TABLE III - EFFECT OF ONO-1714 ON THE DEVELOPMENT OF SMALL INTESTINAL POLYPS IN THE $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Genotype	Treatment (no. of mice)	Multiplicity of small intestinal polyps at:			
		Proximal	Middle	Distal	Total
$Apc^{Min/+}$	1% DSS (12)	1.60 ± 1.51[1]	10.60 ± 7.23	40.40 ± 19.06	52.60 ± 24.92
	1% DSS + 50 ppm ONO-1714 (10)	1.78 ± 1.20	9.22 ± 6.28	26.11 ± 19.23	37.11 ± 23.91
	1% DSS + 100 ppm ONO-1714 (10)	2.00 ± 1.50	7.56 ± 4.85	32.89 ± 16.47	42.44 ± 20.91
$Apc^{+/+}$	1% DSS (5)	0	0	0	0
	1% DSS + 50 ppm ONO-1714 (5)	0	0	0	0
	1% DSS + 100 ppm ONO-1714 (5)	0	0	0	0

[1]Mean ± SD.

TABLE IV - INFLAMMATION SCORE OF THE LARGE INTESTINE IN THE $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Genotype	Treatment (no. of mice)	Inflammation score at:		
		Colon	Cecum	Large bowel (total)
$Apc^{Min/+}$	1% DSS (12)	3.67 ± 0.49[1]	3.92 ± 0.29	3.79 ± 0.26
	1% DSS + 50 ppm ONO-1714 (10)	3.10 ± 0.74	3.40 ± 0.84	3.30 ± 0.59[2]
	1% DSS + 100 ppm ONO-1714 (10)	2.90 ± 0.74[2]	3.10 ± 0.99[2]	3.05 ± 0.55[3]
$Apc^{+/+}$	1% DSS (5)	3.60 ± 0.55	3.80 ± 0.45	3.70 ± 0.27
	1% DSS + 50 ppm ONO-1714 (5)	3.20 ± 0.84	3.40 ± 0.55	3.30 ± 0.57
	1% DSS + 100 ppm ONO-1714 (5)	2.80 ± 0.84	3.00 ± 1.00	2.90 ± 0.89

[1]Mean ± SD.–[2,3]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Tukey's multiple comparison post test ($^2p < 0.05$ and $^3p < 0.01$).

Score for inflammation in the large bowel

Table IV summarizes data on colonic inflammation scores in the large intestine. DSS administration caused inflammation in the colonic mucosa (Fig. 1*a*). No significant differences in the degrees of colonic mucosal inflammation were noted between the $Apc^{Min/+}$ and $Apc^{+/+}$ mice irrespective of the treatments. In the large bowel of $Apc^{Min/+}$ mice, the value was significantly decreased by administration of 50 ppm ONO-1714 ($p < 0.05$; Fig. 1*d*) and 100 ppm ONO-1714 ($p < 0.01$; Fig. 1*g*) when compared to the DSS alone group (Fig. 1*a*). Furthermore, treatment with 100 ppm ONO-1714 significantly decreased the value in the colon ($p < 0.05$) and cecum ($p < 0.05$). The scores of $Apc^{+/+}$ mice that received DSS and ONO-1714 were relatively low when compared with DSS-treated $Apc^{Min/+}$ mice, but the differences did not reach the statistical significance.

Immunoreactivity for COX-2 and iNOS was observed in the colonic mucosa in all groups of each phenotype. COX-2 (Figs. 1*e* and 1*h*) and iNOS reactivity (Figs. 1*f* and 1*i*) of colonic mucosa in administration of DSS and ONO-1714 were decreased, when compared with the in DSS-treated group in the $Apc^{Min/+}$ mice (Fig. 1*b* for COX-2 and Fig. 1*c* for iNOS).

