MINI-REVIEW

Cancer Chemoprevention by Phytochemicals and their Related Compounds

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Abstract

Cancer chemoprevention by phytochemicals may be one of the most feasible approaches for cancer control. For example, phytochemicals obtained from vegetables, fruits, spices, teas, herbs and medicinal plants, such as carotenoids, phenolic compounds and terpenoids, have been proven to suppress experimental carcinogenesis in various organs. These candidates should be evaluated by intervention studies, before acceptance as cancer preventive agents for human application. Phytochemicals may also be useful to develop "designer foods" or "functional foods" for cancer prevention. We are now planning animal foods, such as meats, eggs and milk, which contain anti-carcinogenic phytochemicals. In prototype experiments, expression of genes for synthesis of phytochemicals, such as phytoene and limonene, has been successful in cultured animal cells.

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Key words: chemoprevention, phytochemicals, carotenoids, green tea polyphenols, curcumin, glycyrrhizin, designer foods, functional foods

Introduction

Cancer prevention, one of the most promising strategies for cancer control, may be accomplished by various methods. Among these chemoprevention by phyto-chemicals obtained from vegetables, fruits, spices, teas, herbs and medicinal plants seems one of the most feasible approaches. In the Asian and Pacific area, a wide variety of plants have been traditionally used as foods and/or medicine. Analysis of these plant materials has revealed that various principles for health promoting action, including cancer prevention, are included in them. We are continuing to survey useful anti-carcinogenic agents from such plant materials, and part of our findings are summarized in this paper.

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(1) Carotenoids

Epidemiological investigations have shown that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits (Peto et al., 1981, Hirayama, 1979). Since β-carotene is present in abundance in these vegetables and fruits, it has been proposed as an important factor for cancer prevention. However, it was recently reported that supplements of β-carotene increased the incidence of lung cancer among heavy smokers. One possible explanation is that in epidemiologic studies, β-carotene merely acts as a marker for cancer

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chemopreventive agents which co-exist with β-carotene in green and yellow vegetables and fruits. In fact, in the case of carotenoids, β-carotene is usually associated with other natural carotenoids, such as α-carotene, lutein, lycopene and β-cryptoxanthin, which might be the actual principles for cancer preventive effect of vegetables and fruits. Thus, we are assessing the cancer chemopreventive potency of various natural carotenoids, in addition to continuing evaluation of β-carotene.

Palm fruits, most of which are produced in the Asian and Pacific area, are known to be carotenoid-rich material. Analysis has revealed relatively high amounts of α-carotene, besides β-carotene. Thus, at first, we examined the anti-carcinogenic effects of palm fruit carotenes.

They were found to show potent anti-tumor promoting activity in a two-stage carcinogesis experiment in skin, initiated with dimethylbenz[a]-antracene (DMBA) and promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA) (see Table I for the experimental design). In the control group, the first tumor appeared within 6 weeks of promotion; at the end of the experiment, 97% of mice developed skin tumors, with an average number of 2.6 per mouse. In contrast, treatment with palm fruits carotene at the dose of 0.6 μmol per painting resulted in the complete suppression of tumor formation, i.e., no skin tumors developed during the whole period of the experiment.

Anti-tumor promoting activity of palm fruit carotenes was confirmed in another two-stage carcinogenesis experiment. We examined their effects on the promotion of lung tumor formation by glycerol in 4-nitroquinoline 1-oxide (4NQO)-initiated mice (see Table II). Oral administration of palm fruit carotenes (at a dose of 0.005% in drinking water, ad libitum) decreased the mean number of tumors per mice to about 19 % of that in the control group ($p<0.001$). Significantly decrease in the percentage of tumor-bearing mice to about 33 % of that in the control group ($p<0.05$) was also observed.

In spontaneous liver carcinogenesis in C3H/He male mice, the mean number of hepatomas was significantly decreased by oral administration of palm fruit carotenes at a dose of 0.005% in drinking water, ad libitum) as compared with the control group; from 6.3 to 3.6 tumors/ mouse ($p<0.01$).

