carcinogenesis (33,34). COX-1 is also involved in carcinogenesis in the colon (35,36) and the tongue (T. Tanaka, Y. Yasui, M. Kim, in preparation). Therefore, both isoforms are involved in carcinogenesis (37). PGE_2 manifests its biological activities through four known G-protein-coupled membrane receptors, EP_1, EP_2, EP_3 and EP_4 (38). Recently reported findings suggest that these PGE_2 receptors contribute to murine tumorigenesis in the colon (37), skin (39) and mammary gland (40). We have recently reported that an EP_1-selective antagonist, ONO-8711, can effectively inhibit 4-NQO-induced tongue carcinogenesis in rats (41), thus suggesting that certain EP receptors are involved in chemically induced tongue carcinogenesis. However, detailed investigations on the expression of all EP receptors are scarce.

In the current study, we investigated the susceptibility of 4-NQO-induced tongue carcinogenesis in male Tg mice to inspect our previous finding that the tongue of human c-Ha-*ras* proto-oncogene-carrying transgenic rats is highly susceptible to 4-NQO-induced carcinogenesis. In addition, we immunohistochemically examined the different expression of four EP receptors in normal squamous epithelium, in dysplasia and in neoplasms in the tongue of Tg mice that are adequately evaluated for consideration for carcinogenicity testing of pharmaceutical candidates (42). Subsequently, the chemopreventive potential of a lipophilic statin, pitavastatin (43), against 4-NQO-induced carcinogenesis in the Tg mice was investigated to determine whether this animal model can be utilized for preclinical animal studies for cancer chemoprevention. Pitavastatin affects the expression of $p21^{waf1}$ that is involved in oral cancer development (21,44), and ras p21 is a target for cancer chemoprevention by statins (45). In addition, pitavastatin is able to inhibit NAD(P)H oxidase activity (46), which is involved in 4-NQO-induced mutagenicity, carcinogenicity and oxidative stress (47). Statins modulate PGs biosynthesis and downregulate COX-2 expression (48). We thus suspected that pitavastatin affects oral carcinogenesis in the Tg mice treated with 4-NQO.

Materials and methods

Animals, diets and carcinogen

Male CB6F1-Tg *ras*H2@Jcl mice (Tg) and non-Tg males bred by CLEA Japan (Tokyo, Japan) at 6 weeks of age were obtained and maintained in plastic cages in an experimental room controlled at 23 ± 2°C temperature, 50 ± 10% humidity and lighting (12 h light/dark cycle). They were all allowed free access to a powdered basal diet of CRF (Charles River Formula)-1 (Oriental Yeast Co., Ltd, Tokyo, Japan) and to tap water. The experiments were conducted according to the 'Guidelines for Animal Experiments in Kanazawa Medical University'. A carcinogen, 4-NQO (98% pure, CAS no. 56-57-5, Wako Pure Chemical Ind., Osaka, Japan) was used to induce tongue and/or esophageal tumors in this study.

Experimental procedure for developing the rasH2 tongue and esophageal carcinogenesis model

A total of 22 Tg and 24 non-Tg male mice were transferred to the experimental room after a 1 week quarantine. They were given tap water containing 20 p.p.m. 4-NQO for 2 (group 1, $n = 5$, each of Tg and non-Tg mice), 4 (group 2, $n = 5$, each of Tg and non-Tg mice), 6 (group 3, $n = 5$, each of Tg and non-Tg mice) and 8 weeks (group 4, $n = 5$, each of Tg and non-Tg mice), and thereafter they received no further treatments. Two *ras*H2 mice (group 5) and four non-Tg mice (group 5) were untreated controls. The animals were killed at 24 weeks to determine the occurrence of preneoplasms and neoplasms in the tongue and esophagus. At killing by exsanguination under a deep ether anesthesia, macroscopic observations were carefully performed and the numbers of grossly visible tumors in the tongue and esophagus were recorded, and then these tissues with or without tumors were processed for histopathological examination after being fixed in 10% buffered formalin. The tongues and esophagus with or without lesions were also processed to assess the expression of EP receptors and a cell proliferation biomarker by immunohistochemistry. For a histological examination, the tissue and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks and then the histological sections were stained with hematoxylin and eosin. Epithelial lesions (dysplasia and neoplasia) in both tissues were diagnosed according to the criteria described by Kramer *et al.* (49). To determine the multiplicity of the tongue and esophageal lesions, the tissue specimens were examined for gross lesions without the use of any magnification aid. Tissue specimens from both the tongue and esophagus were cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin. Each tissue specimen was totally submitted as multiple transverse sections for histological processing. This averaged 5–6 pieces/tissue specimen of the tongue and 10–12 pieces/tissue specimen of the esophagus. The lesions in the tissues were counted on all slides stained with hematoxylin and eosin, and then the sum was divided by the number of slides, and they were expressed as the mean ± SD.

Immunohistochemistry of EP receptors

An immunohistochemical analysis of the EP receptors, EP_{1-4}, of the tongue and esophagus specimens from all the mice was done. Four-micrometer-thick paraffin sections of the 10% buffered formalin-fixed tongue and esophagus were mounted on salinized glass and deparaffinized in xyline and descending strengths of ethanol. The sections were washed in 0.05 M phosphate buffer saline (PBS, pH 7.6). The endogenous peroxidase activity and non-specific binding were blocked by incubations with 0.3% hydrogen peroxide in methanol for 5 min at room temperature. After being rinsed with PBS three times for 9 min and exposed to PBS/1% bovine serum albumin for 5 min at room temperature to reduce non-specific binding, the slides were incubated overnight at 4°C with rabbit polyclonal antibodies against EP_1 (code no. 101740, Cayman Chemical Co., Ann Arbor, MI), EP_2 (code no. 101760, Cayman Chemical Co.), EP_3 (code no. 101750, Cayman Chemical Co.) and EP_4 (code no. 101775, Cayman Chemical Co.), all being diluted at 1:1500 in PBS. The slides were rinsed three times for 9 min in PBS and incubated for 30 min in Dako Envision+ Peroxidase Rabbit (K4003, Dako Japan, Kyoto, Japan). The slides were rinsed three times for 9 min in PBS. Thereafter, they were incubated for 1 min in 3,3′-diaminobenzidine-4HCl and rinsed with PBS. Finally, the sections were counterstained with Mayer's hematoxylin. The negative controls were prepared by substituted primary antibody with buffered saline. To estimate the degree of stainability of the EP receptors in the lesions, the grading system (grade 0–5) was used: grade 0–10, no immunoreactivity; grade 1, very weak immunoreactivity in 11–20% of cells; grade 2, weak immunoreactivity in 21–30% of cells; grade 3, weak immunoreactivity in 31–40% of cells; grade 4, moderate immunoreactivity in 41–50% of cells and grade 5, marked immunoreactivity in 51–100% of cells. The results of the immunohistochemical analysis were blindly scored as a 'normal' appearing squamous epithelium, severe dysplasia, squamous cell papilloma (PAP) and SCC that developed in all groups.

Preclinical chemoprevention study of pitavastatin using rasH2 male mice

Since no tumors developed in the tongue and esophagus of non-Tg mice, a preclinical chemoprevention study for evaluating possible inhibitory effects of pitavastatin on 4-NQO-induced tumorigenesis was conducted in male Tg mice. Forty-two Tg males were divided into six experimental and control groups. Groups 1 through 4 were given 4-NQO (20 p.p.m. in drinking water) for 8 weeks. Group 1 received no further treatment. Starting 1 week after the cessation of 4-NQO exposure, animals in groups 2, 3 and 4 were fed the diets containing pitavastatin at dose levels of 1, 5 and 10 p.p.m. for 15 weeks, respectively. Group 5 was given the diet mixed with 10 p.p.m. pitavastatin. Group 6 was an untreated control. At week 24, all mice were killed by exsanguination under deep ether anesthesia, and the macroscopic inspections were carefully performed. After killing, the number of grossly visible tumors in the tongue, esophagus and other tissues were recorded, photos were taken and then the organs with lesions were processed for histopathological examination after fixation in 10% buffered formalin. Each tongue was cut in half longitudinally: one half was used for histopathology and immunohistochemistry and the remainder for PGE_2 determination. Five serial sections (4 μm each) were made from the tissue specimens after embedding in paraffin. One section was used for histopathology and the others for immunohistochemistry of the EP receptors, EP_1 and EP_2, proliferative cell nuclear antigen (PCNA) and cyclin D1.

The histopathological diagnosis was done on the sections stained with hematoxylin and eosin. Epithelial lesions (dysplasia and neoplasia) in the tongue and esophagus were diagnosed according to the criteria described by Kramer *et al.* (49). The multiplicities of the tongue and esophageal lesions were determined, as described above. An immunohistochemical analysis of EP_1 and EP_2 was performed as described above. The intensity and localization of the immunoreactivity against the EP_1 and EP_2 primary antibodies were determined on the tongue sections containing SCC using a microscope (Olympus BX41, Olympus Optical Co., Ltd, Tokyo, Japan) and evaluated for the intensity of the immunoreactivity of EP_1 and EP_2 on a 0–4+ scale. The overall intensity of the staining reaction was scored with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and <10% positive cells, 2+ mild immunoreactivity and 10–30% positive cells, 3+ moderate immunoreactivity and 31–60% positive cells and 4+ strong immunoreactivity and 61–100% positive cells. To assess the proliferative activity of the tongue SCC developed in groups 1 through 4, the PCNA-labeling index was quantified. The immunohistochemical detection of PCNA-positive nuclei was done using an

immunohistochemical analysis kit (Dako Japan). The labeling indices were calculated by counting the PCNA-positive nuclei in at least 100 cells at three different fields of tongue and esophagus from each mouse. Cyclin D1 immunohistochemistry was also performed for the evaluation of cell cycle activity of tongue SCCs. Briefly, 4 μm paraffin-embedded sections from tongue SCCs developed in groups 1 through 4 were deparaffinized with three changes of xylene and hydrated using a graded series of alcohol. Slides were incubated twice in 1 mM ethylenediaminetetraacetic acid (pH 8.0) at 121°C in an autoclave, 5 min each to effect antigen retrieval before staining, then exposed overnight to 1:100 diluted cyclin D1 mouse monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK). Slides were then developed by the avidin–biotin–peroxidase complex methods. Cells were considered positive for cyclin D1 when definite nuclear staining was identified. The positive cell ratio (percentage) for cyclin D1 was determined by randomly observing 100 cancer cells under magnification ×400 (>25 fields) to score. Positive cell ratios were calculated as numbers per 100 cells.

For the determination of the PGE_2 content, the tongue tissue (~100 mg) without tumors was obtained from groups 1–6 after killing. The samples were then placed into microcentrifuge tubes containing 1 ml of sodium phosphate buffer (10 mmol/l, pH 7.4) and finely minced with scissors for 15 s. The samples were then incubated for 20 min at 37°C in a shaking water bath. Following the incubation period, the samples were centrifuged at 9000*g* for 30 s and the supernatants collected. The supernatants were flash frozen in liquid nitrogen and stored at −80°C for subsequent determination of PGE_2 content. The PGE_2 level was assayed using the PGE_2 ELISA kit (Cayman Chemical Co.) according to the manufacturer's instructions.

Statistical analysis

A statistical analysis of the incidence of lesions was performed using Fisher's exact probability test, and the other results expressed as the mean ± SD were analyzed by Student–Newman–Keuls multiple comparison test using the GraphPad InStat software (version 3.05, GraphPad Software, San Diego, CA). A level of $P < 0.05$ was considered to be statistically significant.

Results

Animal model study

General observation. All Tg and non-Tg mice in groups 1–5 showed a good tolerance for 4-NQO-exposure in their drinking water. The growth curves during the study did not significantly differ among the groups with different treatment periods and between the two phenotypes (data not shown). After killing, the mean body, liver and relative liver weights did not significantly differ among the groups (data not shown).

Tongue and esophageal tumors development. Whereas no tumors or dysplastic lesions developed in any organs, including the tongue, esophagus and forestomach, of the non-Tg mice that received drinking water with or without 4-NQO, exophytic tongue and esophageal tumors developed in the Tg mice that received 4-NQO (Figure 1A). One large forestomach tumor, histologically diagnosed as an SCC, developed in a *ras*H2 mouse that received 4-NQO for 6 weeks. Such neoplasms were not observed in the untreated Tg mice. A large forestomach tumor developed in one mouse of group 3, but no tumors were observed in tissues other than the tongue and esophagus of any animals. The cumulative distribution of the tongue and tumors is illustrated in Figure 1B. When compared with the *ras*H2 mice that received 4-NQO for 2, 4 or 6 weeks, a number of tongue and esophageal tumors developed, distributed in whole parts of the tissues. The tongue and esophageal tumors were histopathologically PAP and SCC (Figure 1C). As summarized in Figure 2A, the number of tumors increased with the increased duration of 4-NQO-exposure: the highest incidence and multiplicity of tongue (100% incidence with a multiplicity of 2.80 ± 1.30 per tongue) and esophageal (60% incidence with a multiplicity of 1.40 ± 1.67 per esophagus) tumors were observed in the Tg mice given 4-NQO for 8 weeks. Preneoplastic lesions that were diagnosed to be dysplasia with various degrees of atypia also developed in the tongue and esophagus with or without tumors in the Tg mice that received 4-NQO (Figure 2B). The incidence and multiplicity of dysplasia were increased when the duration of the 4-NQO exposure was increased. In addition, the histopathological grade of dysplasia depended on the duration of the 4-NQO exposure.

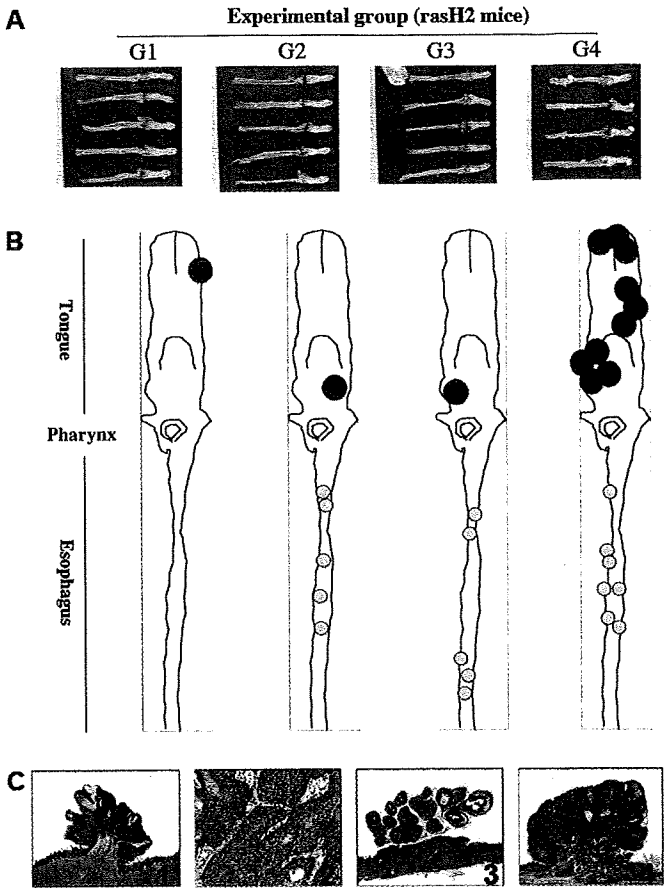

Fig. 1. Tongue and esophageal tumors developed in *ras*H2 mice that received 4-NQO in drinking water for 2 (G1, group 1), 4 (G2, group 2), 6 (G3, group 3) or 8 weeks (G4, group 4). (**A**) Macroscopic view of tongue and esophageal tumors. A large forestomach tumor developed in a *ras*H2 mouse of group 3. (**B**) Tumor distribution in the tongue and esophagus. (**C**) Histopathology of tongue and esophageal tumors: 1, tongue PAP; 2, tongue SCC; 3, esophageal PAP and 4, esophageal SCC. Hematoxylin and eosin stain, original magnification: 1, ×10; 2, ×50; 3, ×20 and 4, ×20.