Real-time quantitative RT-PCR analysis of COX-2, iNOS, IL-1β and TNFa

A RT-PCR analysis demonstrated comparable levels of GAPDH mRNA in the nonlesional colonic mucosa of 5 mice from each group (Table V). The mRNA levels of COX-2, IL-1β and TNFα significantly increased in the $Apc^{Min/+}$ mice treated with DSS alone, in comparison to the wild-type mice treated with DSS alone ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively). Feeding with ONO-1714 at dose levels of 50 and 100 ppm in $Apc^{Min/+}$ mice significantly decreased the mRNA expression of COX-2 ($p < 0.001$ and $p < 0.001$, respectively), IL-1β ($p < 0.01$ and $p < 0.01$, respectively), and TNFα ($p < 0.001$ and $p < 0.05$, respectively), in comparison to that of the DSS alone group. In the $Apc^{+/+}$ mice, there was an increase in IL-1β mRNA and a decrease in TNFα mRNA in the group treated with 50 ppm ONO-1714, but the values were not significantly different among the groups. The mRNA expression of COX-2 was no significant differences among the groups of the $Apc^{+/+}$ mice. As to iNOS, the mRNA expression in the colonic mucosa of $Apc^{Min/+}$ mice treated with DSS and 100 ppm ONO-1714 was up-regulated in comparison to the $Apc^{Min/+}$ mice treated with DSS alone, but this increase was not statistically significant. In the $Apc^{+/+}$ mice, the mRNA level of iNOS significantly increased by 100 ppm ONO-1714 administration ($p < 0.05$) in comparison to the DSS alone group.

Clinical chemistry

The results of clinical chemistry are given in Table VI. Serum level of TG (243.0 ± 115.5 mg/dl) in the $Apc^{Min/+}$ mice that received DSS alone was dramatically increased by almost 6.5 times that of the wild-type counterparts (37.1 ± 32.2 mg/dl, $p < 0.001$). The administration of 50 and 100 ppm ONO-1714 decreased this increase of serum TG ($p < 0.01$ and $p < 0.01$, respectively). However, the treatment with ONO-1714 did not influence the serum TG in the wild type mice. Other serum profiles (Glu, TP, Alb, A/G, T-Bil, BUN and T-Cho) were not influenced by the treatment with DSS and/or ONO-1714 in the $Apc^{Min/+}$ and wild-type mice. The values of AST, ALT, LDH, ALP and creatinine were almost comparable among the groups in the $Apc^{Min/+}$ and $Apc^{+/+}$ mice regardless of the DSS treatment (data not shown).

Discussion

The results of the present work indicated that a specific iNOS inhibitor, ONO-1714, effectively inhibited DSS-induced large bowel carcinogenesis in the $Apc^{Min/+}$ mice without any adverse effects. The suppressive effect of ONO-1714 on the development of large bowel adenocarcinomas closely correlated with the inhibition of the serum TG levels and the inhibition of proinflammatory cytokines (TNFα and IL-1β) and COX-2 mRNA levels. These findings may suggest that dietary ONO-1714 suppresses in the inflammation-associated colon carcinogenesis in the $Apc^{Min/+}$ mice that are model mice for human familial adenomatous polyposis.

The precise mechanisms of the IBD-related carcinogenesis process are largely unclear, while it is generally assumed that IBD-associated colorectal carcinogenesis is promoted by chronic inflammation.[22] Mucosal inflammation may result in colonic carcinogenesis through several proposed mechanisms. In the present study, treatment with ONO-1714 reduced the inflammation score of large bowel mucosa in the $Apc^{min/+}$ mice. The length of the large bowel represents the biological parameter of severity in colonic inflammation.[3,5] In the current study, ONO-1714 treatment prevented the shortening of large bowel by DSS in the $Apc^{min/+}$

