Inhibitory effects of troglitazone, a peroxisome proliferator-activated receptor γ ligand, in rat tongue carcinogenesis initiated with 4-nitroquinoline 1-oxide

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Ligands for peroxisome proliferator-activated receptor (PPAR) γ have been implicated in growth inhibition and cell differentiation in certain malignancies. In this study, the effects of troglitazone, a PPARγ ligand, given during the postinitiation phase of oral carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats. Rats aged 6 weeks were given 4-NQO at 20 ppm for 8 weeks to induce tongue neoplasms. Starting 1 week after the cessation of 4-NQO exposure, animals were fed diets containing 0, 30 or 100 ppm troglitazone for 22 weeks. At the end of the study (week 32), the incidences of 4-NQO-induced tongue neoplasms and preneoplasms were determined histopathologically and cell proliferation activity was estimated by counting bromodeoxyuridine (BrdU)-labeling indices and cyclin D1-positive cell ratios. In addition, immunohistochemical expression of cyclooxygenase (COX)-2 and PPARγ was assessed in the tongue lesions. Feeding with 100 ppm troglitazone significantly decreased the incidence of squamous cell carcinoma when compared to the group without troglitazone treatment (5.0% vs. 45.8%, *P*<0.005). Interestingly, the BrdU-labeling index and cyclin D1-positive cell ratio assessed in the non-lesional tongue squamous epithelium were reduced by dietary administration of troglitazone (*P*<0.0001–0.005). Additionally, the immunoreactivity of COX-2 in the tongue lesions was also decreased by the treatment (*P*<0.01–0.05). These results clearly showed that dietary troglitazone inhibits 4-NQO-induced tongue carcinogenesis and such inhibition is related to suppression of increased cell proliferation and/or COX-2 expression. This study warrants further investigation on the use of PPARγ ligands as a novel preventive approach for oral malignancy. (Cancer Sci 2003; 94: 365–371)

The nuclear receptor superfamily acts as ligand-responsive transcription factors that participate in many processes important for cell and tissue homeostasis. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D or retinoic acid.[1)] At present, several PPARs including PPARα, PPARβ (PPARδ, NUC-1, or FAAR), and PPARγ have been identified.[2)] Of these, PPARγ was initially shown to have regulatory roles in insulin sensitization.[3, 4)] The receptor binds to the promoter region of target genes involved in adipocyte differentiation and lipid storage[4–6)] as a heterodimer with the retinoid X receptor.[7)] Activation of this receptor has been implicated in glucose metabolism, cell cycle control, and macrophage development and function.[8–12)] In addition, recent studies suggest that PPARs play an important role in carcinogenesis.[13)]

15-Deoxy-δ-prostaglandin J_2, a natural-occurring metabolite of prostaglandin D_2, and troglitazone, a thiazolidinedione analogue and a synthetic anti-diabetic drug, have been reported to be selective ligands for PPARγ.[9, 14, 15)] Recent studies indicated that both natural and synthetic ligands for PPARγ stimulate cell differentiation and inhibit cell growth of various types of neoplasia including lung, breast, prostate, colon, pancreas and bladder cancers.[8, 16, 17)] Recent work from our group demonstrated possible inhibitory effects of ligands for PPARα and γ on the early stage of chemically-induced and inflammation-related colon tumorigenesis in rats.[18, 19)] However, there are few *in vivo* data showing that ligands for PPARγ exhibit tumor-preventive potential in experimental animal models. In addition, previous reports indicated that dietary administration of ligands for PPARγ, including troglitazone, enhances polyp formation in $Apc^{Min/+}$ mice,[20)] suggesting that the effects of PPARγ ligands on *in vivo* carcinogenesis are still unclear and controversial.

Oral cancer is a common neoplasm in certain areas, such as Asia, the Pacific Islands, parts of Europe, and parts of Brazil.[21)] Although Japan has one of the lowest incidences of oral, lip, and pharyngeal cancers in the world,[22)] the incidences of these malignancies have recently been increasing.[23)] Generally, neoplasms in the head and neck, including the oral cavity, are multistage and multifocal.[24)] Squamous cell carcinoma (SCC) is a common primary cancer of the tongue and oral cavity, which progresses from hyperplastic lesions through dysplasia and carcinoma *in situ* to invasive carcinoma.[24, 25)] Transition from preneoplastic lesions to invasive carcinoma is noted in 17.5% of leukoplakia with hyperplasia in the absence of proper treatment, and the transition rate from dysplasia to cancer extends to 36% of the lesions.[26)] Unfortunately, progress of therapeutic approaches for this malignancy has not yet been sufficient to control the carcinogenic process and improve the mortality. Hence, it is important to advance a novel strategy to prevent and/or manage oral lesions effectively. One of such promising approaches is chemoprevention, which is a preventive strategy carried out by dietary administration of certain natural or synthetic compounds. We have identified several candidates for chemopreventive agents against oral malignancy using an experimental animal model having 4-nitroquinoline 1-oxide (4-NQO)-induced tongue lesions.[24)] Interestingly, a spectrum of preneoplastic and neoplastic lesions in the oral cavity, especially the tongue, is produced by exposure to 4-NQO in rats. The 4-NQO-induced oral lesions, which consist of ulcerated and endophytic tumors and hyperplastic and dysplastic lesions, are comparable to human lesions, indicating that this model for oral cancer of humans is a good one.[24)]