EP receptors' immunohistochemistry of tongue and esophageal lesions. As indicated in Figure 3, the neoplasms and dysplasias that developed in the tongue and esophagus expressed EP_{1-4} receptors, while the expression in the non-lesional and normal squamous epithelium of the tongue and esophagus was quite low or absent. Among the EP receptors, EP_1 and EP_2 expressed as strongly positive in the dysplastic lesions and neoplasms in these tissues. The expression was observed in the cytoplasm of the cells that composed the lesions.

Chemoprevention study

General observation. All Tg mice belonging to groups 1–5 showed a good tolerance of the treatment with 4-NQO and/or pitavastatin. The mean body weight (29.0 ± 5.5 g) of group 2 (4-NQO → 1 p.p.m. pitavastatin) and the mean body (28.4 ± 4.5 g), liver (1.39 ± 0.23 g) and relative liver weights (4.89 ± 0.23 g/100 g body wt) of group 3 (4-NQO → 5 p.p.m. pitavastatin) were significantly lower than that (body weight, 35.9 ± 6.1 g; liver weight, 1.81 ± 0.29 g and relative liver weight, 5.07 ± 0.31 g/100 g body wt) of group 1 (4-NQO alone) at week 24 ($P < 0.05$, for each comparison). However, the values of group 4 (4-NQO → 10 p.p.m. pitavastatin) were comparable with those of group 1 (data not shown).

Tongue and esophageal tumor development. As summarized in Table I, tongue tumors with a 100% incidence and a multiplicity of 2.20 ± 1.23 developed in *ras*H2 mice that received 4-NQO alone

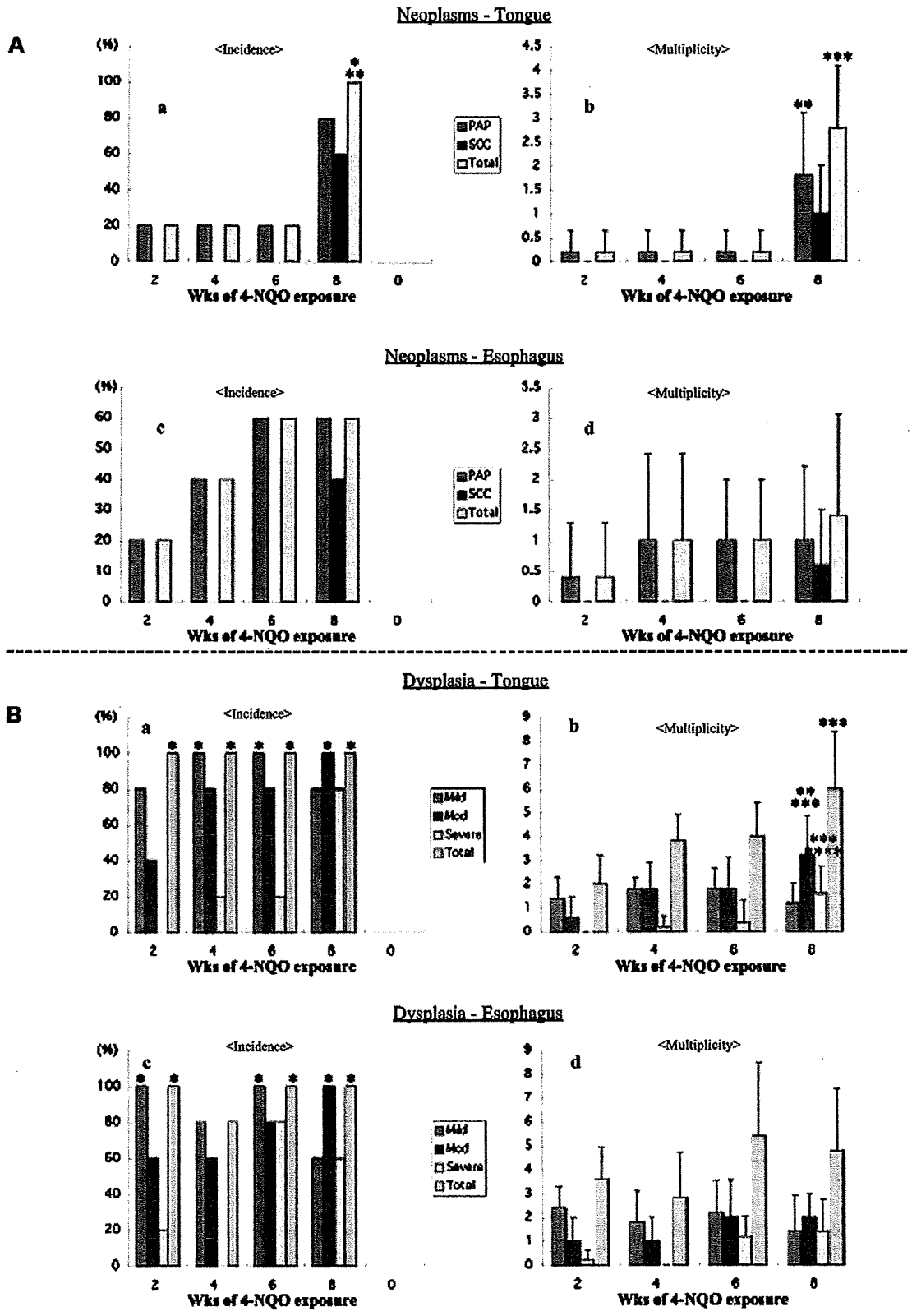

Fig. 2. Incidence and multiplicity of (A) tumors and (B) dysplasia that developed in the tongue and esophagus of *ras*H2 mice that received 4-NQO in their drinking water.

(group 1). Feeding with pitavastatin reduced the incidence and multiplicity of tongue tumors: 5 p.p.m. pitavastatin in the diet after 4-NQO-exposure significantly lowered the incidence and multiplicity of tongue PAP ($P = 0.03316$ and $P < 0.05$, respectively) and 10 p.p.m. pitavastatin in the diet significantly lowered the incidence of tongue PAP ($P = 0.0316$), SCC ($P = 0.0229$), total tumors (PAP + SCC, $P = 0.0065$) and the multiplicity of total tongue tumors ($P < 0.05$). Regarding tongue dysplasia, feeding with 1 and 5 p.p.m. pitavastatin significantly reduced the incidence of mild dysplasia ($P = 0.0065$ and $P = 0.0015$, respectively), and 10 p.p.m. pitavastatin in the diet

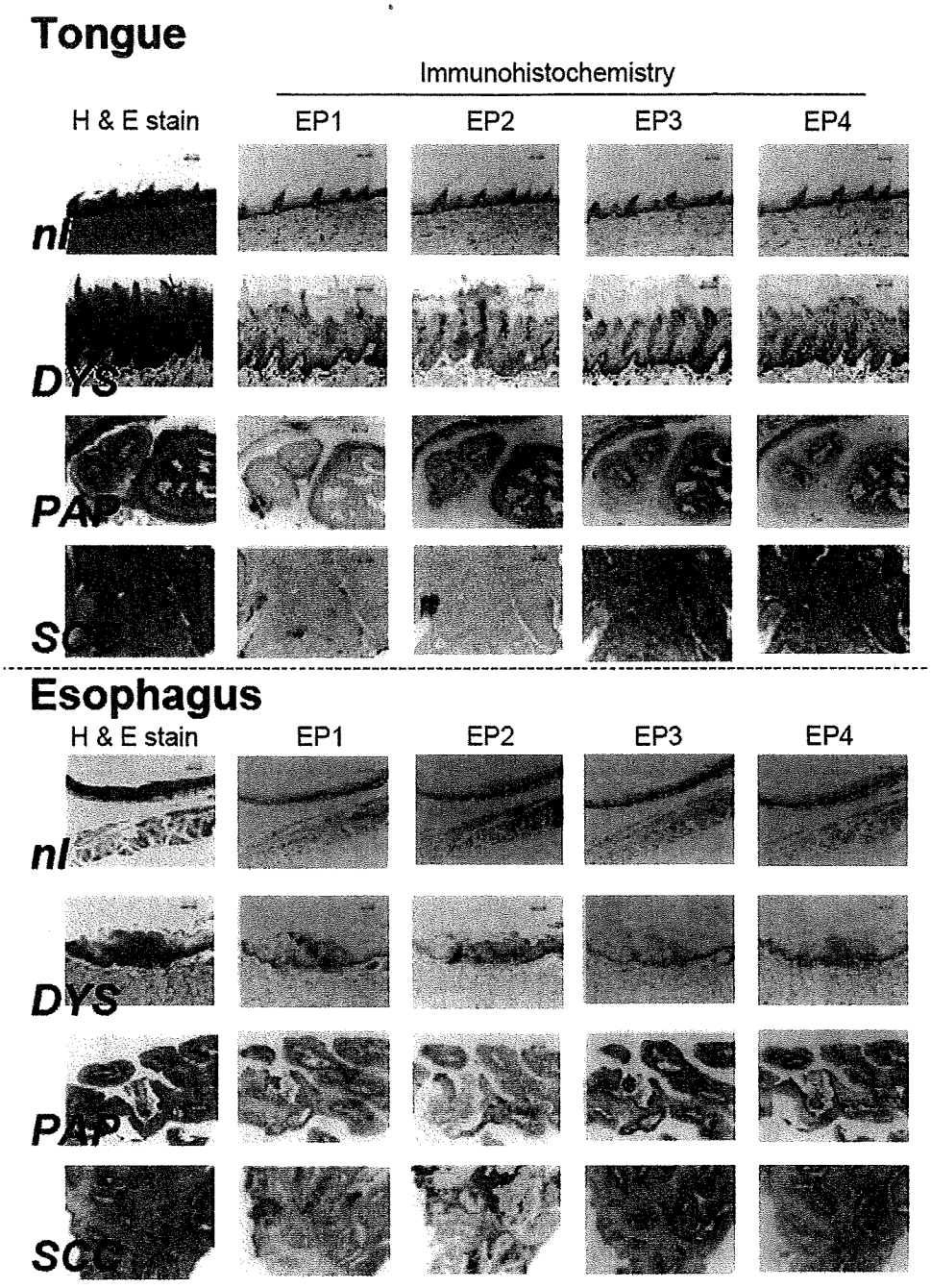

Fig. 3. Immunohistochemical expression of EP_{1-4} receptors in the normal epithelium and lesions induced by 4-NQO in tongue and esophagus. Expression of EP_{1-4} receptors is weak in the normal squamous epithelium of both tissues. Expression of EP_1 and EP_2 is strong in the dysplasia, PAP and SCC, whereas that of EP_3 and EP_4 is weak in the lesions. nl, normal squamous epithelium; DYS, squamous cell dysplasia. Hematoxylin and eosin stain and immunohistochemistry of EP_{1-4}, original magnification ×20.

significantly lowered the incidence of severe tongue dysplasia ($P = 0.0306$, Table I). Similarly, dietary feeding with 5 p.p.m. pitavastatin significantly reduced the multiplicity of mild dysplasia of the tongue ($P < 0.05$), and pitavastatin feeding at all dose levels significantly lowered the multiplicity of total dysplasia (mild, moderate and severe dysplasia) ($P < 0.01$ at 1 p.p.m., $P < 0.001$ at 5 and 10 p.p.m.).

Regarding esophageal tumors, the incidence of SCC was significantly reduced by feeding with 10 p.p.m. pitavastatin ($P = 0.0076$). Although the incidence and multiplicity of esophageal SCC and total tumors (PAP + SCC, Table II) decreased after the administration of pitavastatin at all dose levels, the reduction did not reach statistical significance (Table II). Treatment with pitavastatin at all dose levels lowered the multiplicity of esophageal dysplasia to various degrees; the differences were not significant (Table II).

PCNA-labeling index and cyclin D1-positive index of tongue SCC. The PCNA-labeling indexes of SCCs developed in the Tg mice belonging to groups 1–4 are illustrated in Figure 4. The index of group 1 (Figure 4A) was the greatest and that of group 4 (Figure 4A) was the lowest among the groups: group 1, 74.2 ± 9.9; group 2, 53.6 ± 15.0; group 3, 39.8 ± 7.7 and group 4, 33.4 ± 8 (Figure 4A). The values of groups 2 ($P < 0.01$), 3 ($P < 0.001$) and 4 ($P < 0.001$) were significantly smaller than those of group 1. The index of group 4 ($P < 0.01$) was also significantly lower than that of group 2. As to

Table I. Incidence and multiplicity of tongue dysplasia and neoplasms in the *ras*H2 mice that received 4-NQO and/or pitavastatin

Group no.	Treatment	Dysplasia								Tumor					
		Incidence				Multiplicity				Incidence			Multiplicity		
		Mild DYS[a]	Moderate DYS	Severe DYS	Total	Mild DYS	Moderate DYS	Severe DYS	Total	PAP	SCC	Total	PAP	SCC	Total
1	4-NQO alone	10/10 (100%)	10/10 (100%)	8/10 (100%)	10/10 (100%)	1.10 ± 0.32[b]	1.50 ± 0.71	1.40 ± 1.08	4.00 ± 1.41	9/10 (90%)	7/10 (70%)	10/10 (100%)	1.40 ± 0.84	0.80 ± 0.63	2.20 ± 1.23
2	4-NQO → 1 p.p.m. pitavastatin	3/8 (38%)c	5/8 (63%)	4/8 (50%)	8/8 (100%)	0.50 ± 0.76	0.75 ± 0.71	0.88 ± 1.13	2.00 ± 0.76d	4/8 (50%)	4/8 (50%)	6/8 (75%)	0.63 ± 0.74	0.50 ± 0.54	1.13 ± 0.99
3	4-NQO → 5 p.p.m. pitavastatin	2/8 (25%)e	7/8 (88%)	4/8 (50%)	8/8 (100%)	0.25 ± 0.46f	1.00 ± 0.54	0.63 ± 0.74	1.88 ± 0.64g	3/8 (38%)h	5/8 (63%)	7/8 (88%)	0.38 ± 0.52f	0.63 ± 0.52	1.00 ± 0.53
4	4-NQO → 10 p.p.m. pitavastatin	4/8 (50%)	5/8 (63%)	2/8 (25%)i	7/8 (88%)	0.50 ± 0.53	0.88 ± 0.84	0.38 ± 0.74	1.75 ± 1.04g	3/8 (38%)h	1/8 (13%)j	3/8 (38%)c	0.63 ± 0.92	0.13 ± 0.35	0.75 ± 1.04f
5	10 p.p.m. pitavastatin	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0	0	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0
6	None	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0	0	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0

[a]DYS, squamous cell dysplasia.
[b]Mean ± SD.
Significantly different from group 1 based on the Fisher's exact probability test ([c] $P = 0.0065$, [e] $P = 0.0015$, [h] $P = 0.0316$, [i] $P = 0.0306$ and [j] $P = 0.0229$).
Significantly different from group 1 based on the Tukey–Kramer multiple comparison test ([d] $P < 0.01$, [f] $P < 0.05$ and [g] $P < 0.001$).

Table II. Incidence and multiplicity of esophageal dysplasia and neoplasms in the *ras*H2 mice that received 4-NQO and/or pitavastatin

Group no.	Treatment	Dysplasia								Tumor					
		Incidence				Multiplicity				Incidence			Multiplicity		
		Mild DYS[a]	Moderate DYS	Severe DYS	Total	Mild DYS	Moderate DYS	Severe DYS	Total	PAP	SCC	Total	PAP	SCC	Total
1	4-NQO alone	9/10 (90%)	10/10 (100%)	7/10 (70%)	10/10 (100%)	1.50 ± 0.71[b]	2.60 ± 1.17	1.40 ± 1.27	5.50 ± 2.37	5/10 (50%)	8/10 (80%)	9/10 (90%)	1.00 ± 1.25	1.40 ± 1.17	2.40 ± 1.96
2	4-NQO → 1 p.p.m. pitavastatin	6/8 (75%)	8/8 (100%)	6/8 (75%)	8/8 (100%)	0.25 ± 1.04	2.38 ± 1.06	1.13 ± 0.99	4.75 ± 1.98	3/8 (38%)	5/8 (63%)	6/8 (75%)	0.50 ± 0.76	1.00 ± 0.93	1.50 ± 1.31
3	4-NQO → 5 p.p.m. pitavastatin	5/8 (63%)	8/8 (100%)	6/8 (75%)	8/8 (100%)	1.00 ± 0.93	2.63 ± 0.74	1.50 ± 1.31	5.13 ± 1.55	4/8 (50%)	4/8 (50%)	5/8 (63%)	0.88 ± 0.99	0.63 ± 0.74	1.50 ± 1.41
4	4-NQO → 10 p.p.m. pitavastatin	6/8 (75%)	7/8 (88%)	6/8 (75%)	8/8 (100%)	1.00 ± 0.93	1.63 ± 1.19	1.13 ± 0.84	3.75 ± 2.32	5/8 (63%)	1/8 (13%)[c]	5/8 (63%)	1.16 ± 1.13	0.25 ± 0.71	1.38 ± 1.51
5	10 p.p.m. pitavastatin	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0	0	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0
6	None	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0	0	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0

[a]DYS, squamous cell dysplasia.
[b]Mean ± SD.
[c]Significantly different from group 1 based on the Fisher's exact probability test ($P = 0.0076$).

