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SHORT COMMUNICATION

Inhibitory effects of *d*-limonene on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats

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The modifying effect of the monoterpenoid *d*-limonene in drinking water on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) was investigated in male F344 rats. The effects of *d*-limonene intake on ornithine decarboxylase (ODC) activity and on the silver stained nucleolar organizer region protein (AgNOR) count in the colonic mucosa were also estimated. Animals were given 3 weekly s.c. injections of AOM (15 mg/kg body wt) to induce ACF. These rats were treated with or without 0.5% *d*-limonene in the drinking water, starting 1 week before the first dosing with AOM. All rats were killed 2 weeks after the last AOM injection, to measure the number of ACF, ODC activity and AgNOR count/nucleus in the colon. In rats given AOM and *d*-limonene the frequencies of ACF and aberrant crypts/colon, and aberrant crypts/focus were significantly decreased compared with those of rats given AOM alone ($P < 0.01$, $P < 0.001$ and $P < 0.001$ respectively). Number of AgNOR counts/nucleus of rats treated with AOM and *d*-limonene was significantly smaller than that of rats treated with AOM alone ($P < 0.001$). These results suggest that the monoterpenoid *d*-limonene might be a chemopreventive agent for colonic carcinogenesis in rats.

Limonene (*p*-mentha-1,8-diene) is a monocyclic monoterpene found in the essential oils of citrus fruits, spices and herbs. The limonene content of orange peel, for example, ranges from 90 to 95% (w/w). Because of its citrus fragrance, limonene is a component of many soft drinks, juices, cosmetics and perfumes. This seemingly simple compound possesses an impressive array of anti-tumorigenic activities. Limonene has chemopreventive activity against spontaneous and chemically induced tumors in mammary gland (1,2), skin (3), liver (4), lung and forestomach of rodents (5,6).

Aberrant crypt foci (ACF*) were first observed in the carcinogen-treated rodent colon (7). They are putative preneoplastic lesions of colon cancer in both rodents (8) and humans (9) and have been proposed as intermediate biomarkers for colon cancer (10–14). Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthesis pathway (15). Polyamines play essential roles in cell proliferation and differentiation and participate in macromolecular synthesis. The induction of ODC has been implicated as being important to carcinogenesis (16) and ODC activity is an intermediate biomarker of cell proliferation. Similarly, silver stained nucleolar organizer protein (AgNOR) count/nucleus in the target organ is suggested to be a useful biomarker, since AgNOR count correlated well with the bromodeoxyuridine labeling index, which is the representative method for accurate determination of S phase cells (17). In our previous studies we constructed an *in vivo* short-term model to test chemopreventive agents using a combination of ACF and these cell proliferation biomarkers (10,11,13). In the present study, using our *in vivo* short-term model, the modifying effects of *d*-limonene treatment on the formation of azoxymethane (AOM)-induced ACF, ODC activity and number of AgNOR/nucleus were investigated in male F344 rat colon.

A total of 48 male F344 rats, 4 weeks of age, purchased from Shizuoka Laboratory Animal Center (Hamamatsu City, Japan), were quarantined for 1 week and then randomized into four groups. All animals were housed four to a wire cage. The holding room was controlled at 23 ± 2°C, 50 ± 10% humidity and a 12 h light/dark cycle. Pellet CE-2 (Clea Japan Inc., Tokyo, Japan) was used as the basal diet during the experiment. At 6 weeks of age rats in groups 1 and 2 were given s.c. injections of 15 mg/kg body wt AOM (Sigma Chemical Co., St Louis, MO) once a week for 3 weeks. Animals in groups 2 and 3 were given 0.5% *d*-limonene (Nacalai tesque Co., Kyoto, Japan) in their drinking water for 5 weeks, starting at 5 weeks of age. This concentration is equal to 0.67% in the diet. Groups 1 and 4 were given tap water throughout the experiment (Figure 1). All rats were provided with water and diet *ad libitum* and weighed weekly. All animals were sacrificed by decapitation at 4 weeks after the first dosing with AOM and complete necropsies were performed. At the termination of study the colons of six rats randomly selected from each group were removed, flushed with saline, slit open longitudinally from cecum to anus, placed between two pieces of filter paper and fixed in buffered 10% formalin for 24 h.

