Inhibitory effects of epigallocatechin-3-gallate on N-nitrosomethylbenzylamine-induced esophageal tumorigenesis in F344 rats

ZHI GANG LI, YUTAKA SHIMADA, FUMIAKI SATO, MASATO MAEDA, ATSUSHI ITAMI, JUNICHI KAGANOI, IZUMI KOMOTO, ATSUSHI KAWABE and MASAYUKI IMAMURA

Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, 54-Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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Abstract. The present study was conducted to assess the inhibitory effects of EGCG (epigallocatechin-3-gallate) on NMBA-induced rat esophageal tumorigenesis and to seek the potential mechanisms. In experiment I, 81 F344 rats were randomly divided into seven experimental groups according to the different regiments of NMBA 1 mg/kg subcutaneously (s.c.) and EGCG 4 mg/kg or 10 mg/kg orally or intraperitoneally (i.p.). The experiment was terminated at 24 weeks. In experiment II, 48 rats were allocated into two groups, each group contained 24 rats, in which the rats were injected with NMBA 1 mg/kg only or a combination of NMBA 1 mg/kg and EGCG 4 mg/kg i.p. Six rats from each group were sacrificed at the 12th, 16th, 20th and 24th week, respectively. The expression of cyclin D1 and cyclooxygenases (COX-2 and COX-1) was detected using semiquantitative RT-PCR, and the production of prostaglandin E_2 (PGE_2) was measured by ELISA. In the groups which were treated with EGCG at a dose of 4 mg/kg i.p., or 10 mg/kg both orally and i.p., the mean number of tumors per rat was significantly reduced to 48, 56 and 61%, respectively ($p<0.05$). The incidence rate of esophageal carcinomas in the rats that were treated with EGCG 4 mg/kg i.p., was significantly lower than that in the rats which only received NMBA 1 mg/kg ($p<0.05$). The expression of cyclin D1 and COX-2, and the levels of PGE_2 were also decreased by EGCG treatment. These results indicated that EGCG significantly inhibits the NMBA-induced rat esophageal carcinogenesis and it inhibitory effects may partly target cyclin D1 and COX-2 expression, and PGE_2 production.

Correspondence to: Dr Yutaka Shimada, Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, 54-Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan
E-mail: shimada@kuhp.kyoto-u.ac.jp

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Introduction

Esophageal carcinoma is the eighth most common cancer in the world (1). Higher incidence rates are found in China, Central Asia, India, and South Africa (2). Esophageal cancer is sensitive to dietary influences. Epidemiological data showed that the ingestion of salt-cured, salt-pickled, and moldy foods increased the risk of esophageal cancer (3). It has been determined that nitrosamines are probable etiologic factors in the high-incidence areas in China (4). N-nitrosomethylbenzylamine (NMBA) has been identified to be a class of nitrosamines and was well documented as a complete carcinogen responsible for initiation of esophageal SCC in rat (5). It also has been observed that the changes seen with NMBA treatment of human fetal esophageal epithelium in culture were similar to the alterations of oncogenes and tumor suppressor genes in clinical tumors of esophagus (6).

On the other hand, the high intake of fruits and vegetable was reported to be able to decrease the risk of esophageal cancer (7), and the drinking of tea was strongly suggested to be associated with a lower incidence of human cancers, including esophageal cancer (8). Furthermore, the protective and inhibitory effects of tea against tumorigenesis were already observed in a variety of experimental animals, including skin (9), lung (10), stomach (11), colon and rectum (12). Recently, other researchers have also demonstrated that NMBA-induced esophageal tumorigenesis in rats was significantly inhibited by green tea or green tea polyphenols (GTP) and green tea extract (GTE) (13). Based on these studies, green tea may be a natural preventive agent of esophageal carcinogenesis. However, the effective compounds in green tea should be further identified, especially the involved potential mechanisms should be clarified before they are used in treatment of esophageal cancer.

EGCG is a major catechin polyphenol in green tea, which often is used to investigate the putative cancer-preventive effects of green tea on carcinogenesis of several organs by many laboratory studies, including skin (14), lung (15) and stomach (16), however still unknown on esophageal carcinogenesis. In this study, we examined the effects of EGCG on NMBA-induced rat esophageal tumorigenesis under different dosages and different pathway of administration.

Because human esophageal carcinogenesis is a multifactorial, multistep process, many genetic factors are involved (17). The factors targeted by EGCG should be clarified. Molecular studies of human esophageal cancer have suggested that cyclin D1, an oncogene which has a crucial role in G1 progression of the cell cycle, has been observed to be amplified in carcinomas of some organs, including human esophageal carcinomas (18,19), and in the animal models. The expression of cyclin D1 was up-regulated by NMBA, and was associated the esophageal tumorigenesis (20). In other studies of human esophageal cancer, cyclooxygenase-2 (COX-2), and elevation of COX-2-mediated prostaglandin E_2 (PGE_2) synthesis were demonstrated to be closely associated with esophageal carcinogenesis (21-23). COX-2 is the enzyme of limiting rate of PGE_2 production (24), therefore, a strong correlation may exist between PGE_2 levels and COX-2 levels. Zimmermann *et al* also reported that, in esophageal cancer cells producing a large amount of PGE_2, COX-2 inhibitors induced apoptotic cell death and reduced proliferation through the inhibition of prostaglandin synthesis (25). In the animal experiment, the expression of COX-2 and the production of COX-2-mediated PGE_2 were up-regulated by NMBA, while the levels of PGE_2 were significantly decreased by JTE-522, a selective COX-2 inhibitor (26). Thus, excessively synthesized PGE_2 mediated by overexpression of COX-2 are believed to play an important role in esophageal carcinogenesis. Based on the above understanding, in this study, we investigated whether and how the inhibitory effects of EGCG on NMBA-induced esophageal tumorigenesis are associated with the expression of cyclin D1, COX-2 and COX-1, and the production of PGE_2.

