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Suppression of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol

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Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a natural product occurring in grapes and various other plants with medicinal properties associated with reduced cardiovascular disease and reduced cancer risk. To evaluate the possibility and potential mechanism(s) of which resveratrol inhibits *N*-nitrosomethylbenzylamine (NMBA)-induced rat esophageal tumorigenesis, 96 F344 male rats were divided into 10 groups and resveratrol (1 and 2 mg/kg) was administered orally or intraperitoneally (i.p.). In the groups in which resveratrol was administered at 2 mg/kg (orally, for 16 weeks), 1 and 2 mg/kg (i.p., for 16 weeks) and 1 mg/kg (i.p., for 20 weeks), the number of NMBA-induced esophageal tumors per rat was significantly reduced to 78, 62, 54 and 48, respectively ($P < 0.05$), and the size of maximum tumors in each group with resveratrol treatment was also significantly smaller than that in NMBA alone group ($P < 0.05$). Although the pathological examination did not indicate significantly decreased incidence of carcinomas by administering resveratrol, the tendency of carcinogensis suppression was observed ($P = 0.177$). Semi-quantitative RT–PCR and ELISA analysis demonstrated that following NMBA treatment, the expression of COX-1 mRNA was strongly present in tumor tissues, while weakly present in non-tissues; the expression of COX-2 mRNA was induced in both tumor and non-tumor tissues. The production of prostaglandin E_2 (PGE_2) increased ~6-fold, compared with the normal esophageal mucosa. The higher expression of COX-1, the up-regulated COX-2 expression and the increased levels of PGE_2 synthesis were all significantly decreased by administering resveratrol. Our study suggests that resveratrol suppressed NMBA-induced rat esophageal tumorigenesis by targeting COXs and PGE_2, and therefore may be a promising natural anti-carcinogenesis agent for the prevention and treatment of human esophageal cancer.

Introduction

Esophageal cancer is one of the most lethal carcinomas in human beings. This disease is usually only diagnosed at an advanced stage and leads to high mortality; so early prevention is thought to be a very important approach in reducing the risk of esophageal cancer. To perform this, it is necessary to understand the potential mechanism(s) of esophageal carcinogenesis. Until now, several hypotheses have been postulated, with the main hypotheses being the link between cancer and prostaglandin E_2 (PGE_2). Based on many current studies, overexpression of COX-2 and elevation of COX-2-mediated PGE_2 synthesis are thought to be associated with human esophageal carcinogenesis. COX-2 expression is up-regulated in several types of human cancers, including esophageal cancer (1). It also was observed that overexpression of COX-2 was induced by NMBA in rat esophagi and significantly associated with esophageal tumorigenesis (2). Recent studies demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs), e.g. aspirin, inhibited both COX-1 and COX-2 (3), and long-term intake of aspirin was associated with an up to 90% decreased risk of developing esophageal cancer (4). Our previous paper determined that NMBA-induced rat esophageal tumorigenesis was suppressed by JTE-522 (4-[4-cyclohexyl-2-methyloxazol-5-yl]-2-fluorobenzenesulfonamide), a selective COX-2 inhibitor, by decreasing COX-2-mediated PGE_2 without reduction of COX-2 expression (2). Thus, suppression of COX-2 expression and/or the COX-2-mediated PGE_2 may be effective targets in chemoprevention of esophageal carcinogenesis.

