Jpn. J. Cancer Res. 88, 821–830, September 1997

Chemoprevention of Azoxymethane-induced Rat Colon Carcinogenesis by a Xanthine Oxidase Inhibitor, 1′-Acetoxychavicol Acetate

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In our studies to find natural compounds with chemopreventive efficacy in foods, using azoxymethane (AOM)-induced colonic aberrant crypt foci and colonic mucosal cell proliferation as biomarkers, a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate (ACA), present in the edible plant *Languas galanga* from Thailand was found to be effective. This study was conducted to test the ability of ACA to inhibit AOM-induced colon tumorigenesis when it was fed to rats during the initiation or post-initiation phase. Male F344 rats were given three weekly s.c. injections of AOM (15 mg/kg body weight) to induce colonic neoplasms. They were fed diet containing 100 or 500 ppm ACA for 4 weeks, starting one week before the first dosing of AOM (the initiation feeding). The other groups were fed the ACA diet for 34 weeks, starting one week after the last AOM injection (the post-initiation feeding). At the termination of the study (week 38), AOM had induced 71% incidence of colonic adenocarcinoma (12/17 rats). The initiation feeding with ACA caused significant reduction in the incidence of colon carcinoma (54% inhibition by 100 ppm ACA feeding and 77% inhibition by 500 ppm ACA feeding, $P=0.03$ and $P=0.001$, respectively). The post-initiation feeding with ACA also suppressed the incidence of colonic carcinoma (45% inhibition by 100 ppm ACA feeding and 93% inhibition by 500 ppm ACA feeding, $P=0.06$ and $P=0.00003$, respectively). Such inhibition was dose-dependent and was associated with suppression of proliferation biomarkers, such as ornithine decarboxylase activity in the colonic mucosa, and blood and colonic mucosal polyamine contents. ACA also elevated the activities of phase II enzymes, glutathione *S*-transferase (GST) and quinone reductase (QR), in the liver and colon. These results indicate that ACA could inhibit the development of AOM-induced colon tumorigenesis through its suppression of cell proliferation in the colonic mucosa and its induction of GST and QR. The results confirm our previous finding that ACA feeding effectively suppressed the development of colonic aberrant crypt foci. These findings suggest possible chemopreventive ability of ACA against colon tumorigenesis.

Key Words: Chemoprevention — 1′-Acetoxychavicol acetate — Azoxymethane — Colon tumorigenesis — Rats

Colon cancer is the third most important malignant neoplasm in the world.[1] It is the second leading cause of cancer deaths in USA and the third in Japan. The risk of colon cancer is highly dependent on dietary factors as well as genetic background.[2–4] Since Japanese nutrition has become increasingly "westernized" with a rising fat intake, the incidence of colon cancer has been increasing in Japan.[2,5,6] There is an inverse correlation between the intake of fruits/vegetables and human colon cancer.[7] In this context, chemoprevention of this malignancy is important.[8]

Free radicals, including oxyradicals, may contribute to the development of certain types of cancer.[9] They can induce permanent DNA sequence changes in the form of point mutations, deletions, gene amplification, and rearrangements, which may result in the activation of protooncogenes and/or the inactivation of tumor suppressor genes.[10] Recently, an increased mucosal oxygen free radical activity was found in azoxymethane (AOM)-induced colonic tumor.[11] Oxygen free radical activity was also increased in histologically normal colonic mucosa of AOM-treated rats.[11] A highly versatile enzyme, xanthine oxidase, which is widely distributed among species, including humans,[12] catalyzes the hydroxylation of many purine substrates, and converts hypoxanthine to xanthine and then uric acid in the presence of molecular oxygen to yield superoxide anion (O_2^-).[13] An increase of this

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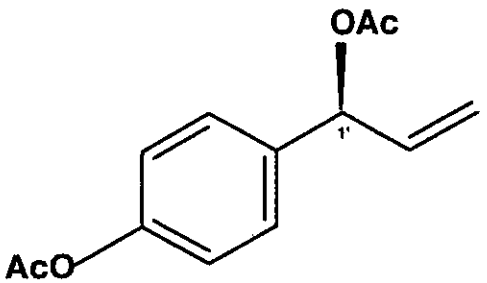

Fig. 1. Chemical structure of 1′-acetoxychavicol acetate (ACA).

enzyme was reported in human brain tumors,[14] chemically induced mouse skin tumors,[15] and mouse skin hyperplasia induced by 12-*O*-tetradecanoylphorbol-13-acetate.[16] These findings suggest that alterations in free radicals, presumably induced by elevated xanthine oxidase activity, may be implicated in tumorigenesis, including colon carcinogenesis. If this is so, xanthine oxidase inhibitors may suppress colon carcinogenesis.

Several natural products in edible plants are known to act as xanthine oxidase inhibitors and are reported to have cancer-chemopreventive activity.[17–19] 1′-Acetoxychavicol acetate (ACA, Fig. 1) is present in seeds or rhizome of *Languas galanga* (Zingiberaceae), which is used as a ginger substitute and as a stomachic medicine in Thailand.[20] ACA is an inhibitor of xanthine oxidase[21] and inhibits tumor promoter-induced Epstein-Barr virus activation *in vitro*.[22] Our recent work has revealed a strong cancer-chemopreventive effect of ACA on 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats.[23]

Aberrant crypt foci (ACF), which are present in carcinogen-treated rodent colon and in the colon of humans at high risk for cancer development, are considered to be preneoplastic lesions for colon cancer.[24–26] This character of ACF favors their use as a biomarker for investigating modulation of colon carcinogenesis.[27] A number of natural compounds that inhibit ACF development induced by exposure to several colon carcinogens have been proved to have chemopreventive activity against colon cancer in rodents.[27,28] In our chemopreventive studies, certain natural antioxidants from edible plants were found to inhibit ACF development induced by colon carcinogens and were proved to be inhibitors of colon carcinogenesis in rats.[17,28–30] However, some investigators have failed to find any correlation between ACF and colon cancer.[31] In a recent study by Kristiansen,[32] a high number of ACF with low crypt multiplicity (1–3 aberrant crypts/focus) in mouse colon after heterocyclic amine treatment was not indicative for the end-point colon cancer. Thus, it might be that only the presence of a high number of ACF with high crypt multiplicity is predictive for colon tumor outcome.[33–35] Recently, protective effect of ACA on the development of ACF with high crypt multiplicity was found in a short-term *in vivo* assay,[36] suggesting a possible inhibitory effect of ACA on colon carcinogenesis.

