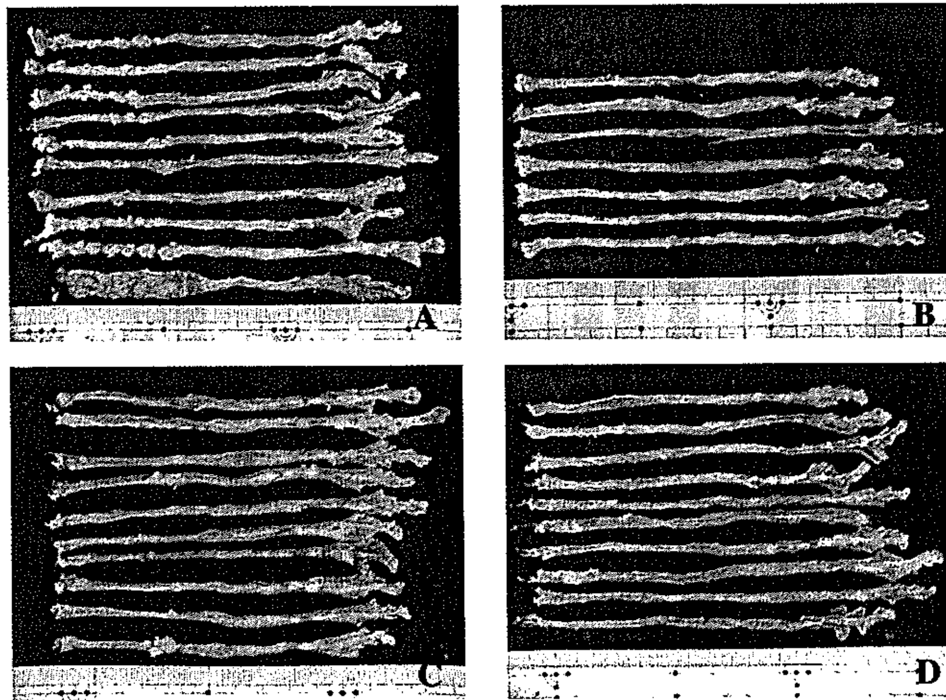

Fig. 2. Macroscopic view of the large bowel treated with AOM and 1% DSS. (A) Numerous colon tumors (2–21 tumors per mouse) develop in all Balb/c mice. (B) One or four colonic tumors are seen in two out of seven C3H/HeN mice. (C) One to five colonic tumors are found in 8 out of 10 C57BL/6N mice. (D) One colonic tumor is present in 2 out of 10 DBA/2N mice.

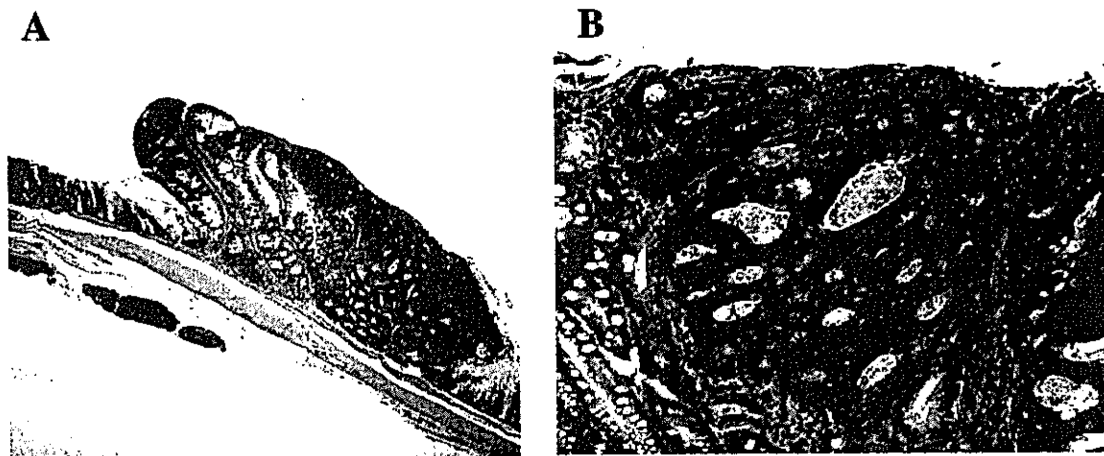

Fig. 3. Histopathology of colonic neoplasms in male Balb/c mice treated with AOM and 1% DSS. (A) Tubular adenoma and (B) moderately-differentiated adenocarcinoma. Hematoxylin and eosin stain. Original magnification, A, 2× and B, 20×.

mice ($P < 0.05$) and DBA/2N mice ($P < 0.01$). The multiplicity of adenocarcinoma in the Balb/c mice (7.7 ± 4.3) was the greatest among the four strains and it was significantly larger than that in the C3H/HeN mice (1.0 ± 1.2, $P < 0.001$).

The scores of inflammation and nitrotyrosine

As shown in Figure 5, the inflammation scores of each strain of mice initiated with AOM and followed by DSS exposure were 1.2 ± 1.1 in Balb/c, 2.3 ± 1.3 in C3H/HeN, 0.4 ± 0.7 in C57BL/6N and 0.6 ± 0.7 in DBA/2N, respectively. The score of C3H/HeN was significantly greater than that for C57BL/6N ($P<0.01$) and DBA/2N ($P<0.01$). As for the mice that received 1% DSS alone, the inflammation score of the C3H/HeN mice (1.4 ± 0.5) was the highest among the strains (1.0 ± 1.2 in Balb/c mice and 0.2 ± 0.4 in DBA/2N mice). C57BL/6N mice given 1% DSS alone had quite a low score of inflammation. The mice treated with AOM alone and the untreated mice demonstrated extremely weak inflammation in the colon.

Nitrotyrosine immunoreactivity was mainly observed in the neoplastic cells, cryptal cells, blood endothelial cells and mononuclear cells, which infiltrated the colonic mucosa (Figure 6). The stainability was relatively weak for infiltrative mononuclear cells in comparison with the cryptal cells and endothelial cells (Figure 6). As shown in Figure 7, the nitrotyrosine immunohistochemistry findings for the Balb/c mice (3.6 ± 0.5) treated with AOM and DSS were significantly higher than those for C3H/HeN (1.7 ± 0.8, $P<0.001$) and DBA/2N mice (1.6 ± 0.5, $P<0.001$). The score of nitrotyrosine-positivity in C57BL/6N mice (3.4 ± 0.5) was

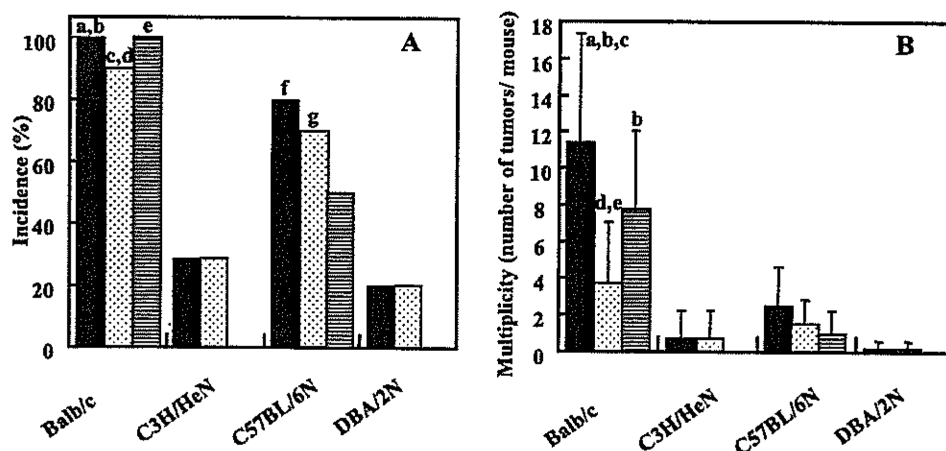

Fig. 4. Incidence and multiplicity of colonic tumors. (A) Incidence of colonic tumors. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN (P=0.0034); b, significantly different from DBA/2N (P = 0.0004); c, significantly different from C57BL/6N (P=0.0175); d, significantly different from DBA/2N (P=0.0027); e, significantly different from C57BL/6N (P=0.0163); f, significantly different from DBA/2N (P=0.0115); and g, significantly different from DBA/2N (P=0.0349). (B) Multiplicity of colonic tumors. Values are the mean ± SD. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN ($P<0.001$); b, significantly different from C57BL/6N ($P<0.001$); c, significantly different from DBA/2N ($P<0.001$); d, significantly different from C3H/HeN ($P<0.05$); and e, significantly different from DBA/2N ($P<0.01$).

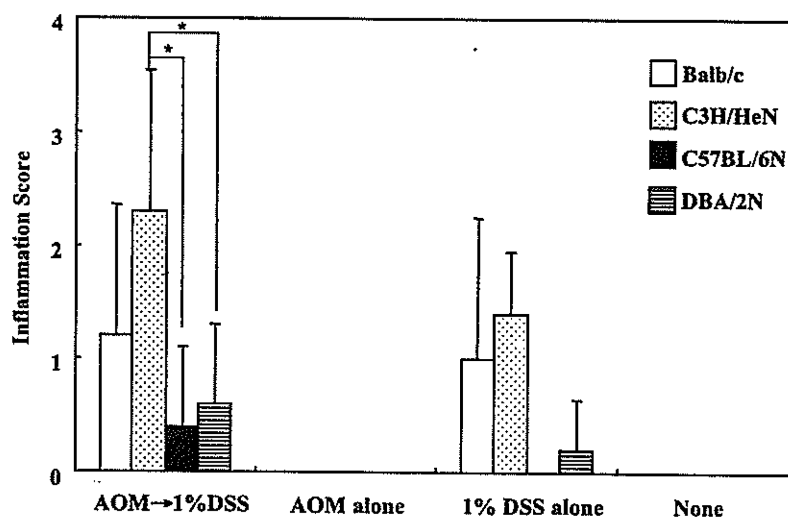

Fig. 5. Inflammation score in the colon for four strains of mice. Values are the mean ± SD. white column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *$P<0.01$.

statistically higher than those in C3H/HeN ($P < 0.001$) and DBA/2N ($P<0.001$) mice. In mice that received 1% DSS alone, the scores in Balb/c (2.8 ± 0.8) and C57BL/6N (2.4 ± 1.1) mice were higher than those in C3H/HeN (1.6 ± 0.5) and DBA/2N mice (1.4 ± 0.5); however, no significant differences were observed among the strains. As for the mice given AOM alone, the scores of nitrotyrosine in the Balb/c mice and C57BL/6N mice were 0.5 ± 0.6 and 0.2 ± 0.4, respectively. C3H/HeN mice and DBA/2N mice treated with AOM alone showed either no or faint stainability of nitrotyrosine. The degree of nitrotyrosine stainability in untreated mice was almost null.

Discussion

The present investigation demonstrated the different susceptibilities of the four strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice to colon tumorigenesis induced by the combination treatments with AOM and DSS. Apparently, Balb/c mice were extremely sensitive to AOM/DSS-induced colon carcinogenesis in the present experimental condition. The sensitivity of Balb/c mice observed in the present study was almost similar to those found in ICR mice (8,32,35). Colonic adenocarcinoma also developed in C57BL/6N, but the incidence was lower than in Balb/c. In contrast, the susceptibility of C3H/HeN and DBA/2N to the administration of AOM and DSS was quite low and only a few colonic adenomas developed in both the strains of mice.

