Chemopreventive Effect of Curcumin on *N*-Nitrosomethylbenzylamine-induced Esophageal Carcinogenesis in Rats

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Modifying effects of curcumin (derived from the rhizome of *Curcuma longa* L.) during the initiation or post-initiation phase of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal carcinogenesis were investigated in male F344 rats. Five-week-old rats were divided into 5 groups, and groups 1, 2 and 3 were given intraperitoneal injections of NMBA (0.5 mg/kg body weight/injection 15 times) for 5 weeks from 7 weeks old to induce esophageal neoplasms. Groups 2 and 3 were fed the diet containing 500 ppm curcumin during the initiation and post-initiation phases, respectively. Group 4 was given the diet containing curcumin throughout the experiment, and group 5 was kept on the basal diet alone and served as an untreated control. Incidence and multiplicity of esophageal neoplasms of group 1 (NMBA alone) were 66.7% and 0.83 ±0.70, respectively. Those of groups 2 and 3 were significantly less than those of group 1 (39.3%, 0.46±0.64, $P<0.05$; 33.3%, 0.36±0.56, $P<0.05$, respectively). Furthermore, the incidence and multiplicity of esophageal preneoplastic lesions (moderate or severe epithelial dysplasia) of group 2 (57.1%, 0.61±0.57; 40%, 0.29±0.46) or 3 (56.7%, 0.67±0.66; 23.3%, 0.23±0.43) were less than those of group 1 (100%, 1.67±0.70; 70.8%, 0.92±0.72) ($P<0.05$). In this experiment, feeding of curcumin significantly decreased the expression of cell proliferation biomarkers (5-bromo-2′-deoxyuridine labeling index) in the non-lesional esophageal epithelium ($P<0.01$). These findings indicate that curcumin inhibits NMBA-induced esophageal carcinogenesis when given during the post initiation as well as initiation phase. This inhibition may be related to suppression of the increased cell proliferation induced by NMBA in the esophageal epithelium.

Key words: Curcumin — Esophageal carcinogenesis — Chemoprevention — *N*-Nitrosomethylbenzylamine — Rat

Esophageal cancer is one of the most lethal carcinomas, and is usually only diagnosed at an advanced stage. An increased risk for developing esophageal cancer has been correlated with smoking and chewing tobacco products,[1, 2] consumption of alcoholic beverages[3] and consumption of salt-cured, salt-pickled and moldy foods, which often contain N-nitroso compounds as contaminants.[4, 5] Esophageal carcinoma is also suggested to be influenced by diets deficient in fresh fruit and vegetables, and possibly infectious agents. Furthermore, diets high in fresh fruit and vegetables are consistently associated with reduced risk for esophageal cancer.[6, 7] Using animal models for esophageal cancer, diallyl sulfide in garlic[8, 9] derivatives of cruciferous vegetable isothiocyanates,[10, 11] green and black tea,[12, 13] selenium,[14] ellagic acid,[15] protease inhibitors[16] and 1′-acetoxychavicol acetate[17] have been reported to inhibit esophageal carcinogenesis by *N*-nitrosomethylbenzylamine (NMBA).[18] Recently, diosmin and hesperidin were also

reported to inhibit esophageal carcinogenesis by *N*-methyl-*N*-amylnitrosamine (MNAN).[19]

Turmeric, the powdered rhizome of *Curcuma longa* L., has been used to treat a variety of inflammatory conditions and chronic diseases.[20, 21] Turmeric has also been used as a coloring and flavoring additive to foods. Curcumin [diferuloylmethane; 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is the main constituent of *Curcuma* species and is composed of two ferulic acids moieties joined by a methylene bridge. This natural dye possesses both anti-inflammatory[22] and antioxidant properties.[23, 24] It has been shown that topical application of curcumin inhibits benzo(*a*)pyrene-induced DNA adduct formation, and development of skin tumors, as well as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal DNA synthesis and tumor promotion in mouse skin.[25–27] Dietary administration of curcumin during the initiation or post-initiation period is also known to inhibit tumorigenesis in forestomach and intestine of mice,[28] and tongue of rats.[29] Huang *et al.* indicated that not only did curcumin reduce the number of tumors per mouse and the percentage of

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mice with tumors, but it also reduced tumor size in forestomach and intestine.[28] Thus, curcumin is now regarded as a promising chemopreventive agent for human cancers.

This study was conducted to examine whether consumption of curcumin has a preventive effect on esophageal carcinogenesis.