Palm fruit carotenes also suppressed N-ethyl-N'-nitrosoguanidine (ENNG)-induced duodenal tumorigenesis. The percentage of tumor-bearing mice was significantly decreased by oral administration (at a dose of 0.05% in drinking water, ad libitum, as compared with that in the control group; 67% of mice in the control group developed tumors, whereas 39% of mice developed tumors in the palm fruit carotene-treated group ($p<0.05$).

As described above, palm fruit carotenes include α-carotene and β-carotene. Thus, we further examined the effect of α-carotene, in comparison with that of β-carotene. It was more found to be more potent at suppressing tumorigenesis in the skin and lung.

In two-stage skin carcinogenesis, α-carotene significantly decreased the mean number of tumors per mouse to about 7% of the control group ($p<0.01$). β-Carotene treatment also decreased the mean number of tumors per mouse, but the difference from the control group was not significant. The percentage of tumor-bearing mice in the control group was 69%, whereas the values in the groups treated with α- or β-carotene were 25% and 31%, respectively. Thus, the former was more inhibitory, though both both carotene forms inhibited skin tumor formation promoted by TPA.

The greater potency of the alpha form in suppressing tumor promotion was confirmed by another two-stage carcinogenesis experiment. We examined the effects of the two types of carotene on the promotion of lung

Table I. Anti-tumorigenic activity of β–cryptoxanthin

Group	(n)	Percentage ofTumor-Bearing Mice	Mean Tumors/ Mouse
Skin Carcinogenesis[1]			
Control	(14)	64	2.7[a]
+ β-Cryptoxanthin	(14)	29	1.6[a]
Colon Carcinogenesis[2]			
Control	(25)	96[b]	1.7
+ β-Cryptoxanthin	(25)	68[b]	1.4

[a]$p<0.05$, [b]$p<0.02$

[1] Tumor initiation was accomplished by a signal application of DMBA (390 nmol) on the shaved backs of female ICR mice. The tumor promoter TPA was applied at a dose of 1.6 nmol / painting twice a week starting 1 week after initiation for 20 weeks. _-Crypto-xanthin (160 nmol) in 200 Π l of acetone was applied 1 h before each application of TPA. Controls were treated with the same amount of vehicle for _-cryptoxanthin.

[2] Male F344 rats were received intrarectal application of 2 mg of N-methylnitrosourea 3 times a week from experimental week 1 to week 5. _-Cryptoxanthin (25 ppm in diet) was applied throughout the experiment (from experimental week 1 to week 30).

tumor formation in 4NQO-initiated mice. Oral administration of α-carotene significantly decreased the mean number of tumors per mouse; the control group developed 4.1 tumors/mouse, whereas the α-carotene-treated group had 1.3 tumors/mouse ($p<0.001$). In contrast, β-carotene showed no suppression under the same experimental conditions.

These results indicate that we should pay more attention to anti-tumorigenic activities of natural carotenoids other than β-carotene. Thus, we expanded the study to test the anti-carcinogenic activity of various natural carotenoids, such as β-cryptoxanthin, lactucaxanthin, lutein, lycopene and fucoxanthin.

First, these natural carotenoids were evaluated using an in vitro screening test system; an assay for inhibitory potency against TPA-enhanced induction of early antigen (EA) of Epstein-Barr virus (EBV) in Raji cells (EBV-EA test system). In this screening test, β-cryptoxanthin obtained from orange, and lactucaxonthin from lettuce, showed potent activity. Therefore, we examined the anti-tumor promoting effect of β-cryptexanthin in in vivo carcinogenesis experiments. In two-stage skin carcinogenesis, treatment with β-cryptexanthin, at the dose of 160 nmol per painting at tumor promoting stage, significantly decreased the mean number of tumors per mouse to about 59% of the control value ($p<0.05$), as shown in Table I. β -Cryptexanthin was also proven to suppress colon carcinogenesis in rats (Table I). Evaluation of lactucaxanthin is a future theme for us.