In the present study, the modifying effect of troglitazone on 4-NQO-induced oral carcinogenesis was investigated in rats to determine whether this compound has preventive efficacy against oral malignancy. In addition, effects of the compound on the expression of proliferation biomarkers such as bromodeoxyuridine (BrdU)-labeling index and cyclin D1-positive ratio

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were assessed to clarify underlying mechanism(s) of the modification. Also, immunohistochemical expression of cyclooxygenase (COX)-2 and PPARγ was investigated in the tongue lesions induced by 4-NQO, since a certain role of COX-2 in head and neck cancers[26, 27] and the relationship between COX-2 and PPAR expressions[28–31] are suggestive.

Materials and Methods

Animals, chemicals, and diets. Male F344 rats, 4 weeks old, were purchased from Charles River Japan (Kanagawa). The rats were housed three or four to a wire cage in a holding room under controlled conditions of a 12-h light/dark cycle, 23±2°C room temperature, and 50±10% relative humidity. Food and water were available *ad libitum*. Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as a basal diet during the experiment. 4-NQO was obtained from Wako Pure Chemical Ind. (Osaka). Troglitazone was kindly supplied by Sankyo Co. (Tokyo). 4-NQO solution, which was dissolved in tap water to a final concentration of 20 ppm, and experimental diets containing troglitazone at a dose of 30 or 100 ppm, were made weekly and stored in a dark, cold room (4°C) until used.

Experimental procedures. After quarantine for 2 weeks, a total of 80 rats were divided into 5 groups as shown in Fig. 1. At 7 weeks of age, rats in groups 1–3 were given 20 ppm 4-NQO for 8 weeks to induce tongue neoplasms. Groups 2 and 3 were fed the diets containing troglitazone at doses of 30 and 100 ppm, respectively, for 22 weeks, starting 1 week after the cessation of 4-NQO treatment. Group 4 was fed the diet containing 100 ppm troglitazone for 22 weeks (from week 10 to week 32) without 4-NQO treatment. Group 5, which was given the basal diet without troglitazone and tap water without 4-NQO throughout the experiment, served as an untreated control. All rats were carefully observed daily. The consumption of the drinking water containing 4-NQO and the diets with troglitazone was recorded to estimate intake of the chemicals. The experiment was terminated at 32 weeks, and all animals were sacrificed. At necropsy, all organs including the oral cavity were carefully inspected to find tongue tumors. For histopathological examination, tissues and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks, and sliced to prepare 5 serial sections (3 μm). The sections were used for hematoxylin and eosin staining and immunohistochemistry of the expression of cell proliferation biomarkers (BrdU-labeling index and cyclin D1-positive cell ratio) and of COX-2 and PPARγ. Tongue epithelial lesions (hyperplasia, dysplasia and neoplasia) in the oral cavity were diagnosed according to the criteria described by Banoczy and Csiba[32] and WHO.[33]

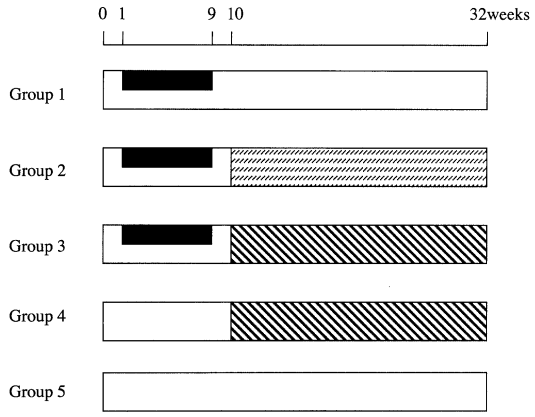

Fig. 1. Experimental protocol. ■, 4-NQO, 20 ppm drinking water; □, basal diet and tap water; ▨, troglitazone, 30 ppm in diet; ▧, troglitazone, 100 ppm in diet.