In the present study, chemopreventive ability of ACA on large bowel tumorigenesis was investigated in a long-term *in vivo* assay using a rat colon carcinogenesis model with AOM as a carcinogen. Cell proliferation biomarkers, such as colonic mucosal ornithine decarboxylase (ODC) activity and blood and colonic mucosal polyamines levels,[8,28,37] were also examined. In addition, activities of glutathione *S*-transferase (GST) and quinone reductase (QR) in the colonic epithelium were assayed to assess whether ACA could modulate the activities of these phase II detoxification enzymes in the liver and colon, since these enzymes divert ultimate carcinogens from reacting with critical cellular macromolecules, and thus their induction is a causal mechanism of protection.[38,39]

MATERIALS AND METHODS

Animals, diet, and chemicals A total of 116 male F344 rats, 4 weeks old, obtained from Japan SLC Inc., Hamamatsu City, were used. Animals were housed three or four to a wire cage in an experimental room under controlled conditions of 23±2°C (SD), 50±10% humidity, and 12 h light/dark cycle. They were allowed *ad libitum* access to diet and water. Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as a basal diet. AOM was purchased from Sigma Chemical Co., St. Louis, MO. ACA (>95% purity) was synthesized according to the method described previously.[22] Experimental diets mixed with ACA at two concentrations (100 and 500 ppm) were prepared by using a V-blender on a weekly basis and stored in a cold room (<4°C) until used. The doses of ACA used were 1/100 (100 ppm) and 1/20 (500 ppm) of the maximum tolerated dose.[36]

Experimental procedure After quarantine for 1 week, rats aged 5 weeks were divided into 7 groups as shown in Fig. 2 and the tables. Starting at 6 weeks of age, animals in groups 1 through 5 were s.c. injected with AOM (15 mg/kg body weight) once a week for 3 weeks. Rats in groups 2 and 3 were given the powdered basal diet containing 100 ppm or 500 ppm ACA for 4 weeks, beginning at 5 weeks of age. Groups 4 and 5 were fed the diets containing 100 ppm and 500 ppm ACA for 34 weeks, respectively, starting one week after the last dosing of AOM. Group 6 was fed the ACA diet (500 ppm) alone throughout the study and did not receive AOM. Group 7 served as an untreated control. Animals were carefully observed daily and weighed weekly until

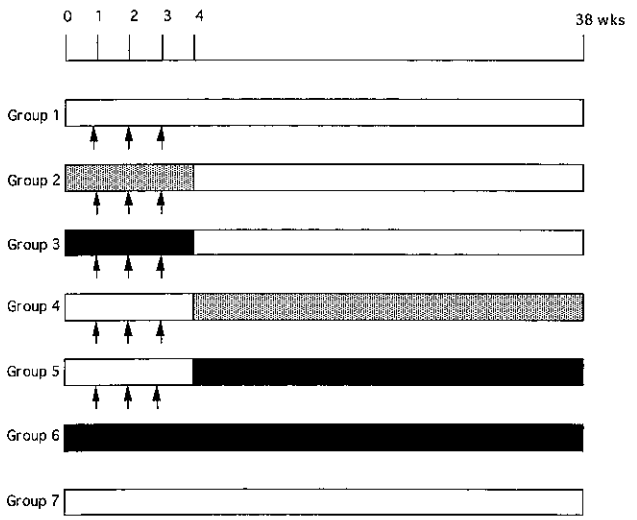

Fig. 2. Experimental protocol. ↑ AOM, 15 mg/kg body weight, s.c. injection, ▨ ACA, 100 ppm in diet, ■ ACA, 500 ppm in diet, □ Basal diet, CE-2.

they reached 14 weeks of age, and then every 4 weeks. The consumption of experimental diets was also recorded. At the termination of the study (week 38), all rats were killed by decapitation. At autopsy, five rats from each group were used for measurement of colonic mucosal ODC activity and polyamine levels in the colonic mucosa and blood. After the macroscopic observation, the colonic tumors were resected for histological examination and the remaining colonic tissues were assayed for ODC and polyamine levels. The intestines of the remaining rats were embedded in paraffin and sliced at 3 μm thickness, after the analysis of ACF on 10% buffered formalin-fixed samples. Intestinal and kidney neoplasms were diagnosed according to the criteria described previously.[40, 41]

ACF analysis The colons other than those used for biochemical assay were used for scoring of ACF. At autopsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. The colons were cut into three sections (about 4 cm each) starting from the anus, and after having been placed between filter papers to reduce mucosal folding, fixed in 10% buffered formalin for at least 24 h. Fixed colon sections were dipped in a 0.2% solution of methylene blue in distilled water for 5 min, briefly washed with distilled water, and placed on a microscope slide with the mucosal surface up. Using a light microscope at a magnification of ×40, ACF were distinguished by their increased size, their more prominent epithelial cells, and their increased pericryptal space from surrounding normal crypts.[24] Crypts overlying lymphoid follicles were excluded from scoring, because normal crypts in this area can sometimes be confused with ACF. The number of ACF observed per colon, the number of aberrant crypts observed in each focus, and the location of each focus were recorded. Colons from all groups were scored blindly for ACF by the same observer (T. T.). After scoring, colons were processed for histopathological examination.

ODC activity For measurement of ODC activity, the colons were rapidly removed, slit open longitudinally, freed from all the contents, and then rinsed in ice-cold saline. They were laid flat on a glass plate with the mucosal side up, and the mucosa was scraped with a stainless steel disposable microtome bladed knife, S35 (Feather Safety Razor Co., Ltd., Osaka). Colon mucosa from each rat was pooled and homogenized in 1.5 ml of 50 m*M* sodium phosphate buffer containing 5 m*M* dithiothreitol, 0.1 m*M* EDTA, and 0.1 m*M* pyridoxal 5′-phosphate using a Polytron. The homogenates were centrifuged for 1 h at 100,000*g* at 4°C. The resulting cytosol fraction was used for determination of ODC activity and protein. ODC activity in the colonic mucosa was determined by the methods described previously. The incubation mixture in a final volume of 250 μl [50 m*M* sodium phosphate, 2 m*M* pyridoxal phosphate, 5 m*M* dithiothreitol, 20 m*M* L-ornithine, and 0.25 mCi of DL-[1-^{14}C]-ornithine (specific activity, 58 mCi/mmol; Amersham)] was incubated for 1 h at 37°C. The reaction was stopped by adding 300 μl of 2 *N* sulfuric acid, and the $^{14}CO_2$ released was collected on barium hydroxide-saturated discs for another 30 min. The ^{14}C, in the form of $Ba^{14}CO_3$, was counted in a scintillation counter. The results were expressed as pmol $^{14}CO_2$/h/mg protein.