In the search for new cancer chemopreventive agents, many plant extracts have been evaluated for their potential to inhibit COXs. Resveratrol is a natural product occurring in grapes and various other plants with medicinal properties associated with reduced cardiovascular disease and reduced cancer risk. It was indicated that resveratrol can block the process of multi-step carcinogenesis (5). Some of this evidence has been observed in the azoxymethane-induced rat colon carcinogenesis experiment and a mouse skin cancer model (5,6). Furthermore resveratrol has shown an anti-proliferative effect on several human cancer cells (7). *In vitro*, many molecular biological properties of resveratrol involved in the transfer of cell signals, the regulation of cell cycle and the induction of apoptosis have been examined (8–10). In addition, it was also observed that resveratrol non-competitively inhibited the cyclooxygenase activity of COX-1 in a concentration-dependent manner (11), and decreased COX-2 transcription and activity in human mammary epithelial cells and colon cancer cells (12,13). Based on the above reviews, we detected the possibility that resveratrol could suppress NMBA-induced rat esophageal tumorigenesis via targeting COXs.

Abbreviations: COX, cyclooxygenase; NMBA, *N*-nitrosomethylbenzylamine; NSAIDs, non-steroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2.

Materials and methods

Animals

A total of 96 F344 male rats (10 weeks of age) were purchased from Japan SLC (Haruno, Shizuoka, Japan). Each cage contained three animals, which were 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.

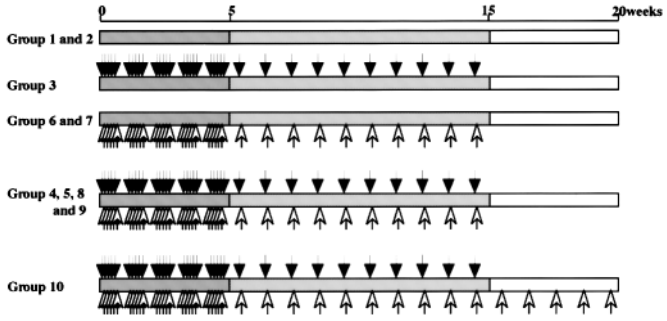

Fig. 1. Schematic representation of experimental protocol. The downward arrows indicate injection of NMBA 1 mg/kg body wt; upward arrows indicate injection of resveratrol at 1 (groups 4, 6, 8 and 10), or 2 mg/kg body wt (groups 5, 7 and 9). The hatched box indicates injection of saline 1 ml/kg body wt or DMSO 5 ml/kg body wt.

Chemical carcinogen and resveratrol

NMBA (*N*-nitrosomethylbenzylamine) was purchased from Nard (Osaka, Japan). Purified resveratrol (3,5,4′-trihydroxy-trans-stilbene; 99% pure) was from Sigma Chemical (St Louis, MO). NMBA was dissolved in saline solution, while resveratrol was first suspended into 20% solution of resveratrol/DMSO and then dissolved in saline solution. NMBA was given at a dose of 1 mg/kg, subcutaneously (s.c.). In our preliminary experiment, rats died early of higher dose of resveratrol 4 mg/kg. So, in this study, administration of resveratrol was regulated into two doses of 1 and 2 mg/kg body wt, per os (p.o.) or intraperitoneally (i.p.). To ensure that the dose was exact, orally administered resveratrol was performed by means of esophageal intubation. To reduce the stress reaction in administration, anesthesia with diethyl ether (Nakarai Tesque, Kyoto, Japan) was given.

Tumorigenesis and tumorisuppression protocol

In this experiment, 96 F344 rats were randomly divided into 10 experimental groups according to the different regiments they were submitted to. This consisted of the following groups: group 1, six rats were injected with saline 1 ml/kg (s.c.); group 2, six rats were administered with saline 1 ml/kg containing DMSO 5 ml (p.o.); group 3, 15 rats were injected with NMBA 1 mg/kg (s.c.); group 4, nine rats received NMBA 1 mg/kg (s.c.) plus resveratrol 1 ml/kg (p.o.); group 5, nine rats received NMBA 1 mg/kg (s.c.) plus resveratrol 2 mg/kg (p.o.); group 6, nine rats received resveratrol 1 mg/kg (i.p.); group 7, nine rats received resveratrol 2 mg/kg (i.p.); group 8, nine rats were administered with NMBA 1 mg/kg (s.c.) plus resveratrol 1ml/kg (i.p.); group 9, nine rats received NMBA 1 mg/kg (s.c.) plus resveratrol 2 ml/kg (i.p.); group 10, 15 rats received NMBA 1 mg/kg plus resveratrol 1 mg/kg (i.p.) (continuous administration). Groups 1 and 2 were negative controls; group 3 was a positive control.