Materials and methods

Animals. A total of 129 F344 male rats (10 weeks of age) were purchased from Japan SLC, Inc. (Haruno, Shizuoka, Japan). The animals were raised 3 per cage and kept in our animal center for 2 weeks before use. Rats were given water and food freely and kept on a 12-h light/12-h dark cycle. Throughout the whole period of the experiment, each rat was weighed once a week. The animals were handled in accordance with the guiding principles in the care and use of animals approved by the Kyoto University Physiological Society. Protocol number was #Med Kyo 01041.

Chemical agents. NMBA was purchased from NARD Co. Ltd. (Osaka, Japan). Purified EGCG (95% pure) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Reagents were prepared immediately before use. NMBA and EGCG were dissolved in sterilized saline solution (0.9% NaCl solution). NMBA was given at a dose of 1 mg/kg body weight, subcutaneously (s.c.); EGCG was administered orally or intraperitoneally (i.p.) at two doses of 4 and 10 mg/kg body weight.

Experimental design. In experiment I, 81 F344 rats were randomly divided into seven experimental groups according to the different regiments. It consisted of the following groups: group 1, 10 rats were injected with saline 1 ml/kg s.c.; group 2, 10 rats were administered orally with EGCG 4 mg/kg alone; group 3, 15 rats were only injected with NMBA 1 mg/kg s.c.; group 4, 15 rats received NMBA 1 mg/kg plus EGCG 4 mg/kg orally; group 5, 15 rats received NMBA 1 mg/kg plus EGCG 10 mg/kg orally; group 6, 8 rats received NMBA 1 mg/kg plus EGCG 4 mg/kg i.p.; group 7, 8 rats received NMBA 1 mg/kg plus EGCG 10 mg/kg i.p. Group 1 and 2 were negative controls, group 3 was positive control. The administration of drugs was scheduled as below: five times weekly for 5 weeks followed by the same dose once per week for another 10 weeks, and then weighted once per week until to the 24th week (the end of this experiment) (Fig. 1).

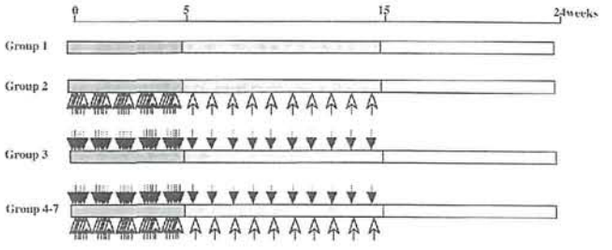

Figure 1. Schematic representation of the experimental protocol. The hatched box indicates injection of saline 1 ml/kg body weight (group 1). The downward arrows indicate injection of NMBA 1 mg/kg body weight (group 3). The upward arrows indicate injection of EGCG at 4 mg/kg body weight (groups 2, 4 and 6), or 10 mg/kg body weight (groups 5 and 7).

In experiment II, 48 rats were allocated into two groups, each group contained 24 rats, in which the rats were injected with NMBA 1 mg/kg only or a combination of NMBA 1 mg/kg and EGCG 4 mg/kg i.p. The administration of drugs was the same as in the above preliminary experiment. Six rats from each group were sacrificed at the 12th, 16th, 20th and 24th week, respectively, after the drug administration began.

At the end of 24 weeks, all surviving animals were sacrificed using diethyl ether in a glass container according to institutional protocols. Esophagi were excised, the total number of tumors >2 mm in diameter was counted and the volume of tumors on each rat esophagus was calculated as length x width x height x 0.52 (27). The tumors were inflated, fixed in 10% phosphate-buffer formalin solution, and routinely embedded in paraffin for H&E staining, and the largest tumors from each group were collected and cut in half, one part was immediately frozen in liquid nitrogen for semi-quantitative RT-PCR assay, the other for PGE_2 analysis.

Pathological diagnosis. Pathological diagnosis of the tumors was determined by a skilled pathologist who did not know about the background of drug administration. According to Xiang *et al* description (28), the histopathological features of NMBA-induced tumors were classified into papilloma, marked endophytic growth of the epithelium; papilloma with atypia, marked precancerous changes; and carcinoma, marked malignant changes of basal cells, malignant changes of papilloma, carcinoma *in situ*, and early infiltrative carcinoma. Representative figures were shown in our previous report (29).

Semi-quantitative RT-PCR for expressions of cyclin D1, COX-2 and COX-1. Total RNA was extracted from non-tumor tissues

Table I. The change of body weight of rats treated.