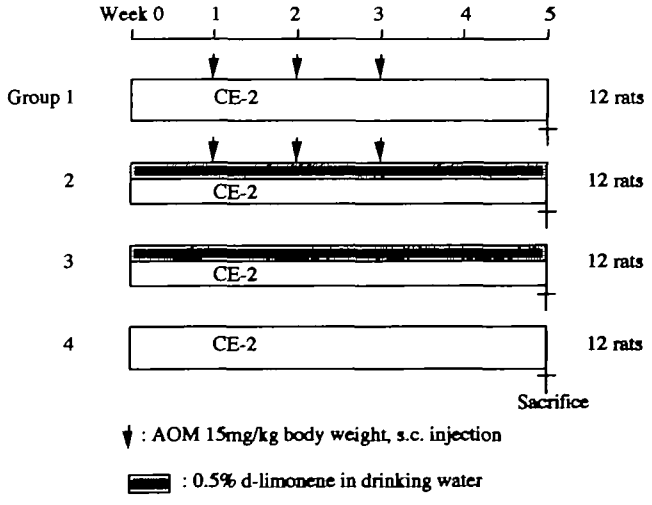

Fig. 1. Experimental protocol

*Abbreviations: ACF, aberrant crypt foci; ODC, ornithine decarboxylase; AgNOR, silver stained nucleolar organizer region protein; AOM, azoxymethane.

Table I. Mean body and liver weights of F344 rats in each group

Group no.	Treatment	Mean body weight (g) of rats						Mean liver weight (g)
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	
1	AOM alone	90 ± 6[a]	121 ± 7	148 ± 10	152 ± 11	192 ± 12	211 ± 10	9.3 ± 0.4
2	AOM + *d*-limonene	90 ± 7	121 ± 11	150 ± 15	168 ± 17	201 ± 19	221 ± 16	9.8 ± 1.2
3	*d*-Limonene alone	95 ± 3	128 ± 5	160 ± 7	182 ± 9	217 ± 11	228 ± 11	10.5 ± 1.6
4	No treatment	85 ± 5	117 ± 6	147 ± 8	172 ± 5	207 ± 4	227 ± 5	10.8 ± 0.4

[a]Mean ± SD.

Table II. Effect of *d*-limonene on AOM-induced ACF in rat colon

Group no.	Treatment	Incidence[a]	ACF/colon	Aberrant crypts/colon	Aberrant crypts/focus
1	AOM alone	6/6	131.3 ± 16.5[b]	234.6 ± 63.2	1.94 ± 0.13
2	AOM + *d*-limonene	6/6	89.5 ± 18.1[c]	157.2 ± 28.2[d]	1.77 ± 0.07[d]

[a]Number of rat colons with ACF divided by total number of colons scored.
[b]Mean ± SD.
[c,d]Significantly different from group 1 (AOM alone) by Student's *t*-test ([c]$P < 0.01$, [d]$P < 0.001$).

Table III. Effect of *d*-limonene on aberrant crypts/focus induced by AOM in rat colon

Group no.	Treatment	ACF/focus				
		1 crypt	2 crypts	3 crypts	4 crypts	5 crypts
1	AOM alone	46.0 ± 6.6[a]	48.5 ± 11.7	28.8 ± 5.4	6.5 ± 1.1	1.5 ± 1.1
2	AOM + *d*-limonene	40.5 ± 11.5	32.8 ± 6.0[b]	14.2 ± 4.1[c]	1.5 ± 0.5[c]	0.5 ± 0.8

[a]Mean ± SD.
[b,c]Significantly different from group 1 (AOM alone) by Student's *t*-test ([b]$P < 0.05$, [c]$P < 0.001$).