MATERIALS AND METHODS

Animals, diets, and carcinogen Male F344 rats, 4 weeks old, were purchased from Japan SLC, Inc. (Hamamatsu). After 1 week of quarantine, rats were transferred to the holding room under controlled conditions at 23±2°C (standard deviation, SD), 50±10% humidity, and a 12-h light/dark cycle and randomized into experimental and control groups. They were housed in wire cages (3 or 4 rats/cage). Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as the basal diet for the experiment. NMBA was obtained from Ash Stevens Inc. (Detroit, MI). Curcumin (CI 75 300 >96% pure) was purchased from Nacalai Tesque, Inc., Kyoto. The diets were stored in a cold room (4°C) and were freely available to the animals.

Experimental procedure A total of 114 rats were randomized into 5 groups as shown in the figure. At 7 weeks of age, rats in groups 1 through 3 received 15 s.c. injections of NMBA over the course of 5 weeks (1.0 mg/kg/dose, 3 doses/week). Rats in group 5 were treated with vehicle, 20% dimethyl sulfoxide (DMSO) in distilled water. All doses of the vehicle or NMBA were administered at a constant volume of 1 ml/kg body weight. Rats in group 2 were given the diet containing 500 ppm curcumin for 7 weeks (starting at 6 weeks of age until 1 week after the last dosing of the carcinogen). They were then switched to the basal diet and maintained on that until the termination. Starting 1 week after the final injection of NMBA, rats in group 3 were fed the diet mixed with cur-

cumin, and the feeding was continued until the termination. Rats in group 4 were fed the diet containing curcumin during the experiment. Rats in group 5 were given the basal diet and tap water throughout the experiment and served as a control.

All rats were carefully inspected daily, and consumption of the experimental diets mixed with test compound was recorded to estimate intake of the agent. The experiment was terminated at 40 weeks after the start, and all animals were killed for evaluation of the frequencies of preneoplastic and neoplastic lesions in the esophagus. At necropsy, all organs, especially esophagus and tongue, were grossly examined. The entire length of the esophagus was removed, opened longitudinally, placed flat on a piece of filter paper with the epithelium exposed and fixed in 10% buffered formalin. Esophageal tumors (≥1 mm in diameter) were quantified, sized and mapped under a dissecting microscope and millimeter ruler. Each esophagus was divided again longitudinally. All other organs were fixed with 10% formalin, embedded in paraffin blocks and processed routinely for histopathological examination.

Determination of proliferative activity in non-lesional esophageal epithelium in terms of 5-bromo-2′-deoxyuridine (BrdU) labeling index To assess the proliferative activity of squamous epithelium of the esophagus, the BrdU-labeling index of five animals from each group was quantified according to the methods described previously.[30] For measurement of BrdU-incorporated nuclei, five animals from each group were given an i.p. injection of 50 mg/kg body weight BrdU (Sigma Chem. Co., Ltd., St Louis, MO) 1 h prior to killing. The esophagi were removed, examined macroscopically, fixed in 10% buffered formalin, and embedded in paraffin wax, and two serial sections (3 μm in thickness) were made. One section was used for immunohistochemical staining analysis with a kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The labeling indices of BrdU were measured by counting labeled nuclei in the esophagus epithelium (400–1000 cells/rat) at 40-fold magnification under a light microscope and were expressed as a percentage. These proliferative measurements were done on 10 selected well-oriented areas of the middle third of the esophagus of each rat without knowledge of the experimental groups. The remaining section was stained with HE for histopathological diagnosis. In this study, dysplasia was categorized as mild, moderate or severe, according to the depth of atypical cells.

Statistical analysis Statistical analysis of the incidence of lesions was performed using Fisher's exact probability test or the χ^2 test, and the data on body weight, liver weight and BrdU-labeling index were compared by means of Student's t test.
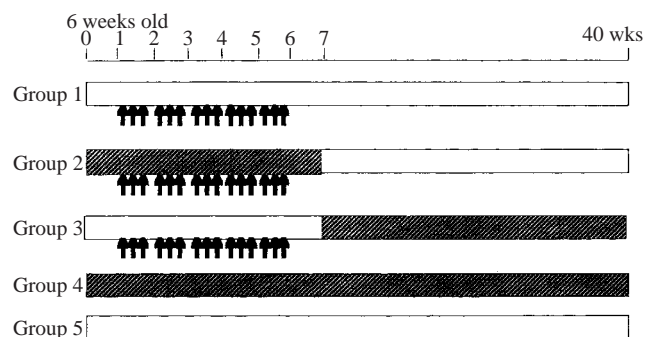

Fig. 1. Experimental protocol. ↑ NMBA, 0.5 mg/kg b.w., 3 days (Monday, Wednesday, Friday)/week basis, NMBA was dissolved in 20% DMSO. A total of 114 male F344 rats were used. Experimental period, 40 weeks. ▨ 500 ppm curcumin in diet.