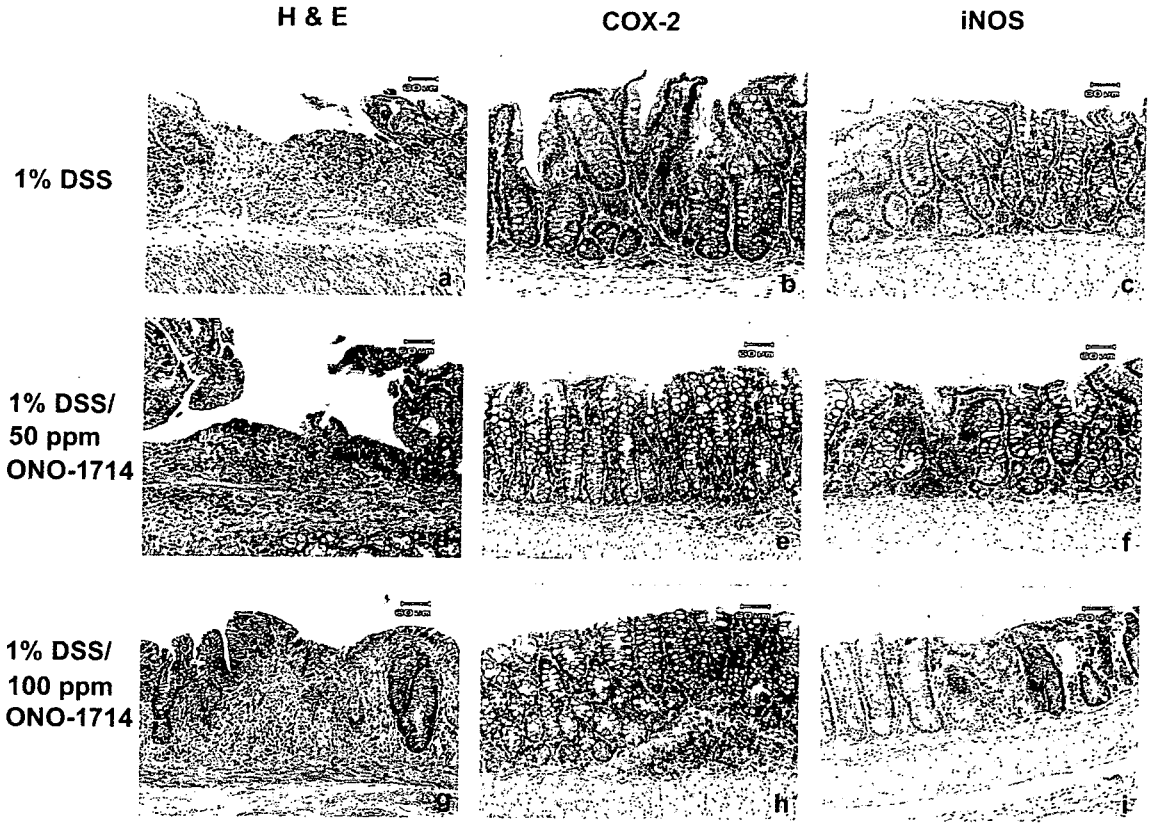

FIGURE 1 – Histopathology and immunohistochemistry of COX-2 and iNOS of colonic mucosa of $Apc^{Min/+}$ mice treated with 1% DSS or 1% DSS/ONO-1714. Histopathological observation revealed mucosal inflammation scored as Grade 3 in 1% DSS group (*a*), 1% DSS/50 ppm ONO-1714 group (*d*) and 1% DSS/100 ppm ONO-1714 group (*g*). While mucosal ulcer of 1% DSS group was not covered by regenerative epithelial cells (*a*), that of 1% DSS/50 ppm ONO-1714 group (*d*) and 1% DSS/100 ppm ONO-1714 (*g*) was covered by regenerative cryptal cells. Expression of COX-2 (*b*) and iNOS (*c*) was strong in the cryptal and inflammatory cells in the mucosa of colon in 1% DSS group, but that in 1% DSS/50 ppm ONO-1714 group (*e* and *f*) and 1% DSS/100 ppm ONO-1714 group (*h* and *i*) was very weak. H&E stain (*a*, *d* and *g*), and COX-2 immunohistochemistry (*b*, *e* and *h*) and iNOS immunohistochemistry (*c*, *f* and *i*). Each bar represents 60 μm.

