Dietary Supplementation with Silymarin Inhibits 3,2′-Dimethyl-4-Aminobiphenyl–Induced Prostate Carcinogenesis in Male F344 Rats

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Abstract

Purpose: Silymarin has been shown to be a potent anticarcinogenic agent. Here, we investigated the modifying effects of dietary feeding with a naturally occurring polyphenolic antioxidant flavonoid silymarin on 3,2′-dimethyl-4-aminobiphenyl (DMAB)–induced prostatic carcinogenesis in male F344 rats.

Experimental Design: Male F344 rats were given s.c. injections of DMAB (25 mg/kg body weight) every other week for 20 weeks. They also received the experimental diet containing 100 or 500 ppm silymarin for 40 weeks, starting 1 week after the last dosing of DMAB. All of the rats were sacrificed 60 weeks after the start of the experiment. Histopathology and immunohistochemistry for proliferative cell nuclear antigen, cyclin D1, and apoptotic indices were done in the prostatic lesions, including invasive adenocarcinomas, intraepithelial neoplasms, and nonlesional glands.

Results: Dietary feeding with 500 ppm silymarin significantly inhibited the incidence of prostatic adenocarcinoma when compared with the DMAB-alone group (17.6% versus 50.0%, $P < 0.05$). The proliferative cell nuclear antigen– and cyclin D1–positive indices in adenocarcinomas, prostatic intraepithelial neoplasm, and nonlesional glands in rats treated with DMAB and silymarin were slightly lower than that of the DMAB-alone group. Also, dietary administration of silymarin increased apoptotic index in prostatic adenocarcinoma by measuring immunohistochemically positive nuclei for ssDNA.

Conclusions: Our results indicate that silymarin exerts chemopreventive ability against chemically induced prostatic carcinogenesis through apoptosis induction and modification of cell proliferation.

Prostate cancer is the most common type of cancer found in older men and the leading cause of cancer mortality in men (1). In Japan, the incidence and mortality rates of this malignancy are lower compared with Western populations (2), but they have gradually increased (3). Furthermore, migrant studies have shown that the incidence of prostate cancer increases generation by generation after immigration in Japanese-Americans (4). These observations strongly suggest that the wide disparity in prostate cancer incidence worldwide is attributable to dietary habits, among which are a regimen rich in several flavonoids and isoflavones that inhibits the progression of prostate cancer by modulating epigenetic events (5). It is, therefore, necessary to intensify our efforts to better understand this disease and develop novel approaches for its prevention and treatment.

Silymarin, the collective name for an extract from the milk thistle [*Silybum marianum* (L.) Gaertneri], is a naturally occurring polyphenolic flavonoid antioxidant. It is composed mainly of silibinin (~80%, w/w; also called silybin, silibin, or sibilinin) with smaller amounts of other stereoisomers (isosilybin, dihydrosibilyn, silydianin, and silychristin, etc.; ref. 6). Silymarin has strong antioxidative properties and is able to scavenge both free radicals and reactive oxygen species (7, 8). In Europe, for over 20 years, silymarin, as an antihepatotoxic, is used clinically for the treatment of alcoholic liver disease (9, 10). In recent years, silymarin has also been used in Asia as a therapeutic agent for liver diseases (6). Silymarin is well tolerated and largely free of adverse effects (6, 11). Silymarin acts as a potent anticarcinogenic agent against *in vitro* and *in vivo* carcinogenesis experiments (12–14). Silymarin and silibinin, which is the major active constituent of silymarin, can inhibit the growth of human prostate carcinoma LNCaP, PC-3, and DU145 cells in culture (15–17). Moreover, silymarin and

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silibinin inhibit cell growth and induce apoptosis in rat prostate cancer cell lines (18). However, chemoprevention studies using silymarin in rodent were limited to skin (13, 19). Silymarin inhibits tumor promoter-caused induction of ornithine decarboxylase activity and mRNA expression in mouse epidermis (20). Silymarin inhibits mRNA expression of endogenous tumor promoter tumor necrosis factor-α (21). More recently, silymarin has been reported to inhibit activation of erbB1 signaling, induce cyclin-dependent kinase inhibitors, G_1 arrest, and cause complete inhibition of growth of human prostate carcinoma DU145 cells (17). Also, silymarin, at lower nontoxic concentrations, can inhibit transformation in cultured rat tracheal epithelial cells treated with benzo(*a*)pyrene (22). These findings led us to evaluate the possible suppressing effects of dietary silymarin on the occurrence of chemically induced neoplasms in organs other than skin of rodents. We recently have found the inhibitory effects of dietary administration of silymarin against rat tongue (23), mouse urinary bladder (24), and rat colon (25) carcinogenesis.

In the current study, we investigated the effects of silymarin on 3,2′-dimethyl-4-aminobiphenyl (DMAB)-initiated prostate carcinogenesis in male F344 rats. Also, the modulatory effects of the silymarin on the proliferating cell nuclear antigen (PCNA), cyclin D1, and apoptotic indices were immunohitochemically investigated in the prostatic lesions induced by DMAB.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male F344 rats (Charles River Japan, Inc., Kanagawa, Japan) were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in polycarbonate cages (three or four rats per cage) under controlled conditions of humidity (50 ± 10%), lighting (12-hour light/dark cycle), and temperature (23 ± 2°C). They have free access to drinking water (ion exchange water) and a basal diet, CRF-1 (Oriental Yeast, Co., Ltd., Tokyo, Japan) from which soy constituents were eliminated throughout the study. Animals were quarantined for 7 days and randomized by body weight into experimental and control groups. DMAB and silymarin were obtained from Sigma-Aldrich Japan, K.K. (Tokyo, Japan). The experimental diet containing silymarin were prepared by Oriental Yeast by adding test chemicals to soy protein-free CRF-1.

Experimental procedure. A total of 68 male F344 rats were divided into nine experimental and control groups. The animals in groups 1 through 3 were given DMAB dissolved in DMSO, s.c., at a dose of 25 mg/kg body weight every other week for 20 weeks. DMAB injection was done between 10:00 a.m. and 11:00 a.m. From 1 week after the last injection of DMAB, group 1 was given the basal diet without silymarin, groups 2 and 3 received silymarin-containing diets (100 ppm for group 2 and 500 ppm for group 3), and group 4 was fed the diet containing 500 ppm silymarin for 40 weeks. Group 5 served as an untreated control. The doses of the test compounds were selected based on previous studies (23). All rats were sacrificed at week 60 by ether overdose to assess the pathologic lesions in all organs, including prostate. At autopsy, all organs were carefully inspected and all macroscopic pathologic findings were recorded. All grossly abnormal lesions in any tissue and the organs, such as accessory sex organs including prostate, liver, kidney, lung, and heart, were fixed in 10% phosphate-buffered formalin for 2 weeks. As for the accessory sex organs, two sagittal slices of the ventral prostate, two sagittal slices of the dorsolateral prostate, which included the urethra, and three transverse slices from each side of the seminal vesicles, which included the anterior prostate, were made and embedded in paraffin. They were then sectioned and stained with H&E for histopathologic diagnosis. The prostatic lesions, including prostatic intraepithelial neoplasm (PIN; ref. 26), were histopathologically diagnosed. The diagnosis of PIN was based on the criteria described by Bostwick and Brawer (27): PIN shows the morphologic continuum of cellular proliferations with nuclear atypia that occur within prostatic ducts, ductules, or acini, and is enclosed by a basement membrane. Several architectural patterns, such as flat, tufting, microcapillary, or cribriform, could be seen.