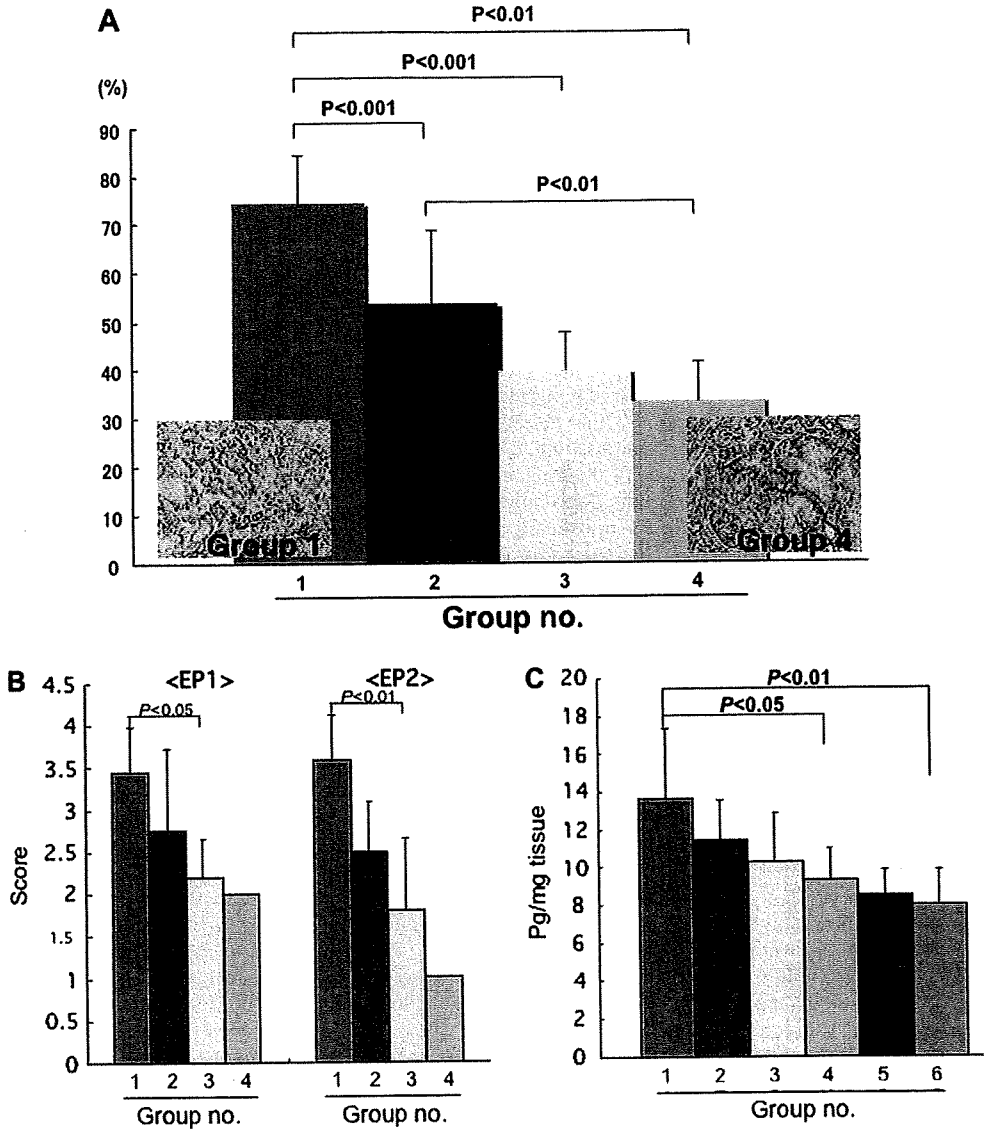

Fig. 4. Immunohistochemical analysis of PCNA-labeling index and EP_1/EP_2 expression of tongue SCCs and PGE_2 levels of the tongue (preclinical chemoprevention study). **(A)** PCNA-labeling indices (mean ± SD) of tongue SCCs in groups 1–4. Inserts are PCNA-immunohistochemistry from a SCC developed in group 1 that received 4-NQO alone (original magnification, ×50) and in group 4 that received 4-NQO and 10 p.p.m. pitavastatin (original magnification, ×50). **(B)** Scores of immunohistochemical expression of EP_1 and EP_2 receptors in tongue SCCs developed in groups 1–4. **(C)** PGE_2 contents in the tongue without tumors.

cyclin D1-positive index (mean ± SD or mean) of SCCs, the values of groups 2 ($n = 5$, 38.00 ± 6.28, $P < 0.01$), 3 ($n = 4$, 24.50 ± 9.26, $P < 0.001$) and 4 ($n = 1$, 24.50) were smaller than that of group 1 (57.00 ± 8.21, $n = 8$).

Scores of EP_1 and EP_2 receptors' immunoreactivities in the tongue SCC. As shown in Figure 4B, the tongue SCC that developed in groups 1–4 expressed EP_1 and EP_4 receptors, the strongest being the SCC of group 1. The dietary pitavastatin reduced the score of both EP_1 and EP_2 reactivity, and the values of group 3 were significantly lower than those of group 1 (EP_1 for $P < 0.05$ and EP_2 for $P < 0.01$). The mean values (2 for EP_1 and 1 for EP_2) of group 4 were also low, but a statistical analysis could not be done because of the small sample size from group 4.

Tongue PGE_2 level. The PGE_2 content of the tongue is illustrated in Figure 4C. The PGE_2 content (13.60 ± 3.66 pg/mg tissue, $P < 0.01$) of group 1 (4-NQO alone) was significantly greater than group 6 (untreated, 8.00 ± 1.83 pg/mg tissue). The pitavastatin feeding reduced the level and the value of group 4 (4-NQO → 10 p.p.m. pitavastatin, 9.25 ± 1.67 pg/mg tissue) was significantly smaller than that of group 1 ($P < 0.05$). The value of group 5 (10 p.p.m. pitavastatin, 8.50 ± 1.29 pg/mg tissue) was comparable with that (8.00 ± 1.80) seen in group 6.

Discussion

In this study, we demonstrated that *ras*H2 mice are highly susceptible to a genotoxic carcinogen, 4-NQO, in drinking water and many neoplasms developed in their tongue and esophagus within the 24 experimental weeks. Interestingly, the EP receptors, especially EP1 and EP2, were immunohistochemically expressed in the lesions (dysplasia and neoplasm) induced by 4-NQO in these tissue specimens. This novel animal carcinogenesis model is useful to investigate field cancerization in the head and neck regions (oral cavity and esophagus) (6,7). The model also can be used to identify cancer chemopreventive agents in these tissues, as we revealed that dietary pitavastatin was

capable of inhibiting the tumor development in the tissue specimens, especially the tongue.

Similar to the high frequency of *p53* mutation in human oral cancers (50), a high percentage of *ras* mutation is also detected in Indian patients (51), but infrequently in white Caucasian populations (52). Especially, a high frequency of mutation in codons 12 and 61 of the H-*ras* gene was observed in chewing tobacco-related oral cancers in India (20). Among the *ras* genes, mutations of the H-*ras* (28%) and K-*ras* genes (33%), but not the N-*ras* gene, were found in oral tumors from the eastern Indian population (51). Therefore, the *ras* gene mutation is relatively high in oral cancer associated with tobacco chewing, and *ras* and *p53* mutational events might be independent and mutually exclusive (53).

Our previous work (24) demonstrated that c-Ha-*ras* proto-oncogene transgenic rats are highly susceptible to 4-NQO-induced carcinogensis in the tongue, but not the esophagus. Other different findings from the c-Ha-*ras* proto-oncogene transgenic rats are that the tongue tumors distributed throughout the dorsal site of the tongue in this study, whereas the tongue tumors developed in the dorsal site of the root of the tongue of the c-Ha-*ras* proto-oncogene transgenic rats that received 4-NQO in their drinking water (24). The reasons for this are not known, but the species difference and/or the difference in the distribution of an enzyme (DT-diaphorase) that catalyzes the conversion of 4-NQO to an ultimate carcinogen, 4-hydroxyaminoquinoline 1-oxide (54), in the tongue may reflect the differences observed.

In the current study, tongue and esophageal tumors developed in the *ras*H2 mice that received 4-NQO (20 p.p.m. in drinking water) for 4–8 weeks. Similar findings have been reported by Tang *et al.* (18), who were able to induce numerous tumors in these tissues of CBA and C57BL/6 mice when given 4-NQO in drinking water for 16 weeks. Their extensive work also indicated alterations in the expression of intermediate filaments (K14 and K1), proliferation activity by estimating bromodeoxyuridine-positive nuclei and a cell cycle inhibitor, p16. In this study, we observed that 4-NQO-induced tongue and esophageal carcinogenesis depends on the duration of a carcinogen treatment and that 4 weeks of treatment is sufficient to induce tumors in two tissues. As Tang *et al.* (18) did not observe any tumors in the tissue specimens other than the tongue and esophagus, we found only one forestomach tumor in a *ras*H2 mouse that received 4-NQO for 6 weeks. In our previous investigation using c-Ha-*ras* proto-oncogene transgenic rats, no tumors developed in their forestomach when given 4-NQO in drinking water. Recently, Fong *et al.* (55) have reported interesting findings that rats given 4-NQO in drinking water and fed a zinc-deficient diet developed tumors in the tongue, esophagus and forestomach, whereas those given 4-NQO and a zinc-sufficient diet had tumors occur only in the tongue, suggesting that dietary modulation, including zinc, influences a manifestation of field cancerization.

Regarding mice genetically modified for tongue carcinogenesis, the *p53* transgenic mice have been reported to be highly susceptible to 4-NQO-induced oral cancer (56). When the palate of the $p53^{Val135/WT}$ mice was treated by the direct application of 4-NQO with a hairbrush, which had been dipped in 4-NQO solution, thrice weekly for 16 weeks and followed by no further treatment for 32 weeks, a greater incidence and multiplicity of squamous cell tumors in the oral cavity, esophagus and forestomach were observed in comparison with the $p53^{WT/WT}$ mice at 48 experimental weeks. Their microarray data suggest the importance of the *p53* mutation, alteration in *p53*-dependent apoptosis and cell proliferation during the carcinogenesis of these tissues. These findings also supported the belief that the crosstalk of apoptosis, cell cycle arrest, transforming growth factor β signaling pathway and *Ras*-mitogen-activated protein kinase pathway may be involved in tumorigenesis. In the current study, we observed numerous tumors mainly in the tongue and esophagus, occurring as early as 24 weeks, although no *p53* mutations were determined. Recently, Caulin *et al.* (57) developed an interesting mouse model without the use of a carcinogen, in which the focal activation of an oncogenic K-*rasG12D* allele in the oral squamous epithelium resulted in the development of a number of oral cavity tumors with an altered expression pattern of keratin 16 weeks after the activation. Taken together, *ras* mutation and/or activation as well as *p53* mutation are therefore considered to be involved in oral cancer development.

Using *ras*H2 mice, we investigated the chemopreventive ability of pitavastatin in 4-NQO-induced tongue and esophageal carcinogenesis. The dietary pitavastatin is therefore considered to have the potential to suppress the development of tongue tumorigenesis, whereas the potential in esophageal carcinogenesis was relatively weak in comparison with that observed in tongue carcinogenesis. The reasons for this are not known, but a different bioavailability of dietary pitavastatin in these tissues is considered to be one possible explanation. In this study, the PGE_2 content in the tongue was lowered by the treatment with pitavastatin. The findings support our previous results that lowering the PGE_2 content by treatment with COX-2 inhibitors (24,32) and a non-steroidal anti-inflammatory drug (58) suppresses 4-NQO-induced carcinogenesis. In this study, the dietary administration of pitavastatin reduced the immunohistochemical expression of EP_1 and EP_2 in the tongue, thus suggesting the involvement of these receptors in tongue carcinogenesis. The inhibitory effects of pitavastatin on carcinogenesis has recently been observed in colon tumorigenesis in our laboratory (43). In addition, an EP_2 antagonist, ONO-8711, in the diet effectively inhibits tongue tumor development in human c-Ha-*ras* transgenic rats initiated with 4-NQO (41). Besides these effects of pitavastatin, we observed that dietary pitavastatin at all doses significantly lowers the PCNA-labeling index of tongue SCCs, thus suggesting that this drug is able to inhibit the growth of tongue SCCs possibly through affecting events during the tumor progression stage. Additionally, feeding with pitavastatin (groups 2–4) reduced the cyclin D1-positive rates of cancer cells when compared with group 1 (4-NQO alone). The findings are of interest, because H-*ras* and cyclin D1, a downstream of the *Ras*, influence the susceptibility of oral cancer (21). Thus, the multiple effects of pitavastatin on the expression of EP_1 and EP_2 receptors, PGE_2 content, proliferation and cell cycle may result in inhibition of tongue carcinogenesis initiated with 4-NQO in *ras*H2 mice.

Using this model, detailed research on molecular and proteomics events, such as the involvement of inflammation in tongue/esophageal carcinogenesis, could be conducted to fight oral and esophageal epithelial malignancies. In addition, the gene–environment and gene–gene interactions in the carcinogenesis (59) of these tissues can be investigated using this *ras*H2 mouse model, since the H-*ras* gene and other members of the *ras* gene family also appear to be a common target for the coding sequence mutations in the initiation of carcinogenesis at several organ sites and in various species by specific carcinogens (60).

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References

1. Parkin,D.M. *et al.* (2005) Global cancer statistics, 2002. *CA Cancer J. Clin.*, **55**, 74–108.
2. Llewellyn,C.D. *et al.* (2004) An analysis of risk factors for oral cancer in young people: a case-control study. *Oral Oncol.*, **40**, 304–313.
3. Mackenzie,J. *et al.* (2000) Increasing incidence of oral cancer amongst young persons: what is the aetiology? *Oral Oncol.*, **36**, 387–389.
4. Scully,C. *et al.* (2000) ABC of oral health. Oral cancer. *BMJ*, **321**, 97–100.
5. Kanojia,D. *et al.* (2006) 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. *Oral Oncol.*, **42**, 655–667.