Regarding the sensitivity of the mice to AOM initiation, the Balb/CJ mice were reported to have a remarkable susceptibility to the formation of distal colon tumors after treatment with AOM (26), whereas C3H, C57BL/6J, and DBA/2 mice were found to have a low incidence of colonic tumors by AOM initiation (25,26,29). Strain differences in the susceptibility to DSS have also been demonstrated: Balb/c, C3H/HeJ and C57BL/6J are relatively susceptible to DSS, whereas DBA/2J mice are virtually resistant based on the frequency of ulceration or the histological score of inflammation in the colon

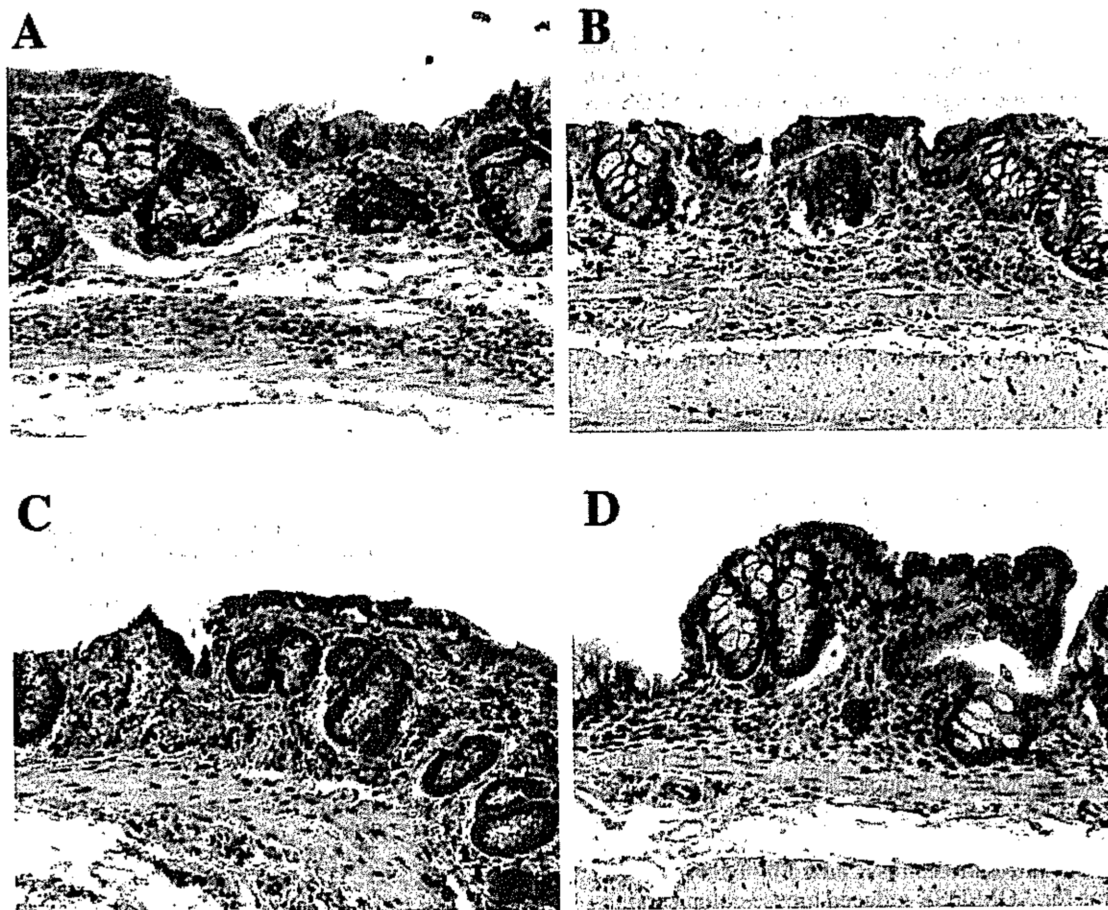

Fig. 6. Nitrotyrosine immunohistochemistry of the colon from four strains of mice given 1% DSS. (A) Balb/c; (B) C3H/HeN; (C) C57BL/6N; and (D) DBA/2N.Original magnification, (A–D), 20×.

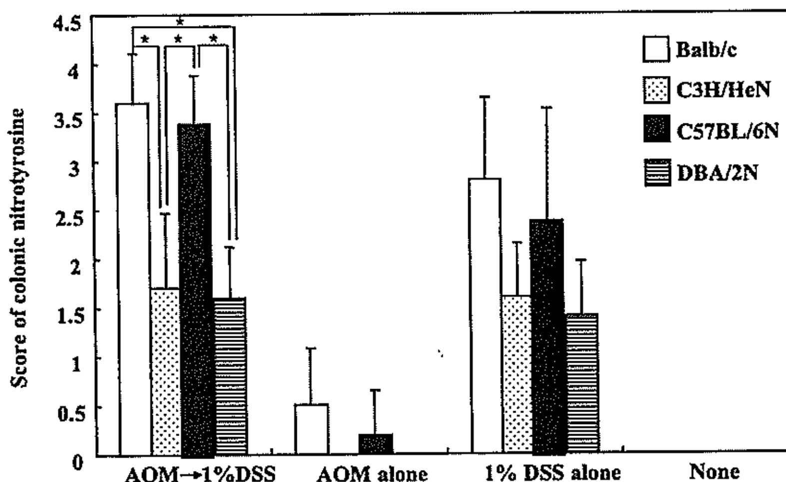

Fig. 7. Score for nitrotyrosine immunohistochemistry. Values are the mean ± SD. White column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *$P < 0.001$.

(27,28). In the current study, the sensitivities of the four strains to DSS were somewhat dissimilar to those of previous studies (27,28). The inflammation score of colonic mucosa revealed a severe and moderate inflammation to be present in the C3H/HeN and Balb/c mice treated with both AOM and DSS, respectively, while C57BL/6N and DBA/2N mice had only a relatively weak inflammation. In the case of the receptivity of C57BL mice to lipopolysaccharide (LPS), C57BL/10ScCr mice were resistant to LPS, whereas C57BL/10ScSn mice responded to LPS (37). Similarly, C3H/HeJ and C3H/HeN are LPS-responder and LPS-non-responder mice, respectively (38,39). As a result, the discrepancy in the response of DSS in mice might be due to differences in the substrains. In the current study, the highest incidence of colonic tumors was

found in Balb/c. C57BL/6N had the second highest incidence among the strains tested. On the other hand, C3H/HeN and DBA/2N had only a few benign colonic tumors (adenomas). The shortening of colon length in the mice that received DSS is one of the biological markers of severity of colonic inflammation (8–10,32,35). When comparing the colon length in mice treated with AOM and DSS with that in untreated mice, the order of the shortening rate of the colon length of mice was Balb/c (7%) > C57BL/6N (4%) > DBA/2N (3%) > C3H/HeN (−6%). These results suggest that the different susceptibilities of the inbred mouse strains to AOM/DSS-induced colon carcinogenesis might correlate with different sensitivities to AOM or DSS, with only slight contradictions among the substrains.

AOM is widely used as a colonic carcinogen to investigate the pathogenesis and modification of colon carcinogenesis in rodents (11–13). AOM requires metabolic activation to exert its carcinogenic action. Cytochrome P450 (CYP) is known to play a prominent role in the modulation of the xenobiotic metabolism, including chemical carcinogens. CYP 2E1 is one of the important factors for converting AOM to methylazoxymethanol, which can produce DNA adduct formation and also produce the initiation event (40,41). Although we did not investigate the activity of CYP 2E1, it may be possible that the expression and/or content of CYP 2E1 differ among the strains examined. This may be indicated by the findings that the relative liver weight of Balb/c, which had the highest susceptibility of AOM/DSS-induced colon carcinogenesis, was higher than that of other strains of mice in the current study (data not shown).

The influence of nitrosation stress caused by DSS is also an important factor for AOM/DSS-induced mouse colon carcinogenesis, since a powerful tumor-promoting activity of DSS has been observed in this model (8,32,35,42). We found a close association between the score of nitrotyrosine and the occurrence of tumors in the current study. Nitrotyrosine-immunohistochemical scores of each strain of mice in the 'AOM → DSS' and 'DSS alone' groups were much greater than those of the 'AOM alone' and 'untreated' groups. The scores of the 'AOM → DSS' group were relatively higher than those of the 'DSS alone' group in all strains of mice and the order was Balb/c > C57BL/6N > C3H/HeN > DBA/2N in these two groups. Such inflammation could influence tumorigenesis, although the inflammation score did not completely correspond with the frequency of colonic tumors in the current study. Indeed, the score of inflammation in the mice receiving both AOM and DSS was higher than that of the mice administered DSS alone. An investigation of additional factors is needed to precisely elucidate the strain differences in the susceptibility to colon carcinogenesis. Recently Greten *et al.* (43) reported interesting findings, namely that a specific inactivation of the IκB kinase (IKK)/NF-κB pathway can attenuate the formation of inflammation-associated colon tumors in *villin-Cre/Ikkβ*$^{F/\Delta}$ mice. They also suggested that IKKβ might be involved in inflammation-related carcinogenesis.

In conclusion, we herein demonstrated the differences in the genetic susceptibility to AOM/DSS-induced colon tumorigenesis among four inbred strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice and found the Balb/c mice to be the most sensitive. Our findings suggest that the genetic background thus plays an important role in the cancer risk in colitis-related colon tumorigenesis. In addition, strain differences in the susceptibility of colon carcinogenesis induced by AOM and DSS might be influenced by the response to nitrosation stress due to inflammation as determined by the genetic background.

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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

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Oral squamous cell carcinoma is one of the most common human neoplasms, and prevention of this malignancy requires a better understanding of its carcinogenesis process. To this end, we tried to establish an animal model using the human c-Ha-*ras* proto-oncogene-carrying transgenic (Tg) rats and the carcinogen 4-nitroquinoline 1-oxide (4-NQO). 4-NQO (20 p.p.m.) was administered to Tg and non-Tg rats for 8 weeks in their drinking water, and then the occurrence of tongue carcinogenesis was compared during the experimental period of 22 weeks. In addition, we determined the DNA ploidy in tongue lesions and examined the immunohistochemical expression of five biomarkers such as cyclin D1, glutathione *S*-transferase placental form, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and β-catenin. Next, the cancer chemopreventive effects of nimesulide, pioglitazone and a synthetic geranylated derivative, which have been reported to be inhibitors of tongue carcinogenesis, were examined in Tg rats treated with 4-NQO. Either during or after treatment with 4-NQO in the drinking water, tongue dysplasia and tumors were observed on the tongues of both Tg and non-Tg rats, with a greater incidence and multiplicity in Tg rats. Histopathologically, squamous cell dysplasia, papilloma and carcinoma with or without invasion were present in the tongue. Immunohistochemistry revealed that expression levels against five biomarkers increase with disease progression, and the changes correlated with those of the DNA ploidy pattern. Interestingly, a strong expression of COX-2, iNOS and β-catenin was observed on the invasive front of squamous cell carcinomas. A subsequent chemoprevention study using Tg rats showed that the chemicals tested suppressed the occurrence of tongue carcinomas when they were administered after 4-NQO-exposure. These results may thus indicate that our 4-NQO-induced Tg rat tongue carcinogenesis model simulates many aspects of human oral carcinogenesis and it can be applied for an analysis of oral cancer development while also helping to identify potentially effective cancer chemopreventive agents against oral cancer.

Abbreviations: CDK, cyclin-dependent kinase; COX, cyclooxygenase; DMBA, 7,12-dimethylbenz(*a*)anthracene; EGMP, ethyl 3-(4′-geranyloxy-3′-methoxyphenyl)-2-propenoate; GST-P, glutathione *S*-transferase placental form; H&E, hematoxylin and eosin; iNOS, inducible nitric synthase; NO, Nitric oxide; 4-NQO, 4-nitroquinoline 1-oxide; PPAR, peroxisome proliferator-activated receptor; SCC, squamous cell carcinoma; Tg rats, Human c-Ha-ras proto-oncogene carrying transgenic rats.

Introduction

Oral cancer, mostly squamous cell carcinoma (SCC), is considered to be one of the most common neoplasms in the world with nearly 390 000 new cases per year (1). This malignancy is particularly common in such developing countries as India, Sri Lanka, Vietnam, the Philippines and Brazil, where it constitutes up to 25% of all types of cancers (2). Recently, the oral cancer incidence and mortality rates have been increasing in the USA, Japan, Germany and Scotland, especially among young males (3–5). In addition, many patients tend to develop secondary primary tumors even if the primary tumors can be treated (6). This suggests the occurrence of a multi-focal tumor development, called 'field cancerization' (7). As a result, the development of cancer chemoprevention is an important strategy for fighting this malignancy (8–10), and an animal model for preclinical studies is warranted to clarify how best to control this epithelial malignancy.

Several animal models for oral carcinoma development were utilized, including hamster, rats and mouse models. The most commonly used model is 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced hamster cheek pouch carcinogenesis model (11) and ~60% of all SCC have a mutation in codon 61 of Ha-*ras* gene (12). Treatment by the administration of 4-nitroquinoline 1-oxide (4-NQO) in drinking water can induce tumors in oral cavities in rats (13) and mice (14). Oral SCCs induced by 4-NQO in rats, which shows morphological and histopathological similarities to those of human tumors, have been extensively used to investigate and test a wide variety of synthetic and natural agents for chemopreventive potential (8).