TABLE V – mRNA EXPRESSION OF COX-2, iNOS, IL-1β AND TNFα IN THE COLONIC MUCOSA

Geno-type	Treatment (no. of mice examined)	mRNA/GAPDH mRNA ratio (10^3)			
		COX-2	iNOS	IL-1β	TNFα
$Apc^{Min/+}$	1% DSS (5)	4.063 ± 1.376[12]	4.062 ± 4.029	20.802 ± 10.759[2]	4.781 ± 2.654[2]
	1% DSS + 50 ppm ONO-1714 (5)	0.622 ± 0.945[3]	4.988 ± 4.964	5.531 ± 6.864[4]	0.723 ± 0.295[3]
	1% DSS + 100 ppm ONO-1714 (5)	0.260 ± 0.215[3]	6.501 ± 4.033	5.662 ± 6.972[4]	1.881 ± 1.247[5]
$Apc^{+/+}$	1% DSS (5)	0.615 ± 1.025	2.136 ± 1.990	5.362 ± 6.881	0.553 ± 0.325
	1% DSS DSS + 50 ppm ONO-1714 (5)	0.556 ± 0.612	1.851 ± 1.092	8.013 ± 10.951	0.251 ± 0.142
	1% DSS DSS + 100 ppm ONO-1714 (5)	0.590 ± 0.242	9.355 ± 6.171[2]	5.765 ± 4.640	0.674 ± 0.343

[1]Means ± SD.–[2]Significantly different from "1% DSS" group of $Apc^{+/+}$ mice by Bonferroni's multiple comparison post test ($^2p < 0.001$).–[3–5]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Bonferroni's multiple comparison post test ($^3p < 0.001$, $^4p < 0.01$ and $^5p < 0.05$).

mice, in line with the reduction in histological inflammation score of the mucosa. Our findings are thus in accordance with those reported by others,[23] in which ONO-1714 ameliorates the DSS-induced colitis in the Balb/c mice. In addition, iNOS induction and the formation of peroxynitrite and the nitration of cellular protein are responsible for the colonic inflammation of IBD.[11] A natural product, auraptene, possessing the radical scavenging activity is formed to inhibit inflammation-related colon carcinogenesis.[24] ONO-1714 also suppresses excessive peroxynitrite generation.[25] These results may suggest that one of the mechanisms by which ONO-1714 exerts a chemopreventive ability might be related to the inhibition of inflammation.

Although the role of iNOS in intestinal tumorigenesis has been disputed, the majority of reports suggest that the NO production of iNOS plays an important role in the processes of inflammation and carcinogenesis.[10] In fact, the expression and activity of iNOS is increased in colonic mucosa in patients with IBD[11] and colonic tumors.[26] Inflammatory damage in UC is also associated with increased production of NO through the iNOS pathway.[27] NO and iNOS are thus involved in colon carcinogenesis with and without colitis.[6,28] Tanaka and coworkers[3–5,29] and Seril *et al.*[6] observed iNOS-positive and nitrotyrosine-positive inflammatory cells in noncancerous colonic mucosa and neoplasms of the mice treated with DSS. Although we did not investigate the immunohistochemical expression of iNOS and nitrotyrosine in this study, we previously observed the positive reaction of both in colonic neoplastic cells as well as their surrounding inflammatory cells in the $Apc^{Min/+}$ mice that received DSS.[8] In addition, iNOS inhibitors could

