.5	0.92
Lung carcinogenesis[b]			
Control	15	66.7	3.07
+Lycopene	13	46.2	1.38

[a]Male C3H/He mice, 6 weeks old, were used. Lycopene, 0.005% in drinking water, was given throughout the experiment (40 weeks).

[b]Male ddY mice were used. 4NQO (10 mg/kg body weight), dissolved in a mixture of olive oil and cholesterol (20:1), was given by a single s.c. injection on the first experimental day. Glycerol (10% in drinking water) was given as tumor promoter from experimental week 5 to week 30 continuously. Lycopene, 0.2 mg in 0.2 ml of mixture of olive oil and Tween 80 (49:1), was given by oral intubation three times a week during tumor promotion stage (25 weeks).

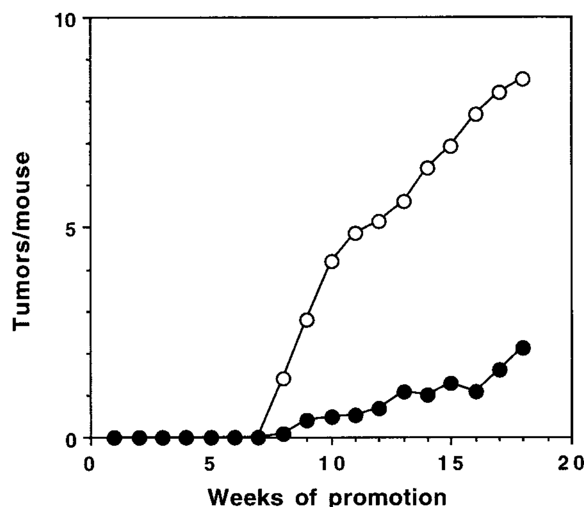

Fig. 3. Effect of lycopene on the promotion of skin tumor formation by TPA in DMBA-initiated female ICR mice. Female ICR mice at the age of 6 weeks were shaved by electric clipper. From 1 week after the initiation by DMBA (100 μg), TPA (1.0 μg = 1.6 nmol) was applied twice a week for 20 weeks. Lycopene (160 nmol, molar ratio to TPA = 100) was applied with each TPA application. o, group treated with DMBA plus TPA (n = 15); o, group treated with DMBA plus TPA and lycopene (n = 15).

TABLE II. Effect of Lycopene and Lutein on Aberrant Crypt Foci Formation in SD Rat Colon Induced by MNU*

Group	Number of aberrant crypt foci/colon		
	With 1–3 aberrant crypts	With 4 or more aberrant crypts	Total
Control	62.2	7.1	69.3
+Lycopene	36.8	5.8	42.7
+Lutein	35.1	5.1	40.2

*Rats received MNU (4 mg, intrarectal administration, 3 times in week 1), and were treated with lycopene or lutein (0.24 mg in 0.2 ml of corn oil, intragastric administration, daily) during weeks 2 and 5.

Anti-Carcinogenic Activity of Lutein

Lutein showed anti-tumor promoting activity in a two-stage carcinogenesis experiment in skin of ICR mice, initiated with diemthylbenz[*a*]anthracene and promoted with TPA and mezerein (Fig. 4)(65% reduction at week 30 of promotion, $P < 0.05$). Lutein also inhibited the development of aberrant crypt foci in SD rat colon induced by MNU (Table II)(42% reduction).

Establishment of Phytoene Mammalian Cells

NIH3T3 cells transfected with pCAcrtB expressed 1.5 kb of mRNA from the crtB gene as a major transcript. Transcripts were not present in cells transfected with the vector alone. Phytoene identified by UV- and field desorption mass-spectra (Table III) was produced by cells containing pCcrtB, but not by control cells.

Analysis of the Properties of Phytoene-Producing Mammalian Cells

Since lipid peroxidation is considered to play a critical role in tumorigenesis, the phospholipid peroxidation level in cells transfected with pCAcrtB was measured. Table IV shows the protective ability of phyotene against oxidative