Mutations in the three *ras* genes and *p53* gene are observed in human cancers, including oral cancer (15–18). Activating mutations in K-*ras* and H-*ras* have been reported in human oral SCCs , primarily in those caused by exposure to carcinogens that are present in betel quid (19). In addition, *ras* activation involves murine oral squamous carcinogenesis (20,21), although some exceptions have been reported (22). The development of oral cancer appears to be a continuum, a progression from the early stage of oral lesions to SCC and metastasis. The results of animal model studies could thus translate directly or indirectly to clinical patients' care initiatives or at least allow targeted studies that make the best use of human clinical trials. 4-NQO-induced rat tongue carcinogenesis is such a model. In addition, genetically modified animal models are also useful

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for elucidating the molecular and cellular processes that lead to cancer initiation, progression and metastasis, and on the suitability to undergo therapeutic and chemopreventive trials (23–26).

The current study was conducted using the human c-Ha-*ras* proto-oncogene-carrying transgenic (Tg) rats highly susceptible to a variety of chemical carcinogens (27) to establish a more realistic animal model for oral carcinogenesis, which reveals both histological and immunohistochemical characteristics (28) similar to the human counterpart. In this study, the sequential observation up to 22 weeks of pathological alterations in the tongue of Tg and non-Tg rats treated with 20 p.p.m. 4-NQO in their drinking water for 8 weeks was conducted. The expression of cyclin D1 (28,29), glutathione *S*-transferase placental form (GST-P) (30) cyclooxygenase (COX)-2 (31), inducible nitric synthase (iNOS) (31) and β-catenin (32) in the tongue lesions induced by 4-NQO was immunohistochemically investigated, because these biological and molecular markers have been characterized in experimental models for oral tumors (12,30,32). A DNA ploidy pattern was also determined in the observed tongue lesions. In addition, to assess the utility of human c-Ha-*ras* proto-oncogene Tg rats in preclinical chemoprevention study on tongue cancer, a chemoprevention study was conducted using three agents, a COX-2 inhibitor nimesulide (31), a ligand for peroxisome proliferator-activated receptor (PPAR)γ pioglitazone (33) and a synthetic geranylated derivative ethyl 3-(4′-geranyloxy-3′-methoxyphenyl)-2-propenoate (EGMP) (34), which have been reported to exert cancer chemopreventive ability against 4-NQO-induced colon carcinogenesis in F344 rats. We have found previously the cancer chemopreventive ability of another PPARγ ligand troglitazone in 4-NQO-induced rat tongue carcinogenesis (29).

Materials and methods

Animals

Human c-Ha-*ras* proto-oncogene Tg and non-Tg rats bred by CLEA Japan (Tokyo, Japan) (35) 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). The animals were all allowed free access to a powdered basal diet CRF-1 (Oriental Yeast, Tokyo, Japan) and to tap water. The experiments were conducted according to the 'Guidelines for Animal Experiments in Kanazawa Medical University'.

Development of animal model

A total of 69 Tg and non-Tg rats were used for the experiment after 1 week quarantine. In this experiment, which was designed to monitor the development of preneoplastic and neoplastic tongue lesions, 33 Tg rats (14 males and 19 females) and 36 non-Tg rats (20 males and 16 females) were given tap water containing 20 p.p.m. 4-NQO (98% pure, CAS no. 56-57-5, Wako Pure Chemical, Osaka, Japan) for 8 weeks, and thereafter they received no further treatments (Figure 1). The animals were sequentially killed at Week 8 (2 Tg males, 2 Tg females, 2 non-Tg males and 2 non-Tg females), Week 10 (1 Tg male, 1 Tg female, 2 non-Tg males and 1 non-Tg female), Week 12 (2 Tg males, 2 Tg females, 2 non-Tg males and 2 non-Tg females), Week 14 (2 Tg males, 3 Tg females, 2 non-Tg males and 2 non-Tg females), Week 18 (2 Tg males, 3 Tg females, 2 non-Tg males and 2 non-Tg females) and Week 22 (5 Tg males, 8 Tg females, 5 non-Tg males and 7 non-Tg females) to determine the occurrence of tongue preneoplasms and neoplasms. After killing by exsanguination under deep ether anesthesia, macroscopic observations were performed and the number of grossly visible tumors in the tongue and esophagus were recorded, and then these organs were processed for histopathological examination after being fixed in 10% buffered formalin. The tongues with or without lesions were also processed to assess the expression of cell proliferation biomarkers 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 (H&E). Epithelial lesions (hyperplasia, dysplasia and neoplasia) in the oral cavity were diagnosed according to the criteria described by Banoczy and Csiba (36) and Kramer *et al.* (37). To determine the multiplicity of the tongue lesions, the tongue was examined for gross lesions without the use of any magnification aid. The tongue was cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin. Each tissue was totally submitted as multiple transverse sections for histological processing. This averaged 5–6 pieces/tissue and 10–12 pieces/total tongue. The tongue lesions were counted on all slides stained with H&E, then the sum was divided by the number of slides, and they were expressed as the mean ± SD.

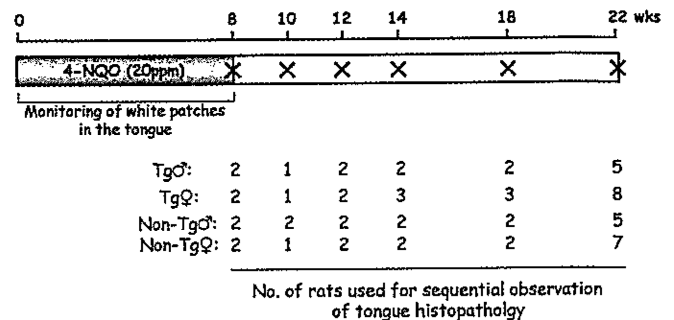

Fig. 1. Experimental protocol. X, killing time points. Nos, number of Tg and non-Tg rats that are killed for tongue histopathology.

Immunohistochemistry for cyclin D1, GST-P, COX-2, iNOS and β-catenin

After killing, the tongues were removed and processed to make tissue sections. Paraffin-embedded tongue tissues were cut and nine serial sections (3 μm) were made. One section was used for the histopathology, and the others for the immunohistochemistry of cyclin D1, GST-P, COX-2, iNOS and β-catenin. Immunohistochemistry for these antibodies was performed using a stain system kit ENVISION+ (K4003 or K4001, DakoCytomation, Kyoto, Japan). Primary antibodies used were as follows: a mouse monoclonal antibody against cyclin D1 (NCL-CYCLIN D1-GM, 1:100 dilution, Novocastra Laboratories, Newcastle upon Tyne, UK), a rabbit polyclonal antibody against GST-P (Cat. no. 311, 1:300 dilution, Medical and Biological Laboratories, Nagoya, Japan), a rabbit polyclonal antibody against COX-2 (Cat. no. 210726, 1:200 dilution, ALEXIS JAPAN, Tokyo, Japan), a rabbit polyclonal antibody against iNOS (Cat. no. ab15326, diluted, Abcam, Cambridge, UK) and a rabbit polyclonal antibody against β-catenin (Cat. no. sc-7199, 1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies were applied to the sections according to the manufacturer's protocol. As negative controls, adjacent sections were processed by omitting incubation with the primary antibodies (cyclin D1, GST-P, COX-2, iNOS and β-catenin). The slides were subsequently reviewed in a blinded fashion.

The cells were considered positive for cyclin D1 when definite nuclear staining was identified. Positive cell ratios for cyclin D1 were calculated by counting at least 50 cells in the tongue lesions of each rat. The immunohistochemical expression of GST-P, COX-2, iNOS and β-catenin was observed with the grading intensity of the immunoreactivity in neoplasms and preneoplastic lesions of the tongue. Lesions with a stainability in >1% of the cells were regarded as positive, whereas all others were regarded as negative. To quantitate the degree of stainability for each antibody, the grading system was used according to the following criteria described by (−), no immunoreactivity; (±, +, ++), increasing degrees of intermediate immunoreactivity; and (+++), extensive immunoreactivity. Care was taken to exclude the possibility of any inflammatory cells that were mistakenly identified as positive epithelial cells.

DNA ploidy analysis

Tongue lesions developed in Tg males that received 4-NQO and then were killed at Week 22 were used for the DNA ploidy analysis. Sections of 5 μm adjacent to H&E and immunohistochemically stained sections were processed for Feulgen's staining (38) after hydrolysis with 1 N HCl. The areas previously selected on the H&E sections were identified on projections of the Feulgen-stained sections. A microspectrophotometer (MMSP, Olympus, Tokyo, Japan) was used for the measurement of nuclear DNA content. The optical setting was as follows: Objective, Plan 40×; Condenser, 20×; Measuring spot, 7–12 μm; Wavelength, 545 nm. More than 50 cells were measured in each lesion and 100 nuclei in SCC. The DNA content of the lymphocytes contained in the section was taken as the diploid (2C) reference value (39). Ploidy histograms

(frequency of occurrence of individual cell ploidy values) were constructed for each histological category. Aneuploidy was objectively computed on the basis of the algorithm (40) as the 5C exceeding rate that is defined as the percentage of cells with a DNA content of >5C. Finally, the mean ploidy and mean rates (%) of <2.5C, 2.5–5C and >5C for each lesion category were calculated.

Chemoprevention study using Tg rats

For 8 weeks, 15 Tg males and 24 Tg females were given 4-NQO (20 p.p.m. in drinking water). Tg males were then divided into two groups: Group 1 (8 males) received no further treatment and Group 2 (7 males) was fed the experimental diet containing 400 p.p.m. nimesulide (Sigma Chemical, St Louis, MO) for 11 weeks, starting 1 week after cessation of 4-NQO exposure. In addition, 24 Tg females were then divided into three groups: Group 3 (7 females) received no further treatment. Groups 4 (9 females) and 5 (8 females) were fed the experimental diets mixed with 500 p.p.m. pioglitazone (Takeda Chemical Industries, Osaka, Japan) and 2% EGMP (96% purity, synthesized from ferulic acid) for 11 weeks, respectively, starting 1 week after the cessation of 4-NQO exposure. At Week 20, all Tg rats were killed by exsanguination under deep ether anesthesia, and macroscopic inspection was done. After killing, number of grossly visible tumors in the tongue and other tissues were recorded, and then these organs with lesions were processed for a histopathological examination after fixation in 10% buffered formalin. For histological examinations, tissue specimens and gross lesions fixed in 10% buffered formalin were embedded in paraffin blocks, and the histological sections were stained with H&E. The tongue was cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin. Epithelial lesions (hyperplasia, dysplasia and neoplasia) in the tongue were diagnosed according to the criteria described by Banoczy and Csiba (36) and Kramer *et al.* (37). To determine the multiplicity of the tongue lesions, the tongue was examined for gross lesions without the use of any magnification aid. Each tissue specimen was totally submitted as multiple transverse sections for histological processing. This averaged 5–6 pieces/tissue and 10–12 pieces/total tongue. The tongue lesions were counted on all slides stained with H&E, the sum was then divided by the number of slides, and the multiplicity was expressed as the mean ± SD.

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

Monitoring of white patches

All rats were well tolerated with oral exposure of 4-NQO in drinking water. During the study, white patches were mainly present in the dorsal site of the root of tongue. When monitored, the number of white patches (clinically called as leukoplakia) in the tongues of Tg and non-Tg rats under light ether anesthesia, starting from Week 2 to Week 8, and the frequency of such white patches gradually increased over time in both Tg and non-Tg rats of either sex between Week 2 and Week 6 (Figure 2). However, the number of lesions in the Tg rats dramatically increased between Week 6 and Week 8 (Figure 2). In addition, the frequency of males was larger than females, in both Tg and non-Tg. At Week 8, five small (<2 mm in diameter) and two large (>3 mm in diameter) white patches in Tg males were biopsied to determine their histopathology. The former were histologically found to be dysplastic leukoplakia while the latter were minute SCC.