Lutein is known to be distributed very widely in various vegetables and fruits. Interestingly, epidemiological studies in the Pacific islands indicate that people with a high intake of all of three carotenoids, α -carotene,β-carotene, and lutein, have the lowest risk of lung cancer (Le Marchant et al., 1993). Since evaluation of β-carotene and α-carotene had been performed as described above, we also examined the effects of lutein on lung carcinogenesis; influence on promotion of lung tumor formation by glycerol in 4NQO-initiated mice was investigated. Administration of lutein [0.2 mg in 0.2 ml of mixture of olive oil and Tween 80 (49:1), given by intragastric gavage, three times a week during the tumor promotion stage] decreased the mean number of tumors per mouse; the control group developed 3.1 tumors/mouse, whereas the lutein-treated group had 2.2 tumors/mouse. Anti-tumor promoting activity of lutein was also observed in a two-stage mouse skin experiment (Nishino, 1997). Furthermore, it was proven that lutein inhibited the development of aberrant crypt foci in the Sprague-Dawley rat colon induced by N-methyl-nitrosourea (Narisawa et al., 1996).

In the case of lycopene and fucoxanthin, anti-carcinogenic activities in various organs have been shown (Nishino, 1995 and 1997, Narisawa et al., 1996 and 1998).

In conclusion, various kinds of natural carotenoids other than β-carotene seem to be valuable chemopreventive agents. Thus, further studies on these agents should be carried out.

(2) Green tea polyphenols

Inhibitory effects of tea preparations and their polyphenols against tumor formation and growth have been reported (Yang and Wang, 1993). Several epidemiological studies have detected a negative association between tea consumption and the development of human cancers.

Concerning lung cancer, a number of investigations have revealed that extracts from various kinds of tea or their major polyphenols inhibited development of lung neoplasms chemically induced in mice. We confirmed the inhibitory effect of Japanese green tea polyphenols on lung carcinogenesis: i.e., Japanese green tea polyphenols suppress lung tumor formation promoted by glycerol in 4NQO-initiated mice (Table II).

It is of interest that extracts of Chinese green teas, as well as Japanese green teas, were also proven to have anti-carcinogenic activity in vivo (Table III) (Bu, 1999).

Green tea is popular in China and Japan. For the past 5000 years, people have been drinking this traditional beverage. The advantages for cancer chemoprevention with green tea components are: it is safe with no harmful effects, cheap and easy to mass produce, and its effectiveness for cancer prevention has already been proven by epidemiologic studies. Such advantages in preventing cancer with green tea extracts should facilitate application in the clinical situation for protective advantage.

Table II. Effect of Japanese green tea polyphenols (GTPs) on lung carcinogenesis

Group	(n)	Percentage ofTumor-Bearing Mice	Mean Tumors/ Mouse
Control	(12)	92	4.9
+ Japanese GTPs	(14)	71	1.9

Male ddY mice were initiated with 4NQO (10 mg / kg body weight), and promoted with glycerol (10%) for 25 weeks (from experimental week 5 to week 30). GTPs (0.05% in drinking water) were applied during the tumor promotion period.

Table III. Effect of Chinese green tea extracts (GTEs) on skin and mammary carcinogenesis

Group	(n)	Percentage of Tumor-Bearing Mice	Mean Tumors/ Mouse
Skin Carcinogenesis[1]			
Control	(15)	100	9.9[a]
+ Chinese GTEs	(15)	100	5.0[a]
Mammary Carcinogenesis[2]			
Control	(10)	80	2.6[b]
+ Chinese GTEs	(10)	40	0.6[b]

[a]$p<0.05$, [b]$p<0.01$

[1] Female ICR mice were initiated with DMBA (390 nmol), and promoted with TPA (1.6 nmol / painting twice a week starting 1 week after initiation for 20 weeks). Chinese GTEs (50 _g) was applied 1h after each application of TPA.