Immunohistochemistry. For the determination of cell proliferation and cell cycle activity of the epithelial cells, PCNA and cyclin D1 immunohistochemistry was done according to the method described previously with some modifications (28, 29). Apoptotic index was also evaluated by immunohistochemistry for ssDNA (28). Immunohistochemistry was done using a stain system kit (DAKO LSAB 2 kit/HRP, DAKO Japan Co., Ltd., Kyoto, Japan). The sections (3 μm in thickness) made from paraffin-embedded tissues were deparaffinized; they were treated sequentially with 0.3% H_2O_2, normal goat serum or horse serum, and first antibodies. A mouse anti-PCNA antibody (1:100 dilution; DAKO Japan), a rabbit polyclonal anti-cyclin D1 antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit polyclonal antibody against ssDNA (1:300; DAKO Japan) were applied to the sections according to the manufacturer's protocol (DAKO LSAB 2 kit/HRP, DAKO Japan). All incubation steps ware carried out for 15 minutes at 37°C. The chromogen used was 3,3′-diaminobenzidine tetrahydrochloride. The tissues were lightly counterstained with hematoxylin to facilitate orientation. Negative controls were stained without the first antibodies. The numbers of cells with positive reactivity for PCNA, cyclin D1, and ssDNA antibody were counted in a total of 3 × 100 cells in three different areas of the tumors, PIN, and nonlesional areas, and expressed as percentage (mean ± SD).

Statistical evaluation. Where applicable, data were analyzed using Fisher's exact probability test, Student's *t* test, or Welch *t* test with $P < 0.05$ as the criterion of significance.

Table 1. Intakes of food and test chemical

Group no.	Treatment	No. rats examined	Daily intake		Total intake of test chemical (mg)
			Food (g/d/rat)	Test chemical (mg/d/rat)	
1	DMAB	18	16.1 ± 2.5*	—	—
2	DMAB → 100 ppm silymarin	17	16.0 ± 2.6	1.60	44.8
3	DMAB → 500 ppm silymarin	17	16.4 ± 2.3	8.20	2,296
4	500 ppm silymarin	8	16.3 ± 2.2	8.15	2,282
5	None	8	15.8 ± 1.7	—	—

*Mean ± SD.

Table 2. Body, liver, prostate, and testicular weights at the end of the study

Group no.	Treatment	No. rats examined	Body weight (g)	Liver weight (g)	Prostate weight (g)	Testes weight (g)
1	DMAB	18	384.8 ± 24.4*	11.82 ± 1.25	3.19 ± 0.61	2.88 ± 0.66
2	DMAB → 100 ppm silymarin	17	386.0 ± 21.3	12.30 ± 0.93	3.18 ± 0.59	2.80 ± 0.55
3	DMAB → 500 ppm silymarin	17	388.7 ± 16.1	12.42 ± 1.00	3.13 ± 0.67	2.79 ± 0.48
4	500 ppm silymarin	8	401.1 ± 11.8	11.76 ± 1.16	3.13 ± 0.45	2.98 ± 0.64
5	None	8	383.9 ± 20.9	12.42 ± 1.19	3.20 ± 0.62	3.03 ± 0.37

*Means ± SD.

Results

General observation. All animals remained healthy throughout the experimental period. During the study, no clinical signs of toxicity were present in any groups. Histologically, there were no pathologic alteration suggesting toxicity of silymarin in the liver, kidneys, lung, and heart. Food consumption (g/d/rat) did not significantly differ among the groups, as shown in Table 1. Estimated intakes of test chemicals were well correlated with doses applied (Table 1). Body, liver, prostate, and testicular weights in all groups at the end of the study are shown in Table 2. The mean body weights, liver, prostate, and bilateral testicular weights did not significantly differ among the groups.

Incidence of neoplasms of prostate and other organs. Table 3 summarizes the data on the incidence of neoplasms of prostate and other tissues. DMAB exposure could induce PIN and adenocarcinomas (Fig. 1A and B) in the ventral lobe of the prostate. Such lesions were not found in other lobes of the prostate and seminal vesicle. Treatment of DMAB alone (group 1) produced 50.0% incidence of well-differentiated prostatic adenocarcinoma (Fig. 1A). The incidence of prostatic adenocarcinoma (Fig. 1B) in group 3 that received DMAB and 500 ppm silymarin (17.6%) was significantly lower than in group 1 (50.0%, $P < 0.05$). Also, feeding with 100 ppm silymarin after DMAB administration (group 2) caused a reduction of incidence of prostatic adenocarcinoma (29.4%), but there was no statistical significance different from group 1. The incidences of prostatic PIN were 50.0% in group 1, 23.5% in group 2, and 64.7% in group 3. These values also did not show statistically significance among the groups 1 through 3. In other organs, a few neoplasms, such as colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma, were noted in a few rats of groups 1 to 3. The incidences of these tumors were not statistically significant among the groups. No prostatic neoplasms were found in groups 4 (500 ppm silymarin alone) and 5 (no treatment).

Immunohistochemical findings. The data on PCNA- (Fig. 1C and D), cyclin D1- (Fig. 1E and F), and apoptosis-positive cells (Fig. 1G and H) in the prostatic lesions are indicated in Table 4. The mean PCNA labeling indices of adenocarcinoma found in group 3 receiving DMAB and 500 ppm silymarin (6.3 ± 1.5) were significantly lower than in group 1 (10.0 ± 2.4, $P < 0.05$). The PCNA labeling indices of PIN in groups 2 (6.8 ± 1.7) and 3 (6.8 ± 2.2) were lower than that of group 1 (8.8 ± 2.9), but the differences were not statistically significant. As for the histologically normal prostatic glands, the PCNA-labeling indices of all groups were comparable. The mean cyclin D1 labeling indices of adenocarcinoma found in groups 2 and 3 were significantly lower than in group 1 ($P < 0.05$ or $P < 0.01$). The cyclin D1 labeling indices of PIN in groups 2 and 3 were slightly lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the cyclin D1 labeling indices of all groups were comparable. The apoptotic index of PIN and adenocarcinoma in group 3 was statistically greater than that of group 1 ($P < 0.05$ and $P < 0.02$, respectively). On the other hand, the apoptotic index of PIN and adenocarcinoma

Table 3. Incidence of pathologic lesions

Group no.	Treatment	No. rats examined	No. rats with incidence		
			Prostate		Others*
			PIN	Adenocarcinoma	
1	DMAB	18	9 (50.0%)	9 (50%)	4 (22.2%)
2	DMAB → 100 ppm silymarin	17	4 (23.5%)	5 (29.4%)	4 (23.5%)
3	DMAB → 500 ppm silymarin	17	11 (64.7%)	3† (17.6%)	2 (11.8%)
4	500 ppm silymarin	8	0 (0%)	0 (0%)	0 (0%)
5	None	8	0 (0%)	0 (0%)	0 (0%)

*Colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma.
†Significantly different from group 1 by Fisher's exact probability test ($P < 0.05$).