Polyamine levels Polyamine levels in the colonic mucosa were assayed by the method of Koide *et al.*[42] For measurement of blood polyamine level, blood was collected from all rats by heart puncture at termination and polyamine levels were assessed.[43]

GST and QR assay To determine whether ACA can modify the activities of GST and QR in the liver and colon, 32 male F344 rats aged 6 weeks were divided into 4 groups and they were gavaged with ACA at a dose of 50, 100 or 200 mg/kg body weight in 0.5 ml of 5% gum arabic (Sigma Chemical Co.) for 5 consecutive days. All rats were killed by decapitation 30 min after the last gavage. The livers and colons were excised immediately. The liver was perfused with saline to remove blood and minced into small pieces. The colon was slit longitudinally and washed with phosphate-buffered saline (pH 7.4) and mucosa was collected by scraping the mucosal surface with a microscope knife. Aliquots of minced liver and mucosal scrapings were processed to obtain the cytosolic fraction as described.[44, 45] The activities of GST with 1-chloro-2,4-dinitrobenzene (CDNB) and/or 1,2-

dichloro-4-nitrobenzene (DCNB) as substrates and QR with NADH and menadione as substrates were determined as described.[46-48] All assays were performed by spectrophotometry at 340 nm and all samples were measured in triplicate. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 μmol of substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method[49] using bovine serum albumin as the standard.

Statistical methods Where applicable, data were analyzed using Fisher's exact probability test, Student's t test or Welch's t test with $P<0.05$ as the criterion of significance.

RESULTS

General observations During the study, clinical signs of toxicity, low survival, and poor condition were not observed in any of the groups. This was confirmed by histopathological examinations in liver, kidneys, and lungs of the rats given the test compound, although mild fatty metamorphosis was seen around the central vein in the liver of a few rats fed 500 ppm ACA alone (group 6). The mean daily intakes of diets with or without ACA per rat were between 15.5–15.9 g/rat (mean intake of ACA/rat/day: 100 ppm diet, 1.54 mg; and 500 ppm diet, 7.68 mg). Mean body and liver weights in all groups are shown in Table I. The mean body weight at termination in groups 4 (AOM→100 ppm ACA) and 5 (AOM→500 ppm ACA) were significantly smaller than that of group 1 (AOM alone) ($P<0.01$ and $P<0.005$, respectively). The mean liver weights of groups 3 (AOM+500 ppm ACA) and 5 were significantly increased when compared with group 1 ($P<0.005$). The mean percent liver weight (g/100 g body weight) of groups 2 (AOM+100 ppm ACA), 3, 4, 5 were significantly greater than that of group 1 ($P<0.001$).

Incidence and multiplicity of intestinal neoplasms Macroscopically, most tumors developed in the large intestine (mainly in the middle and distal colon) and some in the small intestine of rats in groups 1–5. Animals in groups 6 and 7 did not have neoplasms in any of the organs examined. Colon tumors were sessile or pedunculated tumors and histologically tubular adenoma, adenocarcinoma or signet ring-cell carcinoma with a higher incidence of adenocarcinoma. A few rats had renal mesenchymal tumors and/or altered hepatocellular foci in groups 1 through 5, but these lesions were not found in groups 6 and 7. The incidence and multiplicity of intestinal tumors are shown in Table II. The frequencies of large intestinal adenocarcinoma in groups 2 (33%), 3 (16%), and 5 (5%) were significantly smaller than that of group 1 (71%) ($P=0.03$, $P=0.001$, and $P=0.00003$, respectively). The frequency of colon adenocarcinoma in group 4 (39%) was lower than that in group 1, but the difference was not significant ($P=0.06$). The incidences of small intestinal tumors did not significantly differ among the groups. As presented in Table III, in terms of the multiplicity of colon carcinoma (number of carcinomas/rat), significant inhibition in groups 3 and 5 was found when compared with group 1 ($P<0.002$ and $P<0.001$). When colonic adenomas and adenocarcinomas were combined, significant reduction in the incidence or multiplicity of large intestinal tumors was found in rats given 500 ppm ACA during the post-initiation phase (group 5), because the incidence and multiplicity of adenomas were slightly greater in rats of groups 3–5 (Tables II and III).

Frequency of ACF The incidence of ACF at the end of the study is listed in Table IV. ACF developed in rats treated with AOM with or without ACA (groups 1–5), while no ACF were present in rats not treated with AOM (groups 6 and 7). The frequencies of ACF/colon, the number of ACF/area (cm^2), the number of aberrant

Table I. Body, Liver, and Relative Liver Weights of Rats Treated with AOM and/or ACA

Group no.	Treatment	No. of rats examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM alone	17	348±17[a]	11.5±1.1	3.29±0.28
2	AOM+100 ppm ACA	18	332±29	12.3±2.2	3.78±0.48[b]
3	AOM+500 ppm ACA	19	343±22	13.4±2.3[c]	3.91±0.48[b]
4	AOM→100 ppm ACA	18	326±26[d]	12.1±1.8	3.77±0.42[e]
5	AOM→500 ppm ACA	20	331±17[f]	13.3±2.2[c]	4.10±0.51[b]
6	500 ppm ACA	12	344±15	14.3±2.0	4.16±0.60
7	No treatment	12	352±24	14.2±2.4	3.99±0.47

a) Mean±SD.
b–d) Significantly different from group 1 by Welch's t test (*b*) $P<0.001$, *c*) $P<0.005$, and *d*) $P<0.01$).
e, f) Significantly different from group 1 by Student's t test (*e*) $P<0.001$ and *f*) $P<0.005$).

