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

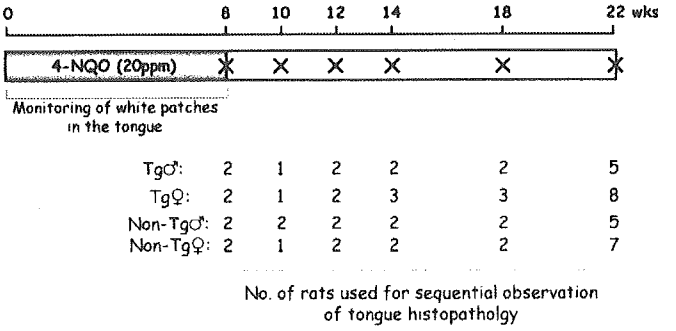

Fig. 1. Experimental protocol. X, killing time points. Nos, number of Tg and non-Tg rats that are killed for tongue histopathology.

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.

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

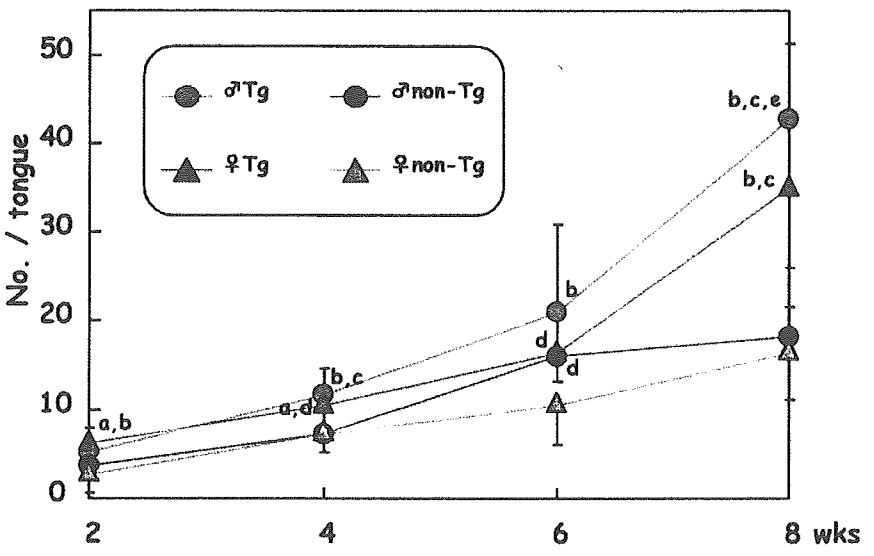

Fig. 2. Mean number of white patches in the tongue that were monitored from Week 2 through Week 8. a, significantly different from male non-Tg ($P < 0.05$); b, significantly different from female non-Tg ($P < 0.001$); c, significantly different from male non-Tg ($P < 0.001$); d, significantly different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

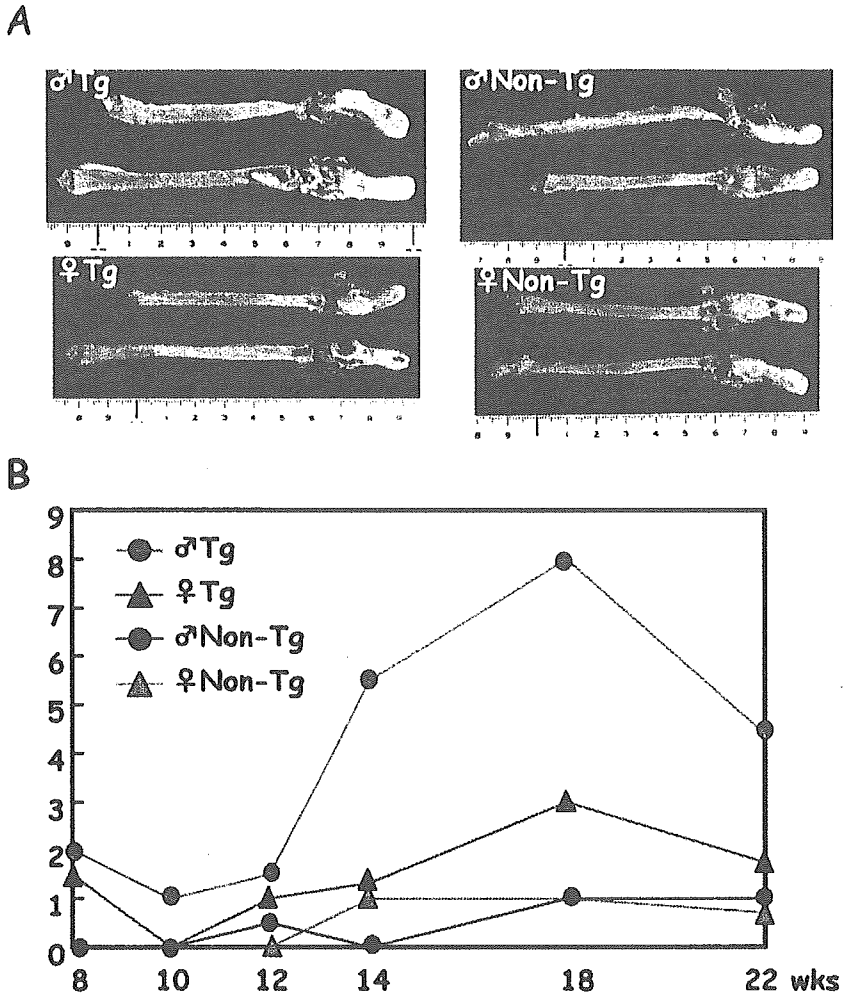

Fig. 3. Tongue neoplasms developed in Tg and non-Tg rats that were treated with 4-NQO. (**A**) Macroscopic view of tongues at Week 22. Large tongue tumors with white patches develop in Tg rats of either sex, whereas only a few small tongue tumors or white patches are found in the tongue in non-Tg rats 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