6. Braakhuis,B.J. *et al.* (2003) A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res.*, **63**, 1727–1730.
7. Ha,P.K. *et al.* (2003) The molecular biology of mucosal field cancerization of the head and neck. *Crit. Rev. Oral Biol. Med.*, **14**, 363–369.
8. van Oijen,M.G. *et al.* (2000) Oral field cancerization: carcinogen-induced independent events or micrometastatic deposits? *Cancer Epidemiol. Biomarkers Prev.*, **9**, 249–256.
9. Brown,K.S. *et al.* (2006) Chemoprevention of squamous cell carcinoma of the oral cavity. *Otolaryngol. Clin. North Am.*, **39**, 349–363.
10. Tanaka,T. (1995) Chemoprevention of oral carcinogenesis. *Eur. J. Cancer B Oral Oncol.*, **31B**, 3–15.
11. Nagao,T. *et al.* (2005) Incidence rates for oral leukoplakia and lichen planus in a Japanese population. *J. Oral Pathol. Med.*, **34**, 532–539.
12. Ha,P.K. *et al.* (2006) Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. *Lancet Oncol.*, **7**, 77–82.
13. Lo Muzio,L. (2001) A possible role for the WNT-1 pathway in oral carcinogenesis. *Crit. Rev. Oral Biol. Med.*, **12**, 152–165.
14. Schwartz,J.L. (2000) Biomarkers and molecular epidemiology and chemoprevention of oral carcinogenesis. *Crit. Rev. Oral Biol. Med.*, **11**, 92–122.
15. Sotiriou,C. *et al.* (2004) Molecular profiling of head and neck tumors. *Curr. Opin. Oncol.*, **16**, 211–214.
16. Mognetti,B. *et al.* (2006) Animal models in oral cancer research. *Oral Oncol.*, **42**, 448–460.
17. Tanaka,T. *et al.* (1991) Alterations of the nucleolar organizer regions during 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. *Carcinogenesis*, **12**, 329–333.
18. Tang,X.H. *et al.* (2004) Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. *Clin. Cancer Res.*, **10**, 301–313.
19. Vered,M. *et al.* (2005) 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. *Oral Oncol.*, **41**, 337–339.
20. Saranath,D. *et al.* (1991) High frequency mutation in codons 12 and 61 of H-*ras* oncogene in chewing tobacco-related human oral carcinoma in India. *Br. J. Cancer*, **63**, 573–578.
21. Sathyan,K.M. *et al.* (2006) Influence of single nucleotide polymorphisms in H-*Ras* and cyclin D1 genes on oral cancer susceptibility. *Oral Oncol.*, **42**, 607–613.
22. Vairaktaris,E. *et al.* (2007) Expression of ets-1 is not affected by N-*ras* or H-*ras* during oral oncogenesis. *J. Cancer Res. Clin. Oncol.*, **133**, 227–233.
23. Rumsby,G. *et al.* (1990) Low incidence of ras oncogene activation in human squamous cell carcinomas. *Br. J. Cancer*, **61**, 365–368.
24. Suzuki,R. *et al.* (2006) An animal model for the rapid induction of tongue neoplasms in human c-Ha-*ras* proto-oncogene transgenic rats by 4-nitroquinoline 1-oxide: its potential use for preclinical chemoprevention studies. *Carcinogenesis*, **27**, 619–630.
25. Saitoh,A. *et al.* (1990) Most tumors in transgenic mice with human c-Ha-ras gene contained somatically activated transgenes. *Oncogene*, **5**, 1195–1200.
26. Pritchard,J.B. *et al.* (2003) The role of transgenic mouse models in carcinogen identification. *Environ. Health Perspect.*, **111**, 444–454.
27. Tamaoki,N. (2001) The rasH2 transgenic mouse: nature of the model and mechanistic studies on tumorigenesis. *Toxicol. Pathol.*, **29** (suppl.), 81–89.
28. Maruyama,C. *et al.* (2001) Overexpression of human H-*ras* transgene is responsible for tumors induced by chemical carcinogens in mice. *Oncol. Rep.*, **8**, 233–237.
29. Yanaida,Y. *et al.* (2002) Dietary silymarin suppresses 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in male F344 rats. *Carcinogenesis*, **23**, 787–794.
30. Smith,W.L. *et al.* (2000) Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.*, **69**, 145–182.
31. Bertagnolli,M.M. (2007) Cox-2 and cancer chemoprevention: picking up the pieces. *Recent Results Cancer Res.*, **174**, 73–78.
32. Yoshida,K. *et al.* (2003) A COX-2 inhibitor, nimesulide, inhibits chemically-induced rat tongue carcinogenesis through suppression of cell proliferation activity and COX-2 and iNOS expression. *Histol. Histopathol.*, **18**, 39–48.
33. Sawhney,M. *et al.* (2007) Expression of NF-kappaB parallels COX-2 expression in oral precancer and cancer: association with smokeless tobacco. *Int. J. Cancer*, **120**, 2545–2556.
34. Vishwanatha,J.K. *et al.* (2003) Modulation of annexin I and cyclooxygenase-2 in smokeless tobacco-induced inflammation and oral cancer. *Mol. Cell Biochem.*, **248**, 67–75.
35. Kitamura,T. *et al.* (2003) Combined effects of prostaglandin E receptor subtype EP_1 and EP_4 antagonists on intestinal tumorigenesis in adenomatous poliposis coli gene knockout mice. *Cancer Sci.*, **94**, 618–621.
36. Kitamura,T. *et al.* (2002) Inhibitory effects of mofezolac, a cyclooxygenase-1 selective inhibitor, on intestinal carcinogenesis. *Carcinogenesis*, **23**, 1463–1466.
37. Mutoh,M. *et al.* (2006) Roles of prostanoids in colon carcinogenesis and their potential targeting for cancer chemoprevention. *Curr. Pharm. Des.*, **12**, 2375–2382.
38. Narumiya,S. *et al.* (2001) Genetic and pharmacological analysis of prostanoid receptor function. *J. Clin. Invest.*, **108**, 25–30.
39. Lee,J.L. *et al.* (2005) Differential expression of E prostanoid receptors in murine and human non-melanoma skin cancer. *J. Invest. Dermatol.*, **125**, 818–825.
40. Chun,K.S. *et al.* (2007) Cyclooxygenase-2 inhibits UVB-induced apoptosis in mouse skin by activating the prostaglandin E2 receptors, EP2 and EP4. *Cancer Res.*, **67**, 2015–2021.
41. Makita,H. *et al.* (2007) A prostaglandin E_2 receptor subtype EP_1-selective antagonist, ONO-8711, suppresses 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Carcinogenesis*, **28**, 677–684.
42. Morton,D. *et al.* (2002) The Tg rasH2 mouse in cancer hazard identification. *Toxicol. Pathol.*, **30**, 139–146.
43. Yasui,Y. *et al.* (2007) A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis. *Int. J. Cancer*, **121**, 2331–2339.
44. Sathyan,K.M. *et al.* (2007) H-*Ras* mutation modulates the expression of major cell cycle regulatory proteins and disease prognosis in oral carcinoma. *Mod. Pathol.*, **20**, 1141–1148.
45. Rao,K.N. (1995) The significance of the cholesterol biosynthetic pathway in cell growth and carcinogenesis (review). *Anticancer Res.*, **15**, 309–314.
46. Yu,H.Y. *et al.* (2005) Statin attenuates high glucose-induced and angiotensin II-induced MAP kinase activity through inhibition of NAD(P)H oxidase activity in cultured mesangial cells. *Med. Chem.*, **1**, 461–466.
47. Huang,Z. *et al.* (2007) Enhanced levels of glutathione and protein glutathiolation in rat tongue epithelium during 4-NQO-induced carcinogenesis. *Int. J. Cancer*, **120**, 1396–1401.
48. Inoue,I. *et al.* (2000) Lipophilic HMG-CoA reductase inhibitor has an anti-inflammatory effect: reduction of MRNA levels for interleukin-1beta, interleukin-6, cyclooxygenase-2. and p22phox by regulation of peroxisome proliferator-activated receptor alpha (PPARalpha) in primary endothelial cells. *Life Sci.*, **67**, 863–876.
49. Kramer,I.R. *et al.* (1978) Definition of leukoplakia and related lesions: an aid to studies on oral precancer. *Oral Surg. Oral Med. Oral Pathol.*, **46**, 518–539.
50. Brinkman,B.M. *et al.* (2006) Disease mechanism and biomarkers of oral squamous cell carcinoma. *Curr. Opin. Oncol.*, **18**, 228–233.
51. Das,N. *et al.* (2000) Ras gene mutations in oral cancer in eastern India. *Oral Oncol.*, **36**, 76–80.
52. Warnakulasuriya,K.A. *et al.* (1992) Point mutations in the Ha-*ras* oncogene are detectable in formalin-fixed tissues of oral squamous cell carcinomas, but are infrequent in British cases. *J. Oral. Pathol. Med.*, **21**, 225–229.
53. Munirajan,A.K. *et al.* (1998) Detection of a rare point mutation at codon 59 and relatively high incidence of H-*ras* mutation in Indian oral cancer. *Int. J. Oncol.*, **13**, 971–974.
54. Sugimura,T. *et al.* (1966) The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. 3. An enzyme catalyzing the conversion of 4-nitroquinoline-1-oxide to 4-hydroxyaminoquinoline-1-oxide in rat liver and hepatomas. *Cancer Res.*, **26**, 1717–1721.
55. Fong,L.Y. *et al.* (2005) Dietary zinc modulation of COX-2 expression and lingual and esophageal carcinogenesis in rats. *J. Natl Cancer Inst.*, **97**, 40–50.
56. Zhang,Z. *et al.* (2006) p53 transgenic mice are highly susceptible to 4-nitroquinoline-1-oxide-induced oral cancer. *Mol. Cancer Res.*, **4**, 401–410.
57. Caulin,C. *et al.* (2004) Inducible activation of oncogenic K-*ras* results in tumor formation in the oral cavity. *Cancer Res.*, **64**, 5054–5058.
58. Tanaka,T. *et al.* (1989) Inhibitory effects of non-steroidal anti-inflammatory drugs, piroxicam and indomethacin on 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in male ACI/N rats. *Cancer Lett.*, **48**, 177–182.
59. Shields,P.G. *et al.* (2000) Cancer risk and low-penetrance susceptibility genes in gene-environment interactions. *J. Clin. Oncol.*, **18**, 2309–2315.
60. Yuspa,S.H. *et al.* (1988) Chemical carcinogenesis: from animal models to molecular models in one decade. *Adv. Cancer Res.*, **50**, 25–70.

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Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice

Shingo Miyamoto, Yumiko Yasui[1], Takuji Tanaka[1], Hajime Ohigashi[2] and Akira Murakami*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan and [1]Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

[2]Present address: Faculty of Biotechnology, Fukui Prefectural University, Fukui 910-1195, Japan

*To whom correspondence should be addressed.
Tel: +81 75 753 6282; Fax: +81 75 753 6284;
Email: cancer@kais.kyoto-u.ac.jp

Adipocytokines are a group of adipocyte-secreted proteins that have significant effects on the metabolism of lipids and carbohydrates, as well as numerous other processes. A number of recent studies have indicated that some adipocytokines may significantly influence the proliferation of malignant cells *in vitro*, whereas it remains unclear whether they have similar roles *in vivo*. In this study, we determined serum levels of adipocytokines in mice with azoxymethane (AOM)- and dextran sulfate sodium (DSS)-induced colon carcinogenesis. Five-week-old ICR mice were given a single intraperitoneal injection of AOM followed by 1% DSS in drinking water for 7 days. Nobiletin (NOB), a citrus flavonoid, was given in the diet (100 p.p.m) for 17 weeks. Thereafter, the incidence and number of colon tumors and serum concentration of adipocytokines were determined at the end of week 20. The serum leptin level in AOM/DSS-treated mice was six times higher than that in untreated mice, whereas there were no significant differences in the levels of triglycerides, adiponectin and interleukin-6. Feeding with NOB abolished colonic malignancy and notably decreased the serum leptin level by 75%. Further, NOB suppressed the leptin-dependent, but not independent, proliferation of HT-29 colon cancer cells and decreased leptin secretion through inactivation of mitogen-activated protein kinase/extracellular signaling-regulated protein kinase, but not that of adiponectin in differentiated 3T3-L1 mouse adipocytes in a dose-dependent manner. Taken together, our results suggest that higher levels of leptin in serum promote colon carcinogenesis in mice, whereas NOB has chemopreventive effects against colon carcinogenesis, partly through regulation of leptin levels.

Introduction

Colorectal cancer was seen in about 1 million new cases throughout the world in 2002, with similar numbers for men and women. In terms of incidence, colorectal cancer ranks fourth in frequency in men and third in women (1). Epidemiological studies have provided abundant evidence that environmental factors, rather than genetic variations between populations, are of prime importance in the etiology of this disease (2,3). One of the most influential factors is obesity, whose prevalence has markedly increased over the past two decades, especially in industrialized countries (4). Obesity is known to increase the risk of several different chronic diseases, such as coronary heart disease, stroke and cancer (5,6). Further, the results of case–control and prospective studies suggest that obesity is a strong risk factor for colorectal cancer, especially in men (7–10). More recently, a prospective population-based study of ~90 000 subjects conducted by the American Cancer Society confirmed that obesity is directly associated with an increased risk of death from colon cancer (11). Animal studies have confirmed that finding and also showed that obesity enhances tumor development (12), whereas calorie restriction inhibits a broad range of spontaneous, transplanted and chemically induced tumors (13). However, the mechanism underlying the development of obesity-associated colon cancer has not been fully elucidated.

Until the discovery of adipocytokines, adipose tissue was only thought to have passive functions as an energy storage depot and mechanical barrier. Adipocytokines are a group of adipose tissue-secreted hormones that were initially reported in the early 1990s when leptin was described (14). Later, it was shown that leptin, resistin, plasminogen activator inhibitor-1, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) had positive relationships to adiposity (15). Since they have some crucial roles in immune regulation, vascular function and adipocyte metabolism, adipocytokines are considered to be central players in the pathogenesis of metabolic syndrome, a cluster of clinical symptoms that include obesity and insulin resistance. Consequently, the regulation of body weight and obesity-related pathology is rapidly becoming a critical concern for public health experts and medical scientists worldwide (16). Most of the studies on the relationship of obesity and colorectal carcinogenesis are using obese animals (e.g. *db/db*, *ob/ob* or high-fat diet consumption mice). However, it is difficult to determine which adipocytokine is involved in colon carcinogenesis in obese animals because, in addition to several adipocytokines, there are a number of altered physiological factors in obese individuals.

Thus, in the present study, we decided to use chemically induced colon carcinogenesis in mice to quantify the serum levels of adipocytokines (17). In addition, the effects of dietary citrus nobiletin (NOB, Figure 1), a candidate chemopreventive agent against cancer in the colon (18,19), toward colon carcinogenesis and the serum level of adipocytokines in mice were investigated to elucidate its regulatory activities.

Abbreviations: AOM, azoxymethane; DMEM, Dulbecco's modified Eagle medium; DSS, dextran sulfate sodium; eIF4B, eukaryotic initiation factor 4B; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; IL-6, interleukin-6; MEK, mitogen-activated protein kinase/extracellular signaling-regulated protein kinase kinase; mTOR, mammalian target of rapamycin; NOB, nobiletin; Ob-R, leptin receptor; TNF-α, tumor necrosis factor-α.

Materials and methods

Mice

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) were obtained at 5 weeks old and maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. On arrival, all mice were randomized and transferred to plastic cages (five mice per cage) and given free access to drinking water and a pelleted basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C). All mice were quarantined for 1 week before starting the experiments.

Chemicals

Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma Chemical Co. (St Louis, MO). Dextran sulfate sodium (DSS) with a molecular weight of 36 000–50 000 was purchased from ICN Biochemicals (Aurora, OH), dissolved in distilled water at a concentration of 1% (wt/vol) and then used to induce colitis. NOB (>98% purity) was obtained from Nard Chemicals (Hyogo, Japan). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and bovine serum were purchased from Gibco BRL (Grand Island, NY). Human recombinant leptin was obtained from R&D Systems (Minneapolis, MN). Antibodies directed against Pi-mitogen-activated protein kinase/extracellular signaling-regulated protein kinase kinase (MEK)1/2 (Ser217/221, #9121), Pi-extracellular signaling-regulated protein kinase (ERK)1/2 (Thr202/Tyr204), Pi-mammalian target of rapamycin (mTOR) (Ser2448, #2971), Pi-S6 (Ser240/244, #2215), Pi-eukaryotic initiation factor 4B (eIF4B) (Ser422, #3591), as well as horseradish peroxidase-conjugated anti-rabbit antibody (#7074), were obtained from Cell Signaling Technology (Beverly, MA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless specified otherwise.

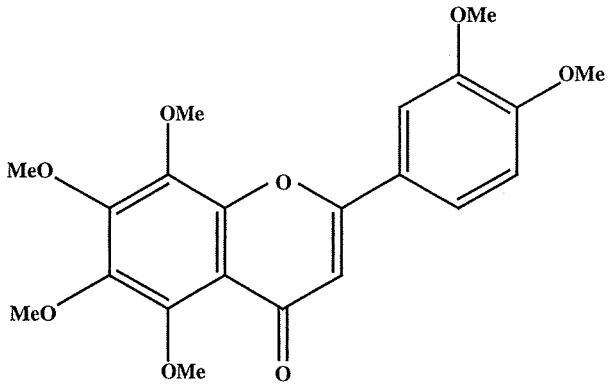

Fig. 1. Chemical structure of NOB.