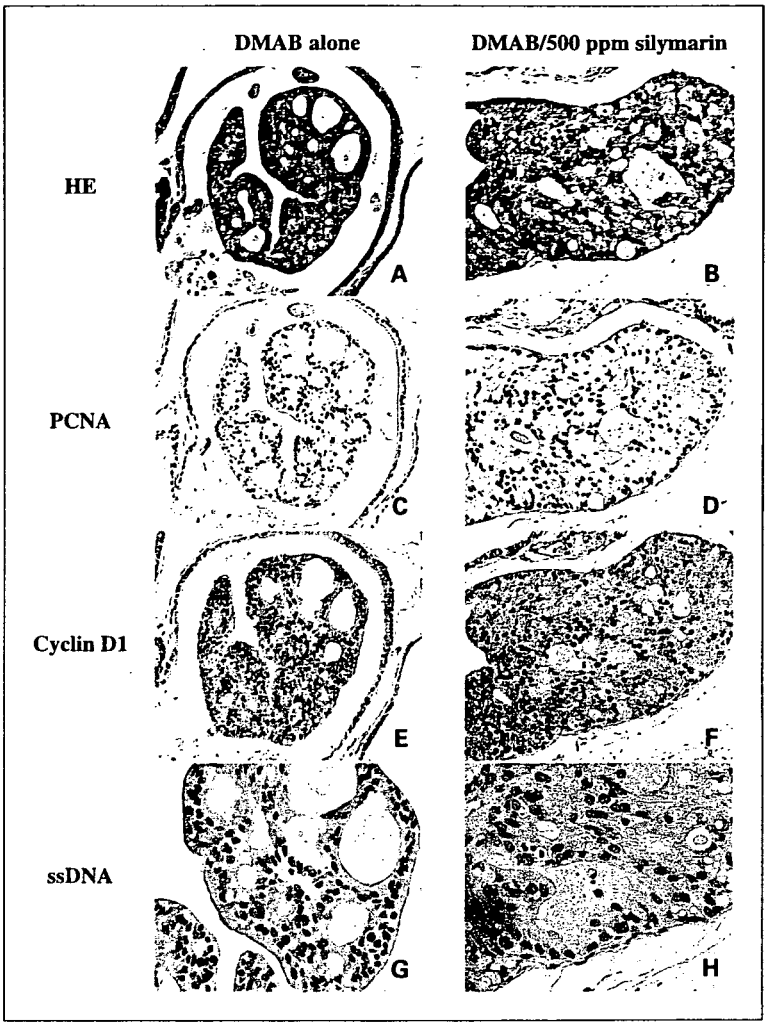

Fig. 1. Histopathology of adenocarcinomas and their immunohistochemistry of PCNA, cyclin D1, and ss DNA. An adenocarcinoma (*A, C, E, G*) from a rat given DMAB alone (group 1) and that (*B, D, F, H*) from a rat given DMAB and 500 ppm silymarin (group 3). H&E stain (*A, B*) and immunohistochemistry for PCNA (*C, D*), cyclin D1 (*E, F*), and ssDNA (*G, H*). Original magnification, ×20 (*A-F*) and ×40 (*G, H*).

in group 2 was lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the apoptotic indices of all groups were comparable.

Discussion

In the present study, dietary administration of 500 ppm silymarin during the promotion phase of DMAB-induced prostatic carcinogenesis significantly inhibited the incidence of prostatic adenocarcinoma. Silymarin is known to inhibit chemically induced carcinogenesis in skin (13), tongue (23), urinary bladder (24), and colon (25). Furthermore, Singh et al. (30) reported that the preventive and therapeutic efficacy of dietary feeding of silibinin on human prostate carcinoma DU145 tumor xenograft in athymic nude mice. These results indicate that silymarin might be a candidate chemopreventive agent against carcinogenesis in multiple organs including prostate.

Several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis could be considered. Cell proliferation plays an important role in multistage carcinogenesis and involves multiple genetic alterations (31, 32). Silymarin and silibinin are reported to suppress the growth of different cancer cells (17, 33-35). Other studies with human prostate cancer cells showed that silymarin and silibinin inhibit the cell growth of androgen-dependent and androgen-independent human prostate carcinomas LNCaP and DU145 cells, respectively (15, 17). Tyagi et al. (18) showed that silymarin and silibinin induce growth inhibition and apoptotic cell death in rat prostate cancer cells. Such effects are considered to occur through perturbation of cell cycle progression, leading to G_1 arrest in a dose- and time-dependent manner, and inhibiting DNA synthesis, possibly because of an effect of G_1 arrest (17, 33, 36, 37). Cyclin D1 is involved in cell cycle during early G_1 phase (38). As the major events leading to cell proliferation occur in the G_1 phase, altered expression of cyclin D1 and their cyclin-dependent kinases might be an important step in carcinogenesis (39). Cyclin D1 overexpression was reported in human cancers (40, 41) and in murine chemically induced carcinogenesis (24, 42). Cyclin D1, which is found to be overexpressed in major of human cancers, has been regarded as a

Table 4. PCNA labeling index, cyclin D1 – positive index, and apoptotic index in the prostatic lesions

Group no.	Treatment	PCNA labeling index (%)			Cyclin D1 positive index (%)			Apoptotic index (%)		
		PIN	ADC	Nonlesional area	PIN	ADC	Nonlesional area	PIN	ADC	Nonlesional area
1	DMAB	8.8 ± 2.9* (9)	10.0 ± 2.4 (9)	4.6 ± 1.5 (5)	28.4 ± 7.2 (9)	35.7 ± 6.0 (9)	4.0 ± 1.6 (5)	1.3 ± 0.3 (9)	2.0 ± 0.5 (9)	1.2 ± 0.3 (5)
2	DMAB → 100 ppm silymarin	6.8 ± 1.7 (4)	7.4 ± 2.4 (5)	4.2 ± 2.4 (5)	25.5 ± 9.9 (4)	27.8 ± 4.4† (5)	3.6 ± 0.5 (5)	1.3 ± 0.3 (4)	2.2 ± 0.4 (5)	1.2 ± 0.3 (5)
3	DMAB → 500 ppm silymarin	6.8 ± 2.2 (11)	6.3 ± 1.5† (3)	4.0 ± 1.2 (5)	24.7 ± 6.3 (11)	23.0 ± 3.6† (3)	3.4 ± 1.1 (5)	1.6 ± 0.3‡ (11)	3.8 ± 0.9‡ (3)	1.1 ± 0.5 (5)
4	500 ppm silymarin	—	—	3.4 ± 1.1 (5)	—	—	0.6 ± 0.5 (5)	—	—	1.2 ± 0.3 (5)
5	None	—	—	3.8 ± 0.8 (5)	—	—	0.6 ± 0.3 (5)	—	—	1.1 ± 0.2 (5)

NOTE: Numbers in parentheses are nos. of lesions or areas examined.
Abbreviation: ADC, adenocarcinoma.
*Mean ± SD.
†Significantly different from group 1 by Student's t test, $P < 0.05$.
‡Significantly different from group 1 by Student's t test, $P < 0.02$.

relevant molecular biomarker in cancer chemoprevention (43, 44). Silymarin and silibinin were reported to decrease in protein levels of cyclin D1 in prostate cancer cells (15, 17). In the present study, silymarin also suppressed cyclin D1 overexpression in prostate adenocarcinoma.

