

Suppression of *N*-Nitrosomethylbenzylamine-induced Rat Esophageal Tumorigenesis by Dietary Feeding of 1'-Acetoxychavicol Acetate

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The modifying effects of 1'-acetoxychavicol acetate (ACA) on *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis were investigated in male F344 rats. At 5 weeks of age, all test animals, except those given the test chemical alone, and the control rats received s.c. injections of NMBA (0.5 mg/kg body weight/injection, three times per week) for 5 weeks. At the termination of the study (20 weeks), 75% of rats treated with NMBA alone had esophageal neoplasms (papillomas). However, the groups given a dose of 500 ppm ACA during the initiation phase developed a significantly reduced incidence of tumors (29%; $P < 0.01$). Exposure to ACA (500 ppm) during the post-initiation phase also decreased the frequency of the tumors (38%; $P < 0.05$). A reduction of the incidence of preneoplastic lesions (hyperplasia or dysplasia) was obtained when ACA was administered in the initiation phase ($P < 0.01$). Cell proliferation in the esophageal epithelium, determined by assay of proliferating cell nuclear antigen (PCNA), was lowered by ACA ($P < 0.05$). Blood polyamine contents in rats given NMBA and the test compound were also smaller than those of rats given the carcinogen ($P < 0.05$). These findings suggest that dietary ACA is effective in inhibiting the development of esophageal tumors by NMBA when given during the initiation or post-initiation phase, and such inhibition is related to suppression of cell proliferation in the esophageal epithelium.

Key words: Chemoprevention — 1'-Acetoxychavicol acetate — *N*-Nitrosomethylbenzylamine — Esophageal tumorigenesis — Rats

Esophageal cancer in humans occurs worldwide and is the seventh most common cancer in terms of incidence.¹ Ninety percent of esophageal cancers are squamous cell carcinomas,² which present a difficult clinical problem. The disease is often accompanied by nutritional dysfunction and advanced cancers readily seed nearby lymph nodes to develop new local, then metastatic disease. Research in China and South Africa has implicated *N*-nitroso compounds and their precursors as probable etiological factors in esophageal cancer.^{3,4} Several of these compounds are regarded as potent inducers of esophageal cancer in animals, principally in rats.⁵ The NMBA-

induced rat esophageal tumor model has been used extensively for investigation of chemical carcinogenesis and cancer prevention,⁶⁻⁹ since animal lesions induced by NMBA are histologically similar to human esophagus cancers and provide opportunities to gain a better understanding of esophageal carcinogenesis.¹⁰

Active primary prevention of cancer is one of the most challenging aspects of medical research.¹¹⁻¹³ Diets high in fresh vegetables are consistently associated with reduced risk for esophageal cancers.¹⁴ In a recent review, Steinmetz and Potter¹⁵ reported that 10 of 11 epidemiological case-control studies found a significant inverse relationship between the intake of fresh fruits or vegetables and esophageal cancer risk. In animal models for esophageal cancer, diallyl sulfide^{16,17} in garlic, derivatives of cruciferous vegetable isothiocyanates,^{18,19} green and black tea,^{8,20} selenium,²¹ ellagic acid,²² protease inhibitors²³ and diosmin and hesperidin²⁴ have been reported to inhibit esophageal carcinogenesis.

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Abbreviations: NMBA, *N*-nitrosomethylbenzylamine; PCNA, proliferating cell nuclear antigen; ACA, 1'-acetoxychavicol acetate; DMSO, dimethylsulfoxide; 4-NQO, 4-nitroquinoline 1-oxide; AOM, azoxymethane; ROS, reactive oxygen species; *O*⁶-MeG, *O*⁶-methylguanine.

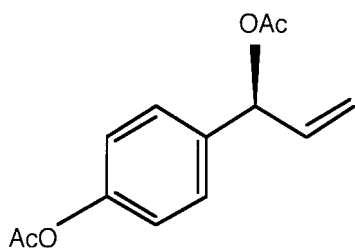


Fig. 1. Chemical structure of ACA.