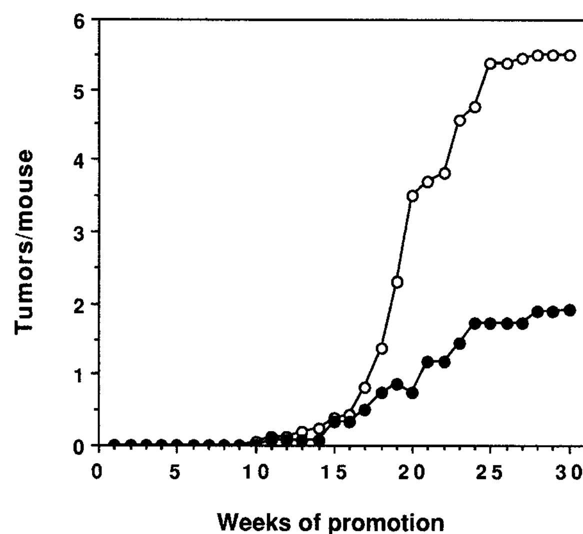

Fig. 4. Effect of lutein on skin tumor formation in female ICR mice, initiated with DMBA and promoted with TPA and mezerein. Female ICR mice, 6 weeks old, were shaved by electric clipper. At 1 week after the initiation by topical application DMBA (100 μg in 100 μl of acetone), TPA (10 nmol in 100 μl of acetone) was applied once, and then mezerein (3 nmol for 15 weeks, and 6 nmol for subsequent 15 weeks in 100 μl of acetone) twice a week. Lutein (1 μmol in 100 μl of acetone, molar ratio to TPA = 100) was applied twice (45 minutes before and 16 hours after TPA application). ○, control group (n = 15); ●, lutein-treated group (n = 15).

TABLE III. Expression of Phytoene Synthase mRNA and Production of Phytoene in Mammalian Cells by Introduction of the crtB Gene From Erwinia Uredovora*

Transfection	Phytoene synthase mRNA	Phytoene (μg/10^7 cells)
Electroporation	+	4.4
Lipofection	+	1.5

*Phytoene synthase mRNA was detected by Northern blot analysis, and phytoene in cell extracts was measured by HPLC.

cellular stress. The phospholipid hydroperoxidation level in phytoene-producing cells was lower (46% reduction) than that in cells transfected with vector alone.

Finally, the rate of H-*ras*-induced transformation in cells transfected with either pCAcrtB or vector alone was compared. The results demonstrate that the rate of formation of transformed foci was lower in the phytoene-producing cells than in control cells (Table V).

DISCUSSION

Recently, we found that various natural carotenoids showed anti-tumor promoter activity in vitro [4]. α-carotene was shown to have higher anticarcinogenic potency in vivo than β-carotene [2]. In the present study, we demonstrate that lycopene and lutein also exhibited potent anticarcinogenic activity in vivo. Phytoene is contained in various vegetables and fruits; for example, a tomato contains about 0.6 μg/g of phytoene in its edible part.

Recent epidemiological investigations have shown that intake of lycopene is associated with a reduced prostate cancer risk [5]. Interestingly, lycopene is the most abundant carotenoid in the prostate gland. Therefore, we are currently investigating the effect of lycopene on prostate carcinogenesis in an animal model system.

In addition to α-carotene, lycopene, and lutein, we have recently found that zeaxanthin has an inhibitory effect on spontaneous liver tumor formation in C3H/He mice (unpublished data). Based on these results, these 4 natural carotenoids in green and yellow vegetables and fruits are being compared for anticarcinogenic activity with that of β-caroten by international collaboration.

Indeed, it appears that more precise investigation of the biological activity of not only β-carotene but also of other carotenoids in daily foods is important for understanding their significance in cancer prevention. Phytoene has been suggested to have anti-oxident activity. In the present study, this hypothesis was confirmed; the level of phospholipid hydroperoxidation induced by the Fe^3/ADP-system was significantly reduced in cells producing phytoene. Furthermore, phytoene-producing cells were proven to acquire resistance against transformation imposed by oncognene H-*ras* transfection. Thus, it is of interest to analyze the relationship between anti-oxidative activity and anti-carcinogenic effect of phytoene.

It may be possible to apply our methods to evaluate the anti-carcinogenic activity of other phytochemicals, since cloning genes to synthesize various substances in vegetables and fruits has already been accomplished. These methods could prove to be particularly useful for evaluating the biological activity of unstable phytochemicals, such as phytoene and other carotenoids.

TABLE IV. Reduction of Oxidative Stress-Induced Lipid Hydroperoxidation Levels in Cells Producing Phytoene*

Transfected plasmid	PCOOH + PEOOH/ PC + PE	(% Inhibition)
Vector	4.6	
crtB	2.5	(46)

*PCOOH: phosphatidylcholine hydroperoxide; PEOOH: phosphatidylethanolamine hydroperoxide; PC: phosphatidylcholine; PE: phosphatidylethanolamine.

TABLE V. Suppression of Transformation Focus Formation Induced by Activated H-*ras* Gene in Cells Producing Phytoene

	Number of transformed foci	
Oncogene	Control	+crtB
ras-1 (pNCO102)	46.5	22.0
ras-2 (pNCO602)	79.5	15.0

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