Groups	Treatment	No. of rats	Body weight (g)	
			Initial	Final
1	Saline 1 ml/kg (s.c.)	10	244±3.5	422±12.2
2	EGCG 4 mg/kg (i.p.)	10	245±5.8	402±12.2
3	NMBA 1 mg/kg (s.c.)	15	245±7.4	394±24.8
4	NMBA 1 mg/kg + EGCG 4 mg/kg (p.o.)	8	245±6.3	387±50.4
5	NMBA 1 mg/kg + EGCG 10 mg/kg (p.o.)	8	245±11.4	381±38.2
6	NMBA 1 mg/kg + EGCG 4 mg/kg (i.p.)	14	243±6.2	371±39.0[a]
7	NMBA 1 mg/kg + EGCG 10 mg/kg (i.p.)	15	246±5.8	370±40.8[a]

[a]$p<0.05$ (Tukey-Kramer test). Group 6 and 7 versus group 1.

(adjacent apparently normal tissues) and tumor tissues of esophagi and cDNA was synthesized from 1 μg total RNA using a First Strand cDNA Synthesis Kit (Pharmacia). The primers for cyclin D1 were designed as: 5'-TGACAACTCT ATCCGCCCCGA-3' (sense) and 5'-GAAAGTGCGTTGT GCGGTAGC-3' (antisense). The primers for COX-2 were designed as: 5'-GGTCTGGTGCCGGGTCTGATGATG-3' (sense) and 5'-GGCCTTTCAAGGAGAATGGAGC-3' (antisense). The primers for COX-1 were designed as: 5'-CTCCAACCTACAACACAGCA-3' (sense) and 5'-ACCG TAGTCCACCAGCATAG-3' (antisense). Aliquots of 1 μl the reserve-transcribed cDNA samples were added to 50 μl of a reaction mixture that contained: 5 μl of 10X buffer, 10 μl of 2 mM dNTP mix, 6 μl of 2.5 mM $MgCl_2$, 0.4 μl of Ex Taq polymerase (Takara), and 1 μl of each primer. Samples were co-amplified for 30 cycles (for cyclin D1, COX-2 and COX-1): denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec (for cyclin D1); 65°C for 20 sec (for COX-2); 56°C for 20 sec (for COX-1), extension at 72°C for 30 sec, and final extension at 72°C for 10 min. Cyclin D1, COX-2 and COX-1 generate 314, 702 and 875 bp PCR products, respectively. Negative control of cyclin D1, COX-2 and COX-1 RT-PCR, which contained no reverse transcriptase, showed no PCR products. GAPDH, constitutively expressed gene, was used as an internal control, generating a 230 bp PCR product. The primers for GAPDH were 5'-AGATGGTGAAGGTCGGTGTG-3' (sense) and 5'-CTGGAAGATGGTGATGGGTT-3' (antisense). The PCR condition for GAPDH was identical to those for cyclin D1 or COX-2 or COX-1. The 10 μl of PCR products were applied to an 8% polyacrylamind gel for cyclin D1 or a 2% agarose gel for COX-2 and COX-1, and then were selectrophoresed. The gel was then stained with ethidium bromide and illuminated on a UV table. Electrophoresed PCR products were scanned using a computer densitometer (NIH image software package) to determine the density of the bands, and the relative value of the cyclin D1 or COX-2 or COX-1 band to GAPDH was calculated in each sample (30).

Measurement of PGE_2 production. The levels of PGE_2 in non-tumor tissues and tumor tissue were determined by ELISA as described previously (31). In brief, to determine basal PGE_2 levels, frozen samples were homogenized on ice in 0.3 ml of 0.1 M Tris-HCl buffer containing 5.6 μM indomethacin (pH 7.4) with a microtube pestel and vortexed thoroughly for 2 min. The quantity of PGE_2 in supernatants was immediately determined with the PGE_2 Monoclonnal Enzyme Immunoassay Kit (Caymen Chemical), according to the manufacturer's instructions. Results were measured using a Dynatech MR5000 microplate reader and normalized to μg of protein.

Statistical analysis. The comparisons of incidence of esophageal tumors in rats between the group which was injected with NMBA and the group which received a combination of NMBA and EGCG treatment were made by means of Chi-square test. Body weights, tumor multiplicity, the expression of cyclin D1, COX-2 and COX-1, and PGE_2 production expressed as the means ± SD. Comparisons between groups were made by means of Tukey-Kramer test. Software used in this study was StatView version 5.0 (SAS. Co.). Differences were considered statistically significant at $p<0.05$.

Results

General observation. In experiment I, 1 rat in group 5 died at the 13th week due to peritonitis; the others survived to the termination of this experiment. We observed the change of the body weight of the rats at critical points (initial and final weeks), and summarized them in Table I. The values of the average body weights of rats at initial week were not significantly different among the groups. The final average body weights of rats in NMBA-untreated groups (groups 1 and 2) were similar. The average body weights of rats which received NMBA (groups 3-7) were reduced in comparison to the saline-treated animals (group 1). Of these, the body weights of rats in groups 6 and 7, which were injected with EGCG i.p., were significantly reduced ($p<0.05$).

Effects of EGCG on multiplicity. In experiment I, the data for the effects of EGCG on NMBA-induced esophageal tumor multiplicity (number and size of tumors in each rat) in each

Table II. The inhibitory effects of EGCG on NMBA-induced esophageal tumorigenesis in rats.