[2] Female C3H/OuJ mice, which develop mammary tumors spontaneously, were treated with or without Chinese GTEs (1% in drinking water) throughout the experiment (for 52 weeks).

(3) Curcumin

Curcumin, a major constituent of turmeric yellow, has proven to exert anti-carcinogenic activities in various organs. For example, it showed anti-tumor promoting activity in mouse skin carcinogenesis induced by DMBA plus TPA. The first tumor appeared at week 6 in the group treated with DMBA plus TPA. In the group treated with DMBA plus TPA and curcumin at the dose of 1 μmol per painting, the first tumor appeared at week 17. A comparison of control and curcumin-treated groups at the end of the experiment (i.e., at week 18) is summarized in Table IV. The percentage of tumor-bearing mice in the group treated with DMBA plus TPA and curcumin was decreased to about 7% of the control group. Curcumin also decreased significantly the average number of tumors per mouse to about 1% of the control group ($p<0.001$). Curcumin-treated mice had smooth skin, while those treated with tumor promoter alone had multiple foci of inflammation, scaring, and necrosis. Therefore, curcumin seemed to prevent inflammatory reactions induced by the tumor promoter.

Curcumin was demonstrated to modulate variety of biological phenomena induced by tumor promoter; for example, suppression of phospholipid metabolism, and aggregation of platelets. Since platelet aggregation is known to be involved in the process of tumor metastasis, curcumin may also have anti-metastatic activity. Curcumin was also proven to interact directly with Ca^{2+}-calmodulin complex, which is known as a modulator for various biological systems, including regulation of cell proliferation.

Since curcumin is widely used as a spice and coloring material in foods as well as medicine. Thus, further investigations to characterize the biological activities of curcumin appear to be worthy of attention for public health.

(4) Glycyrrhizin and its related compounds.

Glycyrrhizin, a triterpenoid saponin, is one of the most important biologically active principles in licorice root (Glycyrrhiza spp.), which has been used widely as a medicine as well as a sweetening material.

Since a protective effect of glycyrrhizin against rat liver carcinogenesis has been reported (Watari et al., 1978), investigation of this compound more extensively as one of the promising agents for the purpose of cancer chemoprevention appears warranted. First, we confirmed that glycyrrhizin suppressed hepatoma formation in C3H/He mice (Table V). When mice were treated with glycyrrhizin, the average number of liver tumors per mouse was decreased to half of the control value. The effects

Table IV. Effect of curcumin on skin carcinogenesis

Group	(n)	Percentage of Tumor-Bearing Mice	Mean Tumors/ Mouse
Control	(15)	96	11.2[a]
+ Curcumin	(15)	7	0.1[a]

[a]$p<0.001$

Female ICR mice were initiated with DMBA (390 nmol), and promoted with TPA (0.8 nmol / painting twice a week starting 1 week after initiation for 18 weeks).

Curcumin (1 μmol) was applied 40 min before each application of TPA.

Table V. Effect of glycyrrhizin on liver carcinogenesis in C3H/He male mice

Group	(n)	Percentage ofTumor-Bearing Mice	Mean Tumors/ Mouse
Control	(15)	80	3.1[a]
+ Glycyrrhizin	(15)	73	1.4[a]

[a]$p<0.05$

C3H/He male mice were treated with or without glycyrrhizin (dissolved in the drinking water at the concentration of 5 mg/100ml, for 40 weeks).

proved to be statistically significant ($p<0.05$, t-test).

Glycyrrhizin also suppressed lung tumor promotion by glycerol in 4NQO-initiated ddY male mice. Thus added to drinking water simultaneously with glycerol, it decreased the average number of lung tumors per mouse.