ACA (Fig. 1) is present in seeds or rhizome of *Languas galanga* (Zingiberaceae), which has been used as a ginger substitute and a stomachic medicine in Thailand.²⁵⁾ ACA is reported to be an inhibitor of xanthine oxidase.²⁶⁾ In a survey of anti-tumor promoters in edible plants of Thailand, this agent was found to inhibit tumor promoter-induced Epstein-Barr virus activation *in vitro*.²⁷⁾ In addition, we have found a strong cancer chemopreventive effect of ACA on 4-NQO-induced oral carcinogenesis²⁸⁾ and AOM-induced colon adenocarcinoma in rats.²⁹⁾

This evidence led us to investigate the modifying effects of ACA on experimental esophageal carcinogenesis. In the present study, the possible inhibitory effects of dietary exposure to ACA during the initiation and post-initiation stages of NMBA-induced esophageal tumorigenesis were investigated in rats.

MATERIALS AND METHODS

Animals, diets, and carcinogens Male F344 rats, 4 weeks old, were purchased from Japan SLC, Inc. (Hamamatsu). After a 2-week quarantine, they were transferred to a holding room under controlled conditions at $23 \pm 2^\circ\text{C}$, $50 \pm 10\%$ humidity, and a 12-h light/dark cycle. They were housed three or four to a wire cage. NMBA (>99% purity) was synthesized by Dr. Stoner. Powdered CE-2 (CLEA Japan, Inc.) was used as the basal diet during the experiment. ACA (>95% purity) was synthesized according to the method described previously.²⁷⁾ Experimental diets mixed with ACA at two concentrations (100 and 500 ppm) were prepared using a V-blender on a weekly basis and stored in a cold room ($<4^\circ\text{C}$) until use.

Experimental procedure The experiment was designed to examine the modifying effects of ACA during the initiation and post-initiation phases of NMBA-induced esophageal tumorigenesis in male F344 rats (Fig. 2). A total of 113 rats were randomized into seven groups. They were provided with a CE-2 diet and water *ad libitum*. After an acclimatization period of 2 weeks, the rats in groups 1 through 5 were treated with NMBA solubilized in 20% DMSO in water by s.c. injection at a dose of 0.5 mg/kg

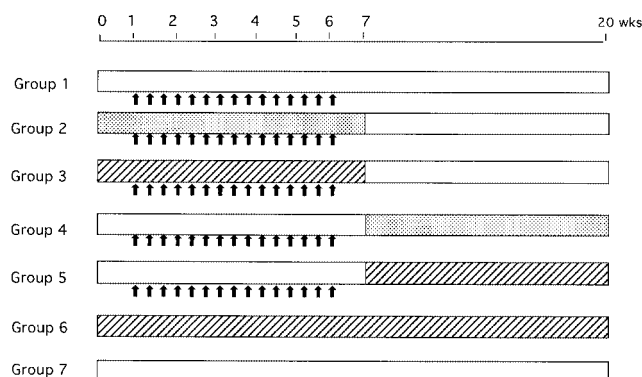


Fig. 2. Experimental protocol. \uparrow NMBA injection (0.5 mg/kg body weight), \square ACA (100 ppm in diet), ▨ ACA (500 ppm in diet), \square basal diet and tap water.

body weight three times (Monday, Wednesday, Friday) per week (0.2 ml) for 5 weeks. Twenty-percent DMSO in water was used as a vehicle control. Groups 2 and 3 were given diets containing 100 and 500 ppm ACA for 7 weeks, respectively, starting at 6 weeks of age until one week after the last carcinogen dose. They were then switched to the basal diet and maintained on this diet for 13 weeks. Groups 4 and 5 were fed the diets mixed with 100 and 500 ppm ACA, respectively, starting one week after the last injection of NMBA, and continued on these diets for 13 weeks. Group 6 was fed the diet containing the test compound (500 ppm ACA) alone during the experiment. Group 7 was given the basal diet and tap water throughout the experiment and served as an untreated control. The experiment was terminated at 20 weeks and all animals were killed by decapitation to evaluate the frequencies of preneoplastic and neoplastic lesions in the esophagus. At necropsy, the esophagus was cut open longitudinally and examined for tumors under the dissecting microscope. Esophageal tumors (equal to or greater than 1 mm in diameter) were quantified, sized, and mapped under a dissecting microscope and a millimeter ruler. All other organs were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed routinely for histopathological examination.