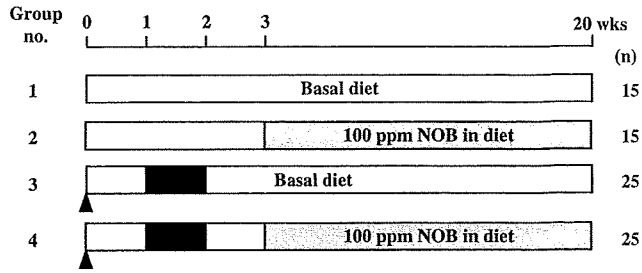

Fig. 2. Experimental protocol for AOM/DSS-induced carcinogenesis. Mice in groups 3 and 4 were given a single intraperitoneal injection of AOM (10 mg/kg body wt). Starting 1 week after the injection, they were administered 1% DSS in drinking water for 7 days (filled rectangle). Mice in groups 2 and 4 were given NOB (100 p.p.m.) in the diet from week 3.

Animal treatment

A total of 80 male ICR mice were divided into one control and three experimental groups (Figure 2). Group 1 served as an untreated control. Group 2 mice were given NOB (100 p.p.m.) in their diet starting from week 3 of the experiment. Mice in groups 3 and 4 were given a single intraperitoneal injection of AOM (10 mg/kg body wt) at the beginning of the experiment and then received 1% DSS in drinking water for 7 days starting 1 week after the injection. Further, group 3 mice were maintained on the basal diet throughout the study, whereas those in group 4 were given the same diet as group 2. The dose of NOB used was determined on the basis of our previous studies (18,19). The animals were sequentially euthanized on weeks 5, 10 and 20 as follows. Three mice each from groups 1 and 2 and five mice each from groups 3 and 4 were euthanized on weeks 5 and 10, whereas nine mice each from groups 1 and 2 and 15 mice each from groups 3 and 4 were euthanized on week 20. The mice were killed under ether anesthesia, and blood samples were immediately collected from the abdominal aorta, after which all organs were removed, with the colons flashed with phosphate-buffered saline, excised, measured in the length (from the ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed again with phosphate-buffered saline. The colons were macroscopically inspected, and whole colons were processed for paraffin embedding after being cut and fixed in 10% buffered formalin for at least 24 h. Histopathological examinations were then done on paraffin-embedded sections after hematoxylin and eosin staining. Colonic neoplasms were diagnosed according to the description by Ward (20). Tissues other than the colon were also evaluated histopathologically.

Clinical chemistry

The collected blood samples were used for clinical chemistry with measurements for triglycerides (Triglyceride E-test, Wako Pure Chemical Industries), adiponectin (Mouse/Rat Adiponectin ELISA Kit, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan), leptin (Quantikine Mouse leptin, ELISA/Assay Kit, R&D Systems), TNF-α (Quantikine Mouse leptin, TNF-α, ELISA/Assay Kit, R&D Systems) and IL-6 (Quantikine Mouse IL-6 ELISA Kit, R&D Systems, respectively) performed. Collected serum samples were examined without dilution to measure triglycerides, TNF-α and IL-6, whereas they were diluted 20- and 2000-fold for leptin and adiponectin measurements, respectively.

Cell culture

HT-29 human colon cancer cells and 3T3-L1 mouse pre-adipocytes were obtained from American Type Culture Collection (Manassas, VA). HT-29 and 3T3-L1 cells were maintained in DMEM supplemented with 10% FBS (HT-29) or 10% bovine serum (3T3-L1), as well as 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation

HT-29 cells (5×10^3/200 μl per well) were seeded into 96-well plates under the growth conditions described above. Twenty-four hours after seeding, the cells were serum starved for 24 h and then treated with leptin (0.01–10 nM) for various time periods (0–72 h), according to a method reported previously by Ogunwobi *et al.* (21), with some modifications. For suppressive experiments, cells were pretreated for 1 h with NOB (0, 10 and 100 μM) before leptin exposure. At various time points, cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Intracellular lipid accumulation and adipocytokines secretion

The 3T3-L1 cells (1×10^4/200 μl per well) were seeded into 96-well plates under the growth conditions described above. After reaching confluence, they were incubated for an additional 24 h (designated as day 0). Then, adipocyte differentiation was induced by treatment with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (10 μg/ml), components of an Adipogenesis Assay Kit (Chemicon International, Temecula, CA), in DMEM containing 10% FBS for 48 h. The medium was then replaced by DMEM containing 10% FBS and insulin (5 μg/ml) and changed to fresh medium every 2 days, according to a method reported previously by Maeda *et al.* (22), with some modifications. On day 2, NOB (0, 10 and 100 μM) was dissolved in dimethyl sulfoxide and then added to DMEM containing FBS and insulin. The final concentration of dimethyl sulfoxide was 0.1%, which was found not to affect cell growth (data not shown). After 12 days, the medium was collected and subjected to enzyme-linked immunosorbent assay to determine the levels of leptin, adiponectin, IL-6 and TNF-α. The cells were stained with the Oil Red-O component of an Adipogenesis Assay Kit according to the manufacturer's instructions. Stained cells were viewed using an inverted microscope (Leica Microsystems, Tokyo, Japan) (original magnification 1:200) and images were captured with a digital camera system. Stained oil droplets in 3T3-L1 cells were extracted with dye extraction solution and then the absorbance of the extracts were measured at 490 nm.

Western blotting

Following treatment with NOB, 3T3-L1 cells were washed with phosphate-buffered saline twice and lysed in lysis buffer [10 nM Tris, pH 7.4, 1% sodium dodecyl sulfate, 1 mM sodium metavanadate (V)] and centrifuged at 3200*g* for 5 min. Denatured proteins (40 μg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h, the membranes were reacted with the appropriate specific primary antibody (1:1000) followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:1000). The blots were developed using ECL western blotting detection reagents.

Statistical analysis

Where applicable, data were analyzed using a Tukey–Kramer multiple comparison test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), Fisher's exact probability test and Student's *t*-test (two sided), with $P < 0.05$ as the criterion of significance.

Results

General observations of mice

Throughout the study, dietary feeding with NOB did not cause clinically harmful symptoms including toxicity. The intake of water and food consumption (grams per day per mice) did not significantly differ among the four groups, and there were no marked changes in the mean relative liver weights and colon lengths (data not shown). In contrast, the mean body weight of group 3 was significantly higher ($P < 0.05$) than that of the groups 1 and 2, and NOB in the diet suppressed that increase by 56%, which was statistically significant (Table I). In parallel, the AOM/DSS treatment led to a notable increase in epididymal fat by 1.6-fold, whereas NOB tended to suppress that increase.

Table I. Body and epididymal fat weights and colon tumor formation in male ICR mice

Group no.	Treatment	Body weight (g)	Epididymal fat weight (g)	Incidence (no. of mice with neoplasms)			Multiplicity (no. of tumors/mouse)		
				Total (%)	Adenoma (%)	Adenocarcinoma (%)	Total	Adenoma	Adenocarcinoma
1	No treatment	41.8 ± 3.2[a]	0.94 ± 0.42	0/6 (0)	0/6 (0)	0/6 (0)	0	0	0
2	100 p.p.m. NOB	43.0 ± 3.2[a]	0.77 ± 0.65	0/6 (0)	0/6 (0)	0/6 (0)	0	0	0
3	AOM/DSS	49.3 ± 6.0[a]	1.52 ± 0.79	5/10 (50)	3/10 (30)	4/10 (40)[b]	2.1 ± 3.7	1.1 ± 2.0	1.0 ± 1.8
4	AOM/DSS + 100 p.p.m. NOB	45.1 ± 4.1	1.30 ± 0.61	1/10 (10)	1/10 (10)	0/10 (0)[b]	0.1 ± 0.3	0.1 ± 0.3	0

Data are shown as the mean ± SD. Body and epididymal fat weights were measured using nine mice from groups 1 and 2 and 15 from groups 3 and 4. Colon tumor formation was analyzed using six mice from groups 1 and 2 and 10 from groups 3 and 4.
[a]Significantly different in Tukey–Kramer multiple comparison post test: $P < 0.05$.
[b]Significantly different in Fisher's exact probability test: $P < 0.05$.

Incidence and multiplicity of colonic neoplasms

We reported previously the significant effects of NOB toward AOM-induced aberrant crypt foci formation (18) and carcinogenesis (19) in rats. In the present study, we attempted to confirm its preventive ability in inflammation-associated colon carcinogenesis model mice and also examined its effects on the serum levels of adipocytokines. Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of mice in groups 3 and 4, which were shown to be tubular adenomas and adenocarcinomas in histopathological findings. As summarized in Table I, the mice in groups 1 and 2 did not develop neoplasms in any of the organs examined, including the colon. In contrast, group 3 had a 50% incidence of colonic tumors and 40% incidence of adenocarcinomas. In group 4, which received AOM/DSS and 100 p.p.m. of NOB in the diet, only a single colonic tumor developed in one mouse, which was shown in histopathological findings to be a tubular adenoma. Thus, the incidence of adenocarcinoma in group 4 (0%) was significantly lower than that in group 3 ($P < 0.05$). The multiplicity of colon adenomas in group 4 was also extremely lower than that in group 3.

Serum levels of leptin, adiponectin, IL-6, TNF-α and triglycerides

We assessed the serum levels of triglycerides and adipocytokines based on a previous report of a positive association of colon cancer with hypertriglycemia in *Apc* knockout mice (23). Interestingly, the serum concentrations of triglycerides, IL-6 and TNF-α in group 3 were elevated by 1.5–1.6-fold as compared with those in group 1, though the differences were not significant. Of note, the serum level of leptin in group 3 increased by 3.1–5.7-fold in a time-dependent manner (from 5 to 20 weeks) and was markedly suppressed by NOB (75–84%). When NOB was given by itself (group 2), it did not affect the level of leptin as compared with the control group.

Effects of leptin and NOB on cell proliferation

Leptin treatment (0.1–10 nM) for 24 h significantly increased HT-29 cell proliferation by 1.3–1.6-fold (Figure 4A) in a time-dependent manner (Figure 4B), which was consistent with previously reported findings (24,25). We also examined the effects of NOB on leptin-dependent and -independent cell growth. As shown in Figure 4C, NOB (10 or 100 μM) abolished leptin-enhanced cell growth, whereas it had no effects on cell proliferation when leptin was not added.

Effects of NOB on Oil Red-O staining and secretion of adipocytokines

We treated differentiated 3T3-L1 adipocytes with NOB (0.1, 1, 10 and 100 μM) to determine its effects on intracellular lipid accumulation and secretion of adipocytokines. Differentiated 3T3-L1 cells were notably loaded with lipid, as detected by Oil Red-O staining. NOB (100 μM) reduced the Oil Red-O staining level of 3T3-L1 cells to 40% (Figure 5A). Further, the flavonoid (1–100 μM) significantly reduced leptin secretion (61–100%, $P < 0.05$, Figure 5B) in a dose-dependent manner. However, it had no effect on the secretion of adiponectin, whereas IL-6 secretion was slightly increased by 10 μM of NOB. The level of TNF-α in media was not detectable following any of the treatments (data not shown).

NOB inhibited leptin secretion partly through suppressed MEK1/2 phosphorylation

The mTOR, a Ser/Thr kinase, is considered to play a crucial role as the regulator of differentiation (26) and leptin secretion (27). We investigated the effects of NOB and rapamycin, an mTOR inhibitor, on the leptin secretion and phosphorylation status of molecules (Figure 6) involved in insulin-signaling pathway (mTOR, eIF4B, S6, Raf, MEK1/2 and ERK1/2). NOB (10 and 100 μM) significantly reduced leptin secretion as well as rapamycin (data not shown). The phosphorylation state of eIF4B and S6, both of which are substrates of mTOR, was abolished in rapamycin-treated cells, whereas NOB selectively decreased the phosphorylation state of only eIF4B. The differing results obtained with NOB and rapamycin led us to examine whether NOB affects the Raf/MEK/ERK pathway. Interestingly, the phosphorylation state of MEK1/2 and ERK1/2, but not Raf, was notably decreased in NOB-treated cells, whereas rapamycin did not affect those of both Raf and MEK1/2 but dramatically increased ERK1/2 phosphorylation. Proposed molecular mechanisms by which NOB suppresses leptin secretion are shown in Figure 7.

Discussion

In the present study, we demonstrated for the first time that serum leptin levels are profoundly increased in mice with chemically induced colon carcinogenesis. In addition, a citrus flavonoid, NOB, lowered not only those levels but also reduced colon tumor development, whereas other adipocytokines (TNF-α, IL-6 and adiponectin) and triglycerides did not significantly alter by the treatment. Further, NOB abolished leptin-stimulated human colon cancer cell proliferation and leptin secretion from insulin-treated adipocytes *in vitro* as well as the flavonoid rutin (28). Together, these results led us to hypothesize that an increased level of leptin promotes colon carcinogenesis in mice and that NOB is able to inhibit that, at least in part, through regulation of leptin levels.

Recently, obesity has become a point of focus in investigations conducted to identify dietary and lifestyle factors related to an increased risk of colorectal cancer (29). Metabolic stress resulting from obesity has been shown to be associated with increased levels of oxidative stress (30), inflammatory cytokines, insulin (31) and lipids (32,33). Of interest, Niho *et al.* (23,34) revealed a hyperlipidemic state in *Apc* gene-deficient mice, used as a model of human familial adenomatous polyposis, as compared with their wild-type counterparts. Our present findings regarding the serum level of triglycerides (Figure 3) are similar to those, though the difference did not reach statistical significance. This discrepancy may attribute to the differences in genetic backgrounds of the mice used and/or the experimental protocols. Obesity is driven by white adipose tissue, from which excess or reduced levels of adipocytokines are secreted (35). Adiponectin is the most abundant cytokine in adipocytes and has been reported to have antidiabetic and anti-inflammatory properties (36),

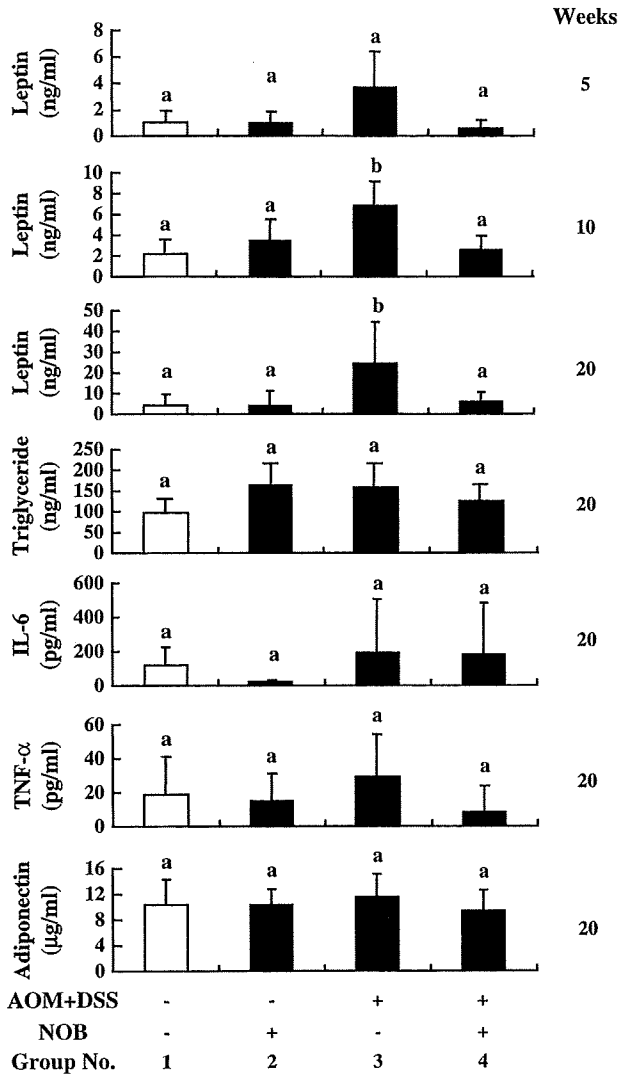

Fig. 3. Levels of serum leptin, triglycerides, IL-6, TNF-α and adiponectin in mice. Serum adipocytokine (leptin, IL-6, TNF-α and adiponectin) levels were quantified by enzyme-linked immunosorbent assay and triglycerides by a Triglyceride E-test. Values are shown as the mean ± SD (n = 3–15). Statistical analysis was performed using a Tukey–Kramer multiple comparison test and the data not sharing a letter, $P < 0.05$.