Incidence and multiplicity of tongue neoplasms and dysplasia

Exophytic papillary or invasive tongue tumors (Figure 3A) were mainly observed in the dorsal site of the root of tongue. Dysplastic lesions with various degrees of atypia (Figure 4B-1–D-1) were also found in the tongue. Tongue tumors were histopathologically squamous cell papilloma and carcinoma with or without invasion (Figure 4E-1–G-1). The incidence and multiplicity of tongue neoplasms at each killing point are given in Table I and is illustrated in Figure 3B. All Tg males killed at Weeks 8, 10, 12, 14, 18 and 22 had tongue neoplasms (squamous cell papilloma and carcinoma). Non-Tg males that were killed at Weeks 8, 10 and 14 did not have any tongue tumors, but they had dysplastic lesions in the tongue. As for non-Tg males, one of two rats killed at Week 12, two of two rats killed at Week 18 and two of five rats killed at Week 22 developed tongue papilloma and/or carcinoma. Tg females killed at Week 8 (one of two rats), Week 12 (one of two rats), Week 14 (two of three rats), Week 18 (all three rats) and Week 22 (five out of eight rats) had tongue neoplasms (squamous cell papilloma and/or carcinoma), but one Tg female killed at Week 10 did not have any tongue tumors. Non-Tg females that were killed at Weeks 8, 10 and 12 did not develop any tongue tumors, but they did have tongue dysplastic lesions. Two of two non-Tg females killed at Week 14, one of two non-Tg females killed at Week 18 and

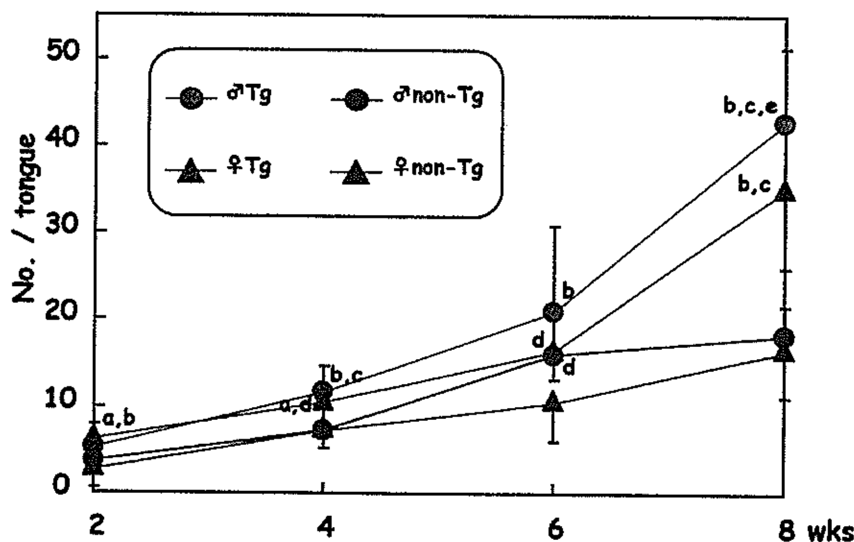

Fig. 2. Mean number of white patches in the tongue that were monitored from Week 2 through Week 8. a, significantly different from male non-Tg ($P < 0.05$); b, significantly different from female non-Tg ($P < 0.001$); c, significantly different from male non-Tg ($P < 0.001$); d, significantly different from female non-Tg ($P < 0.05$); and e, significantly different from female Tg ($P < 0.05$).

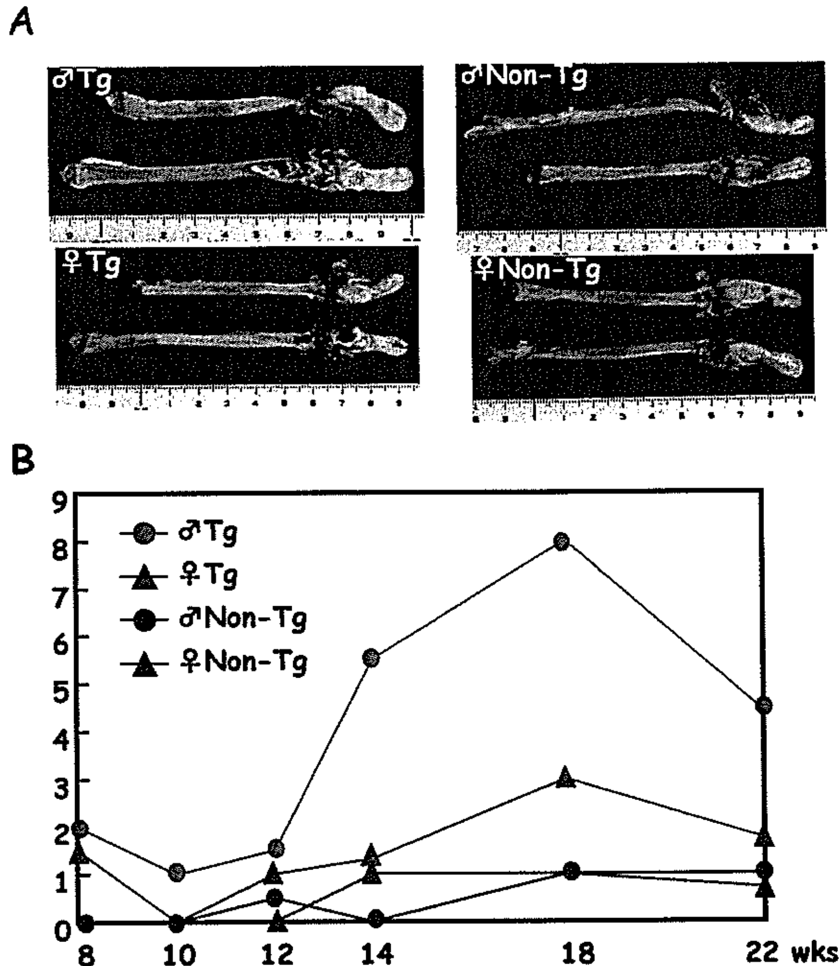

Fig. 3. Tongue neoplasms developed in Tg and non-Tg rats that were treated with 4-NQO. (A) Macroscopic view of tongues at Week 22. Large tongue tumors with white patches develop in Tg rats of either sex, whereas only a few small tongue tumors or white patches are found in the tongue in non-Tg rats of both sexes. (B) Mean multiplicity (number of tumors/rat) of tongue tumors at each killing time point. Number of tongue tumors increased from Week 14 in male Tg rats.

two of seven non-Tg females killed at Week 22 developed papilloma and/or carcinoma. Besides neoplasms, the Tg and non-Tg rats of both sexes also had tongue dysplasia with different degrees of atypia and multiplicity: the occurrence of lesions in Tg rats was earlier than in non-Tg rats, and the frequency in Tg rats was greater than in non-Tg rats (data not shown).

In other organs, esophageal tumors and/or mammary tumors were present in Tg rats. One papilloma and two carcinomas in a Tg male killed at Week 18. In addition, two esophageal papillomas developed in a Tg male and one esophageal carcinoma in each of two Tg males that were killed at Week 22. In two Tg females, esophageal papillomas developed (one had one papilloma and the other two papillomas). However, the non-Tg rats did not develop tumors in tissues other than the tongue.

Immunohistochemistry of cyclin D1, COX-2, iNOS, GST-P and β-catenin

Immunoreactivity against five antibodies (cyclin D1, COX-2, iNOS, GST-P and β-catenin) was closely similar in the non-lesional areas and lesions in the tongue of Tg and non-Tg males and females. Their expression was found in the nucleus, cytoplasm and/or cell membrane (Figure 4).

Cyclin D1 immunohistochemistry revealed that a few basal cells in the non-lesional 'normal' appearing areas were positive for nuclei (Figure 4A-2). Their nuclear stainability increased with the disease progression from dysplasia/ papilloma to carcinoma (Figure 4B-2–G-2). The cyclin D1-positive ratio reflecting the rate of proliferation was the same in preneoplastic lesions dysplasia, and it reached the highest values in tumors (Table II).

Strong GST-P immunoreactivity was present in the nucleus and cytoplasm of all dysplastic lesions and carcinomas developed in rats treated with 4-NQO (Figure 4A-3–G-3). Papilloma cells were also positive for GST-P, but their intensity was weak in comparison with dysplastic and carcinoma cells (Figure 4E-3). Non-lesional cells at the lower part of squamous epithelium were weakly positive for GST-P (Figure 4A-3). The multiplicity of GST-P-positive dysplastic lesions during Week 8 through Week 22 is given in Table III. The highest multiplicity of GST-P-positive dysplastic lesions was observed at Week 12 in Tg males, at Week 8 in Tg females, at Week 18 in non-Tg males, and at Weeks 14 and 18 in non-Tg females.

COX-2 immunoreactivity was weakly present in the basal cells of the 'normal' appearing areas (Figure 4A-4–G-4). Dysplasia with mild, moderate and severe atypia

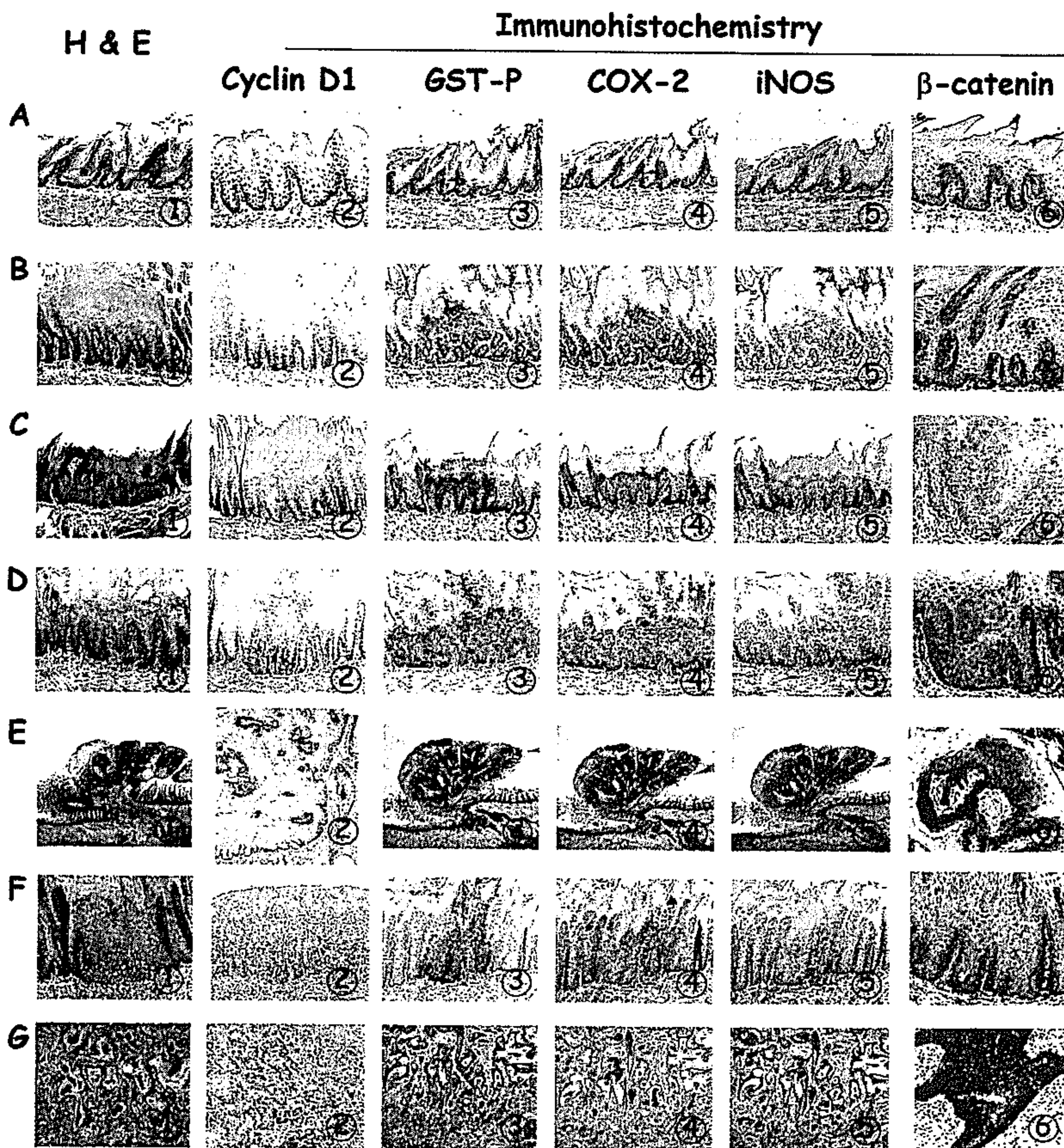

Fig. 4. Histopathology and immunohistochemistry of cyclin D1, GST-P, COX-2, iNOS and β-catenin of tongue lesions in Tg males that received 4-NQO. (A), 'Normal' appearing tongue squamous epithelium; (B) Mild dysplasia; (C) Moderate dysplasia; (D) Severe dysplasia; (E) Squamous cell papilloma; (F) SCC *in situ* and (G) Invasive SCC. (A-1), (B-1), (C-1), (D-1), (E-1), (F-1) and (G-1), H&E stain; (A-2), (B-2), (C-2), (D-2), (E-2), (F-2) and (G-2), cyclin D1 immunohistochemistry; (A-3), (B-3), (C-3), (D-3), (E-3), (F-3) and (G-3), GST-P immunohistochemistry; (A-4), (B-4), (C-4), (D-4), (E-4), (F-4) and (G-4), COX-2 immunohistochemistry; (A-5), (B-5), (C-5), (D-5), (E-5), (F-5) and (G-5), iNOS immunohistochemistry; and (A-6), (B-6), (C-6), (D-6), (E-6), (F-6) and (G-6), β-catenin immunohistochemistry. Five biomarkers (cyclin D1, GST-P, COX-2, iNOS and β-catenin) are expressed in the squamous cell lesions of these tongues and their expression increases with the disease progression. Original magnification, (A-1, A-3–5), (B-1–6), (C-1–6), (D-1–6), (F-1–5), and (G-1–5), 10×; (A-2), (A-6), (E-6), (F-6) and (G-6), 20×; and (E-1–5), 2×.