Evaluation of anti-tumor promoting activity of glycyrrhizin was also carried out in an experimental model system for mouse skin two-stage carcinogenesis. In this experiment, glycyrrhetinic acid, instead of glycyrrhizin, was used because glycyrrhetinic acid may be the active form of glycyrrhizin; in fact, glycyrrhetinic acid, but not glycyrrhizin, showed inhibitory effects on tumor promoter-induced phenomena in vitro. The first tumor appeared at week 6 in the control group. In the group treated with glycyrrhetinic acid at the dose of 10 μmol per painting, no tumor was observed until week 10. At week 18 of promotion, the control group produced 8.8 tumors per mouse, whereas the group treated with glycyrrhetinic acid had 1.5 tumors per mouse ($p<0.01$, t-test).

The results described above show that glycyrrhizin and/ or glycyrrhetinic acid are promising cancer chemopreventive agents. Besides glycyrrhetinic acid, various kinds of natural triterpenoids, which are structurally related to glycyrrhetinic acid, have been identified as biologically active substances. These triterpenoids are distributed in our daily foods and medicinal plants. For example, oleanolic acid is known to be widely present in various vegetables. Thus, we further tested their cancer preventive activity. First, we evaluated anti-tumor promoter activity in in vitro tests. For screening of new anti-tumor promoters, evaluation of the inhibitory potency for TPA-enhanced phospholipid metabolism, as well as the EBV-EA test system, have been shown to be valuable. In fact, it is well established that tumor promoters may primarily induce changes in phospholipid metabolism, which is suggested to play an important role in tumor promotion, and various kinds of chemicals, which modulate cellular phospholipid metabolism, have proven to have anti-tumor-promoting activity in vivo. As shown in Table VI, some natural oleanane-type triterpenoid compounds, such as oleanolic acid, hederagenin, and erythrodiol, were found to have anti-tumor promoter activity. It is of interest that the CH_2OH group in the structure seems to enhance biological activity. Thus, to potentiate anti-tumor promoter activity, a COOH group of hedergenin was converted into CH_2OH by chemical modification, obtaining 18β -olean-12-ene-3β , 23, 28-triol (compound I). As expected, compound I showed higher activity than hederagenin. 18-H also seems to be important for the activity because saikogenin A, which lacks 18-H, showed lower anti-tumor-promoter activity. Furthermore, potentiation of anti-tumor promoter activity by the conversation of 18β–H into 18α–H was demonstrated. Thus 18α–olean-12-ene-3β–, 23, 28-triol (compound II) showed the highest inhibition rate on the TPA action thus far tested. The 50 percent inhibitory dose (ID_{50}) of compound II was calculated to be around 3μg/ ml (6μM), which is about half of the ID_{50} of compound I.

These results in the in vitro screening test prompted us to examine the effects of compound II on in vivo two-stage mouse skin carcinogenesis. The first tumor appeared at week 6 in the control group. In the group treated with compound II at the concentration of 81 nmol per painting, the first tumor appeared at week 11. At week 18, the control group treated with DMBA plus TPA without compound II demonstrated 10.6 tumors per mouse, whereas the group treated with DMBA plus TPA and compound II had 0.6 tumors per mouse ($p<0.001$, t-test). Compared with the results for glycyrrhetinic acid, compound II was much more active in suppressing tumor promotion in vivo; the activity of compound II was more than 100 times that observed for glycyrrhetinic acid.

The mechanisms of glycyrrhetinic acid dose for dose suppression of carcinogenesis have not been elucidated yet. Cytoprotective and anti-inflammatory effects may play an important role. In fact, hepatocellular damage induced by liver carcinogens and skin inflammation caused by tumor promoters were effectively suppressed by glycyrrhizin or glycyrrhetinic acid.