Groups	Treatment	No. of rats	Mean number of tumors per rat[a] (%)	Mean volume of tumors (mm^3)[a] (%)
1	Saline 1 ml/kg (s.c.)	10	0	0
2	EGCG 4 mg/kg (i.p.)	10	0	0
3	NMBA 1 mg/kg (s.c.)	15	6.1±3.2 (100)	32.3±69.7 (100)
4	NMBA 1 mg/kg + EGCG 4 mg/kg (p.o.)	8	4.1± 1.2 (67)	29.8±31.5 (92)
5	NMBA 1 mg/kg + EGCG 10 mg/kg (p.o.)	8	3.4±1.8[b] (56)	25.6±22.6 (79)
6	NMBA 1 mg/kg + EGCG 4 mg/kg (i.p.)	14	2.9±1.5[b] (48)	17.1±18.6 (53)
7	NMBA 1 mg/kg + EGCG 10 mg/kg (i.p.)	15	3.7±2.2[b] (61)	21.9±22.4 (68)

[a]The diameter of tumors >2 mm. [b]Significantly different from group 3 ($p<0.05$). Means ± SD (statistical analysis using Tukey-Kramer test).

Table III. The incidence of esophageal tumors in rats treated with NMBA and a combination of NMBA and EGCG during various stages.

Time of sacrifice	Macro-examination	Treatment		p-value
		NMBA 1 mg/kg (s.c.)	NMBA 1 mg/kg + EGCG 4 mg/kg (i.p.)	
12 weeks	Incidence (%)[a]	3/6 (50)	1/6 (17)	
	No. of total tumors[b]	3	1	
	Mean no. of tumors/rat	0.5±0.5	0.2±0.4	0.26[c]
16 weeks	Incidence (%)	4/6 (67)	4/6 (67)	
	No. of total tumors	5	4	
	Mean no. of tumors/rat	0.8±1.0	0.7±0.5	0.72[c]
20 weeks	Incidence (%)	5/6 (83)	5/6 (83)	
	No. of total tumors	17	10	
	Mean no. of tumors/rat	2.8±1.6	1.7±1.2	0.19[c]
24 weeks	Incidence (%)	6/6 (100)	6/6 (100)	
	No. of total tumors	35	17	
	Mean no. of tumors/rat	5.8±1.8	2.8±1.2	0.007[c]

[a]Percent of animals with esophageal tumors. [b]The diameter of tumors >2 mm. [c]Student's t-test (no pair).

group are summarized in Table II. Compared with group 3, EGCG showed significant inhibitory effects on the mean number of tumors per rat in group 5, 6 and 7 to 56, 48 and 61%, respectively ($p<0.05$). The reduction of the mean volumes of tumors from groups 4-7 by EGCG treatment was also observed, however, the decrease was not statistically significant.

In addition, we further compared the incidence and multiplicity of tumors at the 12th, 16th, 20th and 24th week between the group only NMBA-injected and the group NMBA plus EGCG-treated which were performed in experiment II. Six rats from each group were examined at these check-points. The data are summarized in Table III. The mean number of tumors per rat in the group which were treated with EGCG was fewer than those in the group which were injected with NMBA alone, especially, significant difference was observed at the 24th week ($p<0.05$). The results of the histopathological examination were compared between these two groups, and are summarized in Table IV. We found that NMBA-induced rat esophageal carcinogenesis was significantly inhibited by administering EGCG at the 16th, 20th and 24th week ($p=0.05$, $p=0.017$ and $p=0.017$, respectively). Our data clearly indicated that EGCG suppressed not only the formation of tumors but also carcinoma development.

The effects of EGCG on the expression of cyclin D1, COX-2 and COX-1. The performed serial sacrificial experiment II allowed us to kinetically investigate the association between

Table IV. The pathological examination of esophageal tumors in rats treated with NMBA and a combination of NMBA and EGCG during various stages.

Time of sacrifice	Micro-examination	Treatment		p-value
		NMBA 1 mg/kg (s.c.)	NMBA 1 mg/kg + EGCG 4 mg/kg (i.p.)	
12 weeks	No. of total tumors[a]	3	1	
	Papilloma (%)	3 (100)	1 (100)	
	Papilloma with atypia	0	0	
	Carcinoma	0	0	0.17[b]
16 weeks	No. of total tumors	5	4	
	Papilloma (%)	2 (40)	3 (75)	
	Papilloma with atypia (%)	2 (40)	1 (25)	
	Carcinoma (%)	1 (20)	0	0.05[c]
20 weeks	No. of total tumors	17	10	
	Papilloma (%)	3 (18)	5 (50)	
	Papilloma with atypia (%)	9 (53)	4 (40)	
	Carcinoma (%)	5 (29)	1 (10)	0.017[c]
24 weeks	No. of total tumors	35	17	
	Papilloma (%)	3 (9)	6 (35)	
	Papilloma with atypia (%)	19 (54)	8 (47)	
	Carcinoma (%)	13 (37)	3 (18)	0.017[c]

[a]The diameter of tumors >2 mm. [b]Fisher test. [c]Chi-square test.