(Figure 4B-4–D-4) showed a relatively strong positivity for COX-2 in nucleus and/or cytoplasm of cells in the upper and middle parts of the epithelium. Approximately half of all papilloma cells (Figure 4E-4) and most cancer cells (Figure 4F-4 and G-4) showed COX-2-positivity in the nucleus and/or cytoplasm. Interestingly, a strong expression of COX-2 was found in the invasion front of carcinoma, where a number of mononuclear inflammatory cells were present.

iNOS-immunohistochemistry showed a weak immunoreactivity in the nucleus and/or cytoplasm of dysplastic (Figure 4B-5–D-5) and carcinoma cells (Figure 4F-5 and G-5): the intensity was greater in the carcinoma cells than in the dysplastic cells. Similar to the COX-2 expression, a strong expression of iNOS was noted in the invasion front of cancer tissue, where numerous inflammatory cells had infiltrated. In addition, papilloma cells were positive for iNOS in their

Table I. Incidence and multiplicity of tongue neoplasms in Tg and non-Tg rats that received 4-NQO

Tg/non-Tg	Sex	Incidence of tongue neoplasms (multiplicity, mean or mean ± SD)					
		Week 8	Week 10	Week 12	Week 14	Week 18	Week 22
Squamous cell papilloma							
Tg	Male	2/2 (1)	0/1 (0)	0/2 (0)	2/2 (3)	2/2 (2.5)	5/5 (1.40 ± 0.80)
Tg	Female	2/2 (1)	0/1 (0)	1/2 (0.5)	2/3 (0.67 ± 0.47)	2/3 (0.67 ± 0.47)	3/8 (0.63 ± 0.99)
Non-Tg	Male	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	2/2 (1)	2/5 (0.60 ± 0.80)
Non-Tg	Female	0/2 (0)	0/1 (0)	0/2 (0)	2/2 (1)	1/2 (0.5)	2/7 (0.29 ± 0.45)
Squamous cell carcinoma							
Tg	Male	2/2 (1)	1/1 (1)	2/2 (1.5)	2/2 (2.5)	2/2 (5.5)	5/5 (3.00 ± 1.67)
Tg	Female	1/2 (0.5)	0/1 (0)	1/2 (0.5)	2/3 (0.67 ± 0.47)	3/3 (2.33 ± 0.47)	5/8 (1.13 ± 1.17)
Non-Tg	Male	0/2 (0)	0/2 (0)	1/2 (0.5)	0/2 (0)	0/2 (0)	2/5 (0.40 ± 0.49)
Non-Tg	Female	0/2 (0)	0/1 (0)	0/2 (0)	0/2 (0)	1/2 (0.5)	2/7 (0.43 ± 0.73)

Table II. Cyclin D1-positive index in various tongue lesions from male Tg and non-Tg rats treated with 4-NQO at Week 22

Lesions	Male Tg	Male non-Tg
'Non-lesional' area	2.6 ± 0.9[a] (10)	6 ± 0.9 (10)
Dysplasia		
Mild	5.4 ± 1.1[b] (10)	4.2 ± 0.8 (10)
Moderate	7.6 ± 1.7[c] (10)	6.2 ± 2.4[c] (10)
Severe	27.0 ± 5.6[d,e,f] (10)	24.8 ± 4.4[d,e,f] (10)
Papilloma	17.6 ± 2.1[d,e,f,g] (5)	16.2 ± 4.2[d,e,f,g] (5)
Carcinoma	38.8 ± 4.0[d,e,f,g] (5)	37.4 ± 6.6[d,e,f,g,h] (5)

Numbers in parentheses are number of lesions examined.
[a]Mean ± SD.
[b,c,d]Significantly different from 'non-lesional' area ([b]$P < 0.005$, [c]$P < 0.001$ and [d]$P < 0.001$)
[e]Significantly different from 'mild dysplasia' ($P < 0.001$).
[f]Significantly different from 'moderate dysplasia' ($P < 0.001$).
[g]Significantly different from 'severe dysplasia' ($P < 0.001$).
[h]Significantly different from 'papilloma' ($P < 0.001$).

Table III. Multiplicity of GST-P-positive dysplasia at each killing time point

	Week 8	Week 10	Week 12	Week 14	Week 18	Week 22
Male Tg	5.5[a] (2)	3.0 (1)	6.5 (2)	6.0 (2)	5.5 (2)	4.00 ± 2.28 (5)
Female Tg	6.5 (2)	6.0 (1)	5.0 (2)	4.7 (3)	5.0 (3)	4.00 ± 2.55 (8)
Male non-Tg	0.5 (2)	2.5 (2)	3.5 (2)	4.0 (2)	6.5 (2)	5.80 ± 2.14 (5)
Female non-Tg	3.5 (2)	3.5 (1)	4.0 (2)	5.0 (2)	5.0 (2)	4.57 ± 2.32 (7)

Numbers in parentheses are number of rats examined.
[a]Mean or mean ± SD.

nucleus/cytoplasm, but the intensity was relatively lower than in cancer cells (Figure 4E-5). A faint positive reaction of iNOS was found in the nucleus/cytoplasm of the non-lesional cells at the basal layer (Figure 4A-5).

β-Catenin staining in the non-lesional cells was weakly positive in their cell membrane, but not in the nucleus (Figure 4A-6). Their nuclear/cytoplasm/cell membrane stainability increased with disease progression from dysplasia/papilloma to carcinoma (Figure 4B-6–G-6). Among these lesions, carcinoma cells expressed the strongest intensity of β-catenin staining in their nuclear/cytoplasm/cell membrane (Figure 4F-6 and G-6). Interestingly, cancer cells at the invasion front expressed the cytoplasmic/nuclear pattern of β-catenin. Some papilloma cells were also positive for β-catenin in their nuclear/cytoplasm/cell membrane.

Table IV summarizes the immunoreactive intensity of five antibodies (cyclin D1, COX-2, iNOS, GST-P and β-catenin). Among the antibodies, GST-P positivity was the most prominent of all the lesions.

DNA ploidy of the tongue lesions

When determining the DNA content, ploidy histograms tended to skew to the right from the diploid value in all the histological categories. This skew increased in relation to the severity of the lesions investigated. Figure 5 shows an example of the characteristic histograms of the tongue lesions that developed in the Tg males receiving 4-NQO. Interestingly most histologically normal-appearing tongue squamous epithelium specimens showed a peak with a small deviation within the 2C–4C region and some events exceeded the tetraploid value. The mean percentages of ploidy distribution are shown in Table V. As expected, invasive carcinomas and carcinomas *in situ* exhibited a statistically significant increase in comparison with the non-lesional epithelium specimens. The mean ploidy values were around the tetraploid range. However, a considerable number of cells exhibited an aneuploid DNA content in the dysplasia and carcinoma. The results for mild dysplastic areas and histologically 'normal appearing' areas were remarkable. The mean ploidy value was significantly higher than for the 'normal appearing' areas, and aneuploid cells were detected in all of the evaluated lesions.

Effects of nimesulide, pioglitazone and EGMP on 4-NQO-induced tongue carcinogenesis in Tg rats

The influence of the dietary administration of the three tested chemicals (nimesulide, pioglitazone and EGMP) on tongue carcinogenesis initiated with 4-NQO in Tg rats of both sexes is illustrated in Figures 6 and 7. The administration of all the test chemicals reduced the incidence of tongue carcinoma in comparison with that in the Tg rats, which received 4-NQO alone, but the differences were not statistically significant (Figures 6A and 7A). However, the dietary administration of 400 p.p.m. nimesulide significantly lowered the multiplicity of tongue SCC (73% inhibition, $P < 0.05$, Figure 6B). In addition, a significant inhibition in the multiplicity of tongue SCC by feeding with 500 p.p.m. pioglitazone (61% inhibition, $P < 0.001$) and by dietary feeding with 2% EGMP (34% inhibition, $P < 0.01$) was observed as shown in Figure 7B. In addition, all the test chemicals in the diet reduced the development of severe tongue dysplasia (data not shown).

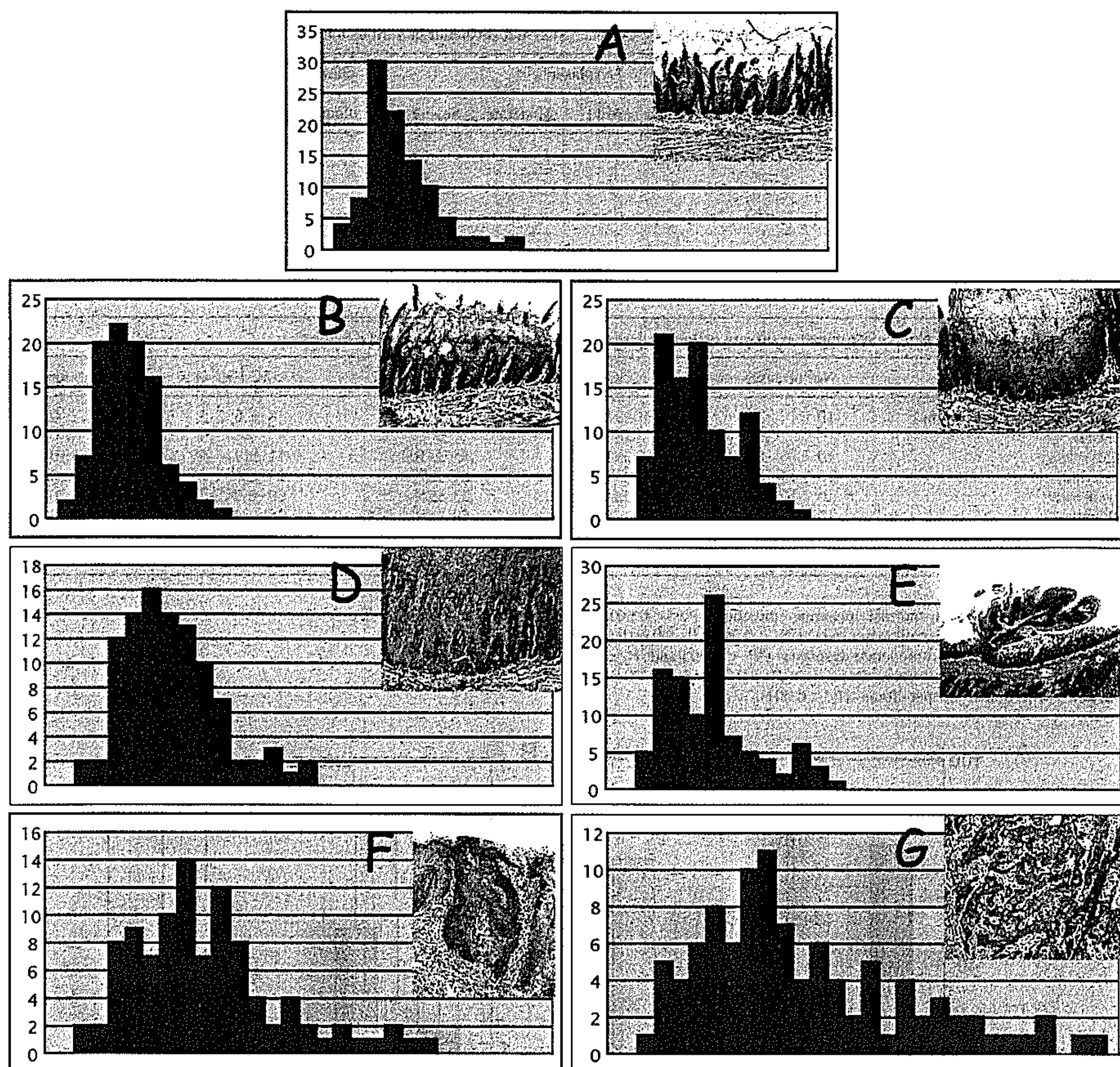

Fig. 5. Representative DNA histograms from various tongue lesions (inserts) in male Tg rats treated with 4-NQO. (A) 'Normal' appearing tongue squamous epithelium; (B) Mild dysplasia; (C) Moderate dysplasia; (D) Severe dysplasia; (E) Squamous cell papilloma; (F) SCC *in situ* and (G) Invasive SCC. DNA aneuploidy is evident with the disease progression. Inserts: tongue lesions stained with H&E. Original magnification, (A)–(D), (F) and (G), 10×; and (E), 2×.