Also, treatment with silymarin inhibits the increase in cell proliferation activity caused by a radical-generating tumor promoter (20). Silymarin is known to exert an antipromoting effect on skin tumorigenesis in mice mediated by impairment of receptor and nonreceptor tyrosine kinase signaling pathway (19). Moreover, in an *in vivo* preclinical prostate cancer model, silibinin inhibits advanced human prostate carcinoma growth (30). In this study, the incidence of adenocarcinoma was decreased by the treatment with silymarin, whereas that of PIN in group 3 was slightly higher than group 1 without statistical significance. The reason for this is unknown, but it may be possible that silymarin feeding at a dose of 500 ppm inhibits progression of PIN to invasive adenocarcinoma. Feeding with silymarin lowered the PCNA labeling indices in the preneoplasms and/or carcinomas of prostate, suggesting that silymarin in diet could suppress the high-proliferative activity of cells initiated with a carcinogen. The other significant finding of this study is the apoptotic index of PIN and adenocarcinoma, which was found to be significantly greater in silymarin-fed rats. The results are in accordance with our previous studies (23, 25) and suggest that, in addition to inhibiting proliferation, apoptosis plays a significant role in inhibition of DMAB-induced prostate carcinogenesis by silymarin. Thus, in the current study, the inhibition of carcinogen-induced prostate malignancies for rats consuming silymarin in part is explained by the alteration of cell proliferating activity and/or apoptosis.

Chemoprevention of cancer might be defined as the deliberate introduction of these selected nontoxic substances into the diet for the purpose of reducing cancer development. Silymarin is clinically used to as antihepatotoxic agents and devoid of any toxicity and untoward effects in both animal and human studies (45). In the present study, the estimated daily silymarin intakes in rats given diet containing 100 and 500 ppm silymarin were ~5 and 25 mg/kg. In a direct extrapolation to a 60 kg person, these doses are equivalent to the estimated doses of clinical use as an antihepatotoxic agent (45). Recently, Singh et al. (30, 46) reported that dietary feeding of silibinin (up to 1%) to nude mice did not show any adverse effect. In the present study, administration of 500 ppm silymarin did not also show any adverse effect on diet consumption, body weight gain, prostate weight, and pathologic alteration for 40 weeks. On the other hand, silibinin is physiologically achievable in different organs including prostate as well as in plasma, and the achievable levels of total slibinin has been found in the range of 15 to 100 μmol/L in plasma by feeding with 0.05% to 1% silibinin/silymarin in rodents (30, 47, 48). The achievable levels (15-100 μmol/L) of silibinin showed inhibition of human prostate cancer cells growth in culture (16, 17, 30). These observations showed that the efficacy of silymarin at dietary dose levels without any adverse effects could have a direct practical and translational relevance to human prostate cancer patients. However, silymarin is a mixture of three structural isomers of flavonoids. Among the flavonoids, silibinin (also called silybin, silibin, or sibilinin) is suggested to be the most active constituent. Because the cancer chemopreventive and anticarcinogenic effects of silymarin seem to be due to the main constituent silibinin (16, 30, 37, 49, 50), further studies of the chemopreventive effects of silibinin itself are necessary.

In conclusion, dietary administration of silymarin significantly suppressed the development of DMAB-induced rat prostate carcinomas. Such cancer protective effect of silymarin might relate to the modulation of cell growth and apoptosis in the prostate neoplastic lesions.

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Lack of Urinary Bladder Carcinogenicity of Sodium L-Ascorbate in Human c-Ha-*ras* Proto-Oncogene Transgenic Rats

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ABSTRACT

Sodium L-ascorbate (Na-AsA) is widely known to be a tumor promoter of rat bladder carcinogenesis but tests negative in standard 2-year bioassays. In the present study, bladder-cancer-susceptible transgenic rats designated Hras128 were used to further examine the tumorigenicity of Na-AsA. A total of 40 7-week-old male transgenic (Tg) and 42 littermate nontransgenic (Non-tg) rats were divided into 4 groups and given powdered MF diet with or without 5% Na-AsA for 57 weeks. Tg rats showed significantly short survival compared with Non-tg, independent of Na-AsA treatment. Tg rats treated with Na-AsA showed a slightly higher incidence of carcinoma (29.6%) as compared to those without Na-AsA treatment (15.4%), but this was without statistical significance. Moreover, the total bladder tumor incidences, including papillomas, did not differ statistically (with Na-AsA, 37.0%; without Na-AsA, 30.8%). No bladder tumor was detected in Non-tg rats. Various kinds of other lesions in various organs were noted in Tg rats treated with or without Na-AsA treatment, but no intergroup differences were evident. In conclusion, Na-AsA did not show tumorigenicity in highly bladder-cancer-susceptible transgenic Hras128 rats. These results suggest that Na-AsA is a pure promoter but not a complete carcinogen in rats.

Keywords. Urinary bladder carcinogenesis; sodium L-ascorbate; human c-Ha-*ras* proto-oncogene transgenic rats; transitional cell carcinoma; in vivo carcinogenicity bioassay; human cancer risk assessment.

INTRODUCTION

Sodium L-ascorbate (Na-AsA), like other sodium salts of saccharin (glutamate and bicarbonate), produces mild superficial urothelial cytotoxicity and regeneration (Fukushima et al., 1983, 1990; Cohen et al., 1995, 1998) when fed at high doses to rats. Na-AsA exerts a promotional effect on rat 2-stage urinary bladder carcinogenesis initiated by genotoxic bladder carcinogens such as *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (Fukushima et al., 1983, 1990; Chen et al., 1999a, 1999b), but demonstrated no tumorigenicity for rat urinary bladder in standard 2-year carcinogenicity tests (Ellwein and Cohen, 1988, 1990; Cohen and Ellwein, 1990; Cohen et al., 1995, 1998). However, Cohen et al. (1998) did demonstrate the carcinogenic potential of Na-AsA in male rats using a 2-generation bioassay, which suggests that the period of Na-AsA treatment and/or the age of the rat at the start of the study may influence the carcinogenic potential of Na-AsA. The purpose of the present study, therefore, was to further evaluate the carcinogenicity of Na-AsA using highly bladder-cancer-susceptible animals.

Various kinds of transgenic and knockout mice are now established as providing good animal models for analysis of gene functions, especially those relevant to carcinogenesis. Some of these animals exhibit high susceptibility to chemical carcinogens in a tissue-specific manner, and now they are receiving much attention with regard to potential application in carcinogenicity tests for risk assessment.

Asamoto et al. (2000) have established a transgenic rat carrying 3 copies of the human c-Ha-*ras* proto-oncogene with its own promoter region, designated Hras128, which is highly susceptible to induction of tumors in mammary tissue by N-methyl-N-nitrosourea (MNU) and 7,12-dimrthylbenz[*a*]anthracene (DBMA) (Asamoto et al., 2000; Tsuda et al., 2001; Fukamachi et al., 2004), and also in the esophagus by N-nitrosomethylbenzylamine (Asamoto et al., 2002) and in the skin by DMBA treatment with or without 12-*O*-tetradecanoylphorbol 13-acetate (TPA) promotion (Park et al., 2004). Moreover, this strain shows remarkable susceptibility to bladder carcinogenesis induced by BBN (Ota et al., 2000).

In the present study, to further assess the tumorigenicity of Na-AsA in the rat urinary bladder, we used this species of cancer-susceptible transgenic rat in a long-term carcinogenicity test.

MATERIALS AND METHODS

Animals

The Hras128 rat line (Asamoto et al., 2000) was generated by injecting copies of the human c-Ha-*ras* proto-oncogene into pronuclei of fertilized rat oocytes from Sprague–Dawley rats obtained from Clea Japan, Inc. (Tokyo, Japan). Subsequent matings were carried out between Tg and Non-tg Sprague–Dawley rats to maintain rat heterozygotes for the transgene (Clea Japan Inc., Tokyo). All the rats subjected to this study were confirmed for the genotype at Clea Japan Inc. (Tokyo, Japan).

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Sodium ascorbate, Na-AsA; *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, BBN; transitional cell carcinoma, TCC.

