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Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation

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The present study was carried out to examine the chemopreventive effects of carotenoids such as fucoxanthin, lycopene and lutein as well as curcumin and its derivative, tetrahydrocurcumin (THC), on development of putative preneoplastic aberrant crypt foci (ACF) in colons of mice initiated with 1,2-dimethylhydrazine dihydrochloride (DMH). Influence on proliferation of colonic crypt epithelial cells was also assessed in terms of 5-bromo-2′-deoxyuridine (BrdU) incorporation. Five-week-old B6C3F$_1$ male mice were divided into three groups, groups 1 and 2 being given DMH (20 mg/kg body wt, s.c.) twice a week for 3 weeks. Animals of group 1 were then treated with one of the test compounds, lycopene (0.005% and 0.0025%) or fucoxanthin (0.01%) in the drinking water and lutein (0.05%), curcumin (0.5%) or THC (0.5% and 0.2%) in the diet from weeks 5–12. Group 2 served as a carcinogen alone control and group 3 mice were given test compounds alone. All animals were killed at week 12. Numbers of ACF/mouse in the group 1 treated with fucoxanthin (47.1 ± 13.7), lutein (42.6 ± 19.6) or 0.5% THC (46.6 ± 17.7) were significantly decreased as compared to the control group 2 value (63.3 ± 19.4) ($P < 0.01$). Numbers of aberrant crypts (ACs)/mouse were also significantly lower after treatment with lutein (79.9 ± 34.7) or 0.5% THC (81.8 ± 32.5) than in the control group (115.1 ± 37.1) ($P < 0.01$). BrdU labeling indices (LI) in mice treated with lutein and 0.5% THC were significantly decreased in both upper and lower half compartments of colonic crypts as compared to the controls ($P < 0.05$ and 0.01, respectively), especially the upper half data corresponding to reduction of ACs/mouse. The results thus suggest that fucoxanthin, lutein, and THC may have potential as chemopreventive agents against colon carcinogenesis.

***Abbreviations:** THC, tetrahydrocurcumin; ACF, aberrant crypt foci; DMH, 1,2-dimethylhydrazine dihydrochloride; BrdU, 5-bromo-2′-deoxyuridine; AC, aberrant crypt; LI, labeling index.

Introduction

Colorectal cancer is a main cause of cancer death in western countries, for example accounting for 15% of all cancers and over 60 000 mortalities annually in the US alone (1). Although the etiology of colon cancer is considered to be multifactorial and complex, dietary factors like a high animal fat intake are considered to be positively linked with an elevated incidence (2–4).

A number of studies have shown that a high consumption of fruits and vegetables causes increase in serum or plasma levels of carotenoids (5,6) and is closely associated with a reduced risk of cancer (7). Naturally occurring carotenoids and curcumin, which are abundant in plant foods, are known to have antioxidant activities and to prevent free radical-induced cellular damage (8–10). Plant constituents such as β-carotene, canthaxanthin and curcumin have in fact already been examined for chemopreventive effects on colon neoplasia (8,11–14). However, little is known about the potential of the related agents, lycopene, lutein and fucoxanthin to inhibit mouse colon carcinogenesis. Recently tetrahydrocurcumin (THC*), a major metabolite and active form of curcumin, found to inhibit mouse skin carcinogenesis (15), was shown to possess more potent antioxidant activity than curcumin (16). In animal studies, colon carcinogenesis models using by azoxymethane or 1,2-dimethylhydrazine dihydrochloride (DMH) with putative preneoplastic aberrant crypt foci (ACF) as end-point marker lesions have been used for assessing the influence of modulatory factors (17,18).

In the present study, chemopreventive effects of lycopene, fucoxanthin, and lutein as well as curcumin and its derivative, THC, were examined in a DMH-initiated colon carcinogenesis model in mice by assessing quantitative values for preneoplastic ACF and cellular proliferative status in terms of 5-bromo-2′-deoxyuridine (BrdU) labeling (19).

Materials and methods

Animals

The animals used were 4 week-old-male B6C3F$_1$ mice (Charles River Japan Co., Ltd., Atsugi, Japan). After acclimatization for 1 week, they were housed five to a plastic cage and fed on basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), in an animal facility controlled at a temperature of 23 ± 2°C, 60 ± 5% humidity, and with a 12 h light and dark cycle.