Administration of all the test chemicals in the diet did not affect the tumor incidence and multiplicity, which were quite low, in organs other than the tongue (data not shown).

Discussion

In the current study, 4-NQO treatment rapidly induced tongue lesions (dysplasia and neoplasms) in Tg males and females, in comparison with non-Tg males and females. Our model can be utilized for the rapid assessment of the modifying effects (inhibition and/or enhancement) of xenobiotics on oral carcinogenesis. In fact, our results in a chemoprevention study using this animal model with a 20 week experimental period, where a COX-2 specific inhibitor, a PPARγ ligand and a synthetic geranylated derivative suppressed the multiplicity of the tongue carcinomas induced by 4-NQO, confirmed our previous findings using a 4-NQO-induced rat tongue carcinogenesis model with a 30 or 32 week experimental period (29,31,34). Another novel finding of the present work is the association between the immunohistochemical overexpression of five biomarkers (cyclin D1, GST-P, COX-2, iNOS and β-catenin) and DNA aneuploidy in dysplastic and neoplastic tongue lesions.

Malignant neoplastic cells possess an indefinite proliferative capability, thus being able to elude a commitment to terminal differentiation and post-mitotic quiescence that normally regulates tissue homeostasis in an organism. In order to achieve a proliferative autonomy, malignant neoplastic cells have to

Table IV. Summary of immunohistochemistry of cyclin D1, GST-P, COX-2, iNOS and β-catenin in various tongue lesions

Antibodies	'Normal' appearing epithelium	Hyperplasia		Dysplasia			Neoplasms		
		Simple	Papillary or nodular	Mild	Moderate	Severe	Papilloma	Carcinoma *in situ*	Invasive carcinoma
Cyclin D1	−	+	+	+	+	+	+	+++	+++
GST-P	−	±	±–+	+	++	++	+	+++	+++
COX-2	−	±	±–+	+	+	++	+	++	+
iNOS	−	±	±–+	±–+	±–+	+	+	+	+++
β-catenin	+	+	+	+	++	+++	+	+++	+++

−, <5% of positive cells; ±, 5–24% of positive cells: +, 25–50% of positive cells; ++, 51–75% of positive cells; and +++, >75% of positive cells.

Table V. Summary of DNA ploidy determination of various tongue lesions of tg males that received 4-NQO alone

Variety of tongue lesions	No. of cases	<2.5C (%)	2.5C–5C (%)	>5C (%)	Mean ploidy
'Normal' appearing-epithelium	10	60 ± 8^{a}	32 ± 6	8 ± 5	2.57 ± 0.24
Dysplasia					
Mild	10	45 ± 11^{b}	45 ± 10^{b}	11 ± 9^{c}	3.15 ± 0.31^{d}
Moderate	10	$25 \pm 4^{b,e}$	51 ± 7^{b}	$24 \pm 6^{b,e}$	$3.48 \pm 0.35^{b,f}$
Severe	10	$18 \pm 4^{b,e,h}$	23 ± 5^{e}	$60 \pm 7^{b,e}$	$4.75 \pm 0.33^{b,e,i}$
Papilloma	10	$25 \pm 5^{b,e,j}$	$50 \pm 5^{b,k}$	$25 \pm 4^{b,e,k}$	$3.73 \pm 0.64^{b,g,h}$
CIS	10	$12 \pm 5^{b,e,i,j,l}$	$25 \pm 8^{b,e,i,l}$	$63 \pm 8^{b,e,i,l}$	$5.01 \pm 0.58^{b,e,i,l}$
Invasive	10	$9 \pm 2^{b,e,i,k,l}$	$27 \pm 5^{e,i,l}$	$65 \pm 5^{b,e,i,l}$	$5.24 \pm 0.45^{b,e,i,j,l}$

[a]Mean ± SD.
[b-d]Significantly different from 'normal appearing-epithelium' ($^{b}P < 0.001$, $^{c}P < 0.005$ and $^{d}P < 0.001$).
[e-g]Significantly different from 'mild dysplasia' ($^{e}P < 0.001$, $^{f}P < 0.001$ and $^{g}P < 0.01$).
[h-i]Significantly different from 'moderate dysplasia' ($^{h}P < 0.005$ and $^{i}P < 0.01$).
[j,k]Significantly different from 'severe dysplasia' ($^{j}P < 0.05$ and $^{k}P < 0.01$).
[l]Significantly different from 'papilloma' ($P < 0.001$).

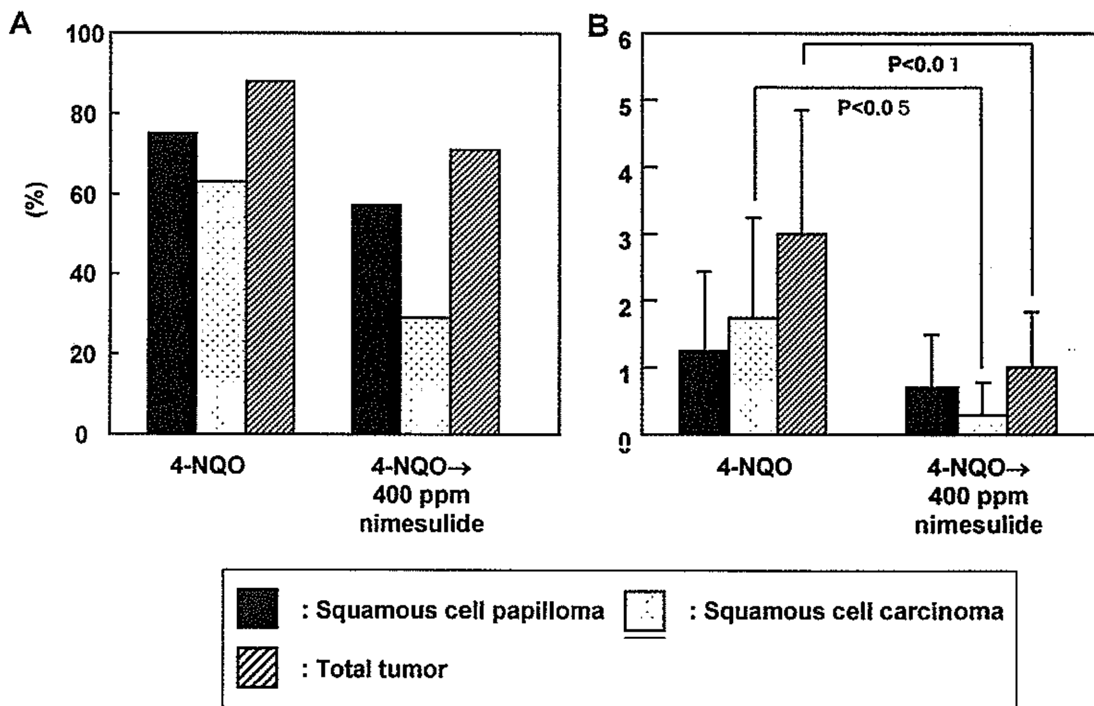

Fig. 6. The effect of dietary nimesulide (400 p.p.m.) on tongue neoplasms in male Tg rat. (A) Incidence (%) of tongue carcinomas and (B) multiplicity (no. of carcinoma/rat) of tongue carcinoma. Treatment with nimesulide reduced the incidence and multiplicity of tongue tumors. The inhibition in the multiplicities of carcinoma and total tumor (papilloma + carcinoma) was significant ($P < 0.05$ for carcinoma and $P < 0.01$ for total tumor).

either switch to an autocrine production of mitogenic factors or acquire activating mutations within the components of the signal transduction pathways that mediate mitogenic signaling. An example of this is the activating mutations of the small GTPase *Ras* (41). The uncontrolled proliferation of malignant neoplastic cells is also frequently achieved by the direct deregulation of cell cycle control. Mitogenic signaling ultimately leads to the upregulated expression of cyclins that

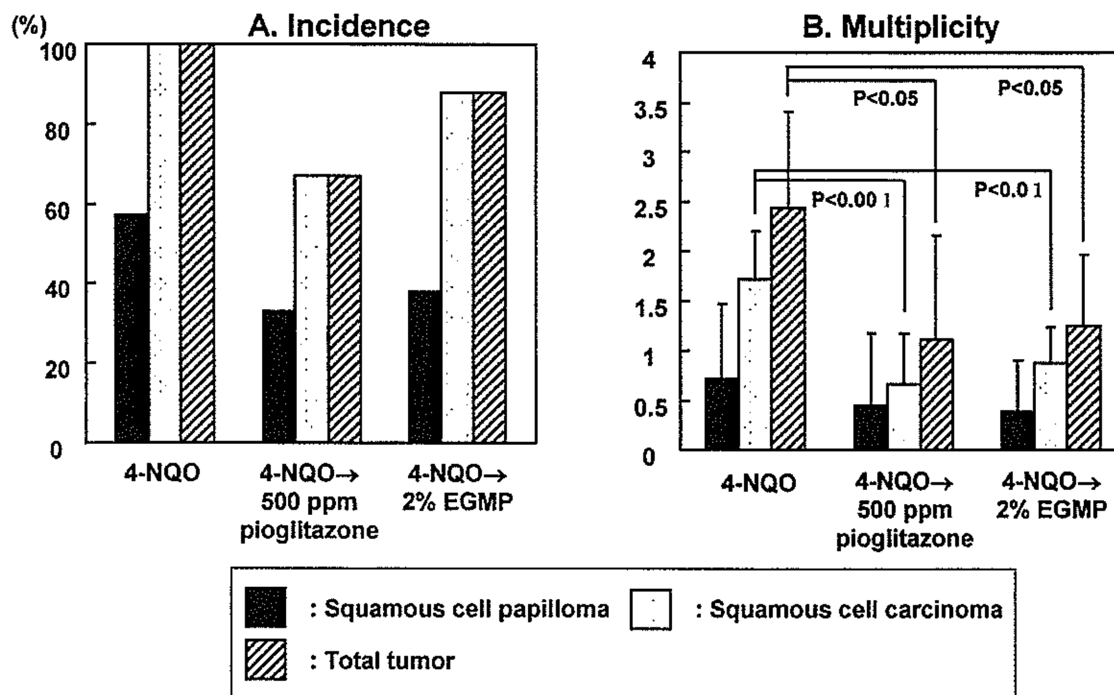

Fig. 7. The effect of dietary feeding with pioglitazone (500 p.p.m.) and EGMP (2%) on tongue neoplasms in female Tg rats. (A) The incidence (%) of tongue carcinomas and (B) multiplicity (number of carcinoma/rat) of tongue carcinoma. Treatment with pioglitazone or EGMP reduced the incidence and multiplicity of the tongue tumors. The inhibition in the multiplicities of carcinoma and total tumor (papilloma + carcinoma) was significant (pioglitazone, $P < 0.001$ for carcinoma and $P < 0.05$ for total tumor; and EGMP, $P < 0.01$ for carcinoma and $P < 0.05$ for total tumor).

together with their catalytic partners, cyclin-dependent kinases (CDKs), hyperphosphorylate the products of the retinoblastoma tumor suppressor gene family.

Cyclin D1 plays an important role in the transition from the G_1 to the S phase of the cell cycle, and perturbations in this control point can lead to neoplastic transformation (42). In fact, cyclin D1 is frequently overexpressed in both human (43) and rat oral tumors (28,29), and it is thought to be an important factor in their development. In this study, we immunohistochemically determined the cyclin D1 expression in sections from tongue lesions induced by 4-NQO in Tg or non-Tg rats and observed a few of cyclin D1-positive cells in the 'normal' appearing basal layer in the tongues of Tg rats after 4-NQO treatment. However, cyclin D1 staining was observed in dysplastic and neoplastic lesions with high expression levels (overexpression) in cancer cells. In contrast, papilloma cells showed a weaker expression of cyclin D1 than did dysplastic and cancer cells. This is in good agreement with our previous findings that were based on male F344 rats treated with 4-NQO (28,29). As a result, an overexpression of cyclin D1 is common in the early lesions that ultimately form malignant oral cancers but not in those that form benign tumors (44). Interestingly, the *ras* oncogene can induce the expression of cyclin D (45,46).