Experimental Protocol

A total of 40 7-week-old male Tg rats were divided into 2 groups. Twenty-seven (group 1) and 13 (group 2) rats were given a powdered MF diet (Oriental Yeast Co., Ltd., Tokyo) with or without 5% Na-AsA (Wako Pure Chemical Industries, Tokyo Japan), respectively. Similarly, a total of 42 7-week-old male Non-tg rats were divided into 2 groups, and 30 (group 3) and 12 (group 4) animals were given a diet with or without 5% Na-AsA, respectively.

Animals were housed in groups of 2 to 3 in plastic cages with hard wood chips for bedding in an animal room with a 12-hour light, 12-hour dark cycle at a temperature of 22 ± 2°C and a humidity of 55 ± 5%. Body weights were measured weekly, and food consumption and water intake were calculated biweekly during the course of the experiment. Animals were carefully monitored until being sacrificed at final week 57 of the experimental period. Those becoming moribund during the course of the study, or when tumors appeared externally, were sacificed under ether anesthesia for autopsy and macroscopic and microscopic examinations.

All the animal experimental procedures used were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Histological Examinations

All the animals were sacrificed under ether anesthesia at 57 weeks from the beginning of the experiment. Major organs including skin/subcutaneous tissue, mammary gland, forestomach, glandular stomach, small and large intestine, liver, pancreas, lung, kidney, testis, prostate, thymus, spleen, and urinary bladder were macroscopically examined. Any tumors apparent in those various organs were collected. Tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned for routine hematoxylin and eosin staining for histopathological analysis. Urinary bladder lesions were classified based on the WHO International Classification of Rodent Tumors (Kunze and Chowaniec, 1990).

Statistical Analysis

Surviving curves were created using the Kaplan–Meier method, and the statistical significance of differences was calculated by the log-rank test. Variations in incidences of tumors between the different treatments or animal types were evaluated with the nonparametric Fisher's exact probability test or the χ^2 test. All the calculations for statistical analysis were performed using the Statview SE+ Graphics, version 5.0 (Abacus Concepts, Berkeley, CA).

RESULTS

Survival Rates

Tg rats harbored various kinds of spontaneous tumors in various organs when the experiment was terminated after 57 weeks. Survival curves for all groups are shown in Figure 1. As a whole, Tg rats showed a short lifetime, regardless of 5% Na-AsA treatment (groups 1 and 2), compared with their littermate Non-tg rats (groups 3 and 4), but no significant differences were noted between group 1 and group 2 ($p = 0.4001$). About half of group 1 had died or been sacrificed due to the weight loss and/or tumor latency, and group 1's survival

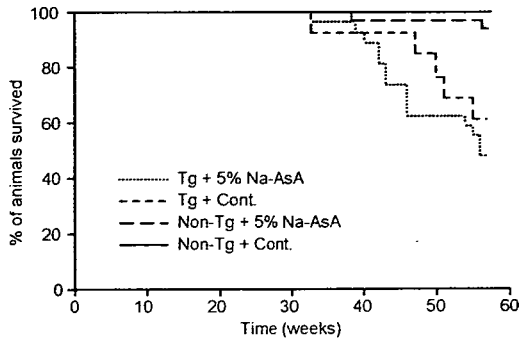

FIGURE 1.—Survival curves of all groups. The experiment was termed at 57 weeks with final sacrificing of all animals.

curve was significantly different from that of group 3 ($p < 0.0001$). The survival curves for groups 3 and 4 were similar. A significant difference was also evident between groups 2 and 4 ($p < 0.05$). No animal died throughout the study in group 4, and 2 animals in group 3 died from pneumonia and fibrosarcoma of subcutaneous tissue with lung metastasis, respectively.

Tumor Incidences

Table 1 summarizes data for incidences of bladder tumors. All the lesions were transitional cell papillomas or carcinomas (Figure. 2A, B), and no bladder tumors occurred in either groups 3 or 4. No difference in the combined overall incidence of transitional cell carcinomas and papillomas was detected between groups 1 (37.0%) and 2 (30.8%). There was a tendency for Tg rats treated with Na-AsA to show a higher incidence of carcinoma (29.6%), compared to those without Na-AsA (15.4%), but no statistical difference was noted. Simple hyperplasia was detected in all the rats treated with Na-AsA, regardless of genotype.

Incidences of tumor-bearing rats in groups 1, 2, 3, and 4 were 70.3, 69.2, 20.0, and 25.0%, respectively (Table 2). The Tg rats showed significantly high incidences of tumors compared with Non-tg rats, both with and without Na-AsA treatment. However, there was no statistical difference between groups 1 and 2. A summary of tumors generated in organs other than the bladder is given in Table 3. Independent of the Na-AsA treatment, transgenic rats exhibited various kinds of malignant tumors in various organs, without significant differences between groups 1 and 2.

DISCUSSION

In 1998, Cohen et al. (1998) examined the tumorigenicity of Na-AsA in male rats using a 2-generation bioassay

TABLE 1.—Tumor incidences in urinary bladder.

Groups	1	2	3	4
No. of rats	27	13	30	12
Papilloma	2 (7.4)[a]	2 (15.4)	0 (0)	0 (0)
TCC	8 (29.6)	2 (15.4)	0 (0)	0 (0)
Total	10 (37.0)	4 (30.8)	0 (0)	0 (0)

[a] % in parenthesis.
TCC, Transitional cell carcinoma.

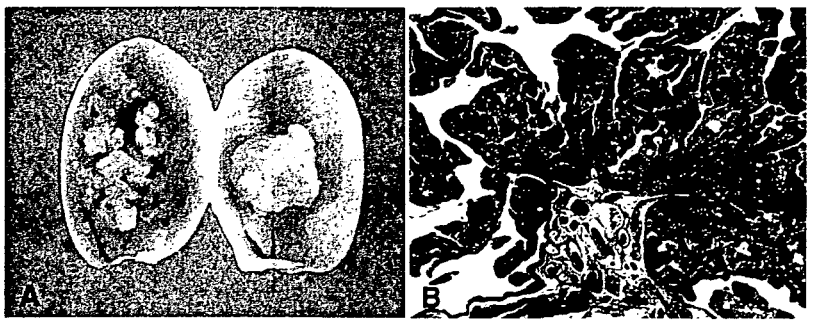

FIGURE 2.—Macroscopic (A) and microscopic (B) appearances of bladder transitional cell carcinoma in Hras128 rat.

that involved feeding to the male and female parental rats for 4 weeks before mating, feeding the dams during gestation and lactation, and then feeding the weaned male F_1 generation rats for the remainder of their lifetime. In that experiment, high-dose (5.0 and 7.0%) treatment of Na-AsA demonstrated an increase in urinary bladder urothelial papillary, nodular hyperplasia, and even papillomas and carcinomas with dose dependence. This implies the possibility of bladder cancer production on the part of this chemical, but no positive cancer induction has been observed thus far in standard, 1-generation-carcinogenicity tests using rodent models.

In the present study, a reexamination of the rat bladder carcinogenesis of the Na-AsA of Hras128 rat, a highly susceptible strain, provided no evidence of cancer induction. The nonsignificant tendency for an increase in the number of malignant tumors in Tg rats suggests the possibility of a weak promotion effect after spontaneous initiation.

Transgenic mice carrying human c-Ha-ras protooncogenes (rasH2) are highly susceptible to various carcinogens and have been demonstrated to be more predictive of human response than the classical cancer test methods (Mitsumori, 2003). This animal model is now regarded as a useful tool for evaluating chemical carcinogenicity, in accordance with the recommendation of the International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use (ICH). However, Na-AsA is not known to exert an enhancing influence on mice bladder carcinogenesis after BBN treatment (Tamano et al., 1993). Therefore, we employed a rat model to evaluate the carcinogenicity of Na-AsA.