Determination of proliferative activity in the tongue epithelium. For measurement of BrdU-incorporated nuclei, all animals were given an intraperitoneal injection of BrdU (50 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) 1 h prior to killing. The immunohistochemical detection of BrdU was done using a commercial kit (DAKO Japan, Kyoto). The labeling indices of BrdU (percentages) were calculated by evaluating approximately 1000 cells in normal or non-lesional tongue epithelium of each rat. For detecting cyclin D1-positive cells, a staining kit (DAKO) was used. A mouse monoclonal antibody against cyclin D1 (Novocastra, Newcastle upon Tyne, UK) was applied to the sections on gelatin-coated glass slides. Positive cell ratios for cyclin D1 were calculated by assessing at least 500 cells in normal or non-lesional tongue epithelium of each rat. Cells were considered positive for BrdU or cyclin D1 when definite nuclear staining was detected.

Immunohistochemistry of COX-2 and PPARγ. Immunohistochemistry of COX-2 and PPARγ were performed using a commercial kit (DAKO). A mouse monoclonal antibody against COX-2 (Transduction Laboratories, Lexington, KY) and a mouse monoclonal antibody against PPARγ (Transduction Laboratories) were used as primary antibodies. For semi-quantitative analysis of COX-2 and PPARγ immunopositivity, the tongue tissue on each slide was evaluated for the overall intensity of immunoreactivity scored with four grades as follow: − (0), no staining; ± (1), weakly positive (weaker than the immunopositivity of macrophages) over less than 10% of the area; + (2), weakly positive over more than 10% of the area; ++ (3), strongly positive (equal or more than the immunopositivity of macrophages) over more than 10% of the area. The mean scores of the preneoplastic and neoplastic tissues were compared among the different groups.

Statistical analysis. Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test. The data of measurements of body and liver weights, the BrdU-labeling index, cyclin D1-positive ratio and immunoreactivity COX-2 and PPARγ were analyzed using Student's or Welch's *t* test and/or the Mann-Whitney *U* test. The results were considered statistically significant if the *P* value was less than 0.05.

Table 1. Body, liver, relative liver, and kidney weights in each group

Group No.	Treatment	No. of rats (final)	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)	Kidney wt (g)	Relative kidney wt (g/100 g body wt)
1	4-NQO alone	24	333.9±14.8[1)]	10.5±0.9	3.16±0.30	2.50±0.59	0.75±0.17
2	4-NQO→30 ppm troglitazone	19	329.3±18.1	10.8±0.9	3.28±0.29[2)]	2.63±0.50	0.80±0.16
3	4-NQO→100 ppm troglitazone	20	329.5±22.2	10.5±1.2	3.17±0.29	2.55±0.60	0.78±0.19
4	100 ppm troglitazone	8	338.8±12.5	10.5±1.1	3.10±0.29	2.35±0.52	0.70±0.13
5	No treatment	8	330.6±13.2	10.3±0.5	3.10±0.11	2.25±0.46	0.68±0.15

1) Mean±SD.
2) Significantly different from group 5 by Welch's *t* test ($P<0.05$).

Results

General observations. Animals in groups 1–3 tolerated well the oral administration of 4-NQO and troglitazone, except for 1 rat in group 2 that died at week 20 for an unknown reason. Average food intakes containing the test chemicals in each group were 16.4–17.6 g/day/animal. The total intakes of troglitazone per mg/day/animal in groups 2 (30 ppm troglitazone) and 3 (100 ppm troglitazone) were 0.33–0.35 and 1.09–1.17, respectively. There were no significant differences in the total intakes of 4-NQO/rat among the three groups (data not shown). The mean body, liver, kidney, relative liver, and relative kidney weights (g/100 g body weight) at the end of the study are shown in Table 1. The differences in mean body weights, liver weights and kidney weights were insignificant among the groups, except for relative liver weight in group 2. Dietary ad-

Table 2. Effect of troglitazone on development of tongue tumors in male F344 rats

Group No.	Treatment	No. of rats	No. of rats with tongue neoplasms (%)			Multiplicity (No. of tumors/rats, mean±SD)	
			Total	Papilloma	SCC	Papilloma	SCC
1	4-NQO alone	24	14 (58.33)	3 (12.5)	11 (45.83)	0.13±0.34	0.54±0.72
2	4-NQO→30 ppm troglitazone	19	5 (26.32)[1)]	0 (0)	5 (26.32)	0	0.26±0.45
3	4-NQO→100 ppm troglitazone	20	3 (15.0)[2)]	3 (15.0)	1 (5.0)[3)]	0.15±0.37	0.05±0.22[4)]
4	100 ppm troglitazone	8	0	0	0		
5	No treatment	8	0	0	0		

1) Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
2) Significantly different from group 1 by Fisher's exact probability test ($P<0.01$).
3) Significantly different from group 1 by Fisher's exact probability test ($P<0.005$).
4) Significantly different from group 1 by Welch's *t* test ($P<0.005$).