GST-P is a useful biomarker for detecting preneoplastic lesions in rat hepatocarcinogenesis (47). In oral carcinogenesis, an alteration in the immunohistochemical expression of GST-P was also reported in the hamster buccal pouch (48) and rat tongue (49), and this expression became stronger with tumor progression. Our findings on GST-P expression in tongue dysplasia and neoplasms are in accordance with these reports. As found in human oral lesions (dysplasia and neoplasms) that are positive for human placental form of GST (GST-π) (49), nuclear/cytoplasmic staining was more frequently noted in severe dysplasia and carcinoma than in papilloma and mild/moderate dysplasia in the present study. This biological marker, thus, can be applied to the early detection of preneoplastic tongue lesions (dysplasia) in humans.

Recently, much attention has been paid to the role of COX-2 in carcinogenesis (50). COX-2 can influence several processes important to cancer development. The inducibility of COX-2 is partly explained by the presence of numerous *cis*-acting elements in the 5′-flanking region of the *COX*-gene (51). COX-2 is generally not found in the normal epithelium but increases in response to mitogens, pro-inflammatory cytokines and growth factors, and it has also been linked to carcinogenesis (52). In the current study, we found the immunohistochemical COX-2 expression to be upregulated in premalignant and malignant lesions, as given by their aberrant DNA content. In addition, a specific COX-2 inhibitor nimesulide in the diet could inhibit 4-NQO-induced tongue carcinogenesis, as shown in this study with Tg rats and in our previous experiment with F344 rats (31). These findings may indicate that an upregulation of COX-2 is related to the development of oral carcinomas (28,29,31,53).

Nitric oxide (NO) plays an important role in both carcinogenesis and tumor progression (50,54–56). NO could stimulate tumor growth and metastasis by promoting the migratory, invasive and angiogenic abilities of tumor cells, which may also be triggered by the activation of COX-2 (57). In fact, a significantly higher expression level of iNOS was found in both the human oral SCC (58) and rat tongue cancer induced by 4-NQO (31,34). As a result, iNOS generating NO in oral cancer progression might be able to play an important role in oral cancer progression. Our data on iNOS immunohistochemistry in this study may suggest a

positive correlation between the positive expression of iNOS and disease progression.

β-Catenin functions as a transcriptional activator of the Wnt signaling pathway in embryonic and tumor development (54). In several types of human cancer, mutations in the *β-catenin* or *APC* gene cause an accelerated tumor cell proliferation and tumor progression through the transcriptional activation of such target genes as *cyclin D1* (59), with the resulting cytoplasmic/nuclear accumulation of β-catenin (60). In the current study, the immunohistochemical β-catenin expression in the non-lesional tongue epithelium was strictly confined to the cell membranes but not nuclei. Whereas some carcinoma cells preserved a weak membranous expression, the membranous expression of β-catenin decreased while the cytoplasmic/nuclear expression increased in line with the disease progression, and carcinoma cells at the invasion front showed a cytoplasmic/nuclear pattern of β-catenin. We did not perform a gene mutation analysis in the current study, but no mutations of *β-catenin* gene were observed in the rat tongue carcinomas induced by 4-NQO (32). These findings are in line with those reported in human oral cancers (43). As a result, it may, thus, be possible that molecular events other than mutations in *β-catenin* and *APC* are responsible for the activation of the Wnt/β-catenin signaling pathway and the cytoplasmic/nuclear expression of β-catenin in tongue carcinogenesis.

Oral lesions with an aberrant DNA content represent an increased risk of cancer (44,61). The value of the DNA content is useful as an early biomarker of oral cancer (61). In this study, the histological grading of 4-NQO-induced tongue lesions correlated to DNA ploidy. The quantification of the histological evaluation of tissue architecture may show a certain correlation among the degree of dysplasia, COX-2 expression and DNA ploidy (62,63). In the current study, the COX-2 expression was upregulated in DNA aneuploid tongue dysplastic and neoplastic lesions. In contrast, non-lesional 'normal' appearing tongue epithelium specimens that showed a weak COX-2 expression in the basal layer had a diploid DNA content. These findings may indicate that COX-2 is upregulated during malignant transition of the tongue epithelium, and this could be in some manner related to the development of genomic instability (64,65).

Oral cancer is a disfiguring disease that continues to increase in incidence, particularly in the young, and to an extent that cannot be fully explained by an increased exposure to the known risk factors. For such malignancies, a chemopreventive approach to oral cancer most likely should encompass a combination of chemicals targeting the metabolic pathways relevant to oral carcinogenesis. Candidate chemicals include retinoids, selective inhibitors of COX-2 and ligands of PPARs, some of which have been tested for their efficacy by our research group. Although the efficiency of any chemical for chemopreventive use should be assessed through a prospective randomized trial and then evaluated only by a definitive end-point for the prevention of cancer, our Tg rat model using intermediate biomarkers (expression of GST-P, cyclin D1, COX-2, iNOS and β-catenin in tongue lesions and white patches) was, thus, found to be effective for a preclinical evaluation of candidate chemopreventive agents against oral cancer development within a short-term period of time.

In conclusion, we established an animal model of oral SCC using Tg rats and the carcinogen 4-NQO. In the model, we observed dysplasia and tumors on the tongues of transgenic rats after treatment with 4-NQO in drinking water, however, the incidence and multiplicity were greater in Tg rats than in non-Tg rats, and the onset of carcinogenesis was earlier in Tg rats. In addition, a series of pathological and immunohistochemical studies revealed that tongue squamous cell dysplasia, papilloma, carcinoma *in situ* and invasive carcinoma occurred in Tg rats. Five biomarkers (cyclin D1, GST-P, COX-2, iNOS and β-catenin) were expressed in these tongue squamous cell lesions and their expression increased with the disease progression. The changes of these markers were correlated with those of DNA ploidy patterns. Finally, we found that three reported cancer chemopreventive agents could inhibit the carcinogenesis in the Tg rat tongue caused by the treatment of 4-NQO. Taken together, we concluded that this Tg rat model could thus be used in the analysis and the chemoprevention/treatment of oral carcinogenesis.

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Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice

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We previously reported the chemopreventive ability of a prenyloxycoumarin auraptene in chemically induced carcinogenesis in digestive tract, liver and urinary bladder of rodents. The current study was designed to determine whether dietary feeding of auraptene and its related prenyloxycoumarin collinin can inhibit colitis-related mouse colon carcinogenesis. The experimental diets, containing the compounds at 2 dose levels (0.01 and 0.05%), were fed for 17 weeks to male CD-1 (ICR) mice that were initiated with a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg body weight) and promoted by 1% (w/v) DSS in drinking water for 7 days. Their tumor inhibitory effects were assessed at week 20 by counting the incidence and multiplicity of colonic neoplasms and the immunohistochemical expression of proliferating cell nuclear antigen (PCNA)-labeling index, apoptotic index, cyclooxygenase (COX)-2, inducible nitric oxide (iNOS) and nitrotyrosine in colonic epithelial malignancy. Feeding with auraptene or collinin, at both doses, significantly inhibited the occurrence of colonic adenocarcinoma. In addition, feeding with auraptene or collinin significantly lowered the positive rates of PCNA, COX-2, iNOS and nitrotyrosine in adenocarcinomas, while the treatment increased the apoptotic index in colonic malignancies. Our findings may suggest that certain prenyloxycoumarins, such as auraptene and collinin, could serve as an effective agent against colitis-related colon cancer development in rodents.
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Key words: auraptene; azoxymethane; collinin; colitis-related carcinogenesis; dextran sodium sulfate

Colorectal cancer (CRC) is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease.[1] Long-term UC patients have high risk of developing CRC, when compared with the general population.[2] The precise mechanisms of the IBD-related carcinogenesis process are largely unclear, although it is generally assumed that chronic inflammation influences the development of IBD-related carcinogenesis.[3]

Fighting IBD-related CRC as well as sporadic CRC, by cancer chemoprevention strategy, is important to reduce the risk, and thus primary prevention of CRC in IBD has recently been receiving more attention. Previous experimental and epidemiological investigations suggest that several agents, such as folic acid,[4] conjugated linoleic acid,[5] ursodeoxycholic acid,[6] 5-aminosalicylic acid[7] and aspirin, may reduce the occurrence of CRC in patients with IBD.[8,9] Consistent with these data, several nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase (COX)-2 inhibitors, suppressed the development of chemically induced colon carcinomas in rats[10] and intestinal polyps in *Min* mice, with a nonsense mutation of the *Apc* gene.[11] In addition, clinical trials demonstrated that intake of a NSAID, sulindac, causes regression of adenomas in patients with familial adenomatous polyposis.[12]

Epidemiological studies indicate an inverse correlation between the intake of fruits/vegetables and human colon cancer.[13] Thus, primary prevention, including chemoprevention, using the active compounds in fruits and vegetables is also important for reducing the risk of this malignancy. Citrus fruit contains several chemopreventive compounds against colon cancer.[14–17] Prenyloxycoumarins, including auraptene (Fig. 1*a*) and collinin (Fig. 1*b*), are candidates of such chemopreventers. They are secondary metabolites, mainly found in plants belonging to the families of Rutaceae and Umbelellierae. Several of these coumarins were shown to possess valuable pharmacological properties. These compounds were reported to have anti-inflammatory activity.[18] Auraptene significantly attenuated the lipopolysaccharide (LPS)-induced protein expression of inducible nitric oxide synthase (iNOS) and COX-2, with decreases in production of nitric anion and prostaglandin E_2 (PGE_2), and yet suppressed the release of tumor necrosis factor (TNF)-α and IκB degradation.[19,20] Furthermore, auraptene and collinin also cause complete inhibition of platelet aggregation, induced by arachidonic acid and platelet activated factor *in vitro*.[21] We have previously found that a citrus auraptene suppresses chemically induced carcinogenesis in rodents.[22–24]

For understanding the pathogenesis of IBD and IBD-related CRC, several animal models have been established. Most used is a mouse model with dextran sodium sulfate (DSS).[25] Modifying effects of several xenobiotics on CRC-related colon carcinogenesis have been reported,[26,27] using this model. However, this colitis model using DSS, with or without carcinogen, needs to a long period repeated administration of DSS to induce colitis and colitis-related CRC that mimic human UC. To investigate the pathogenesis in IBD-related CRC and search novel and effective chemopreventive agents against this type of malignancy, we developed a novel colitis-related mouse CRC model, using a colon carcinogen azoxymethane (AOM) and DSS, in which large bowel adenocarcinomas develop within a short-term period, and their histology and biological alteration resemble to those found in humans.[28] Our animal model indicates that in the large bowel, inflammation induced by DSS strongly promotes the development of epithelial malignant neoplasia. Oxidative/nitrosative stress caused by DSS exposure may contribute the development of high incidence of colonic adenocarcinomas.[29,30] Recently, we demonstrated that dietary administration of COX-2 inhibitor and peroxisome proliferator-activated receptor ligands suppressed colitis-related colonic carcinogenesis, using our mouse colon carcinogenesis model.[31]

As a part of our search for safer chemopreventive agents against colitis-related colon cancer, we examined, in the present study, the

Abbreviations: AOM, azoxymethane; CRC, colorectal cancer; COX, cyclooxygenase; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; H & E, hematoxylin and eosin; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; NSAIDs, nonsteroidal anti-inflammatory drugs; PCNA, proliferating cell nuclear antigen; PGE_2, prostaglandin E_2; ssDNA, single stranded DNA; TNF, tumor necrosis factor; UC, ulcerative colitis.

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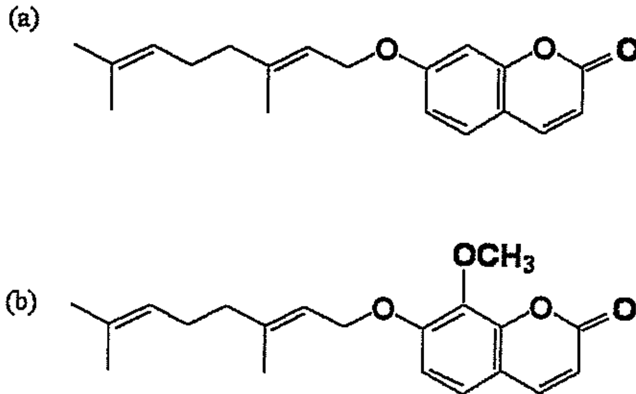

FIGURE 1 – Chemical structures of (*a*) auraptene and (*b*) collinin.