The administration of drugs in groups 1–10 was scheduled as follows: five times weekly for 5 weeks followed by the same dose once per week for another 10 weeks, and then weighted once per week for 5 weeks (for groups 1–9); the administration of resveratrol in group 10 was performed continuously once a week until the end of this experiment (illustrated as Figure 1).

At the end of 20 weeks, all surviving animals were killed 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×width×height×0.52 (14). Forty-one tumors from group 3 which received NMBA only, and 16 tumors from group 10 which were treated by resveratrol were inflated, fixed in 10% phosphate-buffer formalin solution, and routinely embedded in paraffin for H&E staining. Four normal mucosa of rat esophagus from group 1 and the largest tumors from each group of groups 3, 8, 9 and 10 were collected and cut in half. One part was immediately frozen in liquid nitrogen for semi-quantitative RT–PCR assay, the other was for PGE_2 analysis.

Pathological diagnosis

Pathological diagnosis of tumors was determined by a skilled pathologist, who did not know about the background of the drugs administered. According to Xiang *et al*. (15), the histopathological features of NMBA-induced tumors in the rat esophagus were classified into papilloma, marked endophytic growth of the epithelium; papilloma with atypia, marked pre-cancerous changes; and carcinoma, marked malignant changes of basal cells, malignant changes of papilloma, carcinoma *in situ*, and early infiltrative carcinoma.

Semi-quantitative RT–PCR for COX-1 and COX-2 expression

Total RNA was extracted from tumor tissues and non-tumor tissues of esophagi and cDNA was synthesized from 1 mg total RNA using a First Strand cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden). The primers for COX-1 were designed as: 5′-CTCCAACCTACAACA CAGCA-3′ (sense) and 5′-ACCGT-AGTCCACCAGCATAG-3′ (antisense). The primers for COX-2 were designed as: 5′-GGTCTGGTGCCGGGTCTGATGATG-3′ (sense) and 5′-GGCCTTTC-AAGGAGAATGGAGC-3′ (antisense). Aliquots of 2 µl of the reserve-transcribed cDNA samples were added to 50 ml of a reaction mixture that contained: 5 ml of 10× buffer, 10 µl of 2 mM dNTP mix, 6 µl of 2.5 mM $MgCl_2$, 0.4 µl of Ex *Taq* polymerase (Takara, Shuzo, Japan) and 1 µl of each primer. Samples were co-amplified for 30 cycles (for COX-1) and 25 cycles (for COX-2): denaturation at 94°C for 20 s, annealing at 56°C (for COX-1); 65°C (for COX-2) for 20 s, extension at 72°C for 30 s and final extension at 72°C for 10 min. Negative control of COX-1 and COX-2 RT–PCR, which contained no reverse transcriptase, showed no PCR products. A constitutively expressed gene, GAPDH, was used as an internal control, generating a 230 bp PCR product. The primers for GAPDH were 5′-AGATGGTGAAGGTCGGT GTG-3′ (sense) and 5′-CTGGAAGATGGTGATGGGTT-3′ (antisense). The PCR condition for GAPDH was identical to those for COX-1 or COX-2. The 10 µl of PCR products were applied to a 2% agarose gel and electrophoresed. The gel was then stained with ethidium bromide and illuminated on an 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 COX-1 or COX-2 band to GAPDH was calculated in each sample (16).

Measurement of PGE_2 production

To determine basal PGE_2 levels, frozen samples were homogenized on ice in 0.5 ml of 0.1 M Tris–HCl buffer containing 5.6 mM 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, Ann Arbor, MI), according to the manufacturer's instructions. Results were measured using a Dynatech MR5000 microplate reader and normalized to microgram of protein.