Table 3. Incidence of preneoplastic lesion of tongue in rats of each group

Group No.	Treatment	No. of rats	No. of rats with preneoplastic lesions (%)					
			Total (%)	Hyperplasia (%)		Dysplasia (%)		
				Simple	Papillary	Mild	Moderate	Severe
1	4-NQO alone	24	24 (100)	12 (50.0)	19 (79.17)	6 (25.0)	11 (45.83)	15 (62.5)
2	4-NQO→30 ppm troglitazone	19	14 (73.68)[1)]	9 (47.37)	8 (42.12)[1)]	4 (21.05)	6 (31.56)	5 (26.32)[1)]
3	4-NQO→100 ppm troglitazone	20	15 (75.0)[2)]	11 (55.0)	11 (55.0)	9 (45.0)	9 (45.0)	3 (15.0)[2)]
4	100 ppm troglitazone	8	0	0	0	0	0	0
5	No treatment	8	0	0	0	0	0	0

1) Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
2) Significantly different from group 1 by Fisher's exact probability test ($P<0.01$).

Table 4. Multiplicity of preneoplastic lesion of tongue in rats of each group

Group No.	Treatment	No. of rats	Multiplicity (No. of preneoplastic lesions/rats, mean±SD)				
			Hyperplasia		Dysplasia		
			Simple	Papillary	Mild	Moderate	Severe
1	4-NQO alone	24	0.50±0.59	0.79±0.64	0.25±0.44	0.46±0.51	0.63±0.58
2	4-NQO→30 ppm troglitazone	19	0.47±0.51	0.42±0.61	0.21±0.42	0.32±0.58	0.26±0.45[1)]
3	4-NQO→100 ppm troglitazone	20	0.55±0.76	0.55±0.51	0.45±0.76	0.45±0.60	0.15±0.37[2)]
4	100 ppm troglitazone	8	0	0	0	0	0
5	No treatment	8	0	0	0	0	0

1) Significantly different from group 1 by Student's *t* test ($P<0.05$).
2) Significantly different from group 1 by Student's *t* test ($P<0.01$).

Table 5. BrdU-labeling index and cyclin D1-positive ratio on non-lesional area of tongue squamous epithelium

Group No.	Treatment	BrdU-labeling index		Cyclin D1-positive ratio	
		No. of rats examined	Mean±SD	No. of rats examined	Mean±SD
1	4-NQO alone	14	12.88±3.06[1)]	24	16.13±2.79[1)]
2	4-NQO→30 ppm troglitazone	6	8.31±2.05[2)]	19	12.95±2.10[4)]
3	4-NQO→100 ppm troglitazone	8	6.77±1.47[3)]	20	11.59±2.13[5)]
4	100 ppm troglitazone	8	5.41±1.45	8	9.20±3.13
5	No treatment	8	5.10±0.53	8	8.66±2.90

1) Significantly different from group 5 by Welch's *t* test ($P<0.001$).
2) Significantly different from group 1 by Student's *t* test ($P<0.005$).
3) Significantly different from group 1 by Welch's *t* test ($P<0.001$).
4) Significantly different from group 1 by Student's *t* test ($P<0.0005$).
5) Significantly different from group 1 by Student's *t* test ($P<0.0001$).

Table 6. COX-2 immunohistochemical staining of tongue lesions

Group No.	Treatment	Preneoplastic lesions					Neoplasms				
		No. of lesions examined	No. of lesions with COX-2 antibody staining[1] (%)				No. of lesions examined	No. of lesions with COX-2 antibody staining[1] (%)			
			–	±	+	++		–	±	+	++
1	4-NQO alone	32	2 (6.3)	2 (6.3)	19 (59.4)	9 (28.1)	16	0 (0)	0 (0)	4 (25.0)	12 (75.0)
2	4-NQO→30 ppm troglitazone	17	2 (11.8)	3 (17.6)	10 (58.8)	2 (11.8)	5	0 (0)	1 (20.0)	1 (20.0)	3 (60.0)
3	4-NQO→100 ppm troglitazone	22	1 (4.5)[2]	6 (27.3)[2]	13 (59.1)[2]	2 (9.1)[2]	4	0 (0)[3]	0 (0)[3]	4 (100)[3]	0 (0)[3]

1) Staining: –, negative; ±, weakly positive; +, moderately positive; ++, strongly positive.
2) Significantly different from group 1 by Mann-Whitney *U* test ($P<0.05$).
3) Significantly different from group 1 by Mann-Whitney *U* test ($P<0.01$).