They were then stained with 0.5% methylene blue in saline according to the procedure of Bird (7) to observe ACF. The number of ACF/colon, the number of aberrant crypts/colon and the number of aberrant crypts/focus were determined by microscopy at a magnification of ×40. The criteria used to identify an aberrant crypt focus topographically included: (i) increased size; (ii) a thicker epithelial cell lining; (iii) an increased pericryptal zone relative to normal crypts. After counting ACF the colon was embedded in paraffin and two serial sections (3 μm thickness) were taken. One section was used for staining AgNOR and the other was stained with hematoxylin and eosin for histological examination. AgNOR staining was carried out according to the method described previously (17). For determination of AgNOR number on the cell nuclei 20 well-oriented crypts in which the base, lumen and top of the crypts could be seen were used. AgNOR was counted on AgNOR stained sections by microscopy at a magnification of ×400. Data were expressed as number of AgNOR/nucleus. At necropsy of the remaining six rats their colons were immediately removed. The colon was rinsed in saline, slit open longitudinally and freed from all contents. It was laid flat on a glass plate and the mucosa was scraped with a stainless steel, disposable, microtome bladed knife (S35; Feather Safety Razor Co. Ltd, Osaka, Japan). Each colonic mucosa was homogenized in 1.5 ml homogenizing buffer (250 mM sucrose, 50 mM Tris–HCl, pH 7.4, containing 1 mM dithiothreitol, 1 mM EDTA, 0.4 mM pyridoxal 5′-phosphate) using a Polytron homogenizer. The homogenates were centrifuged at 15 000 r.p.m., 4°C for 30 min. The resulting cytosol fraction was used to determine ODC activity and protein concentration. ODC activity in the colon mucosa was determined by a modification of methods described previously (18). The incubation mixture, in a final volume of 40 μl [50 mM *N*-(2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 1 mM EDTA, 0.25 μCi DL-[1-^{14}C]ornithine (sp. act. 42.47 mCi/mmol; NEN, Boston, MA)], was incubated at 37°C for 1 h. The reaction was stopped by adding 20 μl 6 N HCl and the $^{14}CO_2$ released was collected on 10% KOH-saturated discs for another 15 min. ^{14}C in the form of $K_2{}^{14}CO_3$ was counted in a scintillation counter. The results were expressed as pmol $^{14}CO_2$/h/mg protein. The data were analyzed by Student's *t*-test. All statements of significance are at $P < 0.05$.

The body weight gains during the experiment and liver weights at death are presented in Table I. No significant effects of AOM and/or *d*-limonene treatment on body and liver weights were observed. No neoplasms were found on macroscopic and microscopic examinations of all organs of rats in each group. Table II summarizes the mean number of ACF/colon, total number of aberrant crypts/colon and the mean number of aberrant crypts/focus. The rats treated with AOM (groups 1 and 2) showed a 100% incidence of ACF. No ACF were seen in the colons of rats without AOM treatment (groups 3 and 4). *d*-Limonene treatment significantly decreased ACF/colon compared with AOM alone ($P < 0.01$). *d*-Limonene treatment also significantly decreased aberrant crypts/colon and aberrant crypts/focus compared with AOM alone ($P < 0.001$). Mean numbers of ACF, consisting of varying numbers (1–5) of crypts in each group, are presented in Table III. All crypt numbers were decreased by *d*-limonene treatment. The numbers of foci consisting of 2, 3 and 4 crypts, especially, were significantly lower in rats treated with *d*-limonene and AOM

Table IV. Colonic mucosal ODC activity of rats in each group

Group no.	Treatment	n	ODC activity (pmol $^{14}CO_2$/h/mg protein)	
			Mean ± SD	Range
1	AOM alone	6	37.7 ± 20.4	13.2–73.4
2	AOM + *d*-limonene	6	26.3 ± 4.4	19.8–30.4
3	*d*-Limonene alone	6	2.2 ± 2.7	0–5.8
4	No treatment	6	9.3 ± 7.9	0–19.8