Determination of PCNA-labeling index in the nonlesional esophageal epithelium To assess the proliferative activity of the squamous epithelium of the esophagus, 10 (groups 1–5) and 5 (groups 6 and 7) animals were selected randomly and PCNA-labeling cell indices were calculated as percentages of stained nuclei under a light microscope. The tissue sections were deparaffinized in xylene and then passed through a graded series of alcohol. Endogenous peroxidase activity was depleted with 3% hydrogen peroxide in methanol. Normal horse serum

(10%) was used to suppress non-specific protein binding. Tissue sections were incubated at 4°C overnight for PCNA staining with the following antibody: mouse monoclonal anti-PCNA antibody at 1:200 dilution. The sections were then washed and incubated with biotinylated horse anti-mouse IgG at room temperature for 30 min. They were

washed and incubated with avidin-biotin-peroxidase complex at room temperature for 30 min using a LSAB kit (Dako Co., Kyoto). In the scoring of labeled nuclei, the basal epithelial cells of the cross section of an entire esophagus were counted under light microscopy. The labeling index was calculated by dividing the number of labeled cells by the total number of cells, and the result was expressed as a percentage.

Blood polyamine levels Since the amount of esophageal mucosa was too low to allow measurement of tissue polyamine levels, they were measured using the method of Koide *et al.*³⁰ At sacrifice, blood from each of 5 rats in groups 1–7 was collected by heart puncture and the levels of diamine, spermine, and spermidine were determined. The results obtained were confirmed to correlate well with those of high-performance liquid chromatography.

Statistical analysis Statistical analysis was performed using Fisher's exact probability test, the unpaired Student's *t* test or Welch's *t* test. ANOVA or the Kruskal-Wallis test used to compare all pairs. The results were considered significant if the *P* value was 0.05 or less.

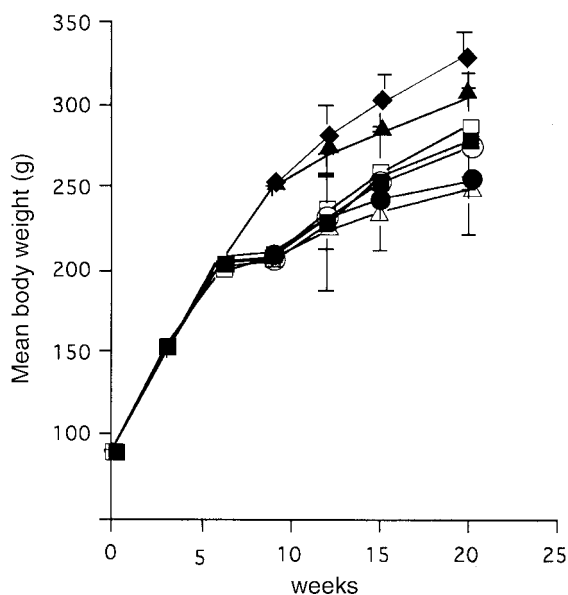


Fig. 3. Body weight gains of each group. ○ G1, ■ G2, □ G3, ● G4, △ G5, ▲ G6, ◆ G7.

RESULTS

General observations Rats in groups 1–7 tolerated the s.c. injection of NMBA and dietary administration of the test compound. There were no pathological alterations in any rats treated with ACA or in the untreated control group. The body weight gains of rats treated with NMBA and/or the test compound are indicated in Fig. 3. The mean body weights of rats in groups 1 and 6 were significantly

Table I. Incidence and Multiplicity of Esophageal Tumors in Rats Treated with NMBA and/or ACA