Chemicals

DMH was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and BrdU from Sigma Chemical Co. (St Louis, MO, USA). The chemical structures of the test compounds used for the present study are shown in Figure 1. Lycopene (93% pure) (LycoRed Natural Products Industries, Ltd., Beer-Sheva, Israel) was isolated from tomatoes. Lutein (95% pure) (Kemin Industries, Inc., Des Moines, IO, USA) and fucoxanthin (92% pure) (Nippon Suisan Kaisha, Ltd., Tokyo, Japan) were prepared from extracts of *Tagetes ercta* and *Hijikia fusiforme*. THC (>93% pure) (Nikken Fine Chemicals Co., Ltd.) was prepared from curcumin (>95% pure) obtained from rhizomes of turmeric by

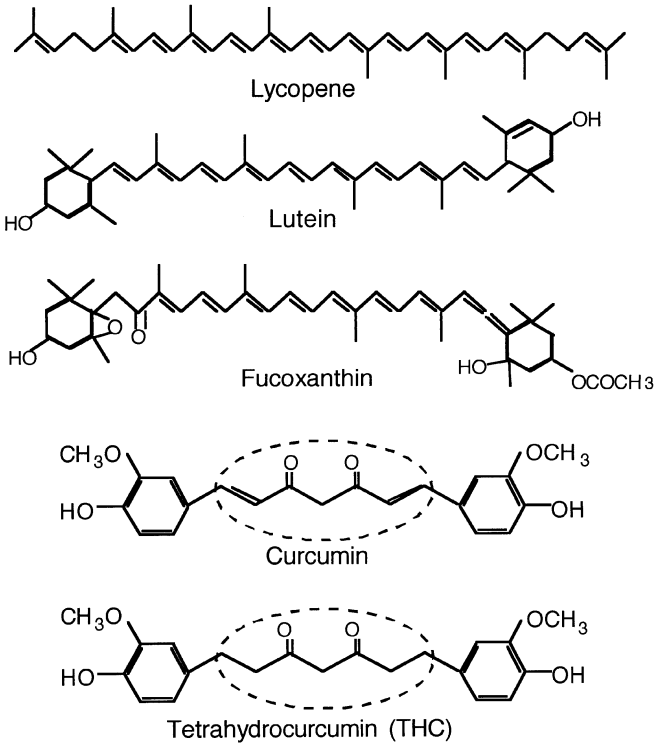

Fig. 1. Structures of the test compounds.

hydrogenating the two double bonds conjugated to β-diketone. Their purities were determined by HPLC on a LiChrosorb RP-8 column (E-Merck, Darmstadt, Germany) and the compounds were stored in sealed vials under anaerobic conditions at –80°C in the dark. Fresh solutions were prepared every three days. Under these conditions it was confirmed that 0.0025 and 0.005% solutions of lycopene remained stable at a room temperature of 22°C, with an average of 89.3% on day 3 of the control solution stored at –80°C as determined by HPLC on a Cosmosil 5C18 column (Nacalai Tesque, Kyoto, Japan) (measurements performed by Lion Corporation, Research Center, Tokyo).

Experimental protocol

Mice of groups 1 and 2 received s.c. injections of DMH (20 mg/kg body wt) twice a week for 3 weeks. At week 5, group 1 animals were divided into 7 treatment subgroups: (a) 0.005% and (b) 0.0025% lycopene, (c) 0.01% fucoxanthin (a–c, in the drinking water), (d) 0.05% lutein, (e) 0.5% curcumin, (f) 0.5% and (g) 0.2% THC (d–g, in the basal diet). The administered doses of test compounds were selected on the basis of results of preliminary and previous studies (20–22). Test compounds were given for 7 weeks. Group 2 served as a DMH alone control and group 3 mice were given the test compounds without prior carcinogen treatment. At week 12, all surviving animals were killed and colons were immediately removed and flushed with 10% formalin solution for 10 min, cut longitudinally from anus to cecum, then placed on flat filter paper for further fixation. After staining with 0.25% methylene blue solution for 15 min, ACFs and aberrant crypts (ACs) were counted under a light microscope as previously described (13,18,20,23–25).

Immunohistochemistry and BrdU labeling index (LI)

BrdU (100 mg/kg body wt) was injected i.p. into 5 mice from each subgroup, 1 h before being killed. After counting ACFs, portions of colon, 2–6 cm from the anus, were taken for paraffin embedding then sectioned for BrdU immunostaining. Mouse anti-BrdU IgG (Dako A/S, Denmark) was applied at a dilution of 1:500 and binding sites were visualized by the avidin-biotin complex immunoperoxidase technique (ABC kit; Vector Laboratories, Burlingame, CA, USA), followed by light counterstaining with hematoxylin (26). The BrdU LI for each group was scored as the number of BrdU positive cells relative to the total number of epithelial cells in the crypts. Five hundred crypts from 5 mice (100 each) were included for counting ACF and ACs. Differences in numbers of ACF, ACs and BrdU LI as well as body and organ wts for each group were analyzed using the Student's *t*-test.