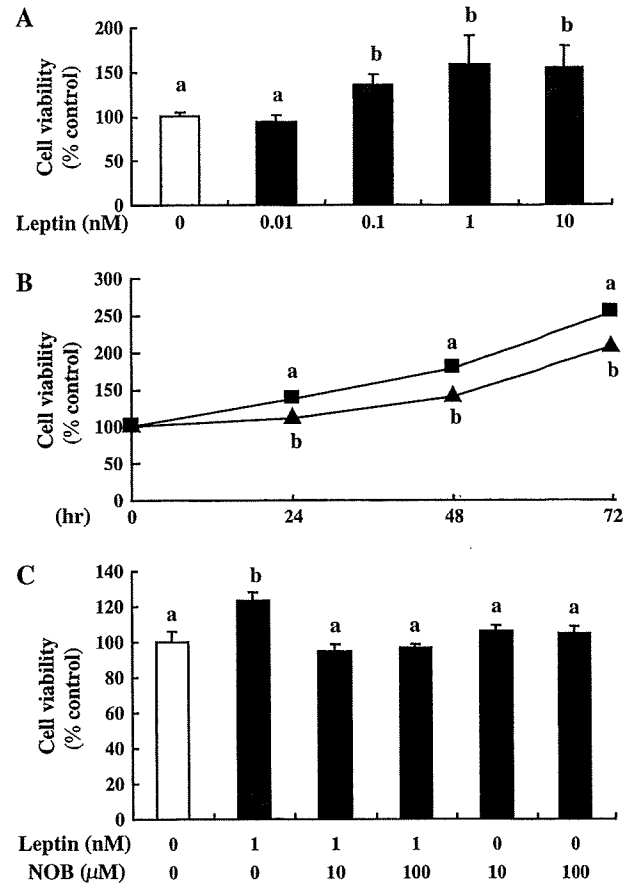

Fig. 4. Effects of leptin on proliferation of HT-29 human colon cancer cells and suppression by NOB. Twenty-four hours after seeding, cells were serum starved for 24 h and then treated with (**A**) leptin (0.01–10 nM) for 24 h (**B**) dimethyl sulfoxide (filled triangle) or 1 nM leptin (filled square) for different time periods (0–72 h) or (**C**) leptin in the presence of NOB (0, 10 and 100 μM) for 24 h. Cell numbers were determined using a Cell Counting Kit-8. Values are shown as the mean ± SD (n = 3). Statistical analysis was performed using a Student's t-test and the data not sharing a letter (a and b), $P < 0.05$ (at the same time point in panel B).

and low levels of adiponectin have been shown to be associated with an increased risk of colorectal cancer in humans (37). Several classical proinflammatory cytokines, e.g. TNF-α and to a large extent IL-6, are also secreted from adipocytes (38) and may participate in the regulation of obesity (39). In addition, epidemiological studies have revealed the roles of TNF-α and IL-6 in the onset of several types of cancer (40). However, there have been no results published regarding the hormonal role of leptin in chemically induced carcinogenesis in rodents.

Leptin is a 16 kDa protein encoded by the *ob* gene and was first revealed in 1994 as a regulator of body weight and energy balance, with its activities displayed in the hypothalamus (14). It is well known that serum leptin levels are highly elevated in obese individuals (41,42) and that leptin is secreted mainly by white adipocytes (43). C57BL/KsJ-*db/db* (*db/db*) mice have a defect in the leptin receptor (*Ob-R*) gene (44), which leads to leptin regulatory impairments of food intake thereby resulting in hyperinsulinemia, hyperglycemia and hyperleptinemia in subjects with extreme obesity (45). In the present study, the mean body and epididymal fat weights in AOM/DSS-treated mice were greater than those in control mice while NOB feeding decreased those (Table I). These results raise the possibility that the elevation of serum leptin levels seen in carcinogenesis model mice is in part due to increases in body and fat weights, though the underlying mechanism is unclear. On the other hand, several studies have reported that mesenteric adipose tissue in inflammatory bowel disease patients overexpress *leptin* mRNA (46,47). A DSS-induced colitis animal model is considered to be very reliable and useful for elucidating the mechanism underlying the onset of inflammatory bowel disease (48), thus DSS treatment may be associated with elevated serum leptin levels in AOM/DSS-treated mice. This issue is now being addressed in our laboratory. Our results (Figure 4) as well as those of several other studies (21,24,25) indicate that leptin acts as a mitogenic factor in cultured human colon cancer cells. However, it is well known that obese animals with elevated leptin levels, e.g. wild mice fed a high-fat diet and *db/db* mice, are highly susceptible to chemically induced carcinogenesis (49,50). Collectively, it is considered that obesity-associated colon carcinogenesis is partly mediated through a leptin-involved mechanism.

NOB (Figure 1), a polymethoxylated flavonoid predominant in citrus fruit peels (Figure 1) (51), has been reported to inhibit the proliferation of a variety of human cancer cell lines (52) and suppress colon carcinogenesis in rats (18,19). Although several reports have implied the preventive mechanism of NOB toward colon carcinogenesis, those results are not definitive. For example, NOB inhibited inducible nitric

oxide synthase and cyclooxygenase-2 expression in macrophages (53) and reduced prostaglandin E_2 levels in rat colonic mucosa treated with AOM (18,19). In the present study, we showed that dietary NOB decreased body and epididymal fat weights, which had been increased by treatment with AOM/DSS. These effects may contribute to its colon cancer preventive activities. In accordance with this notion, Saito *et al.* (54) recently reported that NOB enhanced both the differentiation and the lipolysis of adipocytes via activation of signaling cascades mediated by cyclic adenosine 3′,5′-monophosphate and cyclic adenosine 3′,5′-monophosphate-responsive element-binding protein. Our findings showing that NOB reduced the accumulation of intracellular lipids (Figure 5A) were similar to those in that study.

In addition, it should be pointed out that NOB inhibited leptin-stimulated but not basal HT-29 colon cancer cell proliferation, though the mechanism is not fully understood. The biological activities of leptin are mediated through its receptor Ob-R, which consists of six splicing variants (Ob-Ra through Ob-Rf) (55). The long (Ob-Rb) and short (Ob-Ra) isoforms transduced leptin signals through ERK1/2- and c-Jun NH_2-terminal kinase 1/2-dependent pathways in human Kupffer and peripheral blood mononuclear cells (56). Also, leptin was reported to stimulate HT-29 cell proliferation through the activation of ERK1/2 and c-Jun NH_2-terminal kinase 1/2 (21,25). Importantly, we recently reported that NOB suppressed phorbol ester-induced activation of ERK1/2, c-Jun NH_2-terminal kinase 1/2 and c-jun in THP-1 human monocytic cells (57). Thus, this flavonoid may inhibit leptin-induced cell proliferation by disrupting mitogen-activated protein kinase pathways.

In adipocytes, mTOR is a master regulator of protein synthesis (58), adipose tissue morphogenesis (59) and leptin synthesis/secretion (60). Consistent with previous study, rapamycin significantly suppressed leptin secretion from 3T3-L1 cells (data not shown), and the suppressive effect may be due to the suppression of S6 and eIF4B, the substrates of mTOR (Figure 6). Meanwhile, in the present study, NOB suppressed the activation of eIF4B but not S6. Kawabata *et al.* (61) recently showed that eIF4B phosphorylation is dependent not only on mTOR but also on mitogen-activated protein kinase pathway. In this study, NOB notably suppressed the phosphorylation of MEK1/2 and ERK1/2, but not Raf (Figure 6). The MEK1/2 activation may be regulated not only by a conventional Ras/Raf signal pathway but also by their autophosphorylation (62). Furthermore, our findings are consistent with those by Miyata *et al.* (63) who reported that NOB inhibited the auto-phosphorylation of MEK1/2 without the affecting Ras and Raf activity in HT-1080 colon cancer cells. Together, it is likely that NOB inhibits phosphorylation of MEK1/2 and eIF4B for decreasing leptin release. Based on the different mode of actions between NOB and rapamycin, their combination may lead to additive or synergistic leptin suppression.

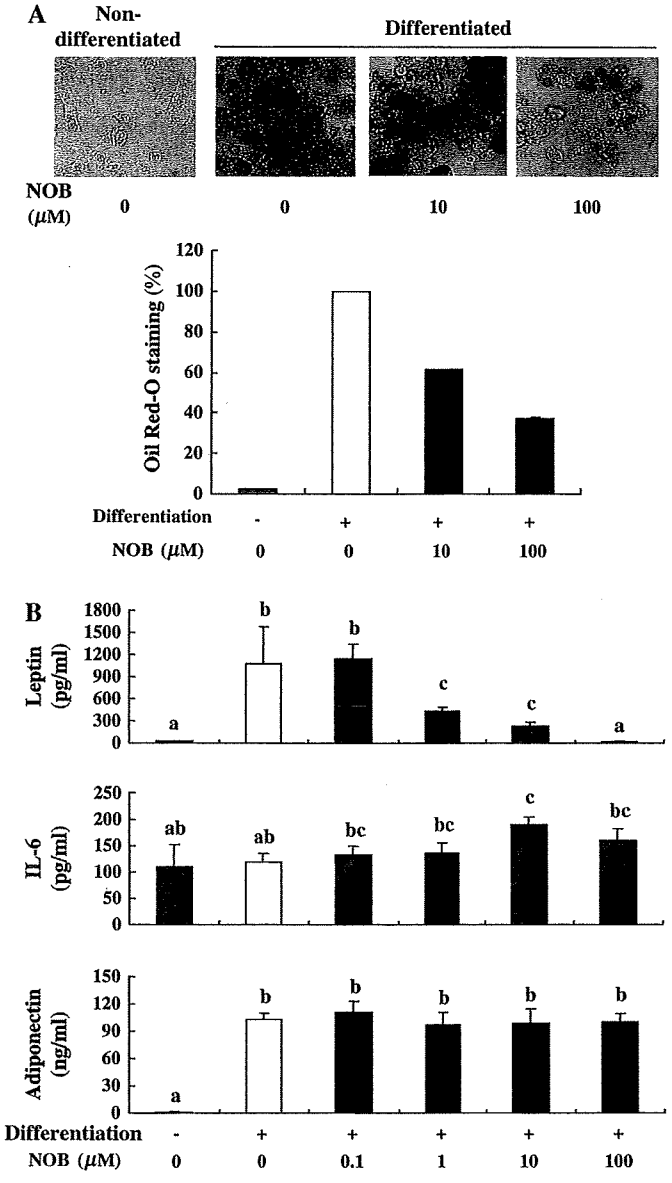

Fig. 5. Effects of NOB on Oil Red-O staining (**A**) and secretion of leptin, IL-6 and adiponectin from differentiated 3T3-L1 cells (**B**). 3T3-L1 mouse preadipocytes were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with dimethyl sulfoxide alone or various concentrations of NOB for 12 days and then the supernatants were removed for measurements of adipocytokines. The cells were washed twice with phosphate-buffered saline and stained with Oil Red-O. Stained cells were then viewed using an inverted microscope (Leica Microsystems) (original magnification 1:200). Leptin, IL-6 and adiponectin secretion were quantified by enzyme-linked immunosorbent assay. Values are shown as the mean ± SD ($n = 6$). Statistical analysis was performed using a Student's *t*-test and the data not sharing a letter, $P < 0.05$.

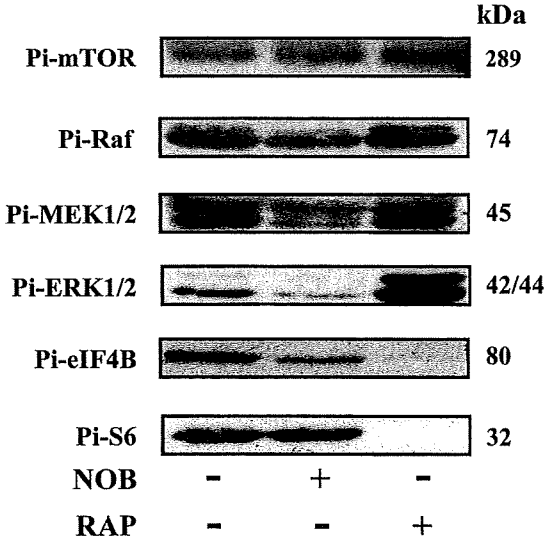

Fig. 6. Effects of NOB or rapamycin on mTOR-signaling pathway in differentiated 3T3-L1 cells. 3T3-L1 mouse pre-adipocytes (1×10^5 cells in 35 mm dish) were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with dimethyl sulfoxide alone, various concentrations of NOB or 100 nM rapamycin for 12 days and then the supernatants were removed for measurements of adipocytokines. The cells were washed twice with phosphate-buffered saline and analyzed by western blotting using specific antibodies. RAP, rapamycin.

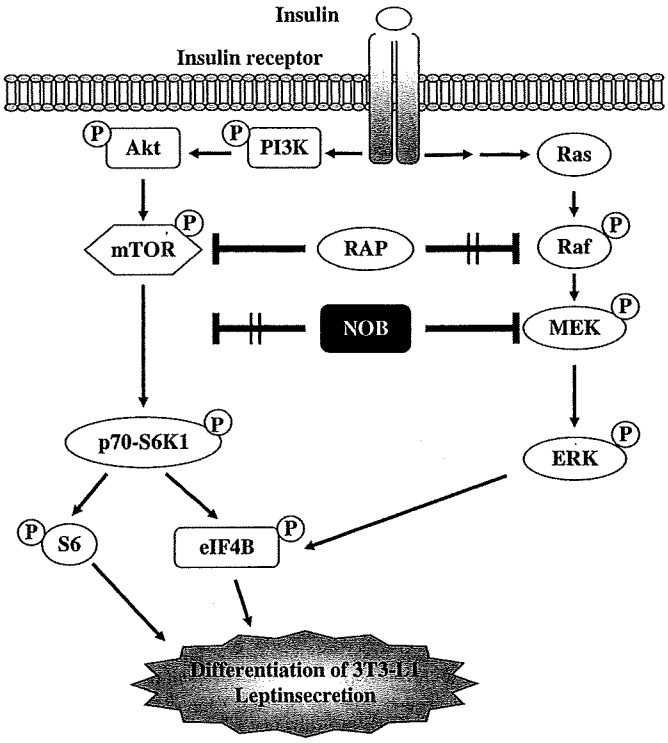

Fig. 7. Proposed schema of molecular mechanisms by which NOB suppresses leptin secretion from 3T3-L1 cells. Differentiation and protein translation are regulated by both Akt/mTOR and MEK/ERK-signaling pathway. mTOR and the downstream factors, such as S6 and eIF4B, are phosphorylated in a constitutive manner. Rapamycin inhibits mTOR and thereby suppressing the activity of downstream molecules for blocking leptin secretion. Meanwhile, NOB induces the dephosphorylation of MEK1/2 without affecting mTOR and Raf and reduces phosphorylation of eIF4B.

In conclusion, the present results suggest that the level of leptin in serum is related to colon carcinogenesis and dietary NOB suppresses carcinogenesis partly through regulation of this hormone. Although additional studies are necessary to confirm our speculation, synthetic drugs or food ingredients targeting leptin secretion and activities may be useful for regulating obesity-associated colorectal cancer development.