Group no.	Treatment	No. of rats examined	Incidence of esophageal tumors (%)			Multiplicity (no. of tumors/rat) of esophageal tumors		
			Total (%)	Squamous cell papilloma (%)	Squamous cell carcinoma (%)	Total	Squamous cell papilloma	Squamous cell carcinoma
1	NMBA alone	20	15 (75)	15 (75)	1 (5)	1.00±0.77 ^{a)}	0.95±0.74	0.05±0.22
2	NMBA + 100 ppm ACA	19	9 (47)	9 (47)	0	0.58±0.67	0.58±0.67	0
3	NMBA + 500 ppm ACA	17	5 (29) ^{b)}	5 (29) ^{b)}	0	0.35±0.59 ^{d)}	0.35±0.59 ^{e)}	0
4	NMBA → 100 ppm ACA	17	8 (47)	8 (47)	0	0.59±0.69	0.59±0.69	0
5	NMBA → 500 ppm ACA	16	6 (38) ^{c)}	6 (38) ^{c)}	0	0.44±0.61 ^{f)}	0.44±0.61 ^{f)}	0
6	500 ppm ACA	12	0	0	0	0	0	0
7	No treatment	12	0	0	0	0	0	0

a) Mean±SD.

b,c) Significantly different from group 1 by Fisher's exact probability test (b) *P*=0.0068, c) *P*=0.0264).

d-f) Significantly different from group 1 by Student's *t* test (d) *P*<0.01, e) *P*<0.02, and f) *P*<0.05).

Table II. Incidence of Esophageal Preneoplastic Lesions in Rats Treated with NMBA and/or ACA

Group no.	Treatment	No. of rats examined	Hyperplasia			Dysplasia			
			Total (%)	Simple (%)	Nodular or papillary (%)	Total (%)	Mild (%)	Moderate (%)	Severe (%)
1	NMBA alone	20	20 (100)	20 (100)	18 (90)	20 (100)	20 (100)	19 (95)	14 (70)
2	NMBA + 100 ppm ACA	19	19 (100)	19 (100)	7 (39) ^{a)}	19 (100)	19 (100)	18 (95)	8 (42)
3	NMBA + 500 ppm ACA	17	17 (100)	17 (100)	8 (47) ^{b)}	16 (94)	14 (82)	8 (47) ^{c)}	5 (29) ^{d)}
4	NMBA → 100 ppm ACA	17	17 (100)	17 (100)	12 (71)	15 (88)	15 (88)	14 (82)	10 (59)
5	NMBA → 500 ppm ACA	16	16 (100)	16 (100)	15 (94)	16 (100)	16 (100)	15 (94)	9 (56)
6	500 ppm ACA	12	0	0	0	0	0	0	0
7	No treatment	12	0	0	0	0	0	0	0

a-d) Significantly different from group 1 by Fisher's exact probability test (a) $P=0.0009$, b) $P=0.0075$, c) $P=0.0018$, and d) $P=0.00218$.

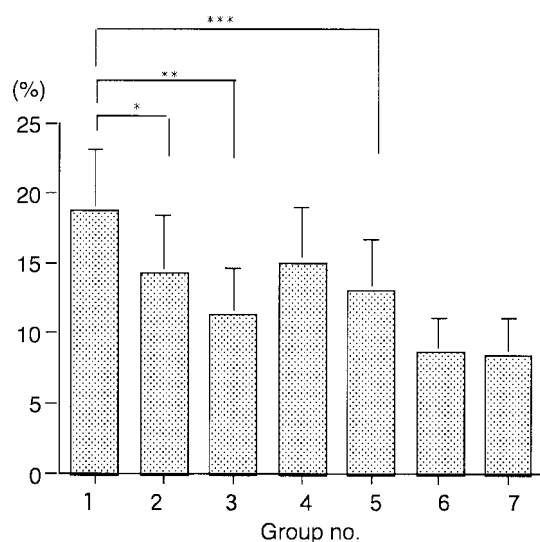


Fig. 4. PCNA-labeling index of esophageal epithelium in NMBA and/or ACA. Data are the means±SD of % accumulation. Significance, determined by using Student's *t* test or Welch's *t* test, is expressed as follows: * group 1 versus group 2, $P<0.05$, ** group 1 versus group 3, $P<0.01$, *** group 1 versus group 5, $P<0.01$.