Table II. Incidence of Intestinal Neoplasms of Rats Fed ACA during or after AOM Exposure

Group no.	Treatment	No. of rats	No. of rats with intestinal neoplasms at: (%)								
			Entire intestine			Small intestine			Large intestine		
			Total	AD[a]	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM alone	17	13 (76)	1 (6)	13 (76)	5 (29)	0	5 (29)	12 (71)	1 (6)	12 (71)
2	AOM+100 ppm ACA	18	9 (50)	2 (11)	7 (39)[b]	3 (17)	1 (6)	2 (11)	7 (39)	1 (6)	6 (33)[c]
3	AOM+500 ppm ACA	19	10 (53)	5 (26)	9 (47)	5 (26)	1 (5)	5 (26)	8 (42)	5 (26)	3 (16)[d]
4	AOM→100 ppm ACA	18	12 (67)	4 (22)	10 (56)	4 (22)	1 (6)	3 (17)	9 (50)	3 (17)	7 (39)
5	AOM→500 ppm ACA	20	6 (30)[e]	3 (15)	3 (15)[f]	2 (10)	0	2 (10)	4 (20)[g]	3 (15)	1 (5)[h]
6	500 ppm ACA	12	0	0	0	0	0	0	0	0	0
7	No treatment	12	0	0	0	0	0	0	0	0	0

a) AD, adenoma; and ADC, adenocarcinoma.
b–h) Significantly different from group 1 by Fisher's exact probability test (*b*) $P=0.02$, *c*) $P=0.03$, *d*) $P=0.001$, *e*) $P=0.005$, *f*) $P=0.0002$, *g*) $P=0.002$, and *h*) $P=0.00003$).

Table III. Multiplicity of Intestinal Neoplasms of Rats Fed ACA during or after AOM Exposure

Group no.	Treatment (no. of rats examined)	Multiplicity (no. of neoplasms/rat) of neoplasms at:								
		Entire intestine			Small intestine			Large intestine		
		Total	AD[a]	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM alone (17)	1.12 ±0.83[b]	0.12 ±0.47	1.00 ±0.69	0.29 ±0.46	0	0.29 ±0.46	0.83 ±0.68	0.12 ±0.47	0.71 ±0.46
2	AOM+100 ppm ACA (18)	0.61 ±0.68	0.11 ±0.31	0.50 ±0.69[c]	0.17 ±0.37	0.06 ±0.23	0.11 ±0.31	0.44 ±0.60	0.06 ±0.23	0.39 ±0.59
3	AOM+500 ppm ACA (19)	0.84 ±1.04	0.37 ±0.58	0.47 ±0.60[d]	0.32 ±0.57	0.05 ±0.22	0.26 ±0.44	0.53 ±0.60	0.32 ±0.46	0.21 ±0.41[e]
4	AOM→100 ppm ACA (18)	0.89 ±0.87	0.22 ±0.42	0.67 ±0.75	0.22 ±0.53	0.06 ±0.23	0.17 ±0.50	0.67 ±0.82	0.17 ±0.37	0.50 ±0.69
5	AOM→500 ppm ACA (20)	0.40 ±0.80[c]	0.25 ±0.70	0.15 ±0.36[f]	0.10 ±0.30	0	0.10 ±0.30	0.30 ±0.78[g]	0.25 ±0.70	0.05 ±0.22[f]
6	500 ppm ACA (12)	0	0	0	0	0	0	0	0	0
7	No treatment (8)	0	0	0	0	0	0	0	0	0

a) AD, adenoma; and ADC, adenocarcinoma.
b) Mean±SD.
c–g) Significantly different from group 1 by Student's *t* test (*c*) $P<0.02$, *d*) $P<0.01$, *e*) $P<0.002$, *f*) $P<0.001$, and *g*) $P<0.05$).

Table IV. Effect of ACA on the Development of AOM-induced ACF

Group no.	Treatment	Incidence (%)	No. of ACF/colon	No. of ACF/cm^2	No. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone	12/12 (100)	93±12[a]	6.88±1.85	287±62	3.09±0.18
2	AOM+100 ppm ACA	13/13 (100)	63±9[b]	5.15±0.81[c]	149±31[b]	2.36±0.24[b]
3	AOM+500 ppm ACA	14/14 (100)	54±2[b]	4.62±0.23[d]	123±16[b]	2.28±0.09[b]
4	AOM→100 ppm ACA	13/13 (100)	66±6[b]	5.60±0.48[e]	156±10[b]	2.35±0.23[b]
5	AOM→500 ppm ACA	15/15 (100)	51±7[b]	4.59±0.52[d]	107±17[b]	2.10±0.33[b]
6	500 ppm ACA	0/7 (0)	0	0	0	0
7	No treatment	0/7 (0)	0	0	0	0

a) Mean±SD.
b–e) Significantly different from group 1 by Student's *t* test or Welch's *t* test (*b*) $P<0.001$, *c*) $P<0.01$, *d*) $P<0.002$, and *e*) $P<0.05$).

crypts/colon, and the number of aberrant crypts/focus in groups 2 through 5 were significantly smaller than those of group 1 ($P<0.001$, $P<0.01$, $P<0.002$ or $P<0.005$).

Colonic mucosal ODC activity As shown in Fig. 3, AOM treatment significantly increased the colonic mucosal ODC activity ($P<0.001$). ACA feeding during both initiation (groups 2 and 3) and post-initiation phases (groups 4 and 5) significantly reduced this increase ($P<0.02$, $P<0.001$, $P<0.01$ or $P<0.002$). The ODC activity of rats in group 6 (ACA alone) was similar to that of group 7 (untreated control).

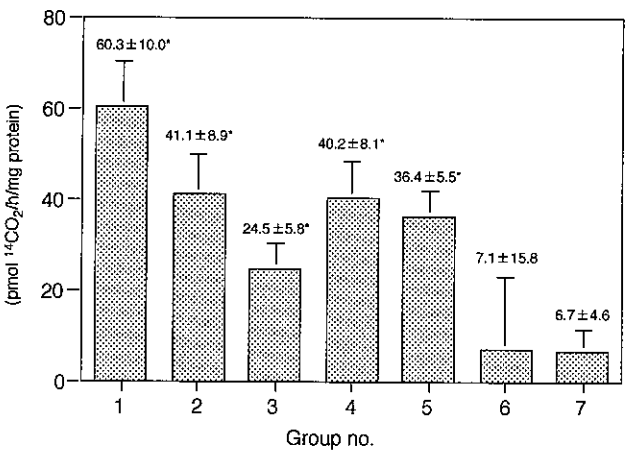

Fig. 3. ODC activity in the colonic mucosa in each group. * $P<0.02$–0.001.