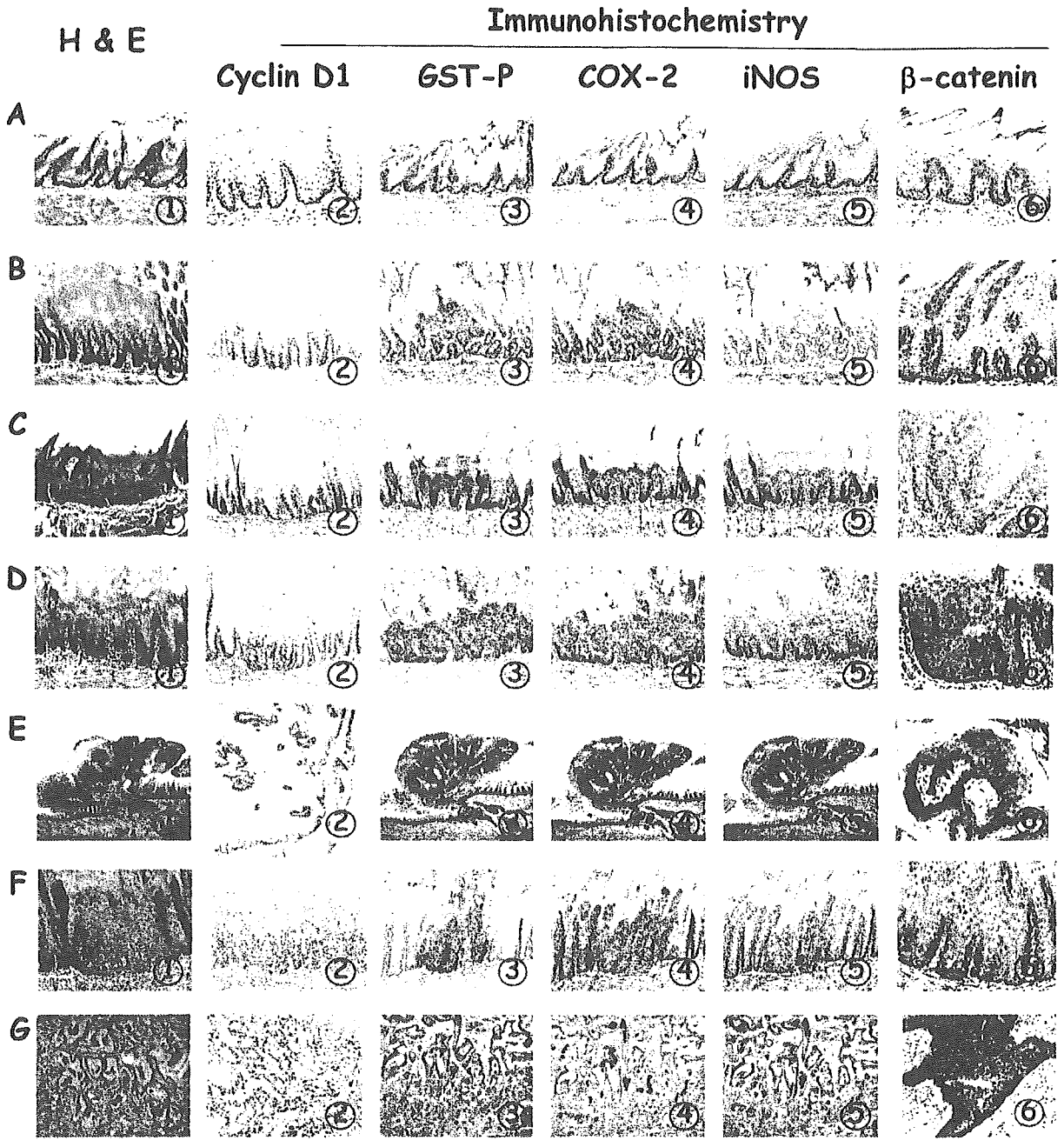

Fig. 4. Histopathology and immunohistochemistry of cyclin D1, GST-P, COX-2, iNOS and β-catenin of tongue lesions in Tg males that received 4-NQO. (**A**), 'Normal' appearing tongue squamous epithelium; (**B**) Mild dysplasia; (**C**) Moderate dysplasia; (**D**) Severe dysplasia; (**E**) Squamous cell 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).

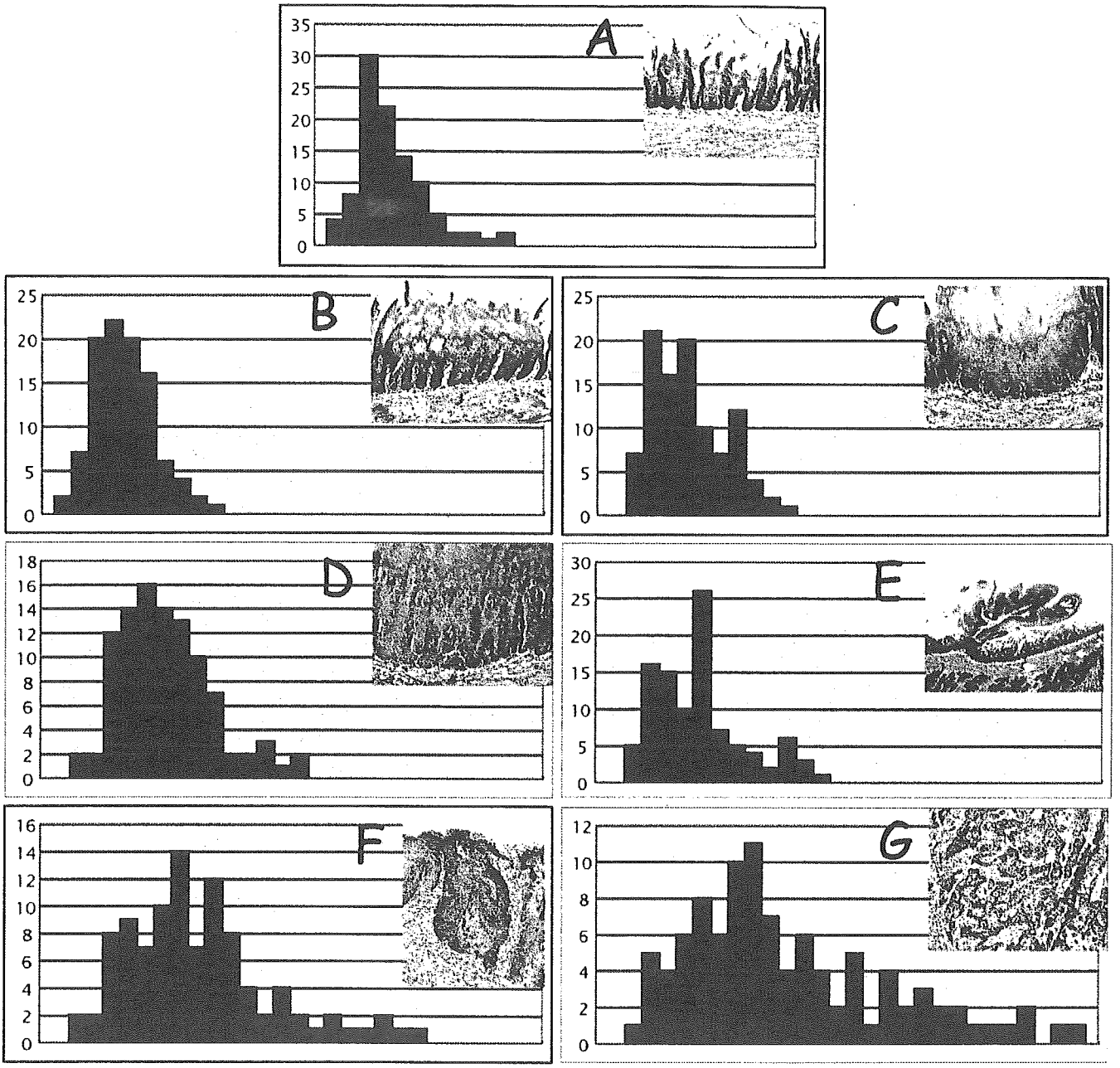

Fig. 5. Representative DNA histograms from various tongue lesions (inserts) in male Tg rats treated with 4-NQO. (**A**) 'Normal' appearing tongue squamous epithelium; (**B**) Mild dysplasia; (**C**) Moderate dysplasia; (**D**) Severe dysplasia; (**E**) Squamous cell papilloma; (**F**) SCC *in situ* and (**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 ± 4[b,e]	51 ± 7[b]	24 ± 6[b,e]	3.48 ± 0.35[b,f]
Severe	10	18 ± 4[b,e,h]	23 ± 5[e]	60 ± 7[b,e]	4.75 ± 0.33[b,e,i]
Papilloma	10	25 ± 5[b,e,j]	50 ± 5[b,k]	25 ± 4[b,e,k]	3.73 ± 0.64[b,g,h]
CIS	10	12 ± 5[b,e,i,j,l]	25 ± 8[b,e,i,l]	63 ± 8[b,e,i,l]	5.01 ± 0.58[b,e,i,l]
Invasive	10	9 ± 2[b,e,i,k,l]	27 ± 5[e,i,l]	65 ± 5[b,e,i,l]	5.24 ± 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' ($P < 0.001$).