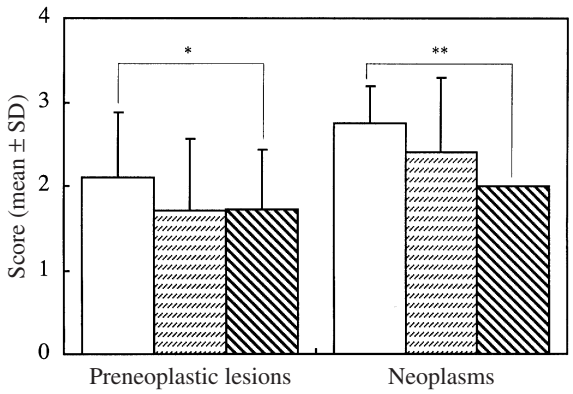

Fig. 2. COX-2 immunoreactivity was analyzed semi-quantitatively in tongue lesions induced by administration of 20 ppm 4-NQO for 8 weeks. The intensity of COX-2 immunohistochemical staining in tongue lesions was determined by scoring with four grades from negative (0) to strong immunoreactivity (3). The open bars represent the mean score of COX-2 immunopositivity in group 1 (4-NQO alone). The thin hatched bars represent that in group 2 (30 ppm troglitazone) and the thick hatched bars indicate that in group 3 (100 ppm troglitazone). The error bars represent SD. *, $P<0.05$. **, $P<0.01$.

ministration of troglitazone did not cause any clinical signs of low survival rate, poor condition, or histological changes in the liver and kidney that would point to toxicity.

Incidence of tumors and preneoplastic lesions. In groups 1–3, endophytic and exophytic tumors developed mainly in the dorsal region of posterior tongues. Histologically, the former tumors were well-differentiated SCCs, and the latter were squamous cell papillomas. In this study, tumors were present only in the tongue, and no metastasis was recognized in other organs. Rats in groups 4 and 5 did not have any neoplastic lesion in any organ. As shown in Table 2, the incidences of total tongue neoplasms (SCC and squamous cell papilloma) were 58.3% (14 of 24 rats) in group 1, 26.3% (5 of 19 rats) in group 2, and 15.0% (3 of 20 rats) in group 3, resulting in a significant reduction of the incidences in groups 2 and 3 ($P<0.05$ and $P<0.01$) as compared to that of group 1. In particular, the incidence and multiplicity of SCC in group 3 (5.0%, 0.05±0.22) were significantly smaller ($P<0.005$) than those in group 1 (45.83%, 0.54±0.72).

In addition to the neoplasms, the incidence and multiplicity of tongue hyperplastic and dysplastic lesions are shown in Tables 3 and 4. The preneoplastic lesions were classified into two categories of hyperplasia (simple and papillary) and three types of dysplasia (mild, moderate, and severe) according to the degree of atypism present.[32, 33] The incidences of total preneoplasia in groups 2 (73.7%) and 3 (75.0%) were significantly lower than that of group 1 (100%, $P<0.05$). Although the incidences of simple hyperplasia of groups 1–3 were comparable, the incidence of papillary hyperplasia in group 2 (42.1%, $P<0.05$) was lower than that in group 1 (79.2%). The incidences of severe dysplasia in groups 2 (26.3%) and 3 (15.0%) were significantly lower than that in group 1 (62.5%) ($P<0.05$ and $P<0.01$, respectively). Similarly, the multiplicities of severe dysplasia in groups 2 (0.26±0.45) and 3 (0.15±0.37) were significantly smaller than that in group 1 (0.63±0.58) ($P<0.05$ and $P<0.01$, respectively).

BrdU-labeling index and cyclin D1-positive cell ratio. The results of BrdU-labeling indices and cyclin D1-positive ratios in the non-lesional squamous epithelium are summarized in Table 5. We counted approximately 1000 cells from each section and expressed the BrdU-labeling index as a percentage. The BrdU-labeling index of group 1 (12.88±3.06) was the highest among the groups and was significantly larger than that of group 5 (untreated control, 5.10±0.53, $P<0.001$). Dietary administration of troglitazone in groups 2 and 3 significantly decreased those values (8.31±2.05 and 6.77±1.47, respectively) when compared with group 1 ($P<0.005$ and $P<0.001$, respectively).