TABLE VI – EFFECT OF ONO-1714 ON THE SERUM BIOCHEMICAL PROFILES IN THE $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Geno-type	Treatment (no. of mice)	Glu (mg/dl)	TP (g/dl)	Alb (g/dl)	A/G	T.Bil (mg/dl)	BUN (mg/dl)	T-Cho (mg/dl)	TG (mg/dl)
$Apc^{Min/+}$	1% DSS (12)	138.8 ± 41.9[1]	4.3 ± 0.3	1.4 ± 0.1	0.45 ± 0.03	0.3 ± 0.2	31.5 ± 6.5	92.0 ± 10.9	243.0 ± 115.5[2]
	1% DSS + 50 ppm ONO-1714 (10)	139.1 ± 72.1	4.2 ± 0.5	1.3 ± 0.2	0.44 ± 0.02	0.2 ± 0.2	30.8 ± 5.5	102.6 ± 16.8	112.5 ± 67.5[3]
	1% DSS + 100 ppm ONO-1714 (10)	127.6 ± 27.8	4.1 ± 0.3	1.2 ± 0.1	0.43 ± 0.02	0.1 ± 0.0	30.1 ± 6.5	106.0 ± 19.0	101.9 ± 75.7[3]
$Apc^{+/+}$	1% DSS (5)	166.9 ± 82.1	4.9 ± 0.5	1.6 ± 0.3	0.50 ± 0.10	0.3 ± 0.2	20.7 ± 5.4	80.4 ± 53.1	37.1 ± 32.2
	1% DSS DSS + 50 ppm ONO-1714 (5)	154.1 ± 66.7	4.7 ± 0.3	1.4 ± 0.1	0.44 ± 0.03	0.2 ± 0.2	22.8 ± 6.5	86.6 ± 48.4	37.4 ± 29.0
	1% DSS DSS + 100 ppm ONO-1714 (5)	196.0 ± 67.5	4.8 ± 0.4	1.4 ± 0.2	0.41 ± 0.03	0.1 ± 0.0	22.0 ± 3.4	96.0 ± 39.0	38.0 ± 21.0

[1]Mean ± SD.–[2]Significantly different from "1% DSS" group of $Apc^{+/+}$ mice by Bonferroni's multiple comparison post test ($^2p < 0.001$).–[3]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Bonferroni's multiple comparison post test ($^3p < 0.01$).

suppress chemically-induced colitis,[9,23] AOM-induced colonic ACF development[30] and tumor load of $Apc^{Min/+}$ mice.[31] We also revealed that a highly selective inhibitor of iNOS, ONO-1714, suppresses AOM-induced ACF formation and decreased the volumes of colorectal tumor in rats.[18] In this study, the incidence of adenocarcinoma decreased by the treatment with ONO-1714, whereas that of adenoma was not affected. The reason for this is unknown, but it may be possible that feeding the rats ONO-1714 may have inhibited the progression of adenomas to adenocarcinomas. Since DSS strongly promoted the progression of dysplastic crypts, which are usually present in untreated $Apc^{Min/+}$ mice, to colonic malignancies,[8] ONO-1714 may thus blocks the progression. In our previous study, the expression of iNOS protein was remarkable in the invasion front of cancer tissue, and a COX-2 inhibitor suppressing this expression caused inhibition of tongue carcinogenesis.[32] Therefore, ONO-1714 may thus be able to potentially suppress cancer invasion.

It is well known that COX-2 expression is markedly elevated in the CRC of both human and rodents, and COX-2 plays an important role in cancer cell proliferation and tumor angiogenesis.[33] Moreover, COX-2 inhibition results in inhibition of CRC development, thus suggesting that the use of COX-2 inhibitors is protective against CRC occurrence.[34] Furthermore, we observed that a COX-2 selective inhibitor (nimesulide) exerts a powerful chemopreventive ability in AOM/DSS-induced colitis-related mouse colon carcinogenesis.[29] In addition, NO is reported to regulate activity and expression of COX-2.[35] iNOS inhibitors reduce not only iNOS activity but also COX-2 activity.[36] In the current study, COX-2 mRNA levels in the large intestine of DSS-treated $Apc^{Min/+}$ mice were much greater than those of wild-type mice. Furthermore, treatment with ONO-1714 reduced mRNA levels for COX-2 in the nonlesional mucosa of large intestine of $Apc^{Min/+}$ mice. Therefore, the down-regulation of COX-2 by an iNOS-inhibitor ONO-1714 might be one of the possible underlying mechanisms of suppression of large intestinal tumor development. Our findings also suggest that NO may regulate the production of prostaglandins, which are known to play a key role in colon tumor development, by affecting COX-2 expression.[37]