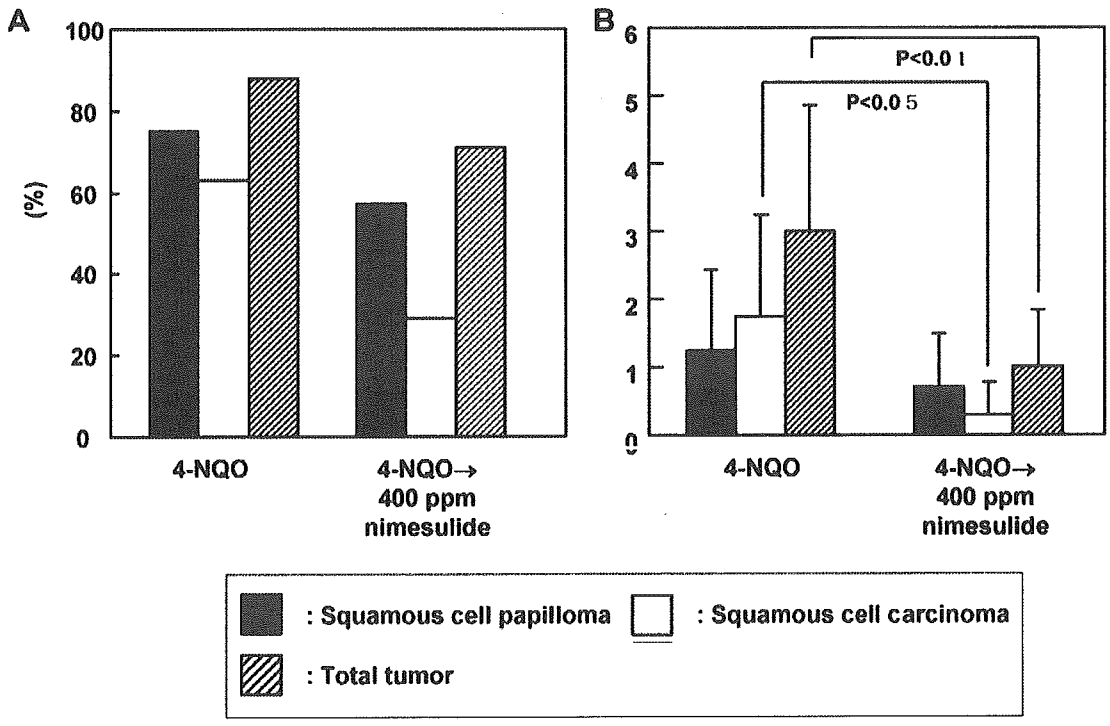

Fig. 6. The effect of dietary nimesulide (400 p.p.m.) on tongue neoplasms in male Tg rat. (**A**) Incidence (%) of tongue carcinomas and (**B**) multiplicity (no. of carcinoma/rat) of tongue carcinoma. Treatment with nimesulide reduced the incidence and multiplicity of tongue tumors. The inhibition in 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

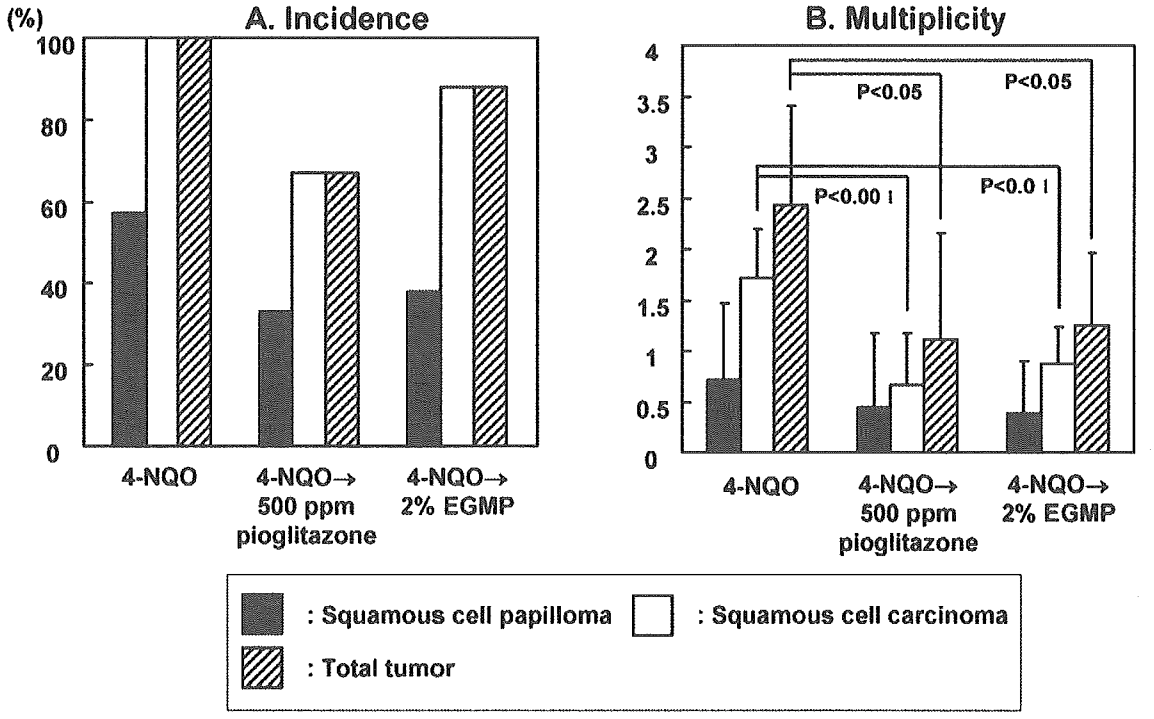

Fig. 7. The effect of dietary feeding with pioglitazone (500 p.p.m.) and EGMP (2%) on tongue neoplasms in female Tg rats. (**A**) The incidence (%) of tongue carcinomas and (**B**) multiplicity (number of carcinoma/rat) of tongue carcinoma. Treatment with pioglitazone or EGMP reduced the incidence and 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.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan; a Grant-in-Aid (no. 15.2052) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant (H2005-6) for the Project Research from the High-Technology Center of Kanazawa Medical University and a grant from the Japan Food Chemical Research Foundation.

Conflict of Interest Statement: None declared.