Similarly, positive cell ratios for cyclin D1 were determined by counting about 1000 cells that were selected randomly, and were calculated as numbers per 100 cells. As shown in Table 5, cyclin D1-positive ratios of groups 2 (12.95±2.10, $P<0.001$) and 3 (11.59±2.13, $P<0.001$) were significantly smaller than that of group 1 (16.13±2.79), which was significantly greater than that of the untreated control (8.66±2.90, $P<0.001$).

Immunohistochemical expression of COX-2 and PPARγ. Immunohistochemical stainability of COX-2 in the tongue lesions is summarized in Table 6 and Fig. 2. Generally, immunohistochemistry for COX-2 revealed that COX-2 expression was multifocal and moderate to strong in intensity in the tongue neoplasms. Weak to moderate immunopositivity of COX-2 was present in preneoplasic lesions (Fig. 3). Faint immunoreactivity for COX-2 was detected in non-lesional tongue squamous epithelium of groups 1–3. Dietary administration of 100 ppm troglitazone (group 3) significantly increased the occurrence of preneoplastic lesions with negative or weak immunoreactivity of COX-2 ($P<0.05$) and significantly decreased that of neoplasms with strong immunoreactivity of COX-2 ($P<0.01$) when compared to group 1. These results indicate that the immunoreactivity of COX-2 was decreased with dietary exposure to troglitazone in both preneoplastic and neoplastic tissues.

Immunohistochemical expression of PPARγ was also examined in normal, preneoplastic, and neoplastic tissues of rat tongues (Fig. 4). The immunoreactivity of PPARγ was mainly recognized in nuclei and/or cytoplasms of cells with a granular pattern. Semi-quantitative analysis of immunohistochemistry showed that there was strong expression of PPARγ proteins in neoplastic cells, moderate to weak in preneoplastic lesions, and weak in non-lesional or normal epithelium. However, there was no statistically significant difference of the PPARγ immunopositivity between the different groups (data not shown).