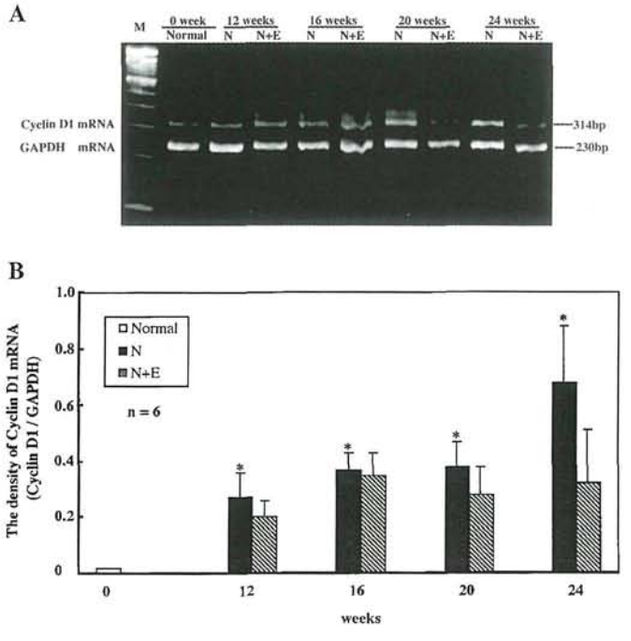

Figure 2. Semi-quantitative RT-PCR assay for the expression of cyclin D1 mRNA in non-tumor tissues of the rat esophagi at different stages of weeks 12, 16, 20 and 24. (A) shows a typical gel, whereas (B) shows the quantification of expression of cyclin D1. N, NMBA 1 mg/kg; N+E, NMBA 1 mg/kg plus EGCG 4 mg/kg. GAPDH was used as the internal standard in normal esophageal mucosa and non-tumor tissues. PCR product sizes: cyclin D1, 413 bp; GAPDH, 230 bp. M, 100 bp molecular marker. The relative value of cyclin D1 band to GAPDH was calculated for each tissue sample. The values represent the means ± SD of data from 6 rats. Bars, SD. *p<0.05 versus control (normal esophageal mucosa).

NMBA-induced rat esophageal tumorigenesis and the expression of cyclin D1, COX-2 and COX-1, which were detected by semi-quantitative RT-PCR, at different stages of weeks 12, 16, 20 and 24. The effects of EGCG on the expression of these factors were also investigated. First, we observed the alteration of cyclin D1, COX-2 and COX-1 expression in the non-tumor tissues of the rat esophagi from the 6 rats of each group. It was observed that the expression of cyclin D1 was slightly present in normal mucosa of esophagi of the rats which were untreated, and then was significantly increased by administering NMBA from weeks 12 to 24. The up-regulated cyclin D1 mRNA expression was decreased by EGCG treatment at each check-point, however, this reduction was not statistically significant (Fig. 2). The expression of COX-2 was not present in normal mucosa of esophagus, but was induced by administering NMBA, and was largely elevated at the 24th week. The increased levels of COX-2 mRNA was significantly decreased by EGCG treatment at the 24th week ($p<0.05$) (Fig. 3). The expression of COX-1 was undetectable at any check-point (data not shown). Second, we examined the levels of cyclin D1, COX-2 and COX-1 mRNA in the paired esophageal samples of the non-tumor tissues and tumor tissues versus the normal esophageal mucosa. The samples were from the rats of group 1 (saline 1 ml/kg), group 3 (NMBA 1 mg/kg alone) and group 6 (NMBA 1 mg/kg plus EGCG 4 mg/kg i.p.). They were sacrificed at the 24th week in experiment I. The expression of cyclin D1 was significantly up-regulated in both non-tumor tissues and tumor tissues by administering NMBA (group 3), as compared with that in the normal mucosa (group 1). The

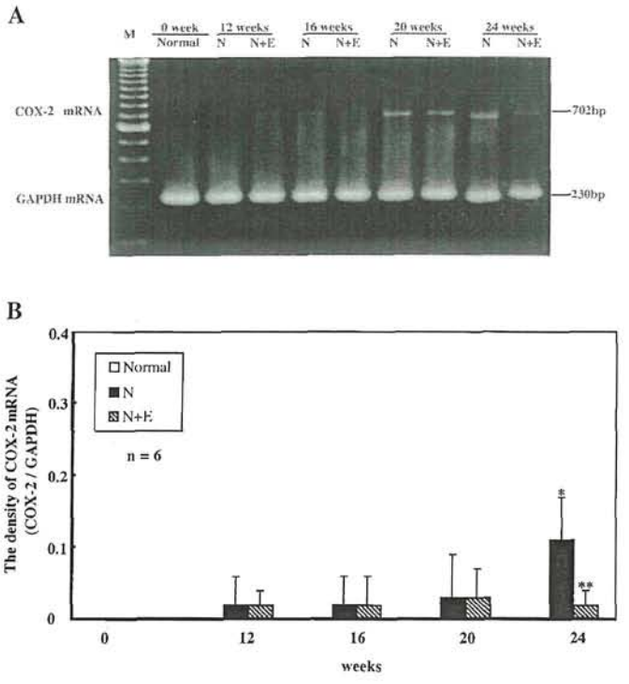

Figure 3. Semi-quantitative RT-PCR assay for the expression of COX-2 mRNA in non-tumor tissues of the rat esophagi at different stages of weeks 12, 16, 20 and 24. (A) shows a typical gel, whereas (B) shows the quantification of expression of COX-2. N, NMBA 1 mg/kg; N+E, NMBA 1 mg/kg plus EGCG 4 mg/kg. GAPDH was used as the internal standard in normal esophageal mucosa and non-tumor tissues. PCR product sizes: COX-2, 702 bp; GAPDH, 230 bp. M, 100 bp molecular marker. The relative value of COX-2 band to GAPDH was calculated for each tissue sample. The values represent the means ± SD of data from 6 rats. Bars, SD. *p<0.05 versus control (normal esophageal mucosa). **p<0.05 versus NMBA 1 mg/kg treatment alone at the same time point.