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References

1. Parkin,D. *et al.* (2005) Global cancer statistics, 2002. *CA Cancer J. Clin.*, **55**, 74–108.
2. Flood,D. *et al.* (2000) Colorectal cancer incidence in Asian migrants to the United States and their descendants. *Cancer Causes Control*, **11**, 403–411.
3. Johnson,I. *et al.* (2007) Review article: nutrition, obesity and colorectal cancer. *Aliment. Pharmacol. Ther.*, **26**, 161–181.
4. National Institutes of Health. (1998) Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults—the evidence report. *Obes. Res.*, **6** (suppl. 2), 51S–209S.
5. Heiat,A. *et al.* (2001) An evidence-based assessment of federal guidelines for overweight and obesity as they apply to elderly persons. *Arch. Intern. Med.*, **161**, 1194–1203.
6. Garofalo,C. *et al.* (2006) Leptin and cancer. *J. Cell. Physiol.*, **207**, 12–22.
7. Kuriyama,S. *et al.* (2005) Obesity and risk of cancer in Japan. *Int. J. Cancer*, **113**, 148–157.
8. MacInnis,R. *et al.* (2006) Body size and composition and colon cancer risk in women. *Int. J. Cancer*, **118**, 1496–1500.
9. Russo,A. *et al.* (1998) Body size and colorectal-cancer risk. *Int. J. Cancer*, **78**, 161–165.
10. Le Marchand,L. *et al.* (1997) Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. *Cancer Res.*, **57**, 4787–4794.
11. McMillan,D. *et al.* (2006) ABC of obesity. Obesity and cancer. *Br. Med. J.*, **333**, 1109–1111.
12. Yakar,S. *et al.* (2006) Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones. *Endocrinology*, **147**, 5826–5834.
13. Hursting,S. *et al.* (2003) Calorie restriction, aging, and cancer prevention: mechanisms of action and applicability to humans. *Annu. Rev. Med.*, **54**, 131–152.
14. Zhang,Y. *et al.* (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature*, **372**, 425–432.
15. Berggren,J. *et al.* (2005) Fat as an endocrine organ: influence of exercise. *J. Appl. Physiol.*, **99**, 757–764.
16. Kopelman,P. (2000) Obesity as a medical problem. *Nature*, **404**, 635–643.
17. Tanaka,T. *et al.* (2003) A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.*, **94**, 965–973.
18. Kohno,H. *et al.* (2001) Dietary administration of citrus nobiletin inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Life Sci.*, **69**, 901–913.
19. Suzuki,R. *et al.* (2004) Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats. *Biofactors*, **22**, 111–114.
20. Ward,J. (1974) Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab. Invest.*, **30**, 505–513.
21. Ogunwobi,O. *et al.* (2007) The anti-apoptotic and growth stimulatory actions of leptin in human colon cancer cells involves activation of JNK mitogen activated protein kinase, JAK2 and PI3 kinase/Akt. *Int. J. Colorectal Dis.* **22**, 401–409.
22. Maeda,H. *et al.* (2006) Fucoxanthin and its metabolite, fucoxanthinol, suppress adipocyte differentiation in 3T3-L1 cells. *Int. J. Mol. Med.*, **18**, 147–152.
23. Niho,N. *et al.* (2003) Concomitant suppression of hyperlipidemia and intestinal polyp formation in Apc-deficient mice by peroxisome proliferator-activated receptor ligands. *Cancer Res.*, **63**, 6090–6095.
24. Hardwick,J. *et al.* (2001) Leptin is a growth factor for colonic epithelial cells. *Gastroenterology*, **121**, 79–90.
25. Rouet-Benzineb,P. *et al.* (2004) Leptin counteracts sodium butyrate-induced apoptosis in human colon cancer HT-29 cells via NF-kappaB signaling. *J. Biol. Chem.*, **279**, 16495–16502.
26. Kim,J. *et al.* (2004) Regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis. *Diabetes*, **53**, 2748–2756.
27. Szkudelski,T. (2007) Intracellular mediators in regulation of leptin secretion from adipocytes. *Physiol. Res*, **56**, 503–512.
28. Hsu,C. *et al.* (2007) Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. *J. Agric. Food Chem.*, **55**, 8404–8410.
29. Calle,E. *et al.* (2004) Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat. Rev. Cancer*, **4**, 579–591.
30. Furukawa,S. *et al.* (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. *J. Clin. Invest.*, **114**, 1752–1761.
31. Kaaks,R. *et al.* (2001) Energy balance and cancer: the role of insulin and insulin-like growth factor-I. *Proc. Nutr. Soc.*, **60**, 91–106.
32. McKeown-Eyssen,G. (1994) Epidemiology of colorectal cancer revisited: are serum triglycerides and/or plasma glucose associated with risk? *Cancer Epidemiol. Biomarkers Prev.*, **3**, 687–695.
33. Järvinen,R. *et al.* (2001) Dietary fat, cholesterol and colorectal cancer in a prospective study. *Br. J. Cancer*, **85**, 357–361.
34. Niho,N. *et al.* (2007) Improvement of hyperlipidemia by indomethacin in Min mice. *Int. J. Cancer*, **121**, 1665–1669.
35. Wellen,K. *et al.* (2003) Obesity-induced inflammatory changes in adipose tissue. *J. Clin. Invest.*, **112**, 1785–1788.
36. Bråkenhielm,E. *et al.* (2004) Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc. Natl Acad. Sci. USA*, **101**, 2476–2481.
37. Wei,E. *et al.* (2005) Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *J. Natl Cancer Inst.*, **97**, 1688–1694.

38. Guzik,T. *et al.* (2006) Adipocytokines—novel link between inflammation and vascular function? *J. Physiol. Pharmacol.*, **57**, 505–528.
39. Borst,S. (2004) The role of TNF-alpha in insulin resistance. *Endocrine*, **23**, 177–182.
40. Yoshimura,A. (2006) Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci.*, **97**, 439–447.
41. Hamilton,B. *et al.* (1995) Increased obese mRNA expression in omental fat cells from massively obese humans. *Nat. Med.*, **1**, 953–956.
42. Kolaczynski,J. *et al.* (1996) Response of leptin to short-term and prolonged overfeeding in humans. *J. Clin. Endocrinol. Metab.*, **81**, 4162–4165.
43. Sinha,M. *et al.* (1996) Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *J. Clin. Invest.*, **97**, 1344–1347.
44. Lee,G. *et al.* (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature*, **379**, 632–635.
45. Frühbeck,G. *et al.* (2001) Rationale for the existence of additional adipostatic hormones. *FASEB J.*, **15**, 1996–2006.
46. Barbier,M. *et al.* (2003) Overexpression of leptin mRNA in mesenteric adipose tissue in inflammatory bowel diseases. *Gastroenterol. Clin. Biol.*, **27**, 987–991.
47. Paul,G. *et al.* (2006) Profiling adipocytokine secretion from creeping fat in Crohn's disease. *Inflamm. Bowel Dis.*, **12**, 471–477.
48. Okayasu,I. *et al.* (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology*, **98**, 694–702.
49. Liu,Z. *et al.* (2001) High fat diet enhances colonic cell proliferation and carcinogenesis in rats by elevating serum leptin. *Int. J. Oncol.*, **19**, 1009–1014.
50. Hirose,Y. *et al.* (2004) Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-db/db mice. *Carcinogenesis*, **25**, 821–825.
51. Chen,J. *et al.* (1997) Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed dancy tangerine peel oil solids. *J. Agric. Food Chem.*, **45**, 364–368.
52. Kandaswami,C. *et al.* (1992) Differential inhibition of proliferation of human squamous cell carcinoma, gliosarcoma and embryonic fibroblast-like lung cells in culture by plant flavonoids. *Anticancer Drugs*, **3**, 525–530.
53. Murakami,A. *et al.* (2000) Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res.*, **60**, 5059–5066.
54. Saito,T. *et al.* (2007) Nobiletin enhances differentiation and lipolysis of 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.*, **357**, 371–376.
55. Tartaglia,L. (1997) The leptin receptor. *J. Biol. Chem.*, **272**, 6093–6096.
56. Frühbeck,G. (2006) Intracellular signalling pathways activated by leptin. *Biochem J.*, **393**, 7–20.
57. Eguchi,A. *et al.* (2006) Nobiletin, a citrus flavonoid, suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cells. *FEBS Lett.*, **580**, 3321–3328.
58. Lynch,C. *et al.* (2002) Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue. *Am. J. Physiol. Endocrinol. Metab.*, **283**, E503–E513.
59. Fox,H. *et al.* (1998) Amino acids stimulate phosphorylation of p70S6k and organization of rat adipocytes into multicellular clusters. *Am. J. Physiol.*, **274**, C206–C213.
60. Roh,C. *et al.* (2003) Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes. *Am. J. Physiol. Endocrinol. Metab.*, **284**, E322–E330.
61. Kawabata,K. *et al.* (2006) Citrus auraptene targets translation of MMP-7 (matrilysin) via ERK1/2-dependent and mTOR-independent mechanism. *FEBS Lett.*, **580**, 5288–5294.
62. Gardner,A. *et al.* (1994) MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity. *Mol. Biol. Cell*, **5**, 193–201.
63. Miyata,Y. *et al.* (2004) Activation of protein kinase C betaII/epsilon-c-Jun NH2-terminal kinase pathway and inhibition of mitogen-activated protein/extracellular signal-regulated kinase 1/2 phosphorylation in antitumor invasive activity induced by the polymethoxy flavonoid, nobiletin. *Mol. Cancer Ther.*, **3**, 839–847.

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A Novel Prodrug of 4′-Geranyloxy-Ferulic Acid Suppresses Colitis-Related Colon Carcinogenesis in Mice

Shingo Miyamoto
Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

Francesco Epifano
Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio," Chieti, Italy

Massimo Curini and Salvatore Genovese
Dipartimento di Chimica e Tecnologia del Farmaco, Sezione di Chimica Organica, Università degli Studi di Perugia, Perugia, Italy

Mihye Kim, Rikako Ishigamori-Suzuki, Yumiko Yasui, Shigeyuki Sugie, and Takuji Tanaka
Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

The inhibitory effects of a novel prodrug, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline (GAP), of the secondary metabolite 4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoic acid (4′-geranyloxy-ferulic acid), on colon carcinogenesis was investigated using an azoxymetahen (AOM)/dextran sodium sulfate (DSS) model. GAP was synthetically derived from ferulic acid. Male CD-1 (ICR) mice initiated with a single intraperitoneal injection of azoxymethane (10 mg/kg body weight) were promoted by 1% (wt/vol) DSS in drinking water for 7 days. They were then given modified AIN-76A diet containing 0.01% or 0.05% GAP for 17 wk. At Week 20, the development of colonic adenocarcinoma was significantly inhibited by GAP feeding at dose levels of 0.01% [60% incidence ($P = 0.0158$) with a multiplicity of and 1.13 ± 1.13 ($P < 0.05$)] and 0.05% [53% incidence ($P = 0.0057$) with a multiplicity of 0.08 ± 1.08 ($P < 0.01$)], when compared to the AOM/DSS group (95% incidence with a multiplicity of 3.10 ± 3.06). Dietary GAP modulated the mitotic and apoptotic indexes in the crypt cells and lowered 8-hydroxy-2′-deoxyguanosine (8-OHdG)-positive cells in the colonic mucosa. Urinary level of 8-OHdG was lowered by GAP feeding. Additionally, dietary GAP elevated the immunoreactivity of an inducible form of heme oxygenase 1 in the colonic mucosa. Our results indicate that GAP is able to inhibit colitis-related colon carcinogenesis by modulating proliferation and oxidative stress in mice.

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Address correspondence to Takuji Tanaka, Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan. E-mail: takutt@kanazawa-med.ac.jp

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the Western countries. Globally, the mortality of CRC was 655,000 deaths per year in 2005 (1). Inflammation was known to be linked with cancer development in several tissues (2). CRC is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease. The risk of CRC increases with increasing extent and duration of the disease (3). For treatment or chemoprevention of IBD and IBD-related CRC, many drugs and chemopreventive agents were introduced (4). A large amount of the drugs are absorbed from the upper gastrointestinal tract, stomach, and small intestine and cause certain side effects. Therefore, it is preferable to deliver the drug site specifically to the colon.

Several synthetic or natural compounds exerting antioxidative and/or anti-inflammatory properties have been proposed as cancer chemopreventive agents (5–7). We previously reported that ferulic acid (R = H, Fig. 1a), abundant in edible plants, such as rice and black raspberries, is able to inhibit chemically induced carcinogenesis in rodents (8). Other investigators have reported data supporting our findings (9,10). A secondary metabolite biosynthetically derived from ferulic acid, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoic acid (R = geranyl, Fig. 1a), is supposed to exert cancer chemopreventive effect (11).

Recently, novel natural and semisynthetic compounds with anti-inflammatory activity (12) have been reported to be effective chemopreventive agents against carcinogenesis in preclinical animal studies, such as collinin (7-geranyloxy-8-methoxy-coumarin) (13), auraptene (13,14) and the ethyl ester

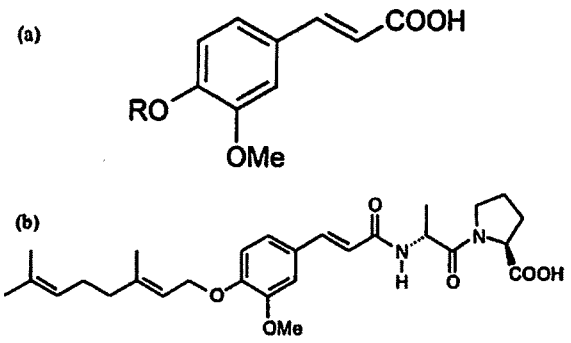

FIG. 1. Chemical structure of (a) ferulic acid. R = H and 3-(4-geranyloxy-3'-methoxyphenyl)-2-*trans*-propenoic acid, R = geranyl, and (b) 3-(4'-geranyloxy-3'-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline (GAP, molecular weight = 498.62).

of 3-(4'-geranyloxy-3'-methoxyphenyl)-2-*trans*-propenoic acid (EGMP) (15,16). Because inflammation is a universal and physiological response in the process of carcinogenesis (2,17–19), the in vivo and in vitro anti-inflammatory properties of these compounds have been demonstrated (20,21). Auraptene and collinin were reported to cause complete inhibition of platelet aggregation induced by arachidonic acid and platelet-activating factor in vitro (22), to act as good chemopreventers in colitis-related mouse colon tumorigenesis (13). In addition, our synthetic derivative, EGMP, has shown various interesting biological effects such as suppression of inducible nitric oxide (iNOS) and cyclooxygenase (COX)-2 protein expression in RAW 264.7 cells induced by lipopolysaccharide and interferon gamma (23) and colon and tongue cancer chemoprevention by dietary feeding in rats (15,16). Furthermore, some myo-inositol esters of 4'-geranoxy-ferulic acid have good inhibitory effects on phorbol ester-induced superoxide generation and Epstein-Barr virus activation (24). All these esters could be hydrolyzed to the parent acid once inside the cells. So the true active compound exerting the above-cited observed biological effects would be 3-(4'-geranoxy-3'-methoxyphenol)-2-*trans*-propenoic acid. Then, it could become a novel candidate as chemopreventive agent of various cancer types and as an anti-inflammatory compound. Pharmacological and chemical properties of the latter acid were recently reviewed (11). To achieve a novel approach in the prevention of CRC by drugs administered in diet, we carried out the synthesis of a novel prodrug, 3-(4'-geranyloxy-3'-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline (GAP, molecular weight = 498.62, Fig. 1b). This novel prodrug of 4'-geranyloxy-ferulic acid was structurally built to be hydrolized by intestinal angiotensin-converting enzyme; this enzyme is an exopeptidase that is quite abundant in the external side of the brush border of the epithelium of the small intestine, and its specificity is to hydrolize the last peptidic link in tripeptides in which +-Ala (or Gly) and L-pro occupy the second-last and last positions, respectively. Based on features of this prodrug, 3-(4'-geranyloxy-3'-methoxyphenyl)-2-*trans*-propenoic acid would be delivered in high concentration in the large bowel (25). Furthermore, its mechanism of activation would ensure chemical and enzymatic stability while passing through the stomach and small intestine by in vitro study (25).

For investigation of the pathogenesis (26–28) and chemoprevention (13,29) of inflammation-related CRC, our mouse model of inflammation related 2-stage colon carcinogenesis with a colonic carcinogen, and a colitis-inducing agent, dextran sodium sulfate (DSS) (15), is useful (30,31). In this model, the powerful tumor promoting effect of DSS is closely related to oxidative/nitrosative stress caused by DSS-induced colitis (26–28). This suggests that oxidative/nitrosative DNA damage by inflammation is involved in carcinogenesis, and thus it is important to control the events leading to inflammation-related carcinogenesis (17). In humans, oxidative stress also plays a key role in the pathogenesis of IBD-related intestinal damage (32). 8-Hydroxy-2'-deoxyguanosine (8-OHdG) production is induced by the oxidation of deoxyguanosine (dG), which is one of the components of DNA. Hydroxyl radicals (•OH) directly act on dG to form 8-OHdG. It is stable in humans and is excised by repair enzymes like 8-oxoguanine DNA glycosylase 1 and excreted in urine. 8-OHdG formation in DNA may also be related to tumorigenesis because many mutagens, tumor promoters, and carcinogens are known to generate oxygen radicals, and this generation of oxygen radicals in vivo is thought to be relevant to carcinogenesis (33). Elevation of urinary and tissue 8-OHdG levels are also known in IBD patients (32).