TNF-α and IL-1β are key cytokines involved in inflammation, immunity and cellular organization.[38] The colonic mucosa in UC patients produces large amounts of proinflammatory cytokines, such as TNF-α and IL-1β,[39] and increases productions of iNOS[11] and COX-2.[40] The increased production of these cytokines correlates with disease activity of IBD,[39] and their synthesis is implicated in the pathogenesis of the disease.[41] In experimental colitis, the expression of TNF-α and IL-1β is also enhanced.[42] Treatment with anti-TNFα monoclonal antibody revealed a more complex role for TNFα in colonic inflammation induced by DSS in mice.[43] Blockage of IL-1 activity decreases colitis in a rabbit model of colitis.[44] In this study, TNFα and IL-1β mRNA expression was substantially up-regulated in the colorectal mucosa of $Apc^{Min/+}$ mice that received DSS when compared with wild-type mice treated with DSS. The mechanisms by which the high-output NO generation through iNOS regulates cytokine release are not clear. However, in the current study, we found that ONO-1714 treatment reduces the mRNA expression of TNFα and IL-1β in the colonic mucosa of DSS-treated $Apc^{Min/+}$ mice. Since the induction of iNOS mRNA is gradual at the initial stage of colitis in $TNF\alpha^{-/-}$ mice when compared to $TNF\alpha^{+/+}$ mice,[45] interaction of iNOS, TNFα and IL-1β may play important roles in tumor promotion of inflammation-related carcinogenesis. Therefore, the suppression of the expression of TNFα and IL-1β by ONO-1714 contribute the low frequency of large tumors observed in this study.

Recent studies on experimental animal models of IBD have indicated that constitutive and inducible NO production seems to be beneficial during acute colitis, but the sustained up-regulation of NO is detrimental.[9] In this study, the results that the expression of iNOS mRNA in colorectal mucosa in both $Apc^{Min/+}$ and $Apc^{+/+}$ mice treated with DSS was increased by treatment with ONO-

1714 are of interest. The mechanisms for this are unclear, but the decreased production of NO by ONO-1714 may induce the expression of iNOS mRNA, because of the production of small quantities of NO maintain intestinal homeostasis and mucosal integrity.[9] Furthermore, NO is synthesized by not only iNOS but also by constitutive NOS, including endothelial NOS (eNOS) and neural NOS (nNOS).[9] The role of constitutively expressed NOS in intestinal inflammation is still not fully understood, but eNOS and nNOS isoforms may have influence on colitis, either by contributing to the inflammation or by affecting mucosal integrity in response to noxious stimuli.

Epidemiologically, a positive association between the hypertriglyceridemia and CRC development has been reported.[46] An experimental rodent study also showed a positive effect of serum TG on the development of ACF.[47] Niho *et al.*[48–50] recently discovered that a hyperlipidemic state is associated with intestinal polyp formation in *Apc*-deficient mice. They also observed that peroxisome proliferator-activated receptors' ligands[48,49] and lipoprotein lipase activator[50] reduce serum TG levels and suppress intestinal polyp formation in *Apc*-deficient mice. In accordance with their findings, the administration of ONO-1714 could therefore improve hyperlipidemia and suppress large bowel adenocarcinoma formation in the $Apc^{Min/+}$ mice in this study. Although we should investigate whether hyperlipidemia is a real risk factor in the CRC occurrence, our findings may suggest that an improvement of hyperlipidemia is beneficial in preventing colon carcinogenesis.

In conclusion, the dietary administration of ONO-1714 could effectively suppress colitis-related colon tumor development in the $Apc^{Min/+}$ mice by affecting multiple factors, including COX-2, TNFα, IL-1β and hyperlipidemia, which are involved in inflammation-related colon carcinogenesis. The clinical significance of our findings therefore merits further investigation.