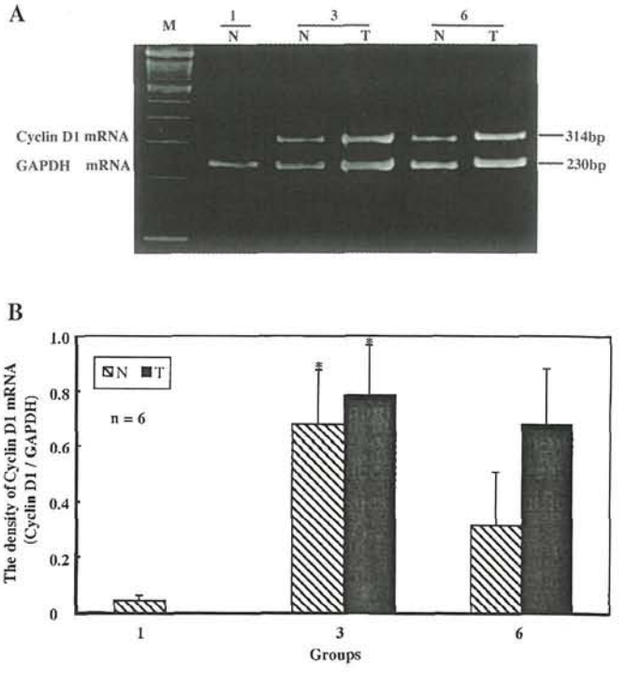

Figure 4. Semi-quantitative RT-PCR assay for the expression of cyclin D1 mRNA in non-tumor tissue and tumor tissues untreated or treated by EGCG at the 24th week. (A) shows a typical gel, whereas (B) shows the quantification of expression of cyclin D1. Results for groups 1 (saline 1 ml/kg), 3 (NMBA 1 mg/kg alone), and 6 (NMBA 1 mg/kg plus EGCG 4 mg/kg). N, non-tumor tissue; T, tumor tissue. GAPDH was used as the internal standard in normal esophageal mucosa and non-tumor tissues. PCR product sizes: cyclin D1, 413 bp; GAPDH, 230 bp. M, 100 bp molecular marker. The relative value of cyclin D1 band to GAPDH was calculated for each tissue sample. The values represent the means ± SD of data from 6 rats. Bars, SD. *p<0.05 versus group 1 (normal esophageal mucosa).

overexpression of cyclin D1 in the tumor tissues was higher than that in the non-tumor tissues. Then, the increased cyclin D1 expression in non-tumor tissues was decreased by the treatment of EGCG, but that in tumor tissue was slightly affected (group 6) (Fig. 4). The expression of COX-2 was not observed in the normal mucosa (group 1), but was significantly induced in both non-tumor tissues and tumor tissues by administering NMBA (group 3). The treatment of EGCG only significantly decreased the elevated COX-2 mRNA expression in non-tumor tissues, but slightly reduced that in tumor tissues (group 6) (Fig. 5). The expression of COX-1 was weakly present in normal mucosa and non-tumor tissues, whereas it was distinctly present in tumor tissues, which were not affected by EGCG treatment (Fig. 6).

The productions of PGE_2 measured by ELISA. To clarify the effects of EGCG on PGE_2 synthesis, we compared the levels of PGE_2 production in paired esophageal samples of the non-tumor tissues and tumor tissues. The detected samples were the other part of same ones that have been used to examine the expression of cyclin D1, COX-2 and COX-1. Fewer products of PGE_2 were present in the normal mucosa of esophagi (group 1). The production of PGE_2 was increased by about three times in non-tumor tissues and over 7-fold in tumor tissues by administering NMBA (group 3), as compared with the normal mucosa of esophagi ($p<0.05$). Compared with group 3, the levels of PGE_2 synthesis in non-tissues (group 6) were significantly reduced by EGCG treatment ($p<0.05$), while those in tumor tissues were not significantly affected by EGCG treatment (Fig. 7).

Discussion

Because current therapies have not effectively improved the survival rates among patients with cancers of the esophagus, primary prevention potentially offers far greater benefits by reducing mortality and the costs of esophageal cancer treatment. Drinking tea is thought to possibly decrease the risk of tumorigenesis of several organs (32), and EGCG has been identified as a major catechin polyphenol in green tea (14). In an experiment using [^{3}H]EGCG, significant radioactivity was observed in the target organs of EGCG (the digestive tract, liver, lung, pancreas and skin) (33). Thus, EGCG has been used to investigate the putative cancer-preventive effects of green tea on carcinogenesis of several organs by many laboratory studies. In addition, the rat model of NMBA-induced esophageal tumorigenesis has been found to be a valuable tool both for understanding the biology of this disease and for assessing the chemopreventive activity of