Polyamine levels in the colonic mucosa and blood As summarized in Table V, the total polyamine levels of colonic mucosa and blood in group 1 were greater than those of the untreated control (group 7). The increase of tissue polyamine was significant ($P<0.001$). The mean polyamine concentrations of colonic mucosa in groups 2, 3, and 5 were significantly lower than that of group 1 ($P<0.001$). Blood polyamine level in group 5 was significantly smaller than that of group 1 ($P<0.05$). Spermidine level of colonic mucosa in group 1 was significantly increased when compared to that of group 7 ($P<0.005$) and ACA feeding significantly reduced this increase ($P<0.02$, $P<0.001$ or $P<0.01$). Blood spermidine level of group 5 was significantly smaller than that of group 1 ($P<0.05$). The two values in groups 6 and 7 were comparable.

GST and QR activities GST and QR activities in the liver and colon of rats gavaged with various dose levels of ACA are presented in Table VI. Dosing of 50, 100, and 200 mg/kg body weight of ACA significantly increased liver GST-CDNB activity ($P<0.01$ or $P<0.001$), although these treatments did not affect the activity of GST-DCNB in the liver. Similarly, gavaged ACA significantly elevated the QR activity in the liver ($P<0.02$, $P<0.002$, and $P<0.001$). In the colonic mucosa, ACA treatment significantly increased the GST-CDNB activity at 50, 100, and 200 mg/kg ($P<0.05$, $P<0.001$, and $P<0.005$, respectively) and the QR activity at 100 and 200 mg/kg ($P<0.002$).

Table V. Tissue and Blood Polyamine Levels of Rats in Each Group

Group no.	Treatment	No. of rats examined (tissue/blood)	Polyamine levels: tissue (nmol/mg protein)/ blood (nmol/ml)			
			Total	Diamine	Spermidine	Spermine
1	AOM alone	5/5	31.1±2.9[a, b]	0.7±1.1	24.0±4.1[c]	6.4±2.2
			/58.1±29.5	/0.0±0.0	/43.3±19.6	/14.8±11.5
2	AOM+ACA (100 ppm)	5/5	17.4±5.0[d]	1.3±2.8	11.6±7.8[e]	4.5±3.1
			/37.0±11.8	/0.0±0.0	/27.7±3.5	/9.3±9.8
3	AOM+ACA (500 ppm)	5/5	14.2±5.4[d]	1.7±3.0	6.0±3.6[d]	6.5±2.8
			/34.1±1.9	/0.1±0.2	/25.9±1.1	/8.2±1.2
4	AOM→ACA (100 ppm)	5/5	24.2±6.5	1.1±1.8	14.4±4.5[f]	8.8±4.3
			/31.5±2.9	/0.0±0.0	/25.6±3.5	/5.9±1.5
5	AOM→ACA (500 ppm)	5/5	18.3±3.2[d]	0.9±2.1	9.4±4.7[d]	7.9±1.7
			/25.1±3.4[g]	/0.1±0.3	/18.5±1.5[g]	/6.5±2.5
6	ACA (500 ppm)	5/5	15.8±2.5	0.1±0.3	9.1±2.3[h]	6.6±1.0
			/26.7±4.2	/0.0±0.0	/21.3±4.2	/5.4±1.9
7	No treatment	4/4	18.9±1.7	0.6±0.6	14.1±2.2	4.3±0.8
			/29.2±2.0	/0.0±0.0	/20.3±2.8	/9.1±1.1

a) Mean±SD.
b, c, h) Significantly different from group 11 by Student's *t* test (*b*) $P<0.001$, *c*) $P<0.005$, and *h*) $P<0.02$).
d–g) Significantly different from group 1 by Student's *t* test (*d*) $P<0.001$, *e*) $P<0.02$, *f*) $P<0.01$, and *g*) $P<0.05$).

Table VI. GST and QR Activities in Liver and Colon of Rats Gavaged with ACA

	Enzyme	Enzyme activities (Mean±SD, mU/mg protein)			
		0 mg[a]	50 mg	100 mg	200 mg
Liver	GST-CDNB	807.6±133.7 (8)	931.4±144.1 (8)	1174.1±164.3[b,c] (8)	1190.1±146.1[b] (8)
	GST-DCNB	38.5±6.2 (8)	39.4±9.1 (8)	43.3±10.7 (8)	43.7±7.2 (8)
	QR	142.1±23.3 (8)	187.4±37.6[d] (8)	226.7±50.0[e] (8)	296.4±67.8[b,f,g] (8)
Colon	GST-CDNB	144.5±9.5 (8)	155.5±7.7[h] (8)	162.1±5.8[b] (8)	166.4±13.9[i] (8)
	QR	506.0±35.5 (8)	538.0±32.3 (8)	582.7±43.4[e] (8)	632.8±75.4[c,e] (8)

Numbers in parentheses are numbers of rats examined.
a) Dose level of ACA (mg/kg body weight/day) for 5 days orally. Animals were killed 30 min after the last administration.
b, d, e, h, i) Significantly different from "0 mg" group by Student's *t* test or Welch's *t* test (*b*) $P<0.001$, *d*) $P<0.02$, and *e*) $P<0.002$).
c, f) Significantly different from "50 mg" group by Student's *t* test or Welch's *t* test (*c*) $P<0.01$ and *f*) $P<0.002$).
g) Significantly different from "100 mg" group by Student's *t* test ($P<0.05$).

DISCUSSION

The results in the present study demonstrated that dietary feeding of ACA during either the initiation or post-initiation phase significantly inhibited AOM-induced large bowel carcinogenesis in rats. The results confirmed our previous study indicating that ACA feeding at 100 ppm and 200 ppm concentrations was effective in the suppression of large ACF (more than 4 crypts) development, which may correlate with tumor incidence,[33] in rats treated with AOM, and the degree of suppressive effect was similar.[36] In the present study, the doses of ACA in the diets were 100 ppm and 500 ppm and the chemopreventive effect of ACA was dose-dependent: 54% reduction at 100 ppm and 77% reduction at 500 ppm by the initiation feeding; and 45% reduction at 100 ppm and 93% reduction at 500 ppm by the post-initiation feeding. Dietary administration of ACA also suppressed cryptal cell proliferation activity increased by AOM.