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Carcinogenesis vol.28 no.3 pp.677–684, 2007
doi:10.1093/carcin/bgl178
Advance Access publication October 19, 2006

A prostaglandin E_2 receptor subtype EP_1-selective antagonist, ONO-8711, suppresses 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis

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We previously reported that certain cyclooxygenase (COX) inhibitors could inhibit chemically induced tongue carcinogenesis. In the present study, we investigated the effects of prostaglandin E_2 (PGE_2) receptor EP_1-selective antagonist ONO-8711 on 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis to know whether an EP_1 receptor involves in oral carcinogenesis. Male Fischer 344 rats were given drinking water containing 4-NQO for 8 weeks (20 p.p.m. for the initial 2 weeks, 25 p.p.m. for 2 weeks, and then 30 p.p.m. for 4 weeks). After 4-NQO treatment, animals were given 400 or 800 p.p.m. ONO-8711 containing diets for 23 weeks. The incidence of tongue squamous cell carcinomas (SCC) in the 4-NQO-treated rats was 64%, while that in the rats given ONO-8711 after 4-NQO exposure was 29 ($P < 0.05$) and 29% ($P < 0.05$) in the 400 and 800 p.p.m. of ONO-8711, respectively. The multiplicity of tongue cancer was also smaller in the 4-NQO + ONO-8711 (400 p.p.m. ONO-8711, 0.35 ± 0.61; and 800 p.p.m. ONO-8711, 0.29 ± 0.47; $P < 0.05$), when compared with the 4-NQO alone group (0.88 ± 0.88). Feeding with ONO-8711 significantly reduced PGE_2 level and cell proliferation activity in the non-tumorous epithelium of the tongue. Also, treatment with ONO-8711 resulted in the decrease in EP_1 immunohistochemical expression in the tongue lesions induced by 4-NQO. The results suggest that EP_1 receptor involves in oral carcinogenesis, and that an EP1-selective antagonist ONO-8711 exerts the cancer chemopreventive effects through the suppression of EP_1 expression, PGE_2 biosynthesis and cell proliferation.

Abbreviations: AOM, azoxymethane; BrdU, 5-bromodeoxyuridine; COX, cyclooxygenase; 4-NQO, 4-nitroquinoline 1-oxide; NSAIDs, non-steroidal anti-inflammatory drugs; ONO-8711, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonyl-amnomethyl)-bicyclo[2.2.2.] octan-2-yl]-5Z-hexenoic acid; PAP, squamous cell papilloma; PGE_2, prostaglandin E_2; SCC, squamous cell carcinoma.

Introduction

Cancer of the head and neck, including oral, laryngeal and pharyngeal sites, is the fifth most common cancer, accounting for ~615 000 new cases annually. About 40% of head and neck malignancies are known to be squamous cell carcinoma (SCC) arising in the oral cavity (1).

Oral cancer is largely related to lifestyle: major etiological factors include high consumption of tobacco and alcohol (2,3). In southern Asia, oral cancer is recognized to result from chewing of betel quid containing lime, areca nut and tobacco together with smoking and alcohol drinking (4). In recent decades, oral cancer incidence and mortality rates have been increasing in USA, Japan, Germany and Scotland, especially among young males (5). In spite of recent advances in surgical procedures, radiotherapy and chemotherapy, the survival rate of patients with head and neck cancer has not been improved, and their treatment often produces dysfunction and distortion in speech, mastication and swallowing. Moreover, a significant number of patients treated primary oral cancer are at high-risk of developing second primary cancer in the head and neck (6), suggesting the concept called 'field cancerization' that is the multi-focal development of premalignant and malignant lesions in the upper aerodigestive tract. Therefore, the prevention of head and neck cancer including oral cancer is highly required. Chemoprevention with appropriate substances is a promising approach and an important strategy for cancer prevention. Numerous chemicals including non-toxic natural or synthetic substances are candidate for chemopreventive agent in cancer development including oral cancer (3).