Statistical analysis

Body weights, tumor multiplicity and PGE_2 production were expressed as the means ± SD. Comparisons between groups were made by means of Tukey–Kramer test. Comparisons of incidence of esophageal tumors in rats treated with NMBA and a combination of NMBA and resveratrol were made by means of χ^2 test. Software used in this study was StatView version 5.0 (SAS, Cary, NC). Differences were considered statistically significant at $P < 0.05$.

Results

General observation

Three rats in group 10 died during weeks 4, 7 and 13, respectively, due to suffocation by anesthesia; the others survived to the termination of this experiment. The body weight of rats treated with saline, NMBA and different doses of resveratrol were recorded and compared throughout the study. The body weight, determined only at critical points, initially and finally, are summarized in Table I. Average body weights of rats in NMBA-untreated groups (groups 1 and 2) were similar, and administration of resveratrol alone (groups 6 and 7) did not produce any effects of toxicity. However, the final average body weights of rats, which received NMBA treatment (groups 3–5 and 8–10) were significantly reduced in comparison to the saline-treated animals ($P < 0.05$). Of these, the reduction of body weights of resveratrol-treated rats (groups 4–5 and 8–10), were less than those of resveratrol untreated rats (group 3). The tumor incidences of groups 4, 5, 8, 9 and 10 (treated with resveratrol) were lower than group 3 (received NMBA alone). The other organs also were macroscopically examined; no tumors were found, and bleeding was not apparent in the gastrointestinal tracts of resveratrol-treated animals.

Effects of resveratrol on tumor multiplicity and histopathological examination

In this experiment, the data for the effects of resveratrol on NMBA-induced esophageal tumor multiplicity (number and

size of tumors in each rat) in each group are summarized in Table II. Compared with group 3 which has been treated by NMBA only administering resveratrol showed significant inhibitory effects on the mean number of tumors per rat in groups 5, 8, 9 and 10 to 78, 62, 54 and 48%, respectively ($P < 0.05$), and on the mean volumes of maximum tumors in groups 4, 5, 8, 9 and 10 to 47, 45, 45, 46 and 38%, respectively ($P < 0.05$). Furthermore, we also examined the histopathological changes of the developed 57 tumors, which were from groups 3 and 10, which was continuously added a low dose of resveratrol 1 mg/kg. We observed and compared the difference in the incidence of papilloma, papilloma with atypia and carcinoma between groups 3 and 10. The representation of the photomicrographs was shown in Figure 2, and the results were summarized in Table III. Although the pathological examination did not indicate significant reduction of carcinomas by administering resveratrol, the tendency in the suppression of carcinogensis was observed ($P = 0.177$).

Table I. The change of body weight of rats treated

Groups	Treatment	No. of rats	Initial weight (g)	Final weight (g)
1	Saline 1 ml/kg	6	255 ± 9.7	406 ± 24.2
2	Saline 1 ml/kg + DMSO 5 μl/kg	6	258 ± 7.2	419 ± 22.2
3	NMBA 1 mg/kg	15	247 ± 6.2	309 ± 50.4[a]
4	NMBA 1 mg/kg+ RV 1 mg/kg (p.o.)	9	249 ± 9.5	335 ± 46.2[a]
5	NMBA 1 mg/kg + RV 2 mg/kg (p.o.)	9	241 ± 8.5	341 ± 39.5[a]
6	RV 1 mg/kg (i.p.)	9	246 ± 7.1	392 ± 11.8
7	RV 2 mg/kg (i.p.)	9	250 ± 3.8	408 ± 9.5
8	NMBA 1 mg/kg + RV 1 mg/kg (i.p.)	9	246 ± 3.4	342 ± 38.6[a]
9	NMBA 1 mg/kg + RV 2 mg/kg (i.p.)	9	245 ± 5.4	354 ± 34.9[a]
10	NMBA 1 mg/kg + RV 1 mg/kg (i.p.) (continuous administration)	12	246 ± 5	344 ± 15[a]

RV, resveratrol.
[a]$P < 0.05$ (Tukey–Kramer test) versus group 1.