Several natural products with antioxidative property exert chemopreventive activity in chemically induced colon carcinogenesis.[28,50] High oxygen free radical activity was found in colon tumors induced by AOM.[11] Histologically normal colonic mucosa of AOM-treated rats also has higher oxygen free radical activity than that of control rats.[11] This suggests that there may be a "field change" in the colonic mucosa as a result of AOM administration, supporting a possible role of oxygen free radical activity in AOM-induced colon carcinoma. In humans, direct measurement of superoxide dismutase and catalase activity in colorectal cancer and normal mucosa showed significantly lower levels of these scavenging enzymes in normal tissue when compared with tumor tissue, suggesting that this depressed scavenging ability may allow unchallenged expression of free radical activity, resulting in DNA damage and cell transformation.[51] ACA could inhibit xanthine oxidase,[21] which generates superoxide anion (known to be associated with tumor development).[9,52] Therefore, it is likely that the chemopreventive effect of ACA found in this study is due to its antioxidative property. Previously, ACA was found to have a powerful inhibitory effect on 4-nitroquinoline 1-oxide-induced rat oral tumorigenesis when fed during both the initiation and post-initiation phases.[23] ACA also reduced the incidence of oral preneoplasia along with a reduction in cell proliferation activity in the oral mucosa.[23] Therefore, ACA might be a possible cancer chemopreventive agent against cancer development in the oral cavity and colon.

Several other mechanisms by which ACA inhibits the development of ACF induced by AOM could be considered from the known metabolism of AOM. The critical steps in the metabolic activation of AOM to reactive species which alkylate DNA, include (i) the hydroxylation of AOM to methylazoxymethanol, which occurs predominantly in the liver, probably via a cytochrome P450-dependent pathway, and to a limited degree in the colonic mucosa[53]; and (ii) the oxidation of methylazoxymethanol to methylazoxyformaldehyde, which is catalyzed by liver and colon microsomes as well as by cytosol alcohol dehydrogenase in these tissues.[54–56] The unstable

compound, methylazoxymethanol, readily yields the methyldiazonium ion, which can alkylate macromolecules by enzymatic and nonenzymatic processes in the liver and colon.[54–56] Alternatively, methylazoxymethanol has been found to be a substrate for NAD^+-dependent dehydrogenase present in the colon and liver, suggesting that the active metabolite of methylazoxymethanol may be the corresponding aldehyde.[57] Disulfiram, known to inhibit the oxidation of dimethylhydrazine and AOM *in vivo*,[58] could inhibit dimethylhydrazine-induced colon carcinogenesis[59] and AOM-induced ACF.[28] Several other colon tumor inhibitors have been shown to interact with AOM metabolism and inhibit AOM carcinogenicity.[60] In the current study, ACA induced phase II detoxification enzymes, GST and QR, in the liver and colon. These results may be correlated with a reduction in the frequency of micronuclei upon feeding of ACA.[36] Thus, it is possible that ACA feeding during the initiation stage modulates the hepatic and colonic metabolism of AOM and decreases the formation of carcinogenic metabolites. This may, in part, explain the tumor-inhibitory effect of ACA which is observed during the initiation phase, although the role of detoxification enzymes in the inactivation of AOM remains to be determined.

The results of the current study also indicated that ACA feeding after carcinogen exposure inhibits colonic neoplasms. In the present study, the expression of known cell proliferation biomarkers, such as ODC activity and polyamine levels (blood and colonic mucosa),[28, 37] was significantly inhibited by the dietary feeding of ACA. Certain chemopreventers could alter (decrease) the expression of these biomarkers in the target tissue.[17, 28, 30] Klaunig[19] reported that a catechin-containing green tea extract prevented the induction of hepatocyte cytolethality caused by oxygen radical-generating compounds including xanthine oxidase and significantly decreased the hepatic cell proliferation in an *in vivo* investigation. Recently, Cohen and Ellwein have highlighted the importance of increased cell proliferation in the carcinogenesis process.[61] Thus, the inhibitory effect of ACA may be due to the modification of cell proliferation in the colonic mucosa through the above-mentioned mechanism(s). In this context, studies on the modulation of colonic mucosal tyrosine protein kinase activity by ACA feeding are interesting, since the phosphorylation of protein at tyrosine residues plays an important role in the regulation of cellular growth and differentiation.[62] In addition, it should be determined whether detoxifying enzymes such as GST and QR play a role in tumor-inhibition by feeding of ACA during the post-initiation stage.

In summary, dietary administration of ACA during either the initiation or post-initiation stage effectively inhibited AOM-induced rat colon carcinogenesis. In addition, ACA significantly suppressed the expression of cell proliferation biomarkers in the colonic mucosa and increased the GST and QR activities in the liver and colonic mucosa. Although the exact mechanisms, including the modulating role of detoxifying enzymes during colon tumorigenesis, of the chemopreventive effects of ACA are not yet known, the results described here suggest a possible chemopreventive action of ACA against colon carcinoma.

ACKNOWLEDGMENTS

We wish to thank Kyoko Takahashi, Chikako Usui, Yuki Ozawa, and Kimiko Nakamura for their technical assistance, and Kazumasa Satoh for care of the animals. This work was supported in part by a Grant-in-Aid for the 2nd Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health and Welfare, a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare in Japan, and a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

(Received June 2, 1997/Accepted July 7, 1997)

REFERENCES

1) Shike, M., Winawer, S. J., Greenwald, P. H., Bloch, A., Hill, M. J. and Swaroop, S. V. Primary prevention of colorectal cancer: the WHO Collaborating Centre for Prevention of Colorectal Cancer. *Bull. World Health Organ.*, **68**, 377–385 (1990).
2) Weisburger, J. H. Nutritional approach to cancer prevention with emphasis on vitamins, antioxidants, and carotenoids. *Am. J. Clin. Nutr.*, **53**, 226S–237S (1991).
3) Dove, W. F., Gould, K. A., Luongo, C., Moser, A. R. and Shoemaker, A. R. Emergent issues in the genetics of intestinal neoplasia. *Cancer Surv.*, **25**, 335–355 (1995).
4) Tanaka, T. Effect of diet on human carcinogenesis. *Crit. Rev. Oncol./Hematol.*, **25**, 73–95 (1997).
5) Haenszel, W., Licks, F. B. and Segi, M. A case-control study of large bowel cancer in Japan. *J. Natl. Cancer Inst.*, **64**, 17–22 (1980).
6) Broder, S. Perspectives on cancer in Japan and the United States. *Jpn. J. Cancer Res.*, **84**, 821–830 (1993).
7) Block, G., Patterson, B. and Subar, A. Fruit, vegetables, and cancer prevention: a review of epidemiological evidence. *Nutr. Cancer*, **18**, 1–29 (1992).
8) Tanaka, T. Chemoprevention of human cancer: biology and therapy. *Crit. Rev. Oncol./Hematol.*, **25**, 139–174 (1997).
9) Cerutti, P. A. Oxy-radicals and cancer. *Lancet*, **344**, 862–863 (1994).