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Received July 4, 2005; revised September 11, 2005;
accepted September 30, 2005

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NITRIC OXIDE
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Suppressive effect of an inducible nitric oxide inhibitor, ONO-1714, on AOM-induced rat colon carcinogenesis

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Received 18 April 2005; revised 8 July 2005
Available online 24 August 2005

Abstract

The expression of inducible nitric oxide synthase (iNOS) is markedly elevated in rat colon cancers induced by azoxymethane (AOM). In addition, iNOS can be detected in most adenomas and dysplastic aberrant crypt foci (ACF), suggesting that iNOS plays an important role in colon carcinogenesis. In the present study, the effect of an iNOS inhibitor, ONO-1714 ((1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride), on AOM-induced rat colon carcinogenesis was investigated. Male F344 rats were treated with 15 mg/kg body weight of AOM once a week, for 2 weeks. ONO-1714 was given to the rats at doses of 10, 20, 50, and 100 ppm in diet for 4 weeks from the day before the first carcinogen treatment. The number of AOM-induced ACF in the rats receiving 10, 20, 50 and 100 ppm ONO-1714 were 94, 73 ($P < 0.05$), 71 ($P < 0.005$), and 53% ($P < 0.0005$), respectively, of the control value. Moreover, the mean number of aberrant crypts per focus was significantly lowered in 100 ppm ONO-1714 group ($P < 0.05$). Then, the effects of long-term treatment (32 weeks) with 50 and 100 ppm ONO-1714 on AOM-induced colorectal tumor development were examined. Although incidences and multiplicities of colon tumors did not significantly differ among the groups, number of tumors developing in the middle part of colon were reduced with both 50 and 100 ppm doses ($P < 0.05$). Furthermore, colon tumor volume tended to be decreased by ONO-1714 treatment, and the number of colon tumors more than 3 mm in diameter was significantly lowered in the 100 ppm ONO-1714 group ($P < 0.01$). These results suggest that iNOS plays roles in both early and late stages of colon carcinogenesis.

Keywords: Colon cancer; iNOS inhibitor; Rat; Azoxymethane; ONO-1714

Chronic infection and inflammation release many cytokines [1] and activate nuclear factor κB (NF-κB) [2], resulting in the expression of NF-κB-regulated, inflammatory-related genes, such as inducible nitric oxide synthase (iNOS) [3]. Resultant overproduction of nitric oxide (NO) contributes to multistage carcinogenesis by inducing DNA mutations and tissue damage [4–6]. Increased expression of iNOS in human cancers, including examples in the colon, stomach, esophagus, lung, pancreas, and prostate, has been described [7–14]. The expression of inducible nitric oxide synthase (iNOS) is markedly elevated in rat colon cancers induced by azoxymethane (AOM) [15]. In addition, iNOS can be detected in most adenomas and dysplastic aberrant crypt foci (ACF), while it is hardly detectable in normal colon mucosa [16]. Thus these previous findings suggest that iNOS plays an important role in colon carcinogenesis.

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1089-8603/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.niox.2005.07.004

We have also shown that iNOS expression can be markedly elevated by transfection of K-*ras* mutant cDNA into IEC-6 rat intestinal epithelial cells in the presence of interleukin-1β (IL-1β) or lipopolysaccharide (LPS) [17] and growth of tumors formed in nude mice by s.c. injection of the K-*ras* mutant-transfected cells was suppressed by feeding diets containing NOS inhibitors, L-N^G-nitroarginine methyl ester (L-NAME) and ONO-1714 ((1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride) [17].

Since K-*ras* mutations are frequently observed in hyperplastic ACF and large tumors in AOM-treated rats [16], as well as in human lesions [18], K-*ras* activation might also contribute to enhancement of their cell proliferation. Indeed, it has been reported that K-*ras* mutations and/or activation increase expression of cyclin D1 and cyclooxygenase-2 (COX-2) in AOM-induced rat colon tumors [19], and K-*ras*-enhanced iNOS expression could conceivably also play a role.

Previously, we have shown that a NOS inhibitor, L-NAME, suppressed ACF formation in the colon of rats treated with AOM [20]. Suppressive effects of iNOS-selective inhibitors, *S*,*S*′-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea (PBIT) and L-N^6-(1-iminoethyl)lysine tetrazole-amide (SC-51), on ACF formation in the rat colon have also been reported [21,22]. However, there has hitherto been no report concerning effects on colon cancer development. It has been reported that ONO-1714 is 10-fold more selective for human iNOS than for human endothelial NOS, very potent with an ID_{50} value of 0.010 mg/kg s.c. and lowly toxic with a maximum tolerated dose of 30 mg/kg i.v. in mice [23,24]. In addition, ONO-1714 is effective even when orally administrated [17]. Therefore in the present study, we examined the influence of ONO-1714, an iNOS-selective inhibitor, on rat colon carcinogenesis induced by AOM, and noted suppressive effects on ACF formation, as well as tumor size.

Materials and methods

Chemicals

ONO-1714 was chemically synthesized at Ono Pharmaceutical (Osaka, Japan). AOM was synthesized at the Nard Institute (Amagasaki, Japan).

Animals

Male F344 rats, purchased from Charles River Japan (Atsugi, Japan) at 6 weeks of age, were used. They were housed in plastic cages in an air-conditioned room with a 12-h light-dark cycle and provided with diet (AIN-76A, Dyets Inc., Bethlehem, PA) and water ad libitum. Body weights and food intake were measured weekly.

Short-term experiment for ACF analysis

Forty five rats were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight, once a week for 2 weeks, and 9 animals each were given a basal diet or diet containing ONO-1714 at doses of 10, 20, 50 or 100 ppm from the day before the first carcinogen treatment until the end of the experiment. The doses were chosen from the results of our previous study in which 50 and 100 ppm ONO-1714 in diet suppressed growth of K-*ras* mutant-transfected rat IEC-6 cells in nude mice [17]. As negative controls, six rats were treated subcutaneously with saline only, and 3 animals each were given a basal diet or diet containing ONO-1714 at a dose of 100 ppm, respectively. Four weeks after the first carcinogen treatment, the rats were sacrificed and their colons were removed, fixed flat between sheets of filter paper in buffered 10% formalin, and stained with 0.2% methylene blue in saline, using the method of Bird [25]. The number of ACF per colon, the number of aberrant crypts (ACs) in each focus, and the location of each focus were determined by microscopy at a magnification of 40×. To categorize the distribution of ACF, we defined the rectum as the segment 2 cm proximal to the anus and divided the remaining colon into three segments of about 6 cm length, the distal colon, the middle colon, and the proximal colon, as described previously [26].

Long-term experiment for analysis of colon cancer

A total of 90 rats were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight once a week for 2 weeks, while 45 rats were similarly given injections of saline without any carcinogen as vehicle-controls. One third of each group was given basal diet or diet containing ONO-1714 at doses of 50 or 100 ppm from the day before the first carcinogen treatment until the end of the experiment. The animals were sacrificed 32 weeks after the first carcinogen treatment and the number, size, and location of all intestinal tumors more than 1 mm in diameter, detected without a microscope, were determined. The volume of the tumors was also assessed as previously described [27]. All tumor samples were fixed in 10% formalin–PBS and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination and tumors were classified according to established criteria [28,29]. The experimental protocol was according to the guidelines for Animal Experiments in the National Cancer Center.

Statistical analysis

Significant differences in the incidences of tumors as well as histological findings were analyzed by the χ^2 test. Group means were compared among the groups using

one-way ANOVA followed by Dunnett's test. A P value of less than 0.05 was regarded as significant. Dose-dependency on tumor volumes per rat was tested using coefficients for linear contrast.