Regarding anti-tumor promoting action, glycyrrhetinic acid has further been proven to inhibit various kinds of biological phenomena induced by TPA-type tumor promoters. For example, it inhibited TPA-stimulated hexose transport, which is one of the protein kinase C-dependent phenomena. Because protein kinase C, a possible receptor protein of TPA, has been suggested to be one of the key enzymes in the signal transduction system, the analysis of the interaction of glycyrrhetinic acid with protein kinase C is worthy of attention. In this context, the effect of glycyrrhetinic acid on the binding of

Table VI. Effects of oleanane-type triterpenoids on the enhanced ^{32}P-incorporation into phospholipids of cultured cells induced by TPA

Compound	Dose (μg/ml)	Inhibition Percentage
Oleanolic acid	25	48
Hederagenin	25	73
Erythrodiol	25	81
Saikogenin A	25	42
18b-Olean-12-ene-	25	100
3b,23, 28-triol	10	62
	5	38
	2.5	20
18b-Olean-12-ene-3	25	100
b23,28 triol	10	94
	5	69
	2.5	40

HeLa cells were incubated with one of the test compounds, and after 1 h, ^{32}P was added with or without TPA (50 nM). Incubation was continued for 4 h, and then the radioactivity incorporated into phospholipid fraction was measured. Data, expressed as percentage of inhibition on TPA-enhanced ^{32}P-incorporation, are mean values of duplicate experiments.

TPA was investigated, and it was proven that glycyrrhetinic acid inhibited TPA binding to mouse skin particulate fraction. Furthermore, it was demonstrated that glycyrrhetinic acid inhibited the activity of protein kinase C.

Whatever the mechanism, glycyrrhetinic acid and its related compounds seem to be useful for the purpose of cancer chemoprevention.

(5) Others

We further expanded the screening study to find possible cancer preventive phytochemicals in foods and medicinal plants. Up to date, we found various promising candidates, for example, allixin, isoliquilitigenin, luteolin, nobiletin, and myo-inositol.

Table VII. Production of phytoene in mammalian cells by introduction of the crtB gene

Cells	Plasmidm	RNA	Phytoene (μg/10^7 cells)
NIH3T3	pCAcrtB	+++	4.4
	LcrtBSH	−	n.d.
HeLa	pCAcrtB	+	1.2
	LcrtBSH	−	n.d.

mRNA was detected by Northern blot analysis.
Phytoene in cell extracts was identified by HPLC. n.d.; not detected.

Discussion

A large number of candidate compounds, which may be practically useful for human cancer prevention, have been found. These should be further evaluated by intervention studies, before acceptance of them as definite cancer preventive agents for human beings. At present, phase I and/or phase II studies are going on in the case of carotenoids, green tea polyphenols, curcumin and glycyrrhizin. Unfortunately such intervention studies take long periods. In this context, intermediate (surrogate) end point biomarkers for the evaluation of cancer preventive potency in human should be developed.

By the way, our future aim of the study on anti-carcinogenic agents is the development of "designer foods" or "functional foods" for cancer prevention; i.e., the creation of new type of foods, which are fortified for cancer preventive function. Food-origin anti-carcinogenic agents seem to be especially useful for this purpose. We are now planning to develop designed meats, eggs and milk, which contain anti-carcinogenic phytochemicals.

As a prototype experiment to accomplish this project, we established mammalian cells synthesizing phytoene, an anti-carcinogenic carotenoid, by introduction of the crtB gene, phytoene synthase gene. Expression plasmids, pCAcrtB, were constructed to transfer the crtB gene to mammalian cells, and transfected into NIH3T3 cells and HeLa cells by electroporation. Phytoene was detected in these cells as shown in Table VII.

Recently, we have also succeeded in expressing the limonene synthase gene in mammalian cells. Since limonene has been suggested to be a possible anti-carcinogenic phytochemical, we will continue this study and design future projects for the development of valuable functional foods.

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Personal Profile: **Hoyoku Nishino**

Pending. Dr Hoshio appreciates the local culture of the Kyoto area, as can be gleaned from this illustration of one of his more relaxed moments