10) Cerutti, P. A. Oxidant stress and carcinogenesis. *Eur. J. Clin. Invest.*, **21**, 1–5 (1991).
11) Qureshi, A., Gorey, T. F., Byrne, P., Kay, E., McKeever, J. and Hennessy, T. P. J. Oxygen free radical activity in experimental colonic carcinoma. *Br. J. Surg.*, **81**, 1058–1059 (1994).
12) Parks, D. A. and Granger, D. N. Xanthine oxidase: biochemistry, distribution and physiology. *Acta Physiol. Scand.*, **548** (Suppl.), 87–99 (1986).
13) McCord, J. M. and Fridovich, I. The reduction of cytochrome C by milk xanthine oxidase. *J. Biol. Chem.*, **243**, 5753–5760 (1968).
14) Kokoglu, E., Belce, A., Ozyurt, E. and Tepeler, Z. Xanthine oxidase levels in human brain tumors. *Cancer Lett.*, **50**, 179–181 (1990).
15) Reiners, J. J., Jr., Thai, G., Rupp, T. and Cantu, A. R. Assessment of the antioxidant/prooxidant status of murine skin following topical treatment with 12-*O*-tetradecanoylphorbol-13-acetate and throughout the ontogeny of skin cancer. Part I. Quantitation of superoxide dismutase, catalase, glutathione peroxidase and xanthine oxidase. *Carcinogenesis*, **12**, 2337–2343 (1991).
16) Pence, B. C. and Reiners, J. J., Jr. Murine epidermal xanthine oxidase activity: correlation with degree of hyperplasia induced by tumor promoters. *Cancer Res.*, **47**, 6388–6392 (1987).
17) Tanaka, T. Cancer chemoprevention by natural products (Review). *Oncol. Rep.*, **1**, 1139–1155 (1994).
18) Wei, H., Bowen, R., Cai, Q., Barnes, S. and Wang, Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. *Proc. Soc. Exp. Biol. Med.*, **208**, 124–130 (1995).
19) Klaunig, J. E. Chemopreventive effects of green tea components on hepatic carcinogenesis. *Prev. Med.*, **21**, 510–519 (1992).
20) Mitsui, S., Kobayashi, S., Nagahori, H. and Ogiso, A. Constituents from seeds of *Alpinia galanga* wild, and their anti-ulcer activities. *Chem. Pharm. Bull.*, **24**, 2377–2382 (1976).
21) Noro, T., Sekiya, T., Katoh, M., Oda, Y., Miyase, T., Kuroyanagi, M., Ueno, A. and Fukushima, S. Inhibitors of xanthine oxidase from *Alpinia galanga*. *Chem. Pharm. Bull.*, **36**, 244–248 (1988).
22) Kondo, A., Ohigashi, H., Murakami, A., Suratwadee, J. and Koshimizu, K. 1′-Acetoxychavicol acetate as a potent inhibitor of tumor promoter-induced Epstein-Barr virus activation from *Languas galanga*, a traditional Thai condiment. *Biosci. Biotech. Biochem.*, **57**, 1344–1345 (1993).
23) Ohnishi, M., Tanaka, T., Makita, H., Kawamori, T., Mori, H., Satoh, K., Hara, A., Murakami, A., Ohigashi, H. and Koshimizu, K. Chemopreventive effect of a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate, on rat oral carcinogenesis. *Jpn. J. Cancer Res.*, **87**, 349–356 (1996).
24) Bird, R. P. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett.*, **93**, 55–71 (1995).
25) Pretlow, T. P., Barrow, B. J., Ashton, W. S., O'Riordan, M. A., Pretlow, T. G., Jurcisek, J. A. and Stellato, T. A. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res.*, **51**, 1564–1567 (1991).
26) McLellan, E. A., Medline, A. and Bird, R. P. Sequential analysis of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Res.*, **51**, 5270–5274 (1991).
27) Pereira, M. A., Barnes, L. H., Rassman, V. L., Kelloff, G. V. and Steele, V. E. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. *Carcinogenesis*, **15**, 1049–1054 (1994).
28) Tanaka, T. and Mori, H. Inhibition of colon carcinogenesis by non-nutritive constituents in foods. *J. Toxicol. Pathol.*, **9**, 139–149 (1996).
29) Mori, H., Tanaka, T., Sugie, S. and Yoshimi, N. Chemopreventive effects of plant derived phenolic, organosulfur and other compounds on carcinogenesis in digestive organs. *Environ. Mutagen. Res. Commun.*, **17**, 127–133 (1995).
30) Tanaka, T., Kojima, T., Kawamori, T. and Mori, H. Chemoprevention of digestive organs carcinogenesis by natural product protocatechuic acid. *Cancer*, **75** (Suppl.), 1433–1439 (1995).
31) Carter, J. W., Lancaster, H. K., Hardman, W. E. and Cameron, I. L. Distribution of intestine-associated lymphoid tissue, aberrant crypt foci, and tumors in the large bowel of 1,2-dimethylhydrazine-treated mice. *Cancer Res.*, **54**, 4304–4307 (1994).
32) Kristiansen, E. The role of aberrant crypt foci induced by the two heterocyclic amines 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the development of colon cancer in mice. *Cancer Lett.*, **110**, 187–192 (1996).
33) Pretlow, T. P., O'Riordan, M. A., Somich, G. A., Amini, S. B. and Pretlow, T. G. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis*, **13**, 1509–1512 (1992).
34) Zhang, X.-M., Stamp, D., Minkin, S., Medline, A., Corpet, D. E., Bruce, W. R. and Archer, M. C. Promotion of aberrant crypt foci and cancer in rat colon by thermolyzed protein. *J. Natl. Cancer Inst.*, **84**, 1026–1030 (1992).
35) Magnuson, B. A., Carr, I. and Bird, R. P. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats fed cholic acid. *Cancer Res.*, **53**, 4499–4504 (1993).
36) Tanaka, T., Makita, H., Kawamori, T., Kawabata, K., Mori, H., Murakami, A., Satoh, K., Hara, A., Ohigashi, H. and Koshimizu, K. A xanthine oxidase inhibitor 1′-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis*, **18**, 1113–1118 (1997).
37) Einspahr, J. G., Alberts, D. S., Gapstur, S. M., Bostick, R. M., Emerson, S. S. and Gerner, E. W. Surrogate endpoint biomarkers as measures of colon cancer risk and