Results

Suppression of ACF formation by ONO-1714 in short-term experiment

The final body weights (g) of the AOM alone, AOM + 10, 20, 50, and 100 ppm ONO-1714, saline alone and saline + 100 ppm ONO-1714 groups were 218.3 ± 2.0 (SE), 215.8 ± 2.0, 207.2 ± 2.3, 207.2 ± 2.2, 204.0 ± 2.8, 230.6 ± 4.7, and 211.6 ± 4.9, respectively. The average food consumption values (g/day/rat) for these groups were 11.8, 11.7, 11.1, 11.1, 11.0, 13.1, and 11.4, respectively. The food consumption did not differ among 20, 50, and 100 ppm ONO-1714 groups. The body weights and food consumption in the 100 ppm ONO-1714 groups were 6.5 and 7.0% lower in AOM-treated groups, and 8.2 and 13% lower in saline-treated groups, respectively, suggesting a link between the two and there were no apparent toxic effects observed during the experiments.

The data for ACF formation in the groups treated with AOM are summarized in Table 1. ACF were found in the colons of all animals treated with AOM. These ACF were mainly located in the distal and middle colon, a few in the rectum and very few in the proximal colon. With administration of 10, 20, 50, and 100 ppm of ONO-1714 to AOM-treated rats, the number of AOM-induced ACF per colon were decreased in a dose-dependent manner to 94, 73, 71, and 53%, respectively, of the control value, 237.4 ± 19.3 (SE). Total number of aberrant crypts (ACs) per colon, and number of ACF consisting of ⩾4 ACs per focus were also decreased by ONO-1714 treatment in a dose-dependent manner. Significant differences were observed for these parameters with 20, 50, and 100 ppm ONO-1714 treatment. Furthermore, treatment with 100 ppm ONO-1714 significantly decreased the mean number of ACs per focus ($P < 0.05$). No ACF were observed in the colons of the saline-injected groups given neither the basal diet nor 100 ppm ONO-1714.

Effects of ONO-1714 on colon tumor development in long-term experiment

The final body weights (g) of the AOM alone, AOM + 50, and 100 ppm ONO-1714, saline alone, and saline + 50, and 100 ppm ONO-1714 groups were 371.0 ± 3.2 (SE), 355.2 ± 4.6, 338.4 ± 3.3, 375.2 ± 7.7, 357.8 ± 5.9, and 354.3 ± 4.2, respectively. The body weights in the 100 ppm ONO-1714 groups with and without AOM were 8.8 and 5.6% lower than the respective control values, this being considered due to the lowered food consumption. During the experiment, one animal in the AOM + 50 ppm ONO-1714 group suffered accidental mortality at week 5 and was excluded from the tumor analysis. Two animals in the AOM group (at weeks 24 and 28), 2 in the AOM + 50 ppm ONO-1714 group (at weeks 28 and 31), and 2 in the AOM + 100 ppm ONO-1714 group (at weeks 28 and 30), which died of tumor development before the termination were, however, included.

The incidences and multiplicities of intestinal tumors (adenomas and carcinomas) at week 32 are summarized in Tables 2 and 3. Colorectal tumor incidences did not significantly differ among the AOM alone, AOM + 50 ppm ONO-1714, and AOM + 100 ppm ONO-1714 groups, being 92, 83, and 87%, respectively. Multiplicities of colorectal tumors were slightly lower in the groups treated with ONO-1714, but the values were not statistically different: being 3.07 ± 0.34 (SE) in the AOM alone group, 2.41 ± 0.36 in the AOM + 50 ppm ONO-1714, and 2.27 ± 0.32 in the AOM + 100 ppm ONO-1714 group. There was no statistically significant variation in the incidences and multiplicities of small intestine and cecum tumors.

Colorectal tumors were located mainly in the distal and middle colon in the AOM alone group, as shown in Fig. 1. Interestingly, number of tumors developing in the middle part of colon were significantly lowered in the 50 and 100 ppm ONO-1714 groups compared with the control ($P < 0.05$) and those in the proximal colon also tended to be decreased, while those in the distal colon and rectum were not altered.

Table 1
Suppression of AOM-induced ACF formation in rat colon by treatment with ONO-1714 for 4 weeks

Dose of ONO-1714 in diet (ppm)	Incidence of rats with ACF	No. of ACF/colon[a] (% of the control)	No. of ACs[a] (% of the control)	Mean no. of ACs/focus[a]	No. of ACF with ⩾ 4 ACs[a] (% of the control)
0	9/9	237.4 ± 19.3 (100)	515.2 ± 47.6 (100)	2.15 ± 0.05	25.2 ± 4.07 (100)
10	9/9	223.9 ± 16.1 (94.3)	458.7 ± 34.8 (89.0)	2.05 ± 0.04	18.0 ± 2.2 (71.4)
20	9/9	174.1 ± 14.9[b] (73.3)	360.4 ± 34.2[b] (70.0)	2.06 ± 0.03	14.9 ± 2.3[b] (59.0)
50	9/9	169.2 ± 7.4[c] (71.3)	342.2 ± 18.1[c] (66.4)	2.02 ± 0.05	10.8 ± 1.7[c] (42.7)
100	9/9	124.7 ± 12.9[d] (52.5)	251.4 ± 28.7[d] (48.8)	1.99 ± 0.05[b]	8.9 ± 1.8[c] (35.2)

[a] Data presented are mean ± SE values.
[b,c,d] Significantly different from the corresponding control values at $P < 0.05$, $P < 0.005$, and $P < 0.0005$, respectively.

Table 2
Effects of ONO-1714 treatment on the incidence of intestinal tumors induced by AOM at week 32

Dose of ONO-1714 in diet (ppm)	Effective no. of animals	No. of animals with tumors in each site (%)				
		Small intestine	Cecum	Colon and rectum		
				Total[a]	Adenoma	Carcinoma
0	30	5 (17)	2 (7)	27 (92)	9 (30)	27 (92)
50	29	8 (28)	4 (14)	24 (83)	7 (24)	24 (83)
100	30	4 (13)	3 (10)	26 (87)	3 (10)	26 (87)

[a] The total represents animals with adenomas and/or carcinomas.

Table 3
Effects of ONO-1714 treatment on the multiplicities of intestinal tumors induced by AOM at week 32

Dose of ONO-1714 in diet (ppm)	Effective no. of animals	No. of tumors per rat[a]				
		Small intestine	Cecum	Colon and rectum		
				Total[b]	Adenoma	Carcinoma
0	30	0.30 ± 0.15	0.07 ± 0.05	3.07 ± 0.34	0.43 ± 0.14	2.63 ± 0.28
50	29	0.31 ± 0.10	0.14 ± 0.06	2.41 ± 0.36	0.28 ± 0.10	2.12 ± 0.33
100	30	0.17 ± 0.08	0.10 ± 0.06	2.27 ± 0.32	0.10 ± 0.06	2.17 ± 0.30

[a] Data presented are mean ± SE values.
[b] The total represents animals with adenomas and/or carcinomas.