Table V. Numbers of cells/crypt column and AgNOR of the colonic epithelium in each group

Group no.	Treatment	n	Cells/crypt column	AgNOR/ nucleus
1	AOM alone	6	31.9 ± 4.9[a]	1.73 ± 0.25
2	AOM + *d*-limonene	6	27.6 ± 4.0	0.87 ± 0.21[b]
3	*d*-Limonene alone	6	22.1 ± 3.9[c]	0.67 ± 0.20
4	No treatment	6	30.7 ± 4.9	1.01 ± 0.28

[a]Mean ± SD.
[b]Significantly different from group 1 (AOM alone) by Student's *t*-test ($P < 0.001$).
[c]Significantly different from group 4 (no treatment) by Student's *t*-test ($P < 0.01$).

than those of rats treated with AOM alone ($P < 0.05$, $P < 0.001$, and $P < 0.001$ respectively). The ODC activity in the colonic mucosa is indicated in Table IV. The mean ODC activity of the colonic mucosa in group 1 (AOM alone) was greater than those of the other groups. In the group of rats treated with AOM and *d*-limonene mean ODC activity in the colonic mucosa was lower than that of rats treated with AOM alone, but the difference lacked statistical significance. The data on numbers of cells/crypt column and AgNOR count/ nucleus of the colonic epithelium in each group are shown in Table V. The mean number of cells/crypt column in group 1 (AOM alone) was the greatest among all groups. The mean numbers of cells/crypt column in groups 2 and 3 (*d*-limonene treatment groups) were lower than in the other groups. The mean number of AgNOR counts/nucleus of the colonic epithelium in group 1 (AOM alone) was greatest among all groups. *d*-Limonene treatment significantly decreased AgNOR count/nucleus of the colonic epithelium compared with AOM alone ($P < 0.001$).

The results of the present study indicate that *d*-limonene treatment inhibited the formation of colonic ACF and reduced AgNOR count/nucleus in the colon induced by AOM using our short-term assay. These results suggest that *d*-limonene had 'blocking' activity against AOM-induced ACF in the colon. ACF are very useful intermediate biomarkers for *in vivo* colon carcinogenesis models. We have previously demonstrated that protocatechuic acid (10), 5-hydroxy-4-(2-phenyl-(*E*)-ethenyl)-2(5*H*)-furanone (13) and s-methylmethane thiosulfonate (14) inhibited the formation of AOM-induced ACF in an *in vivo* short-term assay and reduced the incidences of colonic neoplasms induced by AOM in a long-term assay (12,14,19). Also, *d*-limonene treatment in the drinking water reduced AOM-induced AgNOR count/nucleus in the colon. Cell proliferation might play an important role in the carcinogenic process. Cell kinetic analysis in the colonic mucosa of individuals at increased risk for colon cancer has revealed an anomalous expansion of epithelial cells within the colonic crypts. We have demonstrated that AgNOR count is a useful index for cell proliferation and we have recently used it as a biomarker in chemoprevention studies of liver, tongue and colon carcinogenesis (10,11,13,20,21). Also, *d*-limonene is rapidly and extensively metabolized in mammals. Limonene has been reported to have low or no toxicity (4). In the present study *d*-limonene had no toxicity in our short-term assay.

In conclusion, *d*-limonene treatment in the drinking water inhibited formation of ACF induced by AOM in male F344 rat colon and reduced cell proliferation biomarkers (AgNOR count in the colonic mucosa in our *in vivo* short-term assay). The present study is the first demonstration of a modifying effect of *d*-limonene on the formation of ACF in the colon in male F344 rats. Further studies on the chemopreventive activity of *d*-limonene for colon cancer in long-term *in vivo* animal models are ongoing in our laboratory.

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