In rodent urinary bladder carcinomas, those induced by BBN with and/or without Na-AsA in rats are mostly of the superficial type, while the invasive type is more frequent in mice. Moreover, almost all of the bladder cancers in rats are TCCs, whereas in mice most are squamous cell carcinomas. In the human bladder, TCCs are a major histological type, and this is classified as a "superficial type." They are associated with frequent recurrence and invasion to the muscle layer, resulting in the poor prognosis. However, the reasons for such recurrence and invasive characteristics have not yet been elucidated in detail. We used a rat transgenic animal model representing the superficial type to reexamine the carcinogenic potential of Na-AsA, to match the histological type of human bladder carcinomas.

Ota et al. (2000) examined the susceptibility of bladder carcinogenesis in the same Tg rats using the potent genotoxic carcinogen, BBN, and revealed a high incidence of bladder carcinomas. Also, the Hras128 rat was found to exhibit greater tumor progression as compared to Non-tg counterparts. Mutations of both the exogenous and endogenous c-Ha-*ras* gene are rare, which may indicate that enhanced tumor development was not due primarily to mutations occurring in the transgene. The authors concluded that the overexpression of total c-Ha-ras protein, due to the expression of integrated genes, plays a major role in rat bladder carcinogenesis. Taking these findings into account, the molecular target of Na-AsA in enhancing bladder cancer production may differ from the c-Ha-*ras* overexpression pathway.

TABLE 2.—Effect of 5% Na-AsA in Hras128 rats.

Groups	Strain and treatment	No. of rats	No. of tumor-bearing rats
1	Tg + 5% Na-AsA	27	19 (70.3)[a,b]
2	Tg + Cont.	13	9 (69.2)[c]
3	Non-Tg + 5% Na-AsA	30	6 (20.0)
4	Non-Tg + Cont.	12	3 (25.0)

[a] % in parenthesis.
[b] Statistically significant with its Non-tg + 5% Na-AsA ($p < 0.0001$).
[c] Statistically significant with its Non-tg + Cont. ($p < 0.01$).
Tg, Hras128; Na-AsA, Sodium L-ascorbate in diet; Cont., Control diet.

TABLE 3.—Summary of tumors in various organs.

Groups	1	2	3	4
No. of rats	27	13	30	12
Mammary				
Fibroadenoma	0 (0)[a]	0 (0)	0 (0)	1 (8.3)
Adenocarcinoma	3 (11.1)	1 (7.7)	1 (3.3)	0 (0)
Liver				
Adenoma	1 (3.7)	0 (0)	0 (0)	0 (0)
Cholangiocarcinoma	1 (3.7)	0 (0)	0 (0)	0 (0)
Kidney				
Adenoma	0 (0)	1 (7.7)	0 (0)	0 (0)
Thymus				
Lymphoma	1 (3.7)	0 (0)	0 (0)	0 (0)
Skin and subcutis				
Sebaceous adenoma	1 (3.7)	0 (0)	0 (0)	2 (16.7)
Keratoacanthoma	1 (3.7)	0 (0)	2 (6.7)	0 (0)
Squamous cell papilloma	1 (3.7)	0 (0)	0 (0)	0 (0)
Squamous cell carcinoma	1 (3.7)	3 (23.1)	1 (3.3)	0 (0)
Basal cell carcinoma	1 (3.7)	0 (0)	1 (3.3)	0 (0)
Fibrosarcoma	3 (11.1)	2 (15.4)	1 (3.3)	0 (0)
Malignant fibrous histiocytoma	1 (3.7)	0 (0)	0 (0)	0 (0)

[a] % in parenthesis.

In conclusion, the rat bladder promoter Na-AsA did not show tumorigenicity, even when administered to highly bladder-cancer-susceptible Hras128 rats. However, the tendency for an increase of malignancy in Tg rats' bladder tumors suggests that Na-AsA may have promotional effects. Taking the previous reports into account, these results suggest that Na-AsA is a pure promoter but not a complete carcinogen in rats.

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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

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

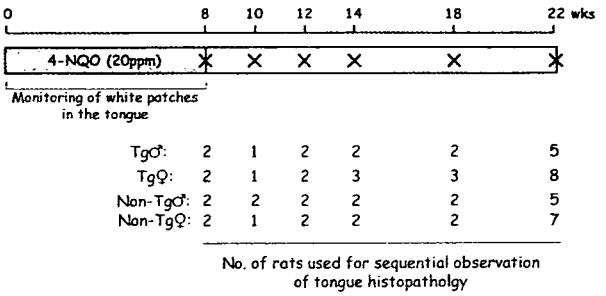

Fig. 1. Experimental protocol. X, killing time points. Nos, number of Tg and non-Tg rats that are killed for tongue histopathology.

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

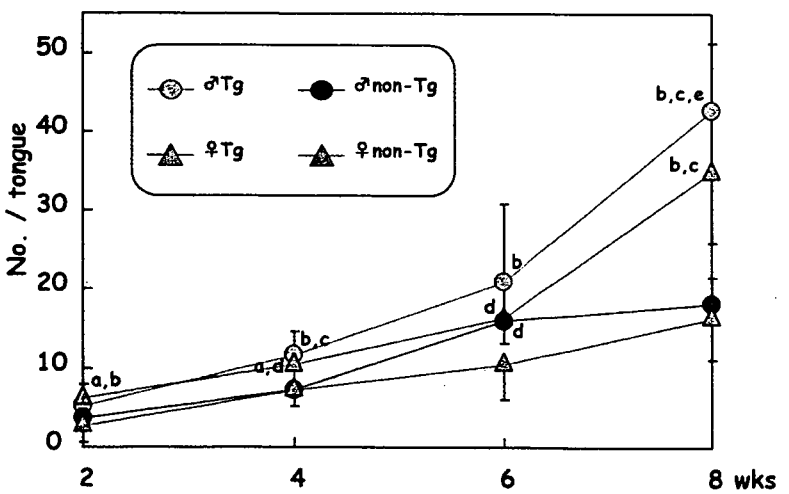

Fig. 2. Mean number of white patches in the tongue that were monitored from Week 2 through Week 8. a, significantly different from male non-Tg ($P < 0.05$); b, significantly different from female non-Tg ($P < 0.001$); c, significantly different from male non-Tg ($P < 0.001$); d, significantly different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