In the current study, we investigated whether dietary GAP exerts cancer chemopreventive ability in colitis-associated colon carcinogenesis using our mouse model (34). Also, effects of GAP on oxidative stress induced by azoxymetahen (AOM) and/or DSS were evaluated by measuring urinary level of 8-OHdG and immunohistochemical expression of 8-OHdG in the colonic mucosa. Additionally, we measured immunohistochemical expression of an important antioxidant enzyme, heme oxygenase (HO)-1, that is involved in the heme degradation process in the colonic mucosa because the significance of targeted induction of HO-1 as a strategy to achieve chemoprevention and chemoprotection is suggested (35).

MATERIAL AND METHODS

Animals, Chemicals, and Diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan), aged 5 wk, were used in this study. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 mice/cage) with free access to tap water and a pelleted basal Charles River Formula-1 diet (Oriental Yeast Co., Ltd., Tokyo, Japan) during quarantine under controlled conditions of humidity (50 ± 10%), lightning (12-h light/dark cycle), and temperature (23°C ± 2°C). They were quarantined for 7 days after arrival and randomized by body

weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DSS with a molecular weight of 36,000 to 50,000 was purchased from ICN Biochemicals (Aurora, OH). DSS for induction of colitis was dissolved in water at 1% (wt/vol). GAP was synthesized, as described previously (25). Experimental diets containing 0, 0.01, and 0.05% GAP in modified AIN-76A (36) were prepared weekly in our laboratory and stored in a cold room. Animals had access to food and water at all times. Food cups were replenished with fresh diet everyday. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

Experimental Procedures

The Institutional Animal Care and Use Committee evaluated all animal procedures associated with the present study and assured that all proposed methods were appropriate.

A total of 60 male ICR mice were divided into 5 experimental and control groups. Mice in Groups 1 through 3 were initiated with AOM by single intraperitoneal injection (10 mg/kg body weight). Starting 1 wk after the injection, 1% DSS in drinking water was administrated to mice for 7 days and then followed without any further treatment for 18 wk. Mice of Group 1 were maintained on modified AIN-76A diet throughout the study. Mice of Groups 2 and 3 were fed modified AIN-76A diets containing 0.01% GAP (Group 2) and 0.05% GAP (Group 3) for 17 wk, respectively, starting 1 wk after the cessation of DSS exposure. Group 4 did not receive AOM and DSS and were fed AIN-76A diet containing 0.05% GAP. Group 5 was fed modified AIN-76A diet and served as an untreated control. At the end of study (Week 20), all mice were sacrificed by CO_2 asphyxiation. They underwent careful necropsy, with emphasis on the colon, liver, kidney, lung, and heart.

At necropsy, the colons were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis, and then washed with saline. They were cut and fixed in 10% buffered formalin for at least 24 h. Histological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H & E) staining. Colonic tumors were diagnosed according to the Ward's (37) description. In brief, if the tumors cells with tubular formation invaded the depth of submucosa, the tumor was diagnosed as adenocarcinoma. When the tumors cells with glandular structure did not invade the submucosa and compressed the surrounding crypts, the tumor was diagnosed as adenoma.

Scoring of Inflammation in the Large Bowel

Inflammation in the large bowel was scored on the H & E-stained sections. For scoring, large intestinal inflammation was graded according to the following morphological criteria (38): Grade 0, normal appearance; Grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa but with retainment of the surface epithelium; and Grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, muscularis propria, and/or subserosa. The scoring was made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

Counting Mitotic and Apoptotic Cells and Crypt Heights

To identify intramucosal apoptotic and mitotic cells in the crypts, paraffin-embedded sections from the distal colon were stained with H & E and evaluated under a light microscope for apoptotic and mitotic cells at a magnification of 400. Apoptotic cells were identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked eosinophilic condensation of the cytoplasm, and sharply delineated cell borders surrounded with a clear halo (39). The apoptotic and mitotic indexes in the colonic crypts were determined on longitudinal sections that allowed evaluation of the whole crypt from the top to the base. One colonic section (from the distal part) per mouse was studied and scored. Randomly chosen crypts (28–56 crypts/colon) with well-oriented crypt structure from the mouth to the base were evaluated for counting apoptosis and mitosis. The apoptotic index (AI) and mitotic index (MI) nuclei were determined by dividing the total number of apoptotic or mitotic cells by the number of epithelial cells evaluated.

Immunohistochemistry of 8-OHdG and HO-1

Immunohistochemistry for 8-OHdG and HO-1 was performed on 4 μm-thick paraffin-embedded sections from the colons of mice in each group. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated through graded ethanol at room temperature. A 0.05 M Tris hydrochloride buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. Sections were treated for 40 min at room temperature, with 2% bovine serum albumin, and incubated overnight at 4°C with primary antibodies such as anti-8-OHdG mouse monoclonal antibody (diluted 1:100; Institute of Aging, Japan) and anti-HO-1 rabbit polyclonal antibody (diluted 1:200, SPA-896; StressGen Biotechnologies, Ann Arbor, MI). To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse immunoglobulin G blocking reagent (Vector Laboratories, Inc., Burlingame, CA) was applied. For 8-OHdG and HO-1 immunohistochemistry, normal rabbit serum was used to block background staining. Staining was performed using a DAKO En Vision kit (DAKO, Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Laboratories). At the last step, the sections were counterstained with hematoxylin. As a negative control, omission of the primary antibody was used. Two observers (T. Tanaka and S. Sugie) were

unaware of the treatment group to which the slide belonged and evaluated the immunoreactivity with grading between 0 and 5: 0 (<15% of the colonic mucosa examined shows positive reactivity), 1 (16–30% of the colonic mucosa examined shows positive reactivity), 2 (31–45% of the colonic mucosa examined shows positive reactivity), 3 (46–60% of the colonic mucosa examined shows positive reactivity), 4 (61–75% of the colonic mucosa examined shows positive reactivity), and 5 (>75% of the colonic mucosa examined shows positive reactivity).

Urinary 8-OHdG Analysis

To determine in vivo oxidative stress, urinary level of 8-OHdG was measured. One day before the sacrifice, 5 animals were selected randomly from each treatment group and placed individually into metabolic cages for urine collection. Urine was collected from each animal over a period of 3 h and frozen at −80°C until analysis. Urinary level of 8-OHdG was determined by competitive enzyme-linked immunoabsorbent assay (Genox, Baltimore, MD) and corrected for urinary creatinine concentrations.

Statistical Evaluation

Where applicable, data were analyzed using 1-way analysis of variance with Tukey–Kramer multiple comparisons test (GraphPad Instat version 3.05, GraphPad Softwear, San Diego, CA) with $P < 0.05$ as the criterion of significance. The Fisher's exact probability test was used for comparison of the incidence of lesions between 2 groups.

RESULTS

General Observation

During the experiment, some animals that received AOM/DSS (Group 1) or AOM/DSS→GAP (Groups 2 and 3) had bloody stool, but the symptom disappeared soon after stopping of DSS treatment. At Weeks 18 to 20, some mice of these groups had bloody stool again and anal prolapse with rectal tumor. There was no significant change between the experimental groups with regards to the parameters tested (body weight, liver weight, relative liver weight, spleen weight, kidney weight, and colon length). Further, no significant pathological alternations were found in these organs except the colon.

Pathological Findings

Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of mice in Groups 1 through 3. These tumors were histopathologically tubule adenoma (Fig. 2A) or adenocarcinoma (well-/moderately differentiated; Fig. 2B). Some adenocarcinomas invaded into submucosa or serosa. Dysplastic crypts (Fig. 2C) were also observed surrounding neoplasms. Enlarged lymph nodes with inflammation were present around the large bowel with tumors. Mice of Groups 4 (GAP alone) and 5 (untreated) had no tumors in all the organs examined including the colon.

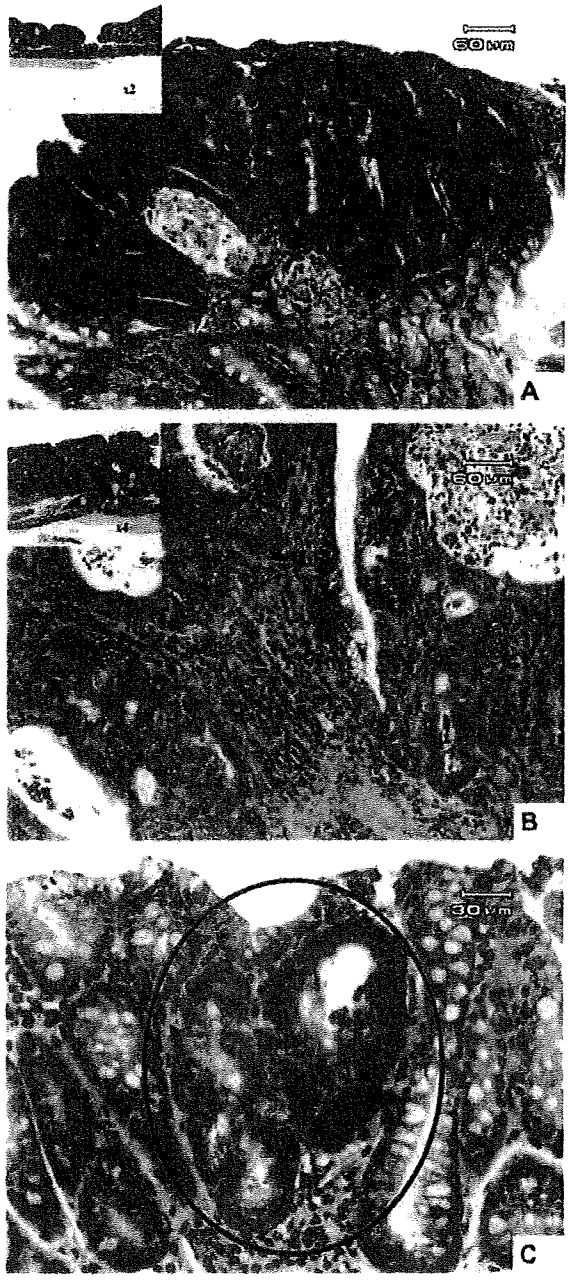

FIG. 2. Representative colonic lesions induced by azoxymetahen/dextran sodium sulfate in mice (Group 1): A: a tubular adenoma, B: a tubular adenocarcinoma with moderately differentiated, and C: dysplastic crypts (circled). Photos inserted in Fig. 2A and 2B are low power of views for each lesion (original magnifications are ×2 in 2A and ×4 in 2B). Figure represents hematoxylin and eosin stain, and bars inserted indicate magnification (μm).

TABLE 1
Incidence and Multiplicity of Colonic Tumors[a]

Group	Treatment	Incidence (No. of Mice With Tumors)			Multiplicity (No. of Tumors/Mouse)[b]		
		Total	AD	ADC	Total	AD	ADC
1	AOM/DSS (20)	20 (100%)	19 (95%)	19 (95%)	5.60 ± 4.81	2.50 ± 2.37	3.10 ± 3.06
2	AOM/DSS → 0.01% GAP (15)	10 (67%)[c]	8 (53%)[d]	9 (60%)[e]	2.33 ± 2.12[f]	1.20 ± 1.27	1.13 ± 1.13[f]
3	AOM/DSS → 0.05% GAP (15)	10 (67%)[c]	8 (53%)[d]	8 (53%)[d]	2.00 ± 2.10[f]	1.20 ± 1.37	0.80 ± 1.08[g]
4	0.05% GAP (5)	0 (0%)	0(0%)	0(0%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	None (5)	0 (0%)	0(0%)	0(0%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] Abbreviations are as follows: AD, adenoma; ADC, adenocarcinoma; AOM, azoxymetahen; DSS, dextran sodium sulfate; GAP, 3-(4'-geranyloxy-3'-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline.

[b] Mean ± SD.

[c] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0002$.

[d] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0057$.

[e] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0158$).

[f] Significantly different from the AOM/DSS group 1-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test, $P < 0.05$.

[g] Significantly different from the AOM/DSS group 1-way ANOVA with Tukey–Kramer multiple comparisons test, $P < 0.01$.

The incidences and multiplicities of colon tumors are listed in Table 1. Group 1 (AOM + DSS) had 95% incidence of colon adenocarcinoma with a multiplicity of 3.10 ± 3.06. The incidences of colonic adenocarcinoma of Groups 2 (AOM/DSS→0.01% GAP, 60%) and 3 (AOM/DSS→0.05% GAP, 53%) were significantly smaller than that of Group 1 ($P = 0.0158$ and $P = 0.0057$, respectively). Also, the multiplicities of colonic adenocarcinoma of Groups 2 (1.13 ± 1.13, $P < 0.05$) and 3 (0.80 ± 1.08, $P < 0.01$) were significantly smaller than that of Group 1.

Inflammation Score in the Colon

Fig. 3A illustrates data on colonic inflammation scores at Week 20. The inflammation score of Group 1 (2.45 ± 0.89) was the greatest. The scores of Groups 2 (1.67 ± 0.82, $P < 0.05$) and 3 (1.07 ± 0.80, $P < 0.001$) were significantly lower than that of Group 1. Colonic inflammation in the mice of Groups 4 and 5 was slight, if present.

Indices of Mitosis and Apoptosis in Colonic Crypts

The data on the epithelial proliferative kinetics in the "normal appearing" distal colon are illustrated in Figs. 3B through 3D. As shown in Fig. 3B, the mean number of crypt cell MI of Groups 1 was significantly higher (4.33 ± 2.16, 2.37-fold increase; $P < 0.001$) than that of Group 5 (1.83 ± 1.60). The dietary administration of GAP (Groups 2 and 3) reduced the mean MI in a dose-dependent manner when compared to Group 1 (4.33 ± 2.16): 27% reduction by 0.01% GAP (Group 2, 3.17 ± 1.17, $P < 0.01$) and 54% reduction by 0.05% GAP (Group 3, 2.00 ± 0.89, $P < 0.001$). Feeding with 0.05% GAP alone (Group 4, 1.83 ± 1.17) did not affect the MI in the crypts when compared to an untreated control (Group 5, 1.83 ± 1.60). As indicated in Fig. 3C, the mean AI of group 1 (1.80 ± 0.84, $P < 0.05$) was significantly greater than that of Group 5 (1.20 ± 0.84). The values of Groups 2 (2.20 ± 0.84) and 3 (3.00 ± 0.71) were larger than that of Group 1, and the increase of Group 3 was statistically significant ($P < 0.001$). The mean AI of Groups 4 (1.40 ± 0.55) and 5 were comparable. As for the crypt column height (number of cells/crypt, Fig. 3D), the value in Group 1 (44.2 ± 4.97, $P < 0.001$), being the lowest among the groups, was significantly smaller than Group 5 (61.8 ± 8.76). The crypt column heights of Groups 2 (45.8 ± 6.06) and 3 (57.4 ± 12.6) were larger than Group 1, and the increase of Group 3 was statistically significant ($P < 0.001$). The value of Groups 4 (58.2 ± 5.81) and 5 were comparable.

Scores of 8-OHdG and HO-1 Immunohistochemistry

Mean scores of HO-1 and 8-OHdG immunohistochemistry are illustrated in Figs. 4A and 4B, respectively. The mean score of HO-1 immunohistochemical positivity of Group 1 (2.10 ± 0.88) was significantly greater than that of Group 5 (0.60 ± 0.89, $P < 0.05$; Fig. 4A). The score of Group 3 (3.40 ± 1.07) was significantly larger than Group 1. The value of Group 2 (3.00 ± 0.82) was greater than that of Group 1, but the increase was insignificant. As shown in Fig. 4B, the mean score of 8-OHdG immunohistochemical positivity of group 1 (3.90 ± 0.88) was significantly greater than that of Group 5 (0.40 ± 0.55, $P < 0.001$; Fig. 4B). The scores of Groups 2 (2.40 ± 0.52, $P < 0.001$) and 3 (1.80 ± 0.79, $P < 0.001$) were significantly lower than Group 1.