Effects of resveratrol on COX-1 and COX-2 mRNA levels

COX-1 and COX-2 mRNA levels were analyzed using a multiplex RT–PCR technique. We compared the levels of COX-1 and COX-2 mRNA in paired samples of esophageal non-tumor tissues (adjacent apparently normal tissues) and the tumor tissues from groups 3, 8, 9 and 10 versus normal esophageal mucosa from group 1. Data are shown in Figures 3 and 4, respectively. The expression of COX-1 was too weak to be observed in both normal mucosa and NMBA-treated non-tumor tissues, whereas a higher expression of COX-1 was observed in NMBA-induced tumor tissues, and then was reduced by administering resveratrol, in particular, continuous administration of resveratrol. On the other hand, there was nearly a 4-fold increase in amounts of COX-2 mRNA in esophageal tumors that received NMBA-treatment alone as compared with normal mucosa; the levels of COX-2 mRNA increased in non-tumor tissues compared with normal mucosa. However, up-regulated COX-2 mRNA expression was decreased by administering resveratrol at different doses (1–2 mg/kg).

Effects of resveratrol on PGE_2 synthesis

In the present study, PGE_2 levels were increased in both non-tumor tissues and tumor tissues in esophagi that received NMBA. The 6-fold increase in production of PGE_2 in tumor tissues that received NMBA alone, compared with the normal esophageal mucosa, was significant ($P < 0.05$). Moreover, the elevated levels of PGE_2 synthesis were significantly inhibited by resveratrol at each of 1 or 2 mg/kg doses, especially a 3-fold decrease was observed in the rats continuously treated with resveratrol 1 mg/kg ($P < 0.01$) (shown in Figure 5).

Discussion

Epidemiological investigations showed that esophageal cancer is sensitive to dietary influences by which the ingestion of salt-cured, salt-pickled and moldy foods increased the risk of esophageal cancer (17). These pickled vegetables and foods have been determined to contain trace amounts of nitrosamines, including NMBA (18). It has been suggested that *N*-nitrosamines (NNO) are risk factors in the development of human esophageal cancer. Exposure to NNO occurs in the higher risk

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

Groups	Treatment	No. of rats	Mean number of tumors per rat[a]	Mean volume of max. tumors (mm^3)[a]
1	Saline 1 ml/kg	6	0	0
2	Saline 1 ml/kg + DMSO 5 μl/kg	6	0	0
3	NMBA 1 mg/kg	15	6.5 ± 1.9 (100%)	55.4 ± 43.4 (100%)
4	NMBA 1 mg/kg + RV 1 ml/kg (p.o.)	9	5.5 ± 2.3 (85%)	25.8 ± 18.4[d] (47%)
5	NMBA 1 mg/kg + RV 2 ml/kg (p.o.)	9	5.1 ± 2.1[b] (78%)	25.2 ± 8.2[d] (45%)
6	RV 1 ml/kg (i.p.)	9	0	0
7	RV 2 ml/kg (i.p.)	9	0	0
8	NMBA 1 mg/kg + RV 1 ml/kg (i.p.)	9	4.0 ± 1.8[b] (62%)	25 ± 11.6[d] (45%)
9	NMBA 1 mg/kg + RV 2 ml/kg (i.p.)	9	3.5 ± 1.6[b] (54%)	25.4 ± 15.9[d] (46%)
10	NMBA 1 mg/kg + RV 1 ml/kg (i.p.) (continuous administration)	12	3.1 ± 1.6[c] (48%)	21 ± 14.4[d] (38%)

RV, resveratrol.
[a]The diameter of tumors >2 mm. Means ± SD (statistical analysis using Tukey–Kramer test).
[b]Significantly different from group 3 ($P < 0.05$).
[c]Significantly different from groups 3, 4 and 5 ($P < 0.05$).
[d]Significantly different from group 3 ($P < 0.05$).