their use in cancer chemoprevention trials. *Cancer Epidemiol. Biomarkers Prev.*, **6**, 37–48 (1997).
38) Talalay, P. Chemical protection against cancer by induction of electrophile detoxification (Phase II) enzymes. *In* "Cellular and Molecular Targets for Chemoprevention," ed. V. E. Steele, G. D. Stoner, C. W. Boone and G. J. Kelloff, pp. 193–205 (1992). CRC Press, Boca Raton, FL.
39) Prochaska, H. J. and Talalay, P. The role of NAD(P)H:quinone reductase in protection against the toxicity of quinones and related agents. *In* "Oxidative Stress: Oxidants and Antioxidants," ed. H. Sies, pp. 195–211 (1991). Academic Press, London.
40) Ward, J. M. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab. Invest.*, **30**, 505–513 (1974).
41) Hard, C. C. and Butler, W. H. Cellular analysis of renal neoplasia: induction of renal tumors in dietary-conditioned rats by dimethylnitrosamine with a reappraisal of morphological characteristics. *Cancer Res.*, **30**, 2796–2805 (1970).
42) Koide, T. The clinical significance of tissue, blood and urine polyamine in renal cell carcinoma. *Jpn. J. Urol.*, **83**, 1228–1237 (1992) (in Japanese).
43) Koide, T., Sakai, S., Takeda, A., Doi, T., Tei, K., Hara, A. and Sawada, H. Detection of polyamines by a new enzymatic differential assay. (4) Fundamental study on a new enzymatic differential assay of blood. *Acta Urol. Jpn.*, **32**, 913–917 (1986) (in Japanese).
44) Benson, A. M., Batzinger, R. P., Ou, S.-Y. L., Bueding, E., Cha, Y.-N. and Talalay, P. Elevation of hepatic glutathione *S*-transferase activities and protection against metabolites of benzo[*a*]pyrene by dietary antioxidants. *Cancer Res.*, **38**, 4486–4495 (1978).
45) De Long, M. J., Prochaska, H. J. and Talalay, P. Tissue-specific induction patterns of cancer-protective enzymes in mice by *tert*-butyl-4-hydroxyanisole and related substituted phenols. *Cancer Res.*, **45**, 546–551 (1985).
46) Prochaska, H. and Fernandes, C. L. Elevation of serum Phase II enzymes by anticarcinogenic enzyme inducers: markers for a chemoprotected state? *Carcinogenesis*, **14**, 2441–2445 (1993).
47) Benson, A. M., Hunkeler, M. J. and Talalay, P. Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc. Natl. Acad. Sci. USA*, **77**, 5216–5220 (1980).
48) Habig, W. H., Pabst, M. J. and Jakoby, W. B. Glutathione *S*-transferases. *J. Biol. Chem.*, **249**, 7130–7139 (1974).
49) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
50) Wattenberg, L. W. Inhibition of carcinogenesis by minor anutrient constituents of the diet. *Proc. Nutr. Soc.*, **49**, 173–183 (1990).
51) Hoffman, C. E. J., Webster, N. R., Wiggins, P. A., Chisholm, E. M., Giles, G. R. and Leveson, S. H. Free radical detoxifying systems in human colorectal cancer. *Br. J. Cancer*, **51**, 127–129 (1985).
52) Guyton, K. Z. and Kensler, T. W. Oxidative mechanisms in carcinogenesis. *Br. Med. Bull.*, **49**, 523–544 (1993).
53) Oravec, C. T., Jones, C. A. and Huberman, E. Activation of the colon carcinogen 1,2-dimethylhydrazine in a rat colon cell-mediated mutagenesis assay. *Cancer Res.*, **46**, 5068–5071 (1986).
54) Fiala, E. S. Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. *Cancer*, **40** (Suppl. 5), 2436–2445 (1977).
55) Fiala, E. S., Kulakis, C., Christiansen, G. and Weisburger, J. H. Inhibition of the metabolism of the colon carcinogen, azoxymethane, by pyrazole. *Cancer Res.*, **38**, 4515–4521 (1978).
56) Fiala, E. S., Caswell, N., Sohn, O. S., Felder, M. R., McCoy, G. D. and Weisburger, J. H. Non-alcohol dehydrogenase-mediated metabolism of methylazoxymethanol in the deer mouse, *Peromyscus maniculatus*. *Cancer Res.*, **44**, 2885–2891 (1984).
57) Zedeck, M. S., Frank, N. and Wiessler, M. Metabolism of the colon carcinogen methylazoxymethanol acetate. *Front. Gastrointest. Res.*, **4**, 32–37 (1979).
58) Fiala, E. S., Bobotas, G., Kulakis, C., Wattenberg, L. W. and Weisburger, J. H. Effects of disulfiram and related compounds on the metabolism *in vivo* of the colon carcinogen, 1,2-dimethylhydrazine. *Biochem. Pharmacol.*, **26**, 1763–1768 (1977).
59) Wattenberg, L. W., Lam, L. K. T., Fladmoe, A. and Borchert, P. Inhibitors of colon carcinogenesis. *Cancer*, **40**, 2435–2445 (1977).
60) Fiala, E. S. Inhibition of carcinogen metabolism and action by disulfiram, pyrazole, and related compounds. *In* "Inhibition of Tumor Induction and Development," ed. M. S. Zedeck and M. Lipkin, pp. 23–69 (1981). Plenum Publ., New York.
61) Cohen, S. M. and Ellwein, L. B. Cell proliferation in carcinogenesis. *Science*, **249**, 1007–1011 (1990).
62) Cantly, L. D., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. Oncogenes and signal transduction. *Cell*, **64**, 281–302 (1991).