RESULTS

Body and liver weights are shown in Table I. There were no marked differences in dietary intake among the groups. Mean body weight in group 4 was slightly smaller than that in group 5 (control group) ($P<0.05$). The body weight in group 3 (given curcumin after the NMBA treatment) was smaller than that in group 1 (given NMBA alone) ($P<0.05$). There were no significant differences in liver weights among all groups.

The incidence and multiplicity (number of tumors/rat) of esophageal neoplasms are indicated in Table II. Those of preneoplastic lesions are also shown in Table III. Esophageal neoplasms and preneoplastic lesions were only found in groups 1–3 exposed to NMBA. The esophageal tumors were solitary or multiple lesions of variable size. They were randomly distributed along the entire length of the esophagus without predilection for the upper, lower or middle regions. Histologically, these tumors were squamous cell papillomas, except for a well-differentiated squamous cell carcinoma in a rat of group 1. In group 1 (MNBA alone), the incidence of total neoplasms was 66.7% (papilloma, 62.5%; carcinoma, 4.17%). The incidences of esophageal papilloma in groups 2 and 3 were 39.3% and 33.3%, respectively. The incidences of total esophageal neoplasms in groups 2 and 3 were significantly

lower than that in group 1 ($P<0.05$). Multiplicities of total neoplasms and papilloma in group 3 (0.36 ± 0.56; 0.36 ± 0.56) were significantly smaller than those in group 1 (0.83 ± 0.70; 0.79 ± 0.72) ($P<0.05$).

Preneoplastic lesions (hyperplasia and dysplasia) were also noted in groups 1–3. There were no differences among groups 1–3 in the incidence and multiplicity of hyperplasia. However, those of moderate, severe dysplasia in groups 2 (57.1%, 0.61 ± 0.57; 40%, 0.29 ± 0.46) and 3

Table I. Body and Liver Weights

Group no.	Treatment	No. of rats examined	Body weight	Liver weight
1	NMBA alone	24	327±28	12.0±1.7
2	NMBA+curcumin (500 ppm)	28	310±42	11.4±1.9
3	NMBA→curcumin (500 ppm)	30	306±32[a]	12±2.2
4	500 ppm curcumin	16	301±33[b]	10.8±0.68
5	No treatment	0	336±27	11.4±1.1

a) Significantly different from group 1 by Student's *t* test ($P<0.05$).
b) Significantly different from group 5 by Student's *t* test ($P<0.05$).

Table II. Effect of Curcumin on Development of Esophageal Tumors in Male F344 Rats

Group no.	Treatment	No. of rats examined	Incidence (%) and multiplicity (no. of tumors/rat, mean±SD) of esophageal tumors		
			Total	Squamous cell papilloma	Squamous cell carcinoma
1	NMBA alone	24	66.7% 0.83±0.70	62.5% 0.79±0.72	4.17%
2	NMBA+curcumin (500 ppm)	28	39.3%[a] 0.46±0.64	39.3% 0.46±0.64	0%
3	NMBA→curcumin (500 ppm)	30	33.3%[a] 0.36±0.56[b]	33.3%[a] 0.36±0.56[c]	0%
4	500 ppm curcumin	16	0%	0%	0%
5	No treatment	7	0%	0%	0%

a) Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
b, c) Significantly different from group 1 by Student's *t* test (*b*) $P<0.01$, *c*) $P<0.05$).

Table III. Incidence and Multiplicity of Preneoplastic Lesions in Rats Given NMBA with or without Curcumin

Group no.	Treatment	Incidence (%) and multiplicity (no./rat, mean±SD) of hyperplasia (HP)/rat	Incidence (%) and multiplicity (no. of rat, mean±SD) of dysplasia (DYS)/rat					
			Mild DYS		Moderate DYS		Severe DYS	
1	NMBA alone	100% 2.625±1.17	100%	2.16±1.00	100%	1.67±0.70	70.8%	0.92±0.72
2	NMBA+curcumin (500 ppm)	96.4% 2.5±1.23	100%	1.46±0.69[e]	57.1%[a]	0.61±0.57[f]	40%[b]	0.29±0.46[g]
3	NMBA→curcumin (500 ppm)	100% 2.8±0.96	100%	1.83±0.46	56.7%[c]	0.67±0.66[h]	23.3%[d]	0.23±0.43[i]
4	500 ppm curcumin	0%	0%		0%		0%	
5	No treatment	0%	0%		0%		0%	

a–d) Significantly different from group 1 by Fisher's exact probability test (*a, c*) $P<0.001$, *b*) $P<0.05$, *d*) $P<0.0005$).
e–i) Significantly different from group 1 by Student's *t* test (*e*) $P<0.01$, *f*) $P<0.0000005$, *g*) $P<0.0005$, *h*) $P<0.000005$, *i*) $P<0.0001$).