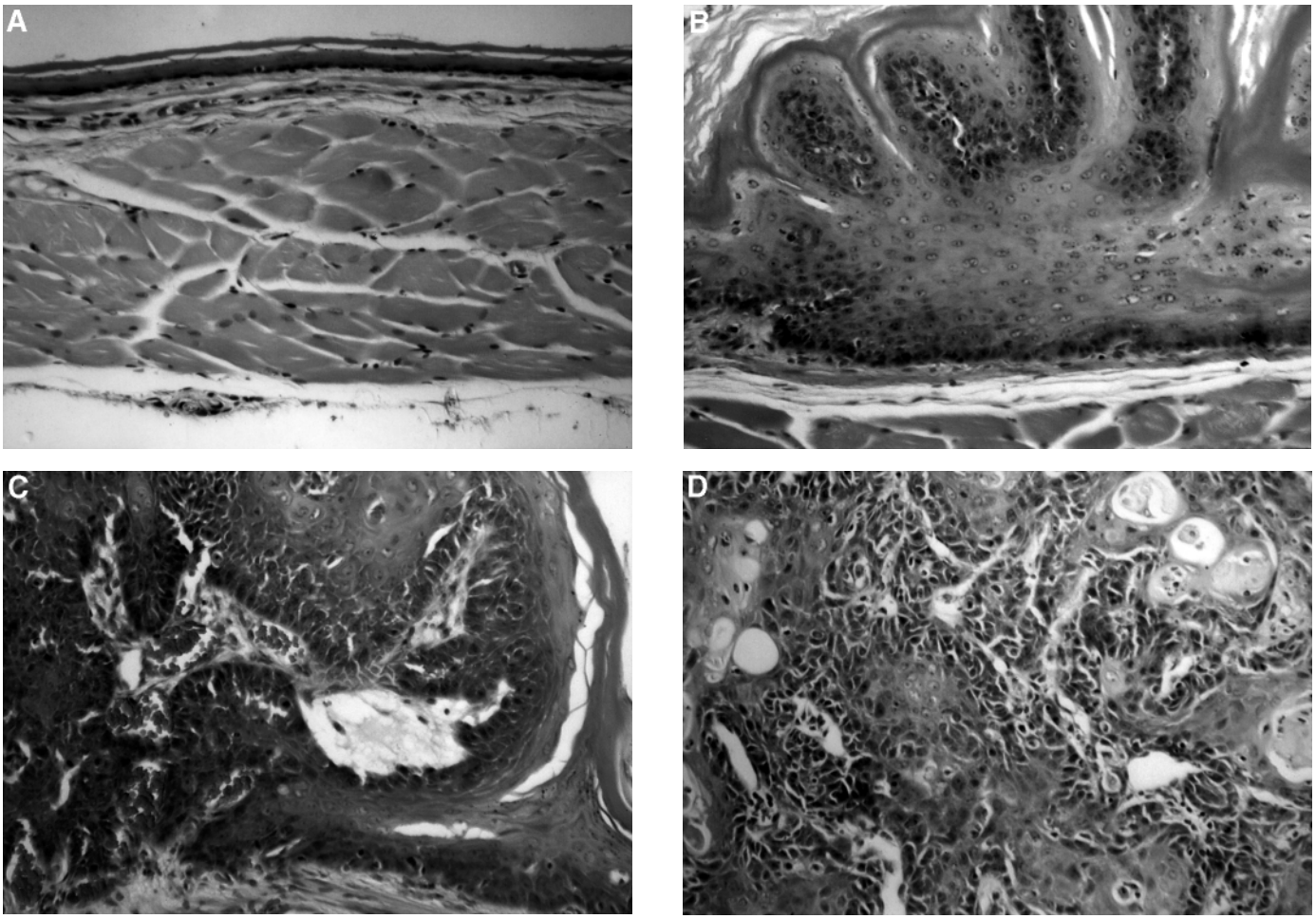

Fig. 2. Micrographs showing H&E stained histopathological representation of NMBA-induced tumorigenesis in the rat esophagus. (**A**) Photomicrograph of a normal rat esophagus (100× magnification). (**B**) Papilloma with a well-developed, peripherally branched stalk covered by acanthotic squamous epithelium (100× magnification). (**C**) Papilloma with atypia (pre-cancerous lesion with multiple keratic pearls) (100× magnification). (**D**) Early endophytic squamous cell carcinoma of the esophagus (100× magnification).

Table III. The incidence of esophageal tumors in rats treated with NMBA and a combination of NMBA and resveratrol

Groups treatment	No. of rats	Number of tumors[a]	Papilloma	Papilloma with atypia	Carcinoma
3 NMBA 1 mg/kg	5	41	17 (41%)	18 (44%)	6(15%)
10 NMBA 1 mg/kg + RV 1 mg/kg (i.p.)	6 (continuously)	16	11 (69%)	4 (25%)	1(6%)

[a]The diameter of tumors >2 mm.
RV, resveratrol; $P = 0.177$ (χ^2 test).

populations in Africa and Linxian, China (19). An animal model, NMBA-induced rat esophageal tumorigenesis, has been well documented as a complete carcinogen responsible for initiation of esophageal squamous cell carcinomas. Recently, a lot of evidence has suggested that human esophageal carcinogenesis may be associated with overexpression of COX-2, and/or elevated levels of PGE_2 product. Moreover, long-term intake of aspirin, one of the NSAIDs that inhibit both COX-1 and COX-2, was associated with an up to 90% decreased risk of developing esophageal cancer (4). We have reported that NMBA-induced esophageal tumorigenesis in rats was