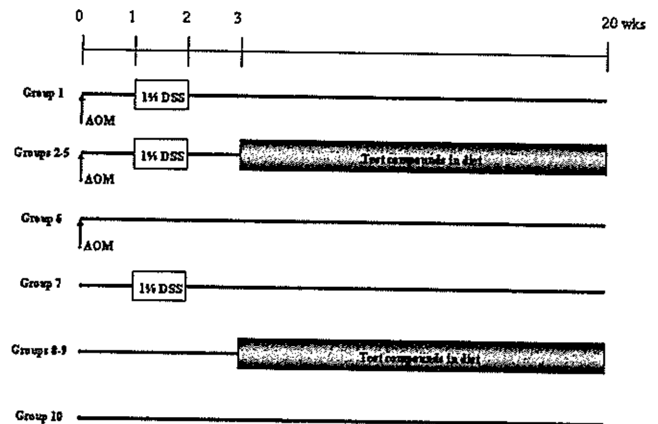

FIGURE 2 – Experimental protocol.

effects of auraptene and collinin on our mouse colon carcinogenesis model.[28]

Material and methods

Animals, chemicals and diets

Male Crj: CD-1 (ICR) (Charles River Japan, Tokyo, Japan), aged 5 weeks, were used in this study. They were maintained at Kanazawa Medical University Animal Facility, according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 or 6 mice/cage), with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan), under controlled conditions of humidity (50 ± 10)%, light (12/12 hr light/dark cycle) and temperature (23 ± 2)°C. They were quarantined for the first 7 days, and then randomized by body weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 was purchased from ICN Biochemicals (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v). Auraptene (99.6% purity)[32] and collinin (99.8% purity)[18] were synthesized, as described previously. Experimental diet, containing auraptene or collinin, was prepared every week by mixing the respective compound in powdered basal diet CRF-1, at a concentration (w/w) of 0.01 or 0.05%. The dose levels of the 2 compounds were selected on the basis of our previous experiments.[18,22–24]

Experimental procedures

A total of 75 male ICR mice were divided into 10 (experimental and control) groups (Fig. 2). Mice in groups 1 through 5 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, animals were administered to 1% DSS in drinking water for 7 days, and then followed without any further treatment for 15 weeks. Mice of group 1 were maintained on basal diet, throughout the study. Mice in groups 2 through 5 were given 0.01% auraptene in diet (group 2), 0.05% auraptene in diet (group 3), 0.01% collinin in diet (group 4) or 0.05% collinin in diet (group 5), respectively, for 17 weeks, starting 1 week after the stop of DSS administration. Group 6 was given a single dose of AOM. Group 7 was given 1% DSS for 7 days. Animals in groups 8 and 9 were given the diets containing 0.05% auraptene and 0.05% collinin alone, respectively. Group 10 consisted of untreated mice. All animals were killed at the end of the study (week 20). Their large bowels were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge) and cut open longitudinally along the main axis, and then washed with saline. The large bowels were macroscopically inspected, cut and fixed in 10% buffered formalin, for at least 24 hr. Histological examination was performed on paraffin-embedded sections, after hematoxylin and eosin (H & E) staining. Colonic neoplasms were diagnosed, according to the description by Ward.[33] Grade of colitis was scored, and the sections were stained with H & E,[29,34] from all groups.

Immunohistochemistry

Immunohistochemistry for proliferating cell nuclear antigen (PCNA), apoptotic nuclei, COX-2, iNOS and nitrotyrosine was performed on 4-µm-thick paraffin-embedded sections, from the colons of mice in each group by the labeled streptavidin biotin method, using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through graded ethanols at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions, and for washes between various steps. Incubations were performed in a humidified chamber. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed.[35] Apoptotic index was also evaluated by immunohistochemistry for single stranded DNA (ssDNA).[35] Sections were treated for 40 min at room temperature, with 2% BSA, and incubated overnight at 4°C with primary antibodies, such as anti-PCNA mouse monoclonal antibody (diluted 1:50; PC10, DAKO Japan), anti-ssDNA rabbit polyclonal antibody (diluted 1:300, DAKO Japan), anti-COX-2 rabbit polyclonal antibody (diluted 1:50, IBL Co., Gunma, Japan), anti-iNOS rabbit polyclonal antibody (diluted 1:1,000, Wako Pure Chemical Industries, Osaka, Japan), and anti-nitrotyrosine rabbit polyclonal antibody (diluted 1:500, Upstate Biotechnology, Lake Placid, NY). To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied for 1 hr. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted.

Intensity and localization of immunoreactivities, against all primary antibodies used, were examined on all sections using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). The PCNA and apoptotic indices were determined, by counting the number of positive cells among at least 200 cells in the lesion, and were indicated as percentages. Each slide for COX-2, iNOS and nitrotyrosine was evaluated for intensity of immunoreactivity on a 0 to 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% of positive cells, 2+ mild immunoreactivity and 10–30% of positive cells, 3+ moderate

immunoreactivity and 31–60% of positive cells, and 4+ strong immunoreactivity and 61–100% of positive cells.

Statistical analysis

Measurements were compared by Bonferroni multiple comparison post test or Fisher's exact probability test. Differences were considered statistically significant at $p < 0.05$.

Results

General observation

Bloody stool was observed in a few mice, which received 1% DSS, and their body weight gains were slightly decreased during the period of treatment. However, thereafter no such clinical symptoms were noted. Body weights, liver weights and relative liver weights, in all groups at the end of the study, are shown in Table I. The mean body weights, liver weights and relative liver weights did not significantly differ among the groups. The mean length of large bowel in groups 2–5 was lower than group 1, but the differences did not reach statistical significance. Histologically, there were no pathological alterations suggesting toxicity of auraptene and collinin in the liver, kidneys, lung and heart. Food consumption (g/day/mice) did not significantly differ among the groups (data not shown).

Pathological findings

Macroscopically, nodular or polypoid colonic tumors were observed in the middle and distal colon of mice in groups 1 through 5. Histopathologically, AOM/DSS treated mice showed dysplasia (Fig. 3*a*), adenoma (Fig. 3*b*) and adenocarcinoma (Fig. 3*c*). These tumors histologically diagnosed as tubular adenoma or well-/moderately-differentiated tubular adenocarcinoma. Animals of groups 6–10 did not have large bowel neoplasms in any organs examined, including the colon. The incidences and multiplicity of colon neoplasma are shown in Table II, respectively. Group 1 (AOM/DSS) induced 100% incidence of colon adenocarcinomas, with a multiplicity of 3.00 ± 1.41. The incidences of colorectal adenocarcinomas in groups 2 (AOM/DSS/0.01% auraptene), 3 (AOM/DSS/0.05% auraptene), 4 (AOM/DSS/0.01% collinin) and 5 (AOM/DSS/0.05% collinin) were significantly smaller than that of group 1 ($p < 0.02$, $p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively). The multiplicity of colon adenocarcinomas in groups 2, 3, 4 and 5 were also significantly lower than that of group 1 ($p < 0.005$, $p < 0.001$, $p < 0.005$ and $p < 0.001$, respectively). Colitis was present with or without colonic dysplasia in the middle or distal colon of mice treated with DSS. As shown in Fig. 4, colonic inflammation scores in groups 3 ($p < 0.05$) and 5 ($p < 0.05$) were significantly decreased, when compared with that in group 1.

Immunohistochemistry for PCNA, ssDNA, COX-2, iNOS and nitrotyrosine in colonic adenocarcinoma

As summarized in Table III, PCNA-labeling index of colonic adenocarcinomas developed in groups 2 ($p < 0.01$), 3 ($p < 0.01$), 4 ($p < 0.01$) and 5 ($p < 0.05$) was significantly smaller than group 1 (Figs. 5*a*–5*c*), and apoptotic index, measured by ssDNA immunohistochemistry in groups 2 ($p < 0.05$), 4 ($p < 0.05$) and 5 ($p < 0.01$), was significantly greater than group 1 (Figs. 5*d*–5*f*). Scores for COX-2 and iNOS expression in colonic adenocarcinomas is also given in Table III. In the positive cases of COX-2 and iNOS expression in the dysplasia and adenocarcinoma, the staining pattern was granular and localized to cytoplasm or nuclei or both. Slight immunoreactivity for COX-2 and iNOS was observed in the superficial layers of the nonlesional colonic mucosa and in parts of basal layer, in all groups. COX-2 expression scores of colonic adenocarcinomas in groups 2 ($p < 0.01$), 3 ($p < 0.05$) and 5 ($p < 0.05$) and that of iNOS in groups 2 ($p < 0.001$), 3 ($p < 0.001$), 4 ($p < 0.01$) and 5 ($p < 0.01$) were significantly decreased, when compared with that in group 1 (Figs. 5*g*–5*i* for COX-2 and Figs. 5*j*–5*l* for iNOS). Nitrotyrosine immunoreactivity (Figs. 5*m*–5*o*) was mainly observed in mononuclear cells infiltrated in the colonic mucosa with the lesions, and the stainability was relatively weak in the neoplastic cells. The score of nitrotyrosine is also given in Table III. The scores of groups 3 ($p < 0.05$) and 5 ($p < 0.05$) were significantly higher than that of group 1. The scores of groups 2 and 4 were also lower than that of group 1, but the differences were insignificant.

Discussion

The results of the present work clearly indicated that 2 prenyloxycoumarins, auraptene and collinin, effectively inhibited AOM/DSS-induced colitis-related colonic carcinogenesis, without any adverse effects in mice. The suppressive effect of auraptene

TABLE I – BODY, LIVER, RELATIVE LIVER WEIGHTS AND LENGTH OF LARGE BOWEL

Group no.	Treatment	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)	Length of colon (cm)
1	AOM + 1%DSS (10)[1]	42.3 ± 2.4[2]	2.5 ± 0.5	5.90 ± 0.96	14.6 ± 1.1
2	AOM + 1%DSS/0.01% auraptene (10)	42.7 ± 2.4	2.6 ± 0.2	6.13 ± 0.55	15.2 ± 0.9
3	AOM + 1%DSS/0.05% auraptene (10)	42.4 ± .3.1	2.7 ± 0.3	6.45 ± 0.56	15.2 ± 0.9
4	AOM + 1%DSS/0.01% collinin (10)	48.1 ± 7.6	2.9 ± 0.6	5.96 ± 0.54	15.0 ± 1.1
5	AOM + 1%DSS/0.05% collinin (10)	45.5 ± 5.7	2.4 ± 0.3	5.20 ± 0.36	15.2 ± 1.2
6	AOM alone (5)	47.9 ± 6.8	3.0 ± 0.5	6.31 ± 0.26	16.5 ± 0.3
7	1% DSS alone (5)	44.6 ± 3.2	2.8 ± 0.3	6.32 ± 0.54	14.9 ± 1.0
8	0.05% auraptene (5)	47.0 ± 5.5	2.6 ± 0.2	5.64 ± 0.38	16.5 ± 0.7
9	0.05% collinin (5)	44.4 ± 3.1	2.8 ± 0.3	6.25 ± 0.64	15.7 ± 0.9
10	None (5)	44.0 ± 2.6	2.8 ± 0.4	6.36 ± 0.61	16.5 ± 1.0

[1]Values in parentheses indicate the numbers of mice examined.–[2]Mean ± SD.

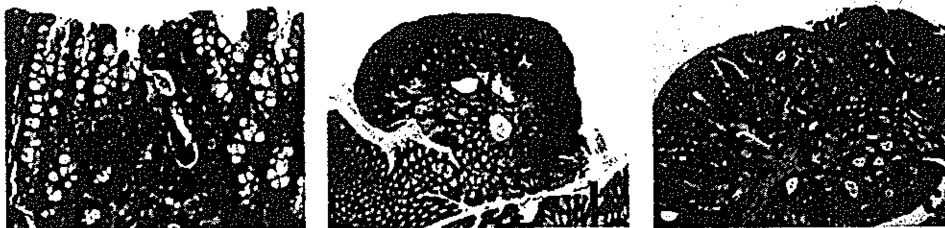

FIGURE 3 – Histopathology of colonic lesions. (*a*) Dyplastic crypts, (*b*) tubular adenoma and (*c*) tubular adenocarcinoma developed in a mouse from group 1. H & E stain, original magnification, (*a*) ×20, (*b*, *c*) ×4.