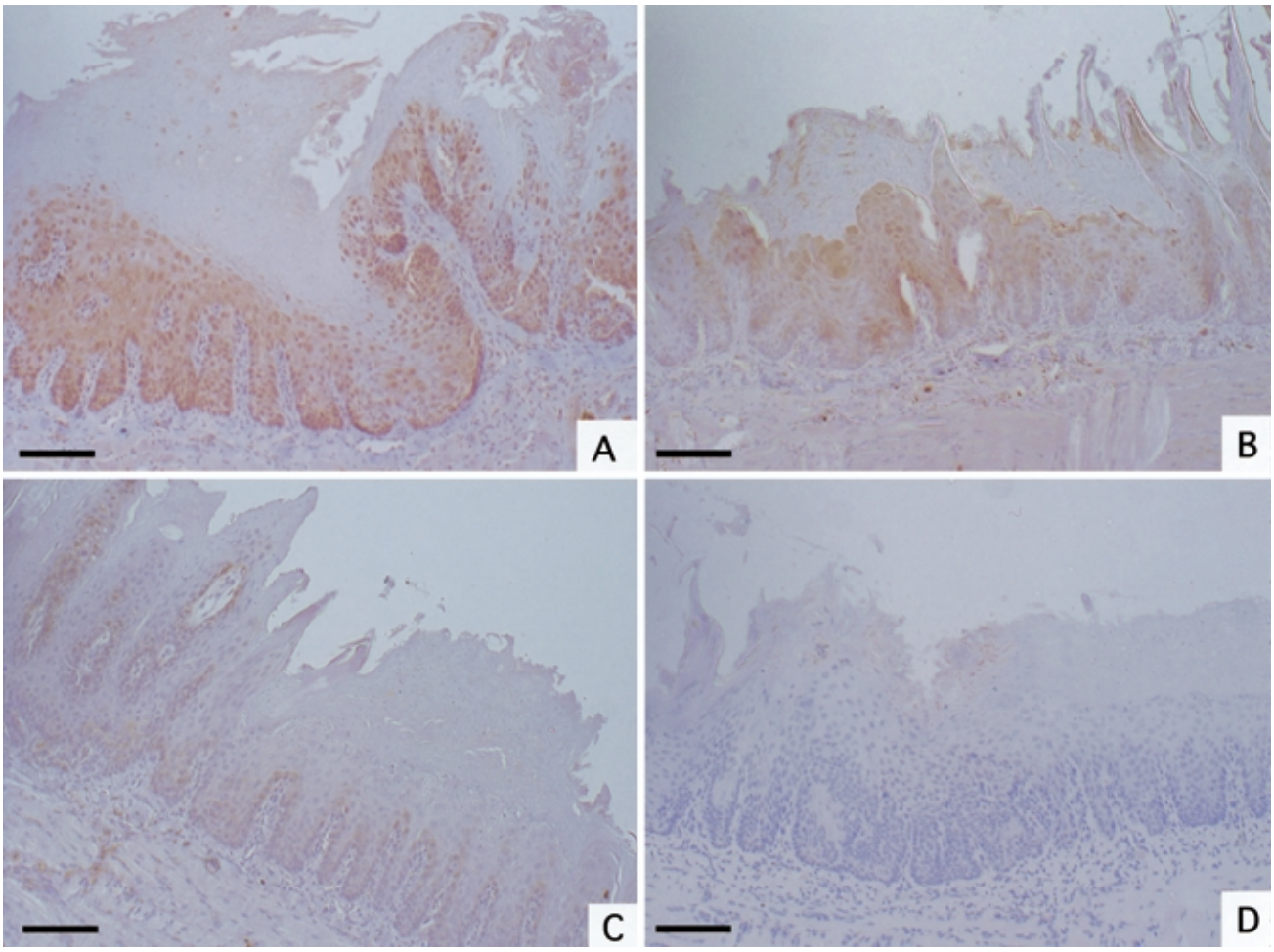

Fig. 3. Immunohistochemistry of COX-2 in tongue lesions induced by administration of 20 ppm 4-NQO for 8 weeks. A–D show representative immunohistochemistry of COX-2 in preneoplastic lesions with different immunopositivity. A, Immunohistochemical expression of COX-2 was strongly positive (equal to or more than the immunopositivity of macrophages) over more than 10% of the area; ++ (3). B, COX-2 expression was moderately positive (weakly positive over more than 10% of the area); + (2). C, COX-2 was expressed weakly (less than 10% of the area of immunohistochemical staining); ± (1). D, No expression of COX-2; − (0). Bars, 100 μm.

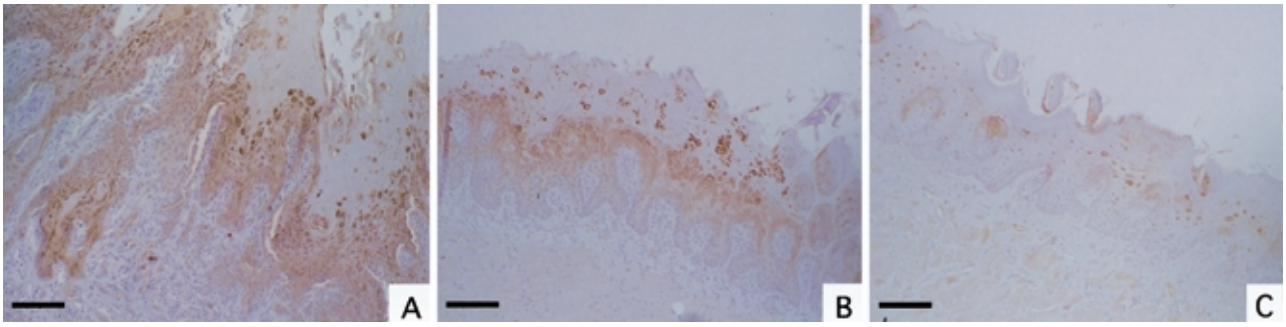

Fig. 4. Immunohistochemistry of PPARγ in squamous cell carcinoma (A), preneoplastic lesion (B) and normal tongue epithelium (C). A, Strong expression of PPARγ was recognized in squamous cell carcinoma. Immunopositivity of PPARγ was detected strongly in nuclei and nuclear membranes and weakly in cytoplasm. B, Moderate PPARγ immunoreactivity was present in preneoplastic lesions. Similarly, the immunoreactivity was localized in nuclei and nuclear membranes and also mildly in cytoplasm. It is apparent that the granular layer of the squamous epithelium had the strongest immunostaining. C, Positive staining of PPARγ in some nuclei of the granular layer was seen in normal squamous epithelium. Bars, 100 μm.

Discussion

In the present study, a significant reduction in the occurrence of tongue neoplasms following 4-NQO treatment was observed in rats fed the diets containing troglitazone. It should be noted that only one SCC developed in the rats with 100 ppm troglitazone. Furthermore, feeding of troglitazone suppressed the development of preneoplastic lesions in tongue tissues. This is the first report demonstrating the inhibitory effect of troglitazone on oral carcinogenesis. Recently, we reported that troglitazone suppresses the formation of aberrant crypt foci, which are recognized as an intermediate biomarker for colon car-

cinogenesis.[18, 19] Additionally, several *in vitro* studies previously demonstrated that troglitazone inhibits cell growth in several human cancer cell lines.[16, 17, 34–37] Although dietary administration of ligands for PPARγ enhances polyp formation in $Apc^{Min/+}$ mice, it is apparent that further analyses of modifying effects of ligands for PPARγ, including troglitazone, on tumorigenesis of various sites are warranted.