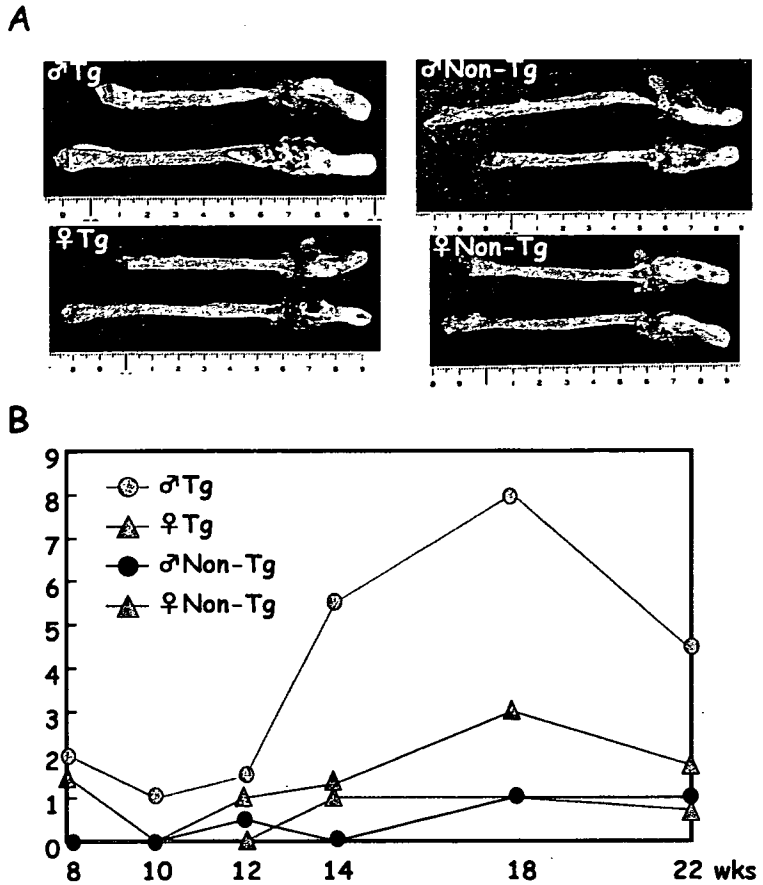

Fig. 3. Tongue neoplasms developed in Tg and non-Tg rats that were treated with 4-NQO. (A) Macroscopic view of tongues at Week 22. Large tongue tumors with white patches develop in Tg rats of either sex. whereas only a few small tongue tumors or white patches are found in the tongue in non-Tg rats of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

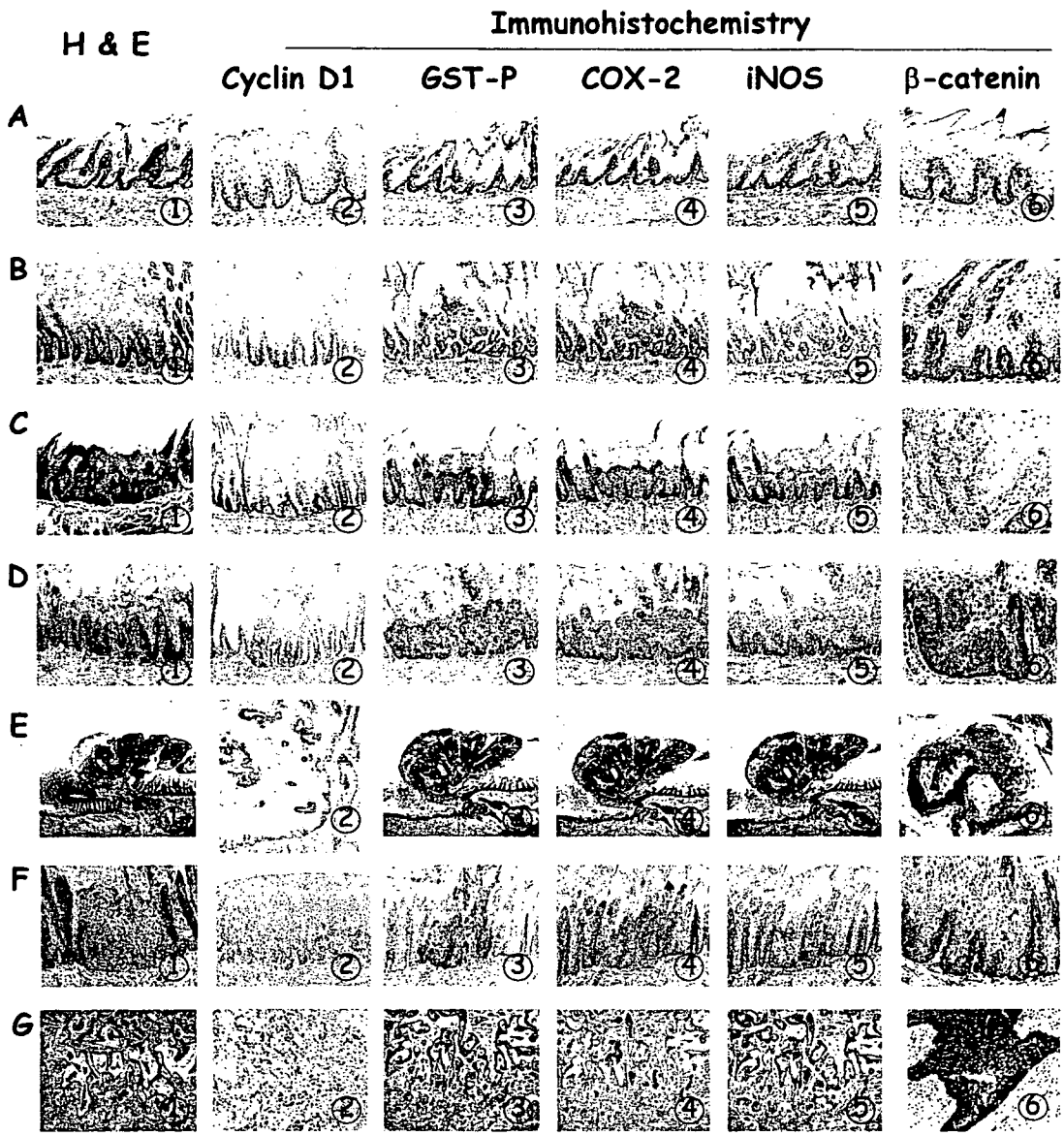

Fig. 4. Histopathology and immunohistochemistry of cyclin D1, GST-P, COX-2, iNOS and β-catenin of tongue lesions in Tg males that received 4-NQO. (**A**), 'Normal' appearing tongue squamous epithelium; (**B**) Mild dysplasia; (**C**) Moderate dysplasia; (**D**) Severe dysplasia; (**E**) Squamous cell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