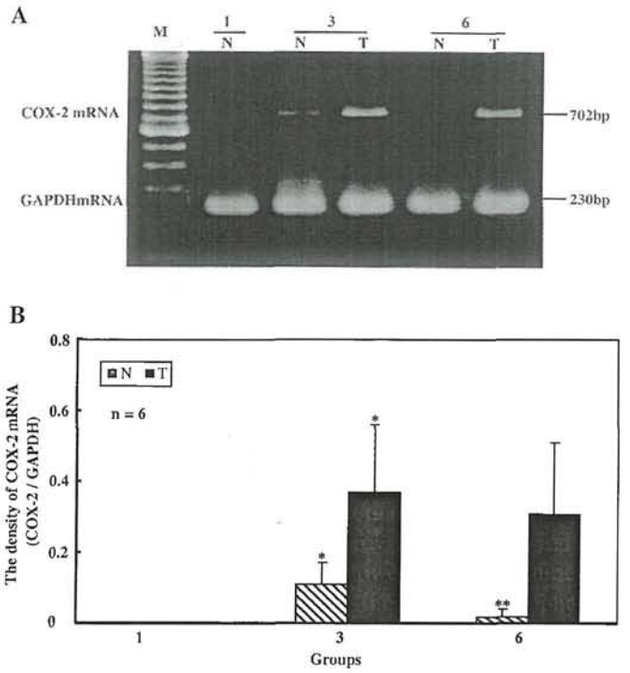

Figure 5. Semi-quantitative RT-PCR assay for the expression of COX-2 mRNA in non-tumor tissue and tumor tissues untreated or treated by EGCG at the 24th week. (A) shows a typical gel, whereas (B) shows the quantification of expression of COX-2. Results for groups 1 (saline 1 ml/kg), 3 (NMBA 1 mg/kg alone), and 6 (NMBA 1 mg/kg plus EGCG 4 mg/kg). N, non-tumor tissue; T, tumor tissue. GAPDH was used as the internal standard in normal esophageal mucosa and non-tumor tissues. PCR product sizes: COX-2, 702 bp; GAPDH, 230 bp. M, 100 bp molecular marker. The relative value of COX-2 band to GAPDH was calculated for each tissue sample. The values represent the means ± SD of data from 6 rats. Bars, SD. *p<0.05 versus group 1 (normal esophageal mucosa). **p<0.05 versus group 3 (non-tumor tissues).

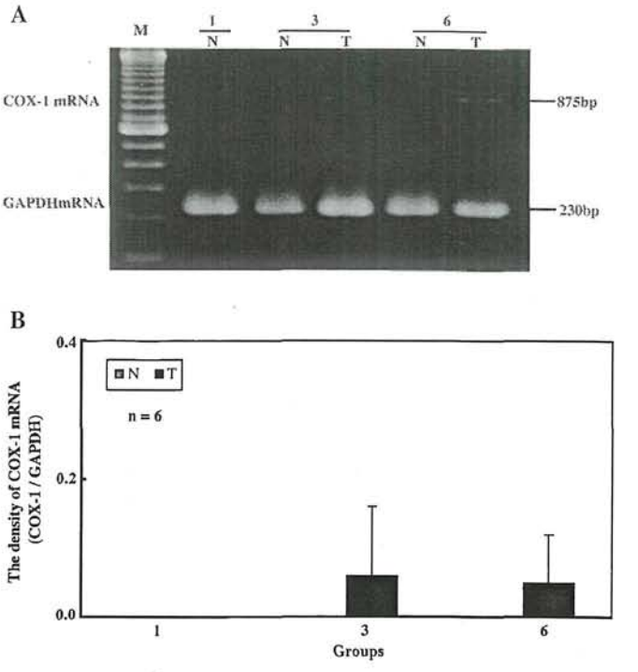

Figure 6. Semi-quantitative RT-PCR assay for the expression of COX-1 mRNA in non-tumor tissue and tumor tissues untreated or treated by EGCG at the 24th week. (A) shows a typical gel, whereas (B) shows the quantification of expression of COX-1. Results for groups 1 (saline 1 ml/kg), 3 (NMBA 1 mg/kg alone), and 6 (NMBA 1 mg/kg plus EGCG 4 mg/kg). N, non-tumor tissue; T, tumor tissue. GAPDH was used as the internal standard in normal esophageal mucosa and non-tumor tissues. PCR product sizes: COX-1, 875 bp; GAPDH, 230 bp. M, 100 bp molecular marker. The relative value of COX-2 band to GAPDH was calculated for each tissue sample. The values represent the means ± SD of data from 6 rats. Bars, SD.

several compounds, including lyophilized black raspberries (34), strawberries (35), diallyl sulfide (36).

In this study, we were interested in a comparison of the tumor multiplicity (number of tumors per rat and size of tumors) in each group in order to understand the effects of EGCG on the formation and growth of NMBA-induced tumors in rat esophagi. Our data showed that the treatment of EGCG significantly reduced the incidence of esophageal tumorigenesis in a dose-dependent manner. These results also indicated that EGCG or green tea as a chemopreventive agent against esophageal carcinogenesis should be used early and long-term. The administration EGCG at a dose 10 mg/kg (orally) or 4 mg/kg (i.p.) was thought to be an appropriate dosage. In contrast, when the dosage of EGCG was 10 mg/kg (i.p.), the toxic reaction was observed. It has been identified that a cup of green tea (2.5 g of dried green tea leaves brewed in 200 ml of water) may contain 142 mg of EGCG (37). Thus, our study provided evidence that drinking 10 cups of green tea daily may prevent carcinogenesis in the human body (38,39). Besides this, we also found that the development of carcinomas was significantly suppressed by EGCG at the earlier period of the formation of the tumors (from week 16).