Table IV. BrdU-labeling Index on Non-lesional Area of Esophagus Squamous Epithelium

Group no.	Treatment	No. of rats examined	BrdU-labeling index (%)
1	NMBA alone	5	24.0±4.5[a]
2	NMBA+curcumin (500 ppm)	5	15.2±2.5[b]
3	NMBA→curcumin (500 ppm)	5	6.7±2.5[c]
4	500 ppm curcumin	4	7.1±1.6
5	No treatment	5	6.0±2.2

a) Significantly different from group 5 by Student's *t* test ($P<0.00005$).
b) Significantly different from group 1 by Student's *t* test ($P<0.01$).
c) Significantly different from group 1 by Student's *t* test ($P<0.0001$).

(56.7%, 0.67±0.66; 23.3%, 0.23±0.43) were significantly less than those in group 1 (100%, 1.67±0.70; 70.8%, 0.92± 0.72). For mild dysplasia, there was a significant difference only between groups 1 and 2 ($P<0.01$) (Table III).

Enumeration of BrdU-labeled cells The results of morphometric analysis of BrdU-labeling indices in the non-lesional squamous epithelium of rats in each group are summarized in Table IV. The mean value of the index in group 1 was significantly larger than that in group 5 ($P<0.00005$). The indices in groups 2 and 3 were significantly smaller than that in group 1 ($P<0.01$ and $P<0.0001$, respectively).

DISCUSSION

In this study, dietary exposure to curcumin during initiation as well as post-initiation reduced the incidence and/or multiplicity of esophageal tumors and preneoplastic lesions (dysplasia). Curcumin is known to be a modifier of phase I enzymes such as P450 1A1/1A2, 2B1/2B2, 2E1. Curcumin could act as an anticarcinogen because of its strong inhibitory activity towards P450 1A1/1A2.[31] Rao *et al*. suggested that the inhibition of azoxymethane (AOM)-induced colon carcinogenesis by curcumin was mediated through modulation of P450 2E1-dependent AOM metabolism by curcumin.[32] It is reported that NMBA requires bioactivation by an esophageal cytochrome P450 isozyme.[33, 34] Thus, the effect of curcumin on the metabolizing enzymes may be important for the blocking effect on NMBA-induced esophageal carcinogenesis, especially in the initiation phase.

Curcumin has a strong free radical scavenging-activity.[35, 36] Shin and Lin[37] have reported that curcumin inhibited TPA-induced lipid peroxidation and 8-hydroxydeoxyguanosine formation in mouse fibroblasts. Such antioxidative properties may be involved in the chemopreventive effect on esophageal carcinogenesis. It is also known that curcumin inhibits benzo(*a*)pyrene-induced DNA adduct formation. It is possible that curcumin inhibits adduct formation by NMBA in the esophagus.

Curcumin has various pharmacological effects, including inhibition of the arachidonic acid pathway (lipoxygenase and cyclooxygenase) and ornithine decarboxylase (ODC) activity.[38–40] The products and intermediates (hydroxyeicosatetraenoic acids and prostaglandin E_2) of the lipoxygenase and cyclooxygenase pathways have been implicated in tumor promotion.[41, 42] Accordingly, such pharmacological effects of curcumin may also be related to the suppressing effect of curcumin on esophageal carcinogenesis.

In this study, dietary exposure to curcumin during the initiation or post initiation phase reduced BrdU-labeling indices in the non-lesional esophageal epithelium. It has been suggested that curcumin suppressed NMBA-induced hyperproliferation of the cells in the esophagus.[43] In esophageal carcinogenesis, as in carcinogenesis in other organs, cell proliferation is suggested to be relevant. For instance, dietary zinc deficiency increases BrdU-labeling indices and NMBA-induced esophageal tumor incidence in mice.[44, 45] In our previous study, diosmin and hesperidine reduced BrdU-labeling indices and development of esophageal neoplasms in MNAN-induced esophageal carcinogenesis in rats.[19] In rat esophageal tumors induced by NMBA, a significant elevation of cyclin D1 levels together with proliferating cell nuclear antigen (PCNA) indices was observed.[46] Control of cell proliferation is, thus, regarded as an important mechanism of the chemopreventive function of curcumin in esophageal carcinogenesis.

In conclusion, we have demonstrated that dietary feeding of curcumin during the initiation as well as post-initiation phase inhibits tumor development and cell proliferation in the target epithelium in the rat model with NMBA. Curcumin, which is widely used as a spice and a coloring agent, is suggested to be a candidate chemopreventive agent against human esophageal cancers.

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