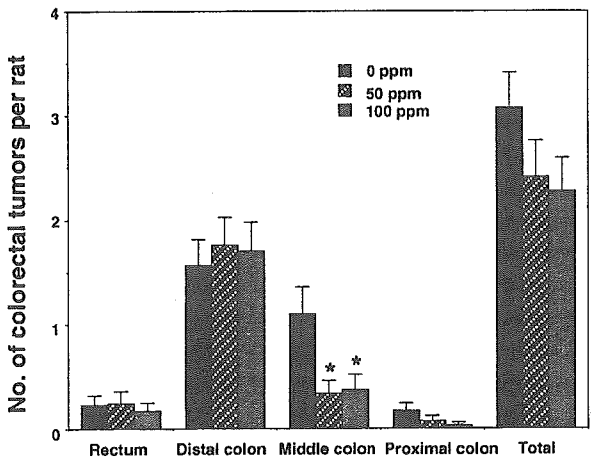

Fig. 1. Effects of ONO-1714 treatment on the location of AOM-induced colorectal tumors. The data for the AOM + 0 ppm ONO-1714 (■), AOM + 50 ppm ONO-1714 (▩) and AOM + 100 ppm ONO-1714 (▩) groups are mean ± SE values. *Significantly different from the respective control at $P < 0.05$.

Colorectal tumor volumes per rat tended to be decreased by ONO-1714 treatment when tested using coefficients for linear contrast ($P < 0.05$), being 70.8 ± 18.5 (SE) mm^3 in the AOM alone group, 47.0 ± 13.8 mm^3 in the AOM + 50 ppm ONO-1714 group, and 30.3 ± 11.0 mm^3 in the AOM + 100 ppm ONO-1714 group, although there was no statistical significance. The number of colorectal tumors ⩾3 mm in diameter was almost the same as that <3 mm, at 1.57 ± 0.25 (SE), and 1.50 ± 0.21, respectively, in the AOM alone group. The number of colorectal tumors more than 3 mm in diameter were lowered by 50 and 100 ppm ONO-1714 treatment to 64 and 46% of the control value, respectively, and a significant difference was observed in the value for the 100 ppm ONO-1714 group ($P < 0.01$), while number of tumors less than 3 mm in diameter did not differ from the control value (Fig. 2A). Especially, the suppressive effect of ONO-1714 on tumor development more than 3 mm in diameter was evident in the middle colon, being 21 and 19% of the control value in 50 and 100 ppm ONO-1714 groups, respectively ($P < 0.01$) (Fig. 2D), and tumor development more than 3 mm in diameter in the rectum and proximal colon also tended to be decreased by ONO-1714 treatment (Figs. 2B and E). Interestingly, number of tumors less than 3 mm in diameter in the middle colon were also decreased to about half by 50 and 100 ppm ONO-1714 treatment, although there was no statistical significance. On the other hand, in the distal colon, the number of tumors more than 3 mm in diameter was slightly decreased by 100 ppm ONO-1714 treatment and that less than 3 mm was slightly increased (Figs. 2C).

Table 4 shows the results of histological examination of AOM-induced colorectal carcinomas. In all groups, most were well-differentiated adenocarcinomas. Signet-ring cell carcinomas were rare and observed mostly in the proximal colon. Compared to the AOM alone group, ONO-1714-treated groups had lower incidences of signet-ring cell carcinomas, although this did not reach statistical significance. The proportions of tumors demonstrating invasion of submucosa (sm) were also slightly, but not significantly, lower in the ONO-1714-treated groups.

Discussion

The present study demonstrated that an iNOS inhibitor, ONO-1714, can effectively decrease development of

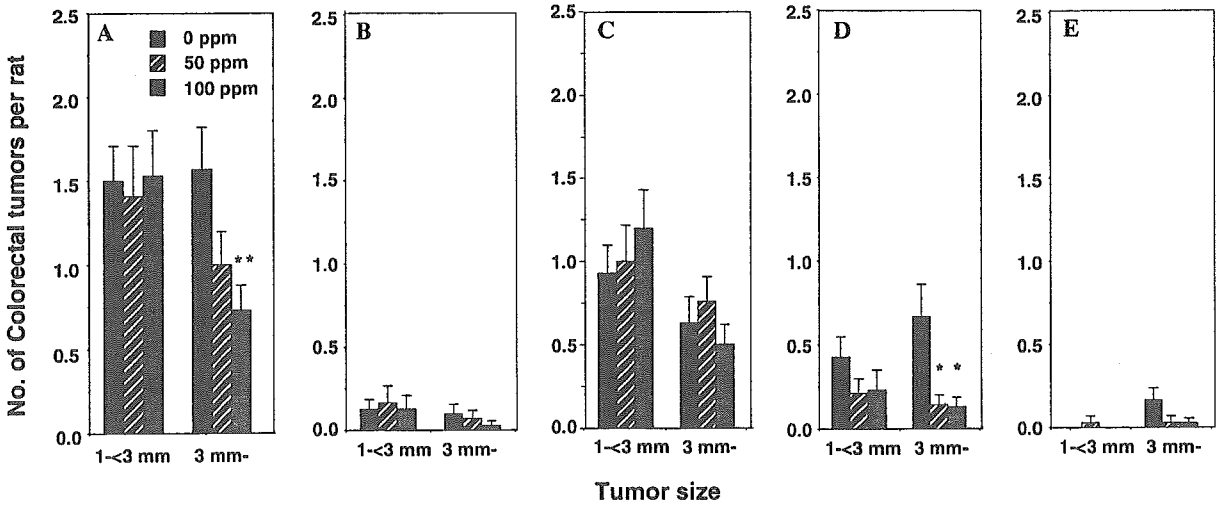

Fig. 2. Effect of ONO-1714 treatment on the size of colorectal tumors. The number of each size of colorectal tumors per rat in the whole colorectum (A), rectum (B), distal colon (C), middle colon (D), and proximal colon (E) for the AOM + 0 ppm ONO-1714 (■), AOM + 50 ppm ONO-1714 (▨) and AOM + 100 ppm ONO-1714 (■) groups are mean ± SE values. *,**Significantly different from the respective control at $P < 0.05$ and $P < 0.01$, respectively.

Table 4
Effects of ONO-1714 treatment on histological types and depth of invasion of AOM-induced colorectal carcinomas at week 32

Dose of ONO-1714 in diet (ppm)	Total no. of carcinomas	No. of carcinomas of each histological type (%)				Depth of invasion (%)		
		Well-differentiated	Moderately-differentiated	Signet-ring cell	Mucinous	m[a]	sm[b]	pm[c]
0	79	72 (93)	0	5 (6)	1 (1)	67 (86)	11 (14)	0
50	62	61 (98)	0	1 (2)	0	57 (92)	5 (8)	0
100	65	64 (99)	0	1 (1)	0	61 (94)	4 (6)	0

[a] Mucosa and muscularis mucosae.
[b] Submucosa.
[c] Muscularis propria.

preneoplastic lesions, ACF, especially large ACF, providing further support for the concept that iNOS plays important roles in the early stage of colon carcinogenesis. Although the colon tumor incidence was not decreased by ONO-1714 treatment, number of colon tumors larger than 3 mm in diameter were lowered, indicating a suppressive effect on growth. Thus, we can conclude that ONO-1714 also impacts on late stages of colon carcinogenesis, even if it not appreciably suppressing malignant tumor development.

Furthermore, ONO-1714 significantly reduced the number of tumors in the middle colon, where relatively large carcinomas often develop. In our previous studies, treatment with docosahexaenoic acid (DHA) also decreased tumors in the middle colon more effectively than in other parts of colorectum [30]. DHA has similarly been reported to down-regulate iNOS expression in colon cancer cells [31]. Further examination of differences in tumor properties in the middle and distal colon appears warranted.