Results

General observations

In group 1, animals treated with fucoxanthin showed significant increases in body and relative kidney wts ($P < 0.05$), as compared to the control group 2 values. The relative liver wts in the lutein and 0.2% THC groups showed significant decreases ($P < 0.05$) throughout the study (data not shown). However, no histopathological evidence of toxicity was found in the livers and kidneys of animals in groups 1 and 3.

ACF and AC counts

All mice receiving DMH had ACF, mostly comprising of 2–3 ACs/focus, but none were found in group 3. Data for mean numbers of both ACF and ACs/colon are summarized in Table I. Numbers of ACF/colon in group 1 animals treated with fucoxanthin, lutein, and 0.5% THC were significantly decreased as compared to group 2 values ($P < 0.01$). Similarly, numbers of ACs in group 1 treated with lutein and 0.5% THC were significantly decreased ($P < 0.01$).

BrdU labeling indices

Epithelial cells labeled with BrdU showed preferential localization in the middle and lower halves of the crypts in all groups. Treatment with all the test compounds except 0.0025% lycopene in group 1 caused significant reduction in the BrdU LI as compared with group 2 ($P < 0.01$) when the whole lengths of the crypts were counted. When the crypts were divided into two compartments, lower and upper halves, with the latter including most of the middle zone cells, all the compounds except for 0.0025% lycopene for the lower and lutein and 0.5% THC for the upper halves showed significant decrease as compared to respective control values (Table II).

Discussion

The present study demonstrated significant inhibition of ACF development in the colons of mice treated with fucoxanthin, lutein, or THC when given during the post-initiation phase. All the other test compounds also showed a tendency for decrease as compared to the group 2 value. Similarly, BrdU LI of crypt cells showed significant reduction in all groups except with the lower dose lycopene. The dose-dependent decreases of BrdU LI observed for lycopene and THC indicate that larger doses might be more effective for inhibition of ACF development. It should be stressed that the inhibition of cell proliferation, as monitored by BrdU LI, ornithine decarboxylase activity or silver-stained nuclear organizer region stains, is known to clearly correlate with suppression of ACF development by chemopreventive agents (24,27,28).

With regard to size of the majority of the ACF observed in the present study, our data are in line with the literature, most reports describing lesions comprising one or two crypts (25,29–31). Although this is relatively small, the results for total number counts of ACs/colon in the lutein and high dose THC groups were also clearly in line with the decrease of BrdU LI in the upper half of the crypts, including the mid-zone crypts, where cell proliferation occurs in carcinogen-treated groups (32,33).

The proposed mechanisms of cancer preventive effects of carotenoids include conversion to retinols inhibiting cellular differentiation (9), up-regulation of cell-to-cell gap junctional communication (34), and stimulation of the immune system (35). Lycopene is known to trap oxygen radicals and inhibit growth of glioma (36), endometrial, mammary and lung cancer

Table I. Data for ACF multiplicity

Group	Treatment	No. of mice	No. of ACF[a]/colon	No. of ACs[b]/colon
1	DMH Lycopene 0.005%	15	50.9 ± 12.7	98.9 ± 27.1
	Lycopene 0.0025%	15	61.0 ± 15.8	119.1 ± 33.0
	Fucoxanthin 0.01%	15	47.1 ± 13.7**	90.0 ± 25.8
	Lutein 0.05%	14	42.6 ±19.6**	79.9 ± 34.7**
	Curcumin 0.5%	14	53.3 ±10.2	100.2 ± 23.3
	THC 0.5%	13	46.6 ± 17.7**	81.8 ± 32.5**
	THC 0.2%	15	51.2 ±13.4	95.5 ± 26.3
2	DMH -	19	63.3 ± 19.4	115.1 ± 37.1
3	Lycopene 0.005%	5	0	0
	Fucoxanthin 0.01%	5	0	0
	Lutein 0.05%	5	0	0
	Curcumin 0.5%	5	0	0
	THC 0.5%	5	0	0

[a]Aberrant crypt foci.
[b]Aberrant crypts.
Data are mean ± SD values. **Significantly different from group 2 at $P < 0.01$.