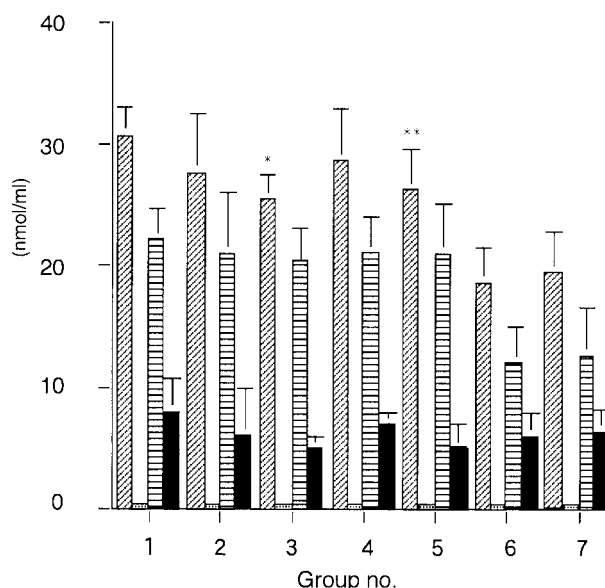


Fig. 5. Blood polyamine levels in NMBA and/or ACA. ▨ total, ▩ diamine, ▤ spermidine, ▥ spermine. Total polyamine level and/or spermidine/spermine ratio reflect proliferation. Total polyamine levels in groups 3 and 5 were significantly lower than that in group 1 (* $P<0.01$, ** $P<0.05$, both versus NMBA-alone group).

cantly smaller than that of group 7 ($P<0.01$). Furthermore, the weight of rats in group 5 was significantly lower than that in group 1 ($P<0.05$). The body weight gains of rats treated with NMBA and fed the experimental diet were slightly lower than those in groups 6 and 7.

Incidences and multiplicities of esophageal tumors and preneoplastic lesions The incidence and multiplicity (no. of tumors/rat) of esophageal tumors are summarized in

Table I. No tumors developed in any rat from groups 6 and 7. In other groups, the esophageal tumors were multiple, variable in size, and randomly distributed along the entire length of the esophagus, without predilection for the upper, lower or middle region. Most of the tumors were histologically squamous cell papillomas. Squamous cell

carcinoma was recognized in only one rat in group 1. In group 1 (NMBA alone), the incidence of papilloma was 75%. The incidences of papilloma of groups 2 and 3 were 47% and 29%, respectively. Those of groups 4 and 5 were 47% and 38%, respectively. The tumor incidence in groups 3 and 5 was significantly lower than that in group 1 ($P=0.0068$ and $P=0.0264$, respectively). The mean multiplicities of esophageal tumors in rats of groups 3 (0.35 ± 0.59) and 5 (0.44 ± 0.61) were significantly lower than that of group 1 (1.00 ± 0.770) ($P<0.01$ – $P<0.05$). Similarly, mean multiplicities of squamous cell papilloma in groups 3 and 5 treated with NMBA and/or ACA were lower than that in group 1 ($P<0.02$ and $P<0.05$, respectively, Table I).

Preneoplastic lesions (hyperplasia and dysplasia) were found in rats in groups 1–5. These lesions were mainly present around the tumors. Hyperplasia was classified as nodular and papillary hyperplasia according to the growth patterns. Dysplasia was categorized as mild, moderate, or severe according to the degree of nuclear atypism. The incidences of these preneoplastic lesions are shown in Table II. The incidences of nodular or papillary hyperplasia in groups 2 (39%) and 3 (47%) were significantly lower than that of group 1 (89%) ($P<0.001$ – $P<0.008$). Frequency of moderate or severe dysplasia of group 3 was significantly lower than that in group 1 ($P=0.0018$ and $P=0.00218$, respectively, Table II).

PCNA-labeling index The results of morphometric analysis of the PCNA-labeling index in the esophageal squamous epithelium are summarized in Fig. 4. The mean number of PCNA-labeling indices of epithelial cell proliferation in the esophagus exposed to NMBA alone (group 1) was significantly higher than that of the untreated control (group 7) ($P<0.001$). The PCNA-labeling indices in groups 3 and 5 were significantly lower than that in group 1 ($P<0.01$), and the difference from group 2 was significant at $P<0.005$. The value in group 6 was comparable to that in group 7.