Although iNOS expression was not detected immunohistochemically in most hyperplastic ACF [16], NOS inhibitors, including iNOS-selective examples, clearly decreased ACF formation in the present and previous studies [20–22]. It is possible that very low levels are present in ACF, many of which possess K-*ras* mutations, and iNOS inhibitors may be more effective on such a low activity state of iNOS. Further mutations such as in the *β-catenin* gene may elevate iNOS expression to a detectable degree [16], and once iNOS expression is elevated, it may be difficult to inhibit its activity completely by iNOS inhibitors. Moreover, in these studies, treatment with iNOS inhibitors was overlapped with AOM treatment. Therefore, it is possible that suppression of ACF formation might be in part due to inhibition of the initiation step, namely DNA alkylation by AOM metabolites or metabolic activation of AOM [32].

It has been reported that angiogenesis is necessary to supply oxygen and nutrients to solid tumors more than 1–2 mm^3 [33] but NO enhances their vascular permeability, partly through activation of matrix metalloproteinases [34]. Thus, suppression of development of tumors more than 3 mm in diameter in ONO-1714-treated groups may be associated with inhibition of angiogenesis by the iNOS inhibitor. NO also enhances activity and expression of COX-2 in several cell lines [35–38] and co-expression of iNOS and COX-2 has been reported for human cancers of the colon [6,38,39], esophagus [9],

stomach [40], pancreas [12], and ovary [41], endometrium [42], and brain [43]. Overexpression of COX-2 promotes angiogenesis through prostanoid-mediated increase in vascular endothelial growth factor (VEGF) [38,40,43]. In the AOM-induced rat colon carcinogenesis model, COX-2 expression is also increased in well-differentiated carcinoma cells of large tumors [16]. It has been reported that iNOS inhibitors, 1400W and SC-51, reduce not only iNOS activity but also COX-2 activity [22,38]. Therefore, reduction of NO-mediated COX-2 activation may be one of the mechanisms underlying suppressive effects of ONO-1714 on colon carcinogenesis.

Our previous studies indicated K-*ras* activating mutations to be frequent in hyperplastic ACF and large tumors [16]. The present study showed that iNOS may contribute to development of preneoplastic lesions, ACF, and expansion of tumor masses in later stage of colon carcinogenesis. These observations in vivo agree with our previous finding that a K-*ras* activating mutation enhances iNOS expression mediated by IL-1β or LPS in cell culture [17]. It should be borne in mind that other cancers with frequent K-*ras* mutations, such as lung and pancreatic examples [44], also show increased iNOS expression [10–13]. Thus, it is suggested that NO production by iNOS is generally involved in tumor-promoting effects of activated K-*ras*, and iNOS-selective inhibitors should be considered as possible candidate agents for prevention of all cancers featuring K-ras activation.

Acknowledgments

We thank Ms. Mika Kawamura, Ms. Yurika Teramoto, and Mr. Naoaki Uchiya for excellent technical assistance. This work was supported in part by Grants-in Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Ministry of Health, Labour and Welfare for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, Japan.

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Carcinogenesis vol.26 no.12 pp.2116–2122, 2005
doi:10.1093/carcin/bgi193
Advance Access publication July 28, 2005

Prostaglandin E receptor *EP3* deficiency modifies tumor outcome in mouse two-stage skin carcinogenesis

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We have recently shown that the prostaglandin E_2 (PGE_2) receptor EP_3 plays an important role in suppression of colon cancer cell proliferation and that its deficiency enhances late stage colon carcinogenesis. Here we examined the effects of *EP3*-deficiency on two-stage skin carcinogenesis. 7,12-Dimethylbenz[*a*]anthracene (50 μg/200 μl of acetone) was thus applied to the back skin of female *EP3*-knockout and wild-type mice at 8 weeks of age, followed by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (5 μg/200 μl of acetone) twice a week for 25 weeks. First tumor appearance was observed in *EP3*-knockout mice at week 10, which was 3 weeks later than in *EP3* wild-type mice, and multiplicity observed at week 11 was significantly lower in the *EP3*-knockout case. However, histological examination showed that the tumor incidence and multiplicity at week 25 were not significantly changed in knockout mice and wild-type mice (incidence, 19/19 versus 23/24; multiplicity, 3.58 ± 0.51 versus 3.17 ± 0.63, respectively). Interestingly, there were no squamous cell carcinomas (SCCs) in the EP_3-knockout mice, while SCCs were observed in 3 out of 24 wild-type mice. Furthermore, benign keratoacanthomas only developed in *EP3*-knockout mice (6/19 versus 0/24, $P < 0.01$). The results suggest that PGE_2 receptor EP_3 signaling might contribute to development of SCCs in the skin.

Introduction

Prostaglandins are lipid autacoids synthesized by cyclooxygenase (COX) in response to numerous growth factors and environmental stimuli. Two isoforms of COXs have been described, the constitutively and ubiquitously expressed COX-1 and the inducible COX-2. Overproduction of prostaglandins attributable to overexpression of COX-2 in various tumors is critical for epithelial carcinogenesis and provides a target for cancer chemoprevention by non-steroidal antiinflammatory drugs (NSAIDs)(1–5). Furthermore, there is a large amount of evidence from epidemiological and pharmacological studies that COX inhibitors exhibit chemopreventive activities for various malignancies in humans, including skin cancer (6,7). Recent studies of chemical carcinogenesis in COX-2-overexpressing transgenic mice demonstrated a promotive role of COX-2 in tumorigenesis in the skin and breast epithelium (8,9). A tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was found to increase the epidermal prostaglandin E_2 (PGE_2) in mouse skin, and thus appears to be critical for TPA-induced hyperproliferation (10). PGE_2 exerts its biological action through binding to four specific receptor subtypes, EP_1, EP_2, EP_3 and EP_4, with seven transmembrane domains. Activation of the EP_1 receptor is associated with increases in intracellular Ca^{2+} and the EP_2 and EP_4 receptors are known to be coupled to Gs protein and stimulate cAMP production by activation of adenylate cyclase. In contrast, the major signaling pathway for the EP_3 receptor is inhibition of adenylate cyclase via Gi. Several isoforms are also generated by alternative splicing from the single *EP3* receptor gene, and these exhibit other functions through activation via G proteins other than Gi (11).

Previously, we examined the role of PGE_2 receptor subtypes in intestinal carcinogenesis using knockout mice, and the results showed that formation of aberrant crypt foci (ACF), putative preneoplastic lesions, induced by azoxymethane (AOM) was decreased in the *EP1* and *EP4* knockout cases, suggesting involvement of the receptors in ACF formation (12,13). Moreover, homozygous deletion of the gene encoding the EP_2 receptor results in decrease of intestinal polyp formation in *Apc* gene-deficient mice (14). Recently, we also examined roles of the EP_3 receptor in AOM-induced long-term colon carcinogenesis using *EP3* receptor-knockout mice, and obtained evidence that it suppresses cell proliferation, down-regulation enhancing late stage colon carcinogenesis (15).

In the present study, we examined two-step skin carcinogenesis with dimethylbenz[*a*]anthracene (DMBA) and TPA (16) in *EP3*-knockout mice. These mice exhibited benign tumor formation, while wild-type mice developed malignant squamous cell carcinomas (SCCs). The results indicate that the EP_3 receptor may play a critical role in the development of malignancies in skin carcinogenesis.