From the point of tumor-preventive mechanisms, supplementation with troglitazone in the diet reduced the expression of biomarkers of cell proliferation, such as BrdU-labeling index and cyclin D1-positive cell ratio. Interestingly, our data showed that troglitazone specifically inhibited 4-NQO-induced hyperproliferation in the non-lesional squamous epithelium, because normal epithelium of negative control rats (group 4) did not show any significant reduction of cell proliferation by troglitazone. This may be due to a difference of PPARγ expression in the tissues between 4-NQO-treated and non-treated rats, since our PPARγ immunohistochemistry findings raise the possibility that PPARγ expression might be increased by 4-NQO treatment. Apparently, further analysis will be needed to prove the point, because limitations of our immunohistochemical method prevented us from quantitating such differences of PPARγ expression.

Cell proliferation is suggested to play an important role in multistage carcinogenesis,[28, 38] including oral tumorigenesis.[24, 39] Accordingly, control of cell proliferation activity is considered to be one of the possible targets of cancer chemopreventive agents.[40] Indeed, we and others have reported that most of the possible chemopreventive agents against 4-NQO-induced oral carcinogenesis suppress cell proliferation activity.[24] Our results imply that the tumor-suppressing effects of troglitazone in the present study might be due to lower cell proliferation. It is interesting to note that troglitazone causes inhibition of cell growth with G1 cell cycle arrest in bladder and hepatocellular carcinoma cells.[16, 37] The regulatory effect of troglitazone on the cell cycle is also accompanied with increased expressions of cyclin-dependent kinase inhibitors, p21 and p18, and reduced cyclin D1 expression.[16, 17] Cyclin D1 is a member of the G1 cyclin family which is involved in regulating the transition through the G1 phase of cell cycle.[41, 42] Cyclin D1 overexpression has been reported in human cancers of the head and neck, including tongue, and in 4-NQO-induced tongue cancers of rats, which leads to dysregulation of cell cycle and uncontrolled cell proliferation.[43–45] Hence, the decreased cyclin D1 expression in the present study may play an important role in the suppression of tongue carcinogenesis by troglitazone.

Another possible mechanism to inhibit the development of 4-NQO-induced neoplasms[27, 28] may be the inhibition of COX-2 activity.[46] An inducible form of cyclooxygenase, COX-2, is linked to regulation of inflammation and is believed to be the target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (NSAIDs).[46] Recently, there are accumulating data showing that NSAIDs also inhibit tumorigenesis of certain organs including the oral cavity. In fact, NSAIDs (indomethacin and piroxicam)[47] and a COX-2-specific inhibitor (nimesulide) inhibit chemically-induced tongue tumorigenesis.[27, 28] It seems likely that the preventive efficacies of NSAIDs or other agents acting against tumorigenesis could be mediated, at least in part, by cyclooxgenase inhibition.[46] Our results suggest that COX-2 protein was down-regulated in rat tongue lesions by dietary exposure to troglitazone, and it seems probable that COX-2 down-regulation tends to result in a reduction in the enzymatic activity of COX-2. Thus, it is quite likely that troglitazone inhibits tongue carcinogenesis by inhibition of COX-2 expression. In support of our results, several reports have described an inhibitory effect of PPARγ ligands on expression[29, 30] and transcriptional activity[31] of COX-2.

Regarding side effects, feeding of troglitazone-containing diets did not cause any retardation of body weight gain in the current study. No significant pathological alterations in the liver, including mild hepatomegaly, were found in rats fed troglitazone. This suggests that the concentration of troglitazone used in this study shows low toxicity under our experimental conditions. Thus, troglitazone might be a promising chemopreventive agent against human cancers including tongue carcinoma. Recent attention, however, has focused on side effects of troglitazone, because of its rare but potentially lethal hepatotoxicity.[48] Accordingly, further studies to overcome such side effects of troglitazone are needed before clinical application.

In conclusion, our results indicate that troglitazone has inhibitory effects on oral carcinogenesis initiated with 4-NQO, and such modifying effects may be related partly to the suppression of cell proliferation and/or inhibition of COX-2 expression. This study suggests the possible effectiveness of a novel preventive approach for oral malignancy by using PPARγ ligands, although further studies will be necessary to evaluate their safety and the precise mechanisms of inhibition.

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