The mechanisms of the inhibitory effects of EGCG on NMBA-induced esophageal tumorigenesis in rats is still

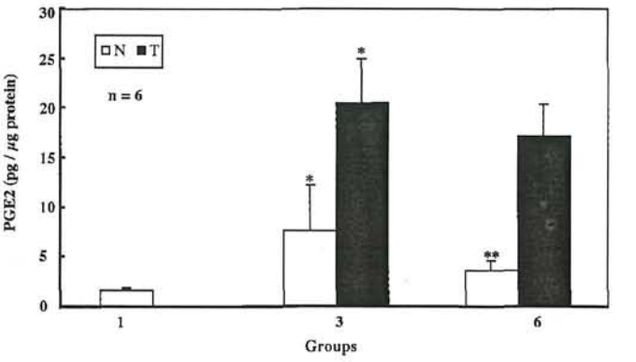

Figure 7. The productions of PGE_2 in non-tumor tissue and tumor tissues untreated or treated by EGCG at the 24th week. Results for groups 1 (normal mucosa), 3 (NMBA 1 mg/kg alone), and 6 (NMBA 1 mg/kg plus EGCG 4 mg/kg). N, non-tumor tissue; T, tumor tissue. Data are the means ± SD of pg/μg protein accumulation. Significance, determined by using Tukey-Kramer test, is expressed as follows: *p<0.05, group 3 (non-tumor tissue and tumor tissue) versus group 1 (normal mucosa); **p<0.05 group 6 (non-tumor tissue) versus group 3 (non-tumor tissue).

unclear. EGCG was shown to inhibit c-jun protein phosphorylation, and results in a decrease in the transcription factor AP-1 activity, which is important for tumor promoter-induced neoplastic transformation, and may be a

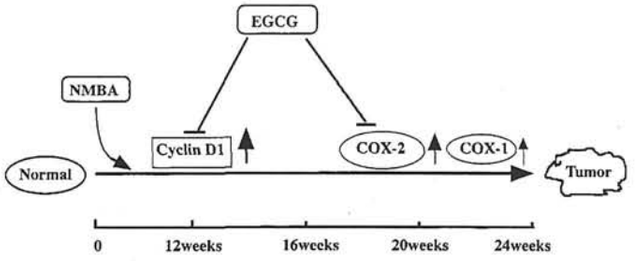

Figure 8. The potential mechanism(s) involved in the inhibitory effects of EGCG on NMBA-induced esophageal tumorigenesis in rats.

key mechanism of cell growth inhibition of Ha-ras gene transformed human bronchial epithelial cells (40). It has been reported that EGCG also resulted in down-modulation of the protein expression of cyclin D1 in human epidermoid carcinoma (A431) cells, prostate carcinoma DU145 cells and breast carcinoma cells, and led to a G0/G1-phase cell cycle arrest and apoptosis (41-43). In addition, to a lesser degree, EGCG decreased COX-2 gene expression in colon cancer cells versus normal cells (44), and inhibited one of the activities of COX-2, which mediates the synthesis of PGE_2, in mouse macrophages (45). Additional studies reported that drinking green tea could reduce the levels of PGE_2 in human skin (46) and rectal mucosa (31).

For understanding the mechanisms involved in the formation of the esophageal tumors induced by NMBA, it is very important to observe the alteration of genetic factors in the non-tumor tissues before tumorigenesis occurs. We found that the expression of cyclin D1 and COX-2 in non-tumor tissues were all induced by NMBA, while the expression of COX-1 was undetectable at any check-point. The increase of cyclin D1 expression was a stepwise change from weeks 12 to 24, while the COX-2 expression was weaker at the 12th and 16th week, then increased strongly from the 20th week. The overexpression of cyclin D1 may be an earlier event during the tumorigenesis than COX-2. We summarized the potential mechanism(s) involved in the inhibitory effects of EGCG on NMBA-induced esophageal tumorigenesis in rats as shown in Fig. 8. We noted that the expression of cyclin D1 and COX-2, and the production of COX-2-mediated PGE_2 synthesis were increased in both non-tumor tissues and tumor tissues by administering NMBA as compared with those in normal esophageal mucosa. The treatment of EGCG reduced the overexpression of cyclin D1 and significantly inhibited the expression of COX-2 and the production of PGE_2 in non-tumor tissues. Thus, the suppression effects of EGCG on the formation of NMBA-induced esophageal tumors were via inhibitory effects of EGCG on the expression of cyclin D1 and COX-2 and production of PGE_2 in non-tumor tissues. Cyclin D1 is the strongest prognostic factor of esophageal cancer (47,48), therefore, the inhibition effect on cyclin D1 is a favorable effect of EGCG. On the other hand, the expression of COX-2 was reported to be associated with the size of tumors. The large carcinomas produce more COX-2 to support their own growth (49). However, in this study, the treatment of EGCG could not significantly reduce the overexpression of COX-2 and the evaluated production of PGE_2 in tumor tissues. These findings maybe explain the reason why EGCG could not inhibit the growth of the formed tumors.

In conclusion, EGCG inhibited NMBA induced rat carcinogenesis via suppression of cyclin D1 and COX-2 expression, and PGE_2 production.

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