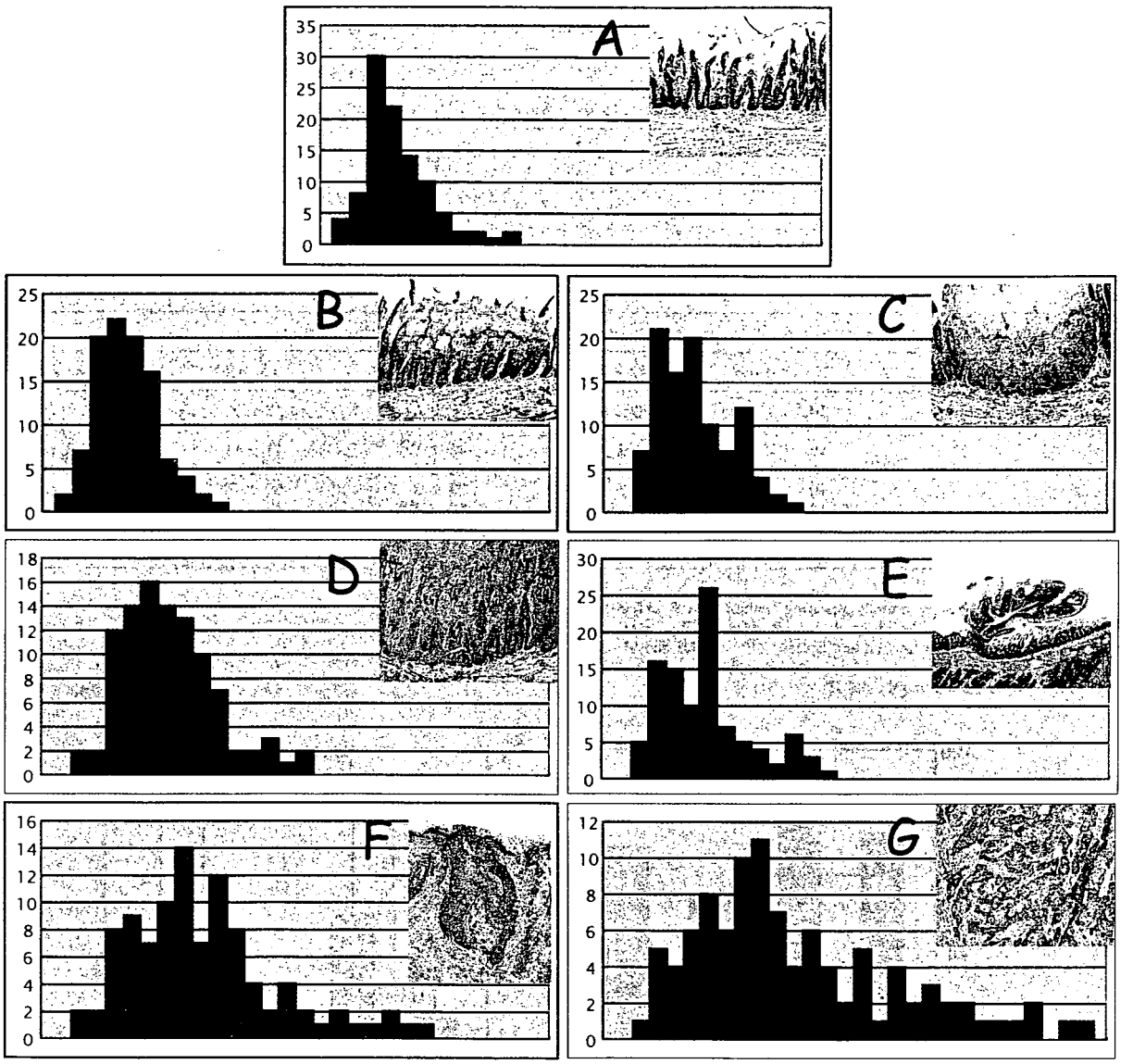

Fig. 5. Representative DNA histograms from various tongue lesions (inserts) in male Tg rats treated with 4-NQO. (**A**) 'Normal' appearing tongue squamous epithelium; (**B**) Mild dysplasia; (**C**) Moderate dysplasia; (**D**) Severe dysplasia; (**E**) Squamous cell papilloma; (**F**) SCC *in situ* and (**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' ($P < 0.001$).

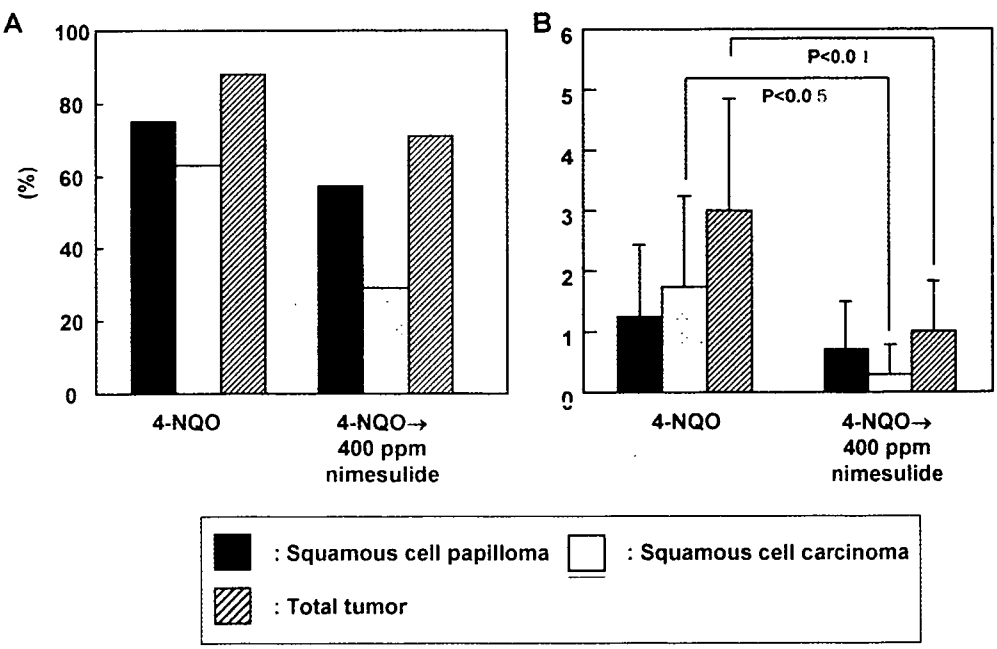

Fig. 6. The effect of dietary nimesulide (400 p.p.m.) on tongue neoplasms in male Tg rat. (A) Incidence (%) of tongue carcinomas and (B) multiplicity (no. of carcinoma/rat) of tongue carcinoma. Treatment with nimesulide reduced the incidence and multiplicity of tongue tumors. The inhibition in the multiplicities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

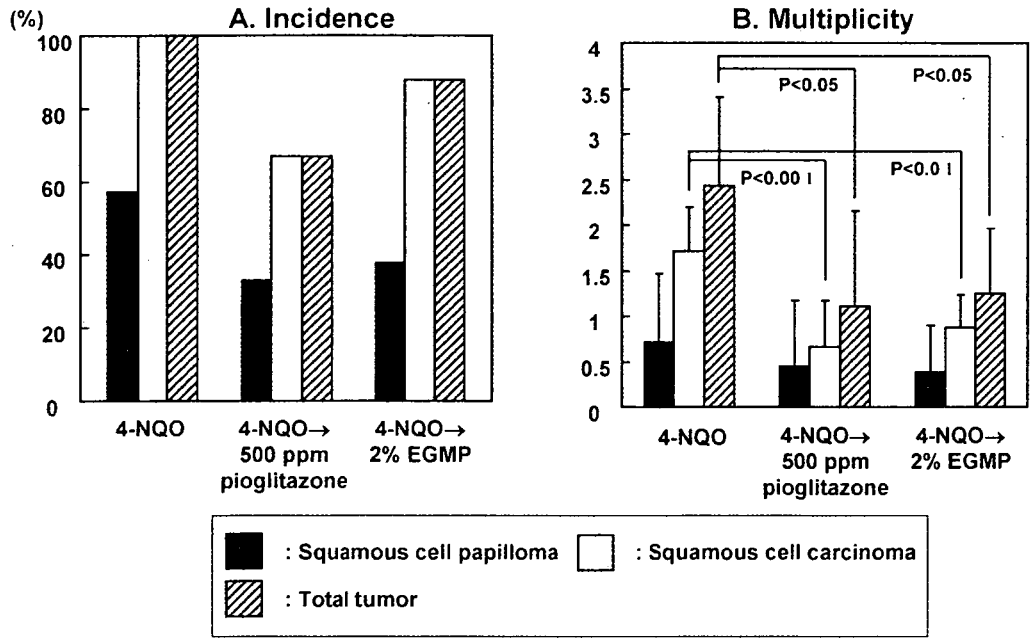

Fig. 7. The effect of dietary feeding with pioglitazone (500 p.p.m.) and EGMP (2%) on tongue neoplasms in female Tg rats. (A) The incidence (%) of tongue carcinomas and (B) multiplicity (number of carcinoma/rat) of tongue carcinoma. Treatment with pioglitazone or EGMP reduced the incidence and multiplicity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

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