Materials and methods

Animals

Mice lacking *EP3* receptor gene were generated as reported previously (17) and were backcrossed to the C57BL/6Cr strain for 10 generations. Female *EP3* receptor-deficient mice were used at 6 weeks of age. Genotypes of the knockout mice were confirmed by PCR according to the method described previ-

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; DMBA, 7,12-dimethylbenz[*a*]anthracene; PGE_2, prostaglandin E_2; SCC, squamous cell carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

ously (12). The animals were housed in plastic cages at 24 ± 2°C and 55% relative humidity with a 12/12 h light/dark cycle. Water and basal diet (AIN-76A; CLEA Japan, Tokyo) were given *ad libitum*. Body weights and food intake were measured weekly.

Two-stage skin carcinogenesis experiments

Initiation was achieved by a single application of 50 μg of DMBA dissolved in 200 μl of acetone to the skin of the backs of female EP_3 receptor-deficient homozygous ($EP_3^{-/-}$) and wild-type mice at 8 weeks of age. From week 1, after the initiation, a 5 μg aliquot of TPA dissolved in 200 μl of acetone was applied to the initiated skin parts of the mice, twice a week for 25 weeks, as described previously (18). Skin tumors were noted grossly for their location, number and diameters, measured with calipers, and digital photographs of the backs of each animal were taken once a week. Mice were killed under ether euthanasia at the end of week 25 and complete autopsy was performed. All skin tumors were subjected to histological examination after routine processing and hematoxylin and eosin staining. The experimental protocol was according to the guidelines for Animal Experiments in the National Cancer Center.

Immunohistochemical staining

Immunohistochemical analyses of skin tumor samples from female $EP_3^{-/-}$ and wild-type mice were performed with the avidin–biotin complex immunoperoxidase technique, as previously reported (19). As primary antibodies, monoclonal mouse anti-β-catenin and mouse anti-E-cadherin antibodies (Transduction Laboratories, Lexington, KY) were used at 100× dilution (20). As the secondary antibody, biotinylated anti-mouse IgG (H+L) raised in a horse, affinity purified, and absorbed with rat serum (Vector Laboratories, Burlingame, CA) was used at 200× dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3′-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As a negative control, duplicate sections were immunostained without exposure to the primary antibody (20).

Analysis of EP receptor expression in skin tumors by RT–PCR

Total RNA was extracted from papillomas (5 mm or more in diameter), keratoacanthoma, SCCs and normal skin tissues by direct homogenization in ISOGEN (Nippon Gene, Tokyo, Japan), and spectrophotometry was used for quantification. Three microgram aliquots of total RNA were subjected to the RT reaction with random 9mer primers using an Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany). After reverse transcription, PCR was carried out with HotStartaq (Qiagen), according to the manufacturer's instructions. To test cDNA integrity, the *cyclopilin* (*PPIA*) gene was amplified for each sample (21). Primers were designed, based on published sequences from Genbank, using the computer program OLIGO 4.0-s (National Biosciences, MD). Primers were designed to cross an exon–exon boundary or insert an intron to ensure that genomic DNA was not being amplified. BLAST searches confirmed that the primers were specific for the target gene. Primers for the *cyclopilin* and *EP receptor* genes are listed in Table I. PCR amplification was performed in a thermal cycler (Gene Amp PCR System 9600, Perkin–Elmer Applied Biosystems, Foster City, CA), with 18–40 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min, using the specific primer sets. The PCR products were then analyzed by electrophoresis on 2% agarose gels.

Quantitative real-time RT–PCR analysis

Quantitative real-time RT–PCR analysis was performed using the Smart Cycler system with an *Ex Taq* R-PCR kit and SYBR Green (Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions. Primers for the *cyclopilin* and EP_3 genes, and the cycle conditions for PCR are listed in Table I. To assess the specificity of each primer set, amplicons generated in PCR reactions were analyzed for their melting point curves and additionally run on 2% agarose gels to confirm the correct sizes of the PCR products. Each PCR product was subcloned into the TA cloning plasmid vector, pGEN-T easy vector (Promega, Madison, WI), and used as a positive control for real-time PCR analyses. The numbers of molecules of specific gene products in each sample were determined using a standard curve generated by amplification of 10^2–10^8 copies of the control plasmid.

Statistical analysis

The significance of differences in the incidences of tumors was analyzed with the χ^2-test and other differences using the Student's *t*-test. Statistical significance was concluded at $P < 0.05$.

Results

Macroscopic findings for skin carcinogenesis

The mean food intake and body weights of EP_3 receptor-knockout mice were comparable with those of wild-type

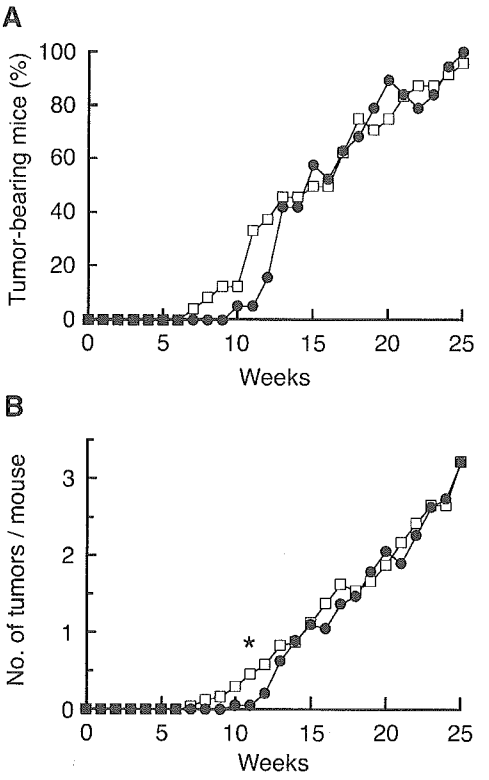

Fig. 1. (**A**) Incidences (percentage of mice with tumors) and (**B**) multiplicities (number of tumors per mouse) of macroscopic skin tumors. Open squares, wild-type; closed circles, $EP_3^{-/-}$ mice. Note the significant differences in multiplicities at week 11 (0.05 ± 0.05 versus 0.46 ± 0.18) between $EP_3^{-/-}$ and wild-type mice. *Significantly different from the corresponding wild-type mice value at $P < 0.05$.

Table I. List of primers used for RT–PCR and real-time RT–PCR

Gene name	Primer sequences (5′→3′)			Product size (bp)	Cycle condition of real-time PCR
Cyclopilin	Forward	ACAAAGTTCCAAAGACAGCAG	(Exon 2)	132	95°C (5 s) → 62°C (20 s) → 72°C (15 s)
	Reverse	TATGGCGTGTAAAGTCACC	(Exon 4)		
EP_1	Forward	TGCCTCATCCATCACTTC	(Exon 2)	159	
	Reverse	ACCACCAACACCAGCAG	(Exon 2–3)		
EP_2	Forward	TATGCTCCTTGCCTTTCAC	(Exon 1)	174	
	Reverse	GACAACAGAGGACTGAGCG	(Exon 2)		
EP_3	Forward	GCTGTCCGTCTGTTGGTC	(Exon 1)	100	95°C (5 s) → 60°C (20 s)
	Reverse	CCTTCTCCTTTCCCATCTG	(Exon 2)		
EP_4	Forward	CATCTTACTCATCGCCACC	(Exon 2)	177	
	Reverse	ATGTAAATCCAGGGGTCCA	(Exon 3)		