suppressed by JTE-522, a selective COX-2 inhibitor, by decreasing COX-2-mediated PGE_2 (2). This well-defined animal model is valuable to mimic as much as possible the clinical setting and modulation of COX-2 expression in which investigation for prevention of human esophageal carcinoma may occur. In the current study, we used this animal model to investigate the action and mechanisms of resveratrol. Our results indicated that resveratrol, being administered p.o. or i.p., time-dependently reduced both the number and size of NMBA-induced esophageal tumors. However, the administration of resveratrol at a high dose of 4 mg/kg led to toxic reaction in animals, and even death, while a low dose and long-term administration of resveratrol, similar to that in group 10 was observed to be effective and safe. In particular, administration of resveratrol at a dose of 2 mg/kg was revealed as the maximum orally tolerated dosage, which significantly suppresses formation and growth of tumors. These findings are perhaps important for future clinical use of resveratrol.

In addition, it was already observed that resveratrol non-

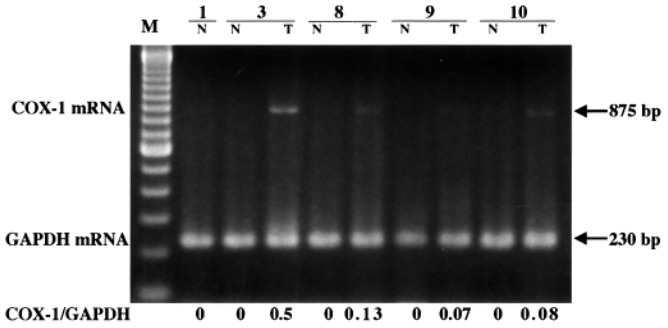

Fig. 3. Multiplex RT–PCR assay for COX-1 mRNA in relation to resveratrol treatment. Results for groups 1 (normal mucosa), 3 (NMBA alone), 8 (NMBA plus resveratrol 1 mg/kg), 9 (NMBA plus resveratrol 2 mg/kg) and 10 (NMBA plus resveratrol 1 mg/kg, continuous administration) are shown. N, non-tumor tissue; T, tumor tissue. GAPDH was used as the internal standard. PCR product sizes: COX-2, 702 bp; GAPDH, 230 bp. M, 100 bp molecular marker.