Blood polyamine levels The results of the blood polyamine assay are illustrated in Fig. 5. The mean amounts of the total polyamine in groups 3 and 5 were significantly smaller than that in group 1 ($P<0.05$, $P<0.01$, respectively). The value in group 6 was comparable to that in group 7.

DISCUSSION

The results of this study demonstrated that dietary ACA (500 ppm) effectively lowers the incidence and multiplicity of esophageal tumors when fed to rats during either the initiation or post-initiation phase. These findings suggest that ACA acts on both phases of NMBA-induced esophageal tumorigenesis, and may support epidemiological observations suggesting an inverse association between the

development of esophageal neoplasms and the consumption of fruits and/or vegetables.^{14, 31)}

Several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis have been suggested. In the present study, ACA (500 ppm) decreased the cell proliferation in the non-lesional esophageal epithelium (Fig. 4). Increased cell proliferation is thought to play an important role in multistage carcinogenesis,^{32, 33)} including esophageal tumorigenesis.³⁴⁾ Studies in human populations at high risk for esophageal cancer have shown that epithelial hyperplasia and dysplasia are early indicators for this malignancy.³⁵⁾ Thus, it is possible that the significant antitumor effects of ACA are partly due to their antiproliferative effects on a carcinogen-exposed squamous epithelium. Patients with esophageal cancer (advanced or early cancer) or severe dysplasia also have increased serum polyamines,³⁶⁾ suggesting that serum polyamines may be good biochemical markers for precursor lesions and early esophageal cancer.^{24, 36–40)} ACA inhibited the increases of blood polyamine levels (Fig. 5) and cell proliferation due to NMBA in this study. Therefore, one of the mechanisms of the chemopreventive activity of ACA during initiation as well as post-initiation may be modification of cell hyperproliferation in the esophageal epithelium exposed to the ultimate carcinogen, NMBA.

As regards preneoplastic lesions, feeding of 500 ppm ACA during initiation significantly reduced the incidence of hyperplasia or dysplasia (Table II). Also, feeding of 100 ppm ACA significantly reduced the incidence of nodular or papillary hyperplasia. Recently, it was reported that the protective effects of ACA might be due to the induction of phase II enzymes in the liver and colonic mucosa.²⁹⁾ Ahn *et al.*⁴¹⁾ reported that the chemopreventive effect of ellagic acid against various chemically induced cancers may involve decreases in the rates of metabolism of these carcinogens by phase I enzymes, due to both direct inhibition of catalytic activity and modulation of gene expression, in addition to effects on the expression of phase II enzymes. It is known that NMBA requires bioactivation by esophageal cytochrome P450 isozymes via benzaldehyde and a methylating species, and preferentially methylates DNA at the O^6 - and N^7 -positions of guanine.^{42, 43)} O^6 -MeG, the major promutagenic DNA adduct, is associated with base mispairing and mutagenesis.^{44–46)} The formation and persistence of O^6 -alkylguanine are considered to be of major importance in the initiation of tumors.⁴⁷⁾ It is not known, however, whether the protective effects of ACA during the initiation stage may be due to inhibition of metabolism and DNA methylation by specific modification of metabolic activation of NMBA or augmentation of carcinogen detoxification, since in the current study we did not measure DNA adducts or the detoxifying enzyme activities in the esophagus and liver. However, in group 2 a significant reduction in the incidence of preneoplastic lesions was

seen, whereas this was not the case in group 4. The role of ACA in the modification of esophageal tumorigenesis in the initiation phase remains unclear. We are planning to measure the CYP/phase II enzyme activities in the esophageal epithelium of rats. ACA has also been referred to as an inhibitor of a superoxide anion-producing enzyme, xanthine oxidase.^{26, 48)} The inhibition of oxidative damage of DNA due to ROS produced during carcinogen metabolism may be important.⁴⁹⁾ The antioxidant properties of ACA may also be effective when this compound is fed to rats during the initiation and post-initiation phases, since oxygen free radicals can stimulate cancer development throughout carcinogenesis.

In conclusion, our results indicated that dietary feeding of ACA decreased the incidence and multiplicity of esophageal neoplasms induced by NMBA in rats. This compound may be a candidate chemopreventive agent against human esophageal cancers.

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