Table II. BrdU LI (%) in the colon crypts

		Colon crypt compartment		
Group	Treatment	Upper half	Lower half	Whole
1	DMH Lycopene 0.005%	1.77 ± 0.78	10.9 ± 3.03**	6.4 ± 1.7**
	Lycopene 0.0025%	1.95 ± 1.04	13.7 ± 3.19	7.8 ± 1.6
	Fucoxanthin 0.01%	1.83 ± 1.39	11.2 ± 3.49**	6.5 ± 1.7**
	Lutein 0.05%	1.56 ± 0.99*	10.3 ±2.54**	5.9 ± 1.4**
	Curcumin 0.5%	1.67 ± 0.91	10.9 ±1.96**	6.3 ± 1.1**
	THC 0.5%	1.39 ± 0.85**	8.63 ± 3.26**	5.1 ± 1.8**
	THC 0.2%	1.70 ± 0.60	9.87 ±1.98**	5.8 ± 1.1**
2	DMH -	2.27 ± 1.17	14.8 ± 3.02	8.5 ± 1.5
3	Lycopene 0.005%	0.72 ± 0.61	7.13 ± 0.94	4.0 ± 0.4
	Fucoxanthin 0.01%	0.40 ± 0.33	8.67 ± 1.62	4.3 ± 0.4
	Lutein 0.05%	0.63 ± 0.35	7.42 ± 0.86	4.1 ± 0.4
	Curcumin 0.5%	0.64 ± 0.59	6.06 ± 1.04	3.4 ± 0.5
	THC 0.5%	0.44 ± 0.20	6.08 ± 1.38	3.3 ± 0.6

BrdU LI for average of 500 crypts (100 crypts each in 5 mice). Data are mean ± SD values. Significantly different from group 2 at $P < 0.05$ (*), 0.01 (**).

cells (37). Its intake is inversely related to the risk of prostate (38,39) and pancreatic cancer (6). Despite its potent cancer chemopreventive effects, the current study indicated that lycopene is less active at inhibiting DMH-induced ACF development and crypt cell proliferation in the mouse colon than the other carotenoid species examined. This might be associated with the fact that lycopene is not converted to retinol and is devoid of the quinone reductase activity involved in detoxification and anti-proliferative actions of vitamins on colon cancer cells (40). Fucoxanthin has been reported to suppress mutagen-induced oncogene expression, cell cycle progression and growth of neuroblastoma cells and exhibit anti-tumorigenic effects on mouse duodenal carcinogenesis (41,42). Lutein was also found to suppress mutagen-induced oncogene expression (43), and Epstein-Barr virus activation in cancer cells (44), while up-regulating gap junctional intercellular communication (34), which might provide a mechanistic basis for its cancer chemopreventive action. However, further studies are needed to clarify how lutein works *in vivo* to complement the only limited data so far available.

Earlier studies demonstrated that 0.2% curcumin in the diet during the initiation period can inhibit azoxymethane-induction of ACF and colon tumors in F344 rats (13,14). In similar studies, feeding of 2% curcumin resulted in decreased numbers of azoxymethane-induced dysplasias and colon tumors in CF-1 mice (12). Accordingly, the main effects of curcumin on colon tumorigenesis may be mediated through direct modulation of azoxymethane metabolism. However, curcumin has been shown to possess anti-inflammatory activity, inhibiting the induction of nitric oxide synthase in activated macrophages (13). It also inhibits azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase and arachidonic acid metabolism and cell proliferation in ACF (22), and might therefore be expected to act as a suppressive agent (7).

The present study in fact demonstrated THC, the new curcumin derivative, to be more active than the parent compound in terms of inhibition of ACF development and cell proliferation. Although THC was reported to be less active than curcumin regarding inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity and tumor promotion in 7,12-dimethylbenz[*a*]anthracene-initiated mouse skin carcinogenesis (15), Osawa *et al*. recently reported that THC, which has both phenolic and β-diketone moieties in the same molecule, exerts a stronger influence on antioxidative activity than curcumin (16,45). Thus, it might be particularly suitable for application as a chemopreventive agent

in vivo carcinogenesis. Further studies of THC action in oxygen radical trapping appear warranted to cast light on the mechanisms.

In conclusion, the present study showed that fucoxanthin, lutein and THC have a potential as chemopreventive agents. Further longer term and higher dose studies are now necessary to confirm that the observed chemopreventive effects on ACFs also extend to colon cancers.

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