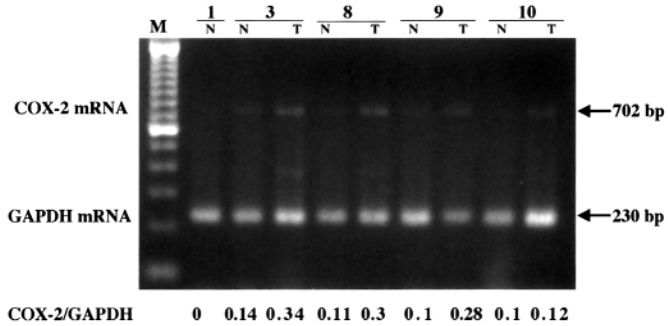

Fig. 4. Multiplex RT–PCR assay for COX-2 mRNA in relation to resveratrol treatment. Results for groups 1 (normal mucosa), 3 (NMBA alone), 8 (NMBA plus resveratrol 1 mg/kg), 9 (NMBA plus resveratrol 2 mg/kg) and 10 (NMBA plus resveratrol 1 mg/kg, continuous administration) are shown. N, non-tumor tissue; T, tumor tissue. GAPDH was used as the internal standard. PCR product sizes: COX-2, 702 bp; GAPDH, 230 bp. M, 100 bp molecular marker.

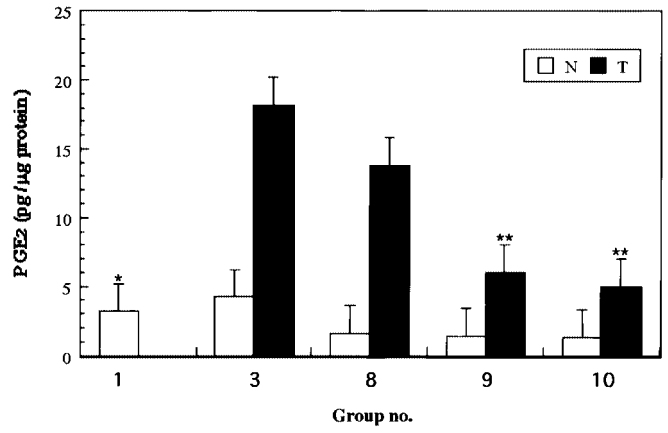

Fig. 5. Effect of resveratrol on NMBA-induced PGE_2 production. Group 1 is the normal esophageal mucosa; group 3 received NMBA alone; groups 8 and 9 were given resveratrol at different doses (1 and 2 mg/kg); group 10 also received resveratrol 1 mg/kg, while administration was continued for 20 weeks. N, non-tumor tissue; T, tumor tissue. Data are the means ± SD of pg/mg protein accumulation. Significance, determined by using Tukey–Kramer test, is expressed as follows: $*P < 0.05$, group 3 (tumor tissue) versus group 1 (normal mucosa); $**P < 0.01$ groups 9 and 10 (tumor tissue) versus group 3 (tumor tissue).

competitively inhibited the cyclooxygenase activity of COX-1 in a concentration-dependent manner (11), and decreased COX-2 transcription and activity in human mammary epithelial cells and colon cancer cells (12,13). In this study, our data indicated that resveratrol suppressed the expression of COX-1 and COX-2, and reduce the production of PGE_2. These findings may partly explain the inhibitory effects of resveratrol on both the number and size of tumors induced by NMBA in this model.

In conclusion, resveratrol may be a promising candidate as a chemopreventive agent against human esophageal cancers.

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