Recently, observational data have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) are associated with the reduced risk of several types of cancers including oral cancer (7–11). Indeed, prostaglandin E_2 (PGE_2) level is elevated in the cancerous tissues when compared with their surrounding tissues (12–16). NSAIDs inhibit cyclooxygenase (COX) activity, and thereby suppress the synthesis of PGE_2, which can stimulate cell proliferation and angiogenesis and inhibit apoptosis and immune surveillance. Recently, two COX enzyme isoforms, known as COX-1 (17) and COX-2 (18), have been identified to be involved in carcinogenesis (19). The inducible form, COX-2 contributes to inflammation and abnormal cell proliferation. Accumulating evidence indicates that COX-2 is involved in carcinogenesis in various organs including oral cavity (15,20), and several COX-2 selective inhibitors have potential roles in the chemoprevention of oral cancer (21–25). On the other hand, a recent study revealed that continuous use of COX-2 selective inhibitor could increase the risk of cardiovascular disease (26).

PGE_2 exerts its biological actions through binding to four specific membrane receptor subtypes known as EP_1, EP_2, EP_3 and EP_4 (21,27). Genetic and pharmacological studies with specific inhibitors have suggested that EP_1 and EP_4 are

important for carcinogenesis in organs where main malignancies are columnar cell origin (28–30). As for EP_3, the receptor signaling suppresses colon carcinogenesis (30), but enhances chemically induced skin carcinogenesis, where most tumor induced are of squamous cell origin (31).

In the previous studies, dietary administration of an EP_1-selective antagonist, ONO-8711, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonyl-amnomethyl)-bicyclo[2.2.2.]octan-2-yl]-5Z-hexenoic acid reduced azoxymethane (AOM)–induced aberrant crypt foci formation in mice and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced breast cancer incidence, multiplicity and volume in rats (32). Moreover, a recent study demonstrated that long-term administration of ONO-8711 in rats reduced AOM-induced colon cancer incidence, multiplicity and volume without toxicity, and EP_1-selective antagonists might be promising candidates for chemopreventive agent (33,34).

In the present study, we investigated the involvement of EP_1 receptor in 4-nitroquinoline 3-1-oxide (4-NQO)-induced tongue carcinogenesis in male F344 rats, and evaluated the modifying effects of the dietary administration with an EP_1-selective antagonist, ONO-8711 on tongue carcinogenesis in rats initiated with 4-NQO. The effects of this chemical on the immunohistochemical expression of EP_1 receptor, PGE_2 biosynthesis and cell proliferation activity in the tongue were assessed to further investigate the efficacy of ONO-8711 in inhibiting carcinogenesis in the tissue other than colon and mammary gland, and to clarify the involvement of EP_1 in tongue carcinogenesis.

Materials and methods

Chemicals

ONO-8711, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonyl-amnomethyl)-bicyclo[2.2.2.]octan-2-yl]-5Z-hexenoic acid, an EP_1-selective antagonist, was chemically synthesized at Ono Pharmaceutical. ONO-8711 was well mixed with a powdered basal diet CE-2 (Japan Clea Company Ltd., Tokyo, Japan) at concentrations of 400 and 800 p.p.m. This chemical proved to be stable for at least 4 weeks at room temperature when added to the basal diet, and the doses used were selected based on the results of previous studies (28,32,33).

Animals, diets and carcinogen

Fischer 344 male rats, 4-week-old, were purchased from Charles River Japan (Kanagawa, Japan). After 2 weeks of quarantine, the rats were randomized into experimental and control groups based on the body weight: 25 rats in Group 1; 17 rats in Group 2; 17 rats in Group 3; 12 rats in Group 4; and 11 rats in Group 5. They were housed three or four to a wire cage in an air-conditioned room with a 12-h light/dark cycle. Food and water were available *ad libitum*. 4-NQO was obtained from Wako Pure chemical Inc. (Osaka, Japan), dissolved in tap water to a final concentration of 20, 25 or 30 p.p.m., and stored in a dark and cold room.

Experimental protocol

A total of 82 rats were divided into five groups as shown in Figure. 1. At 6 weeks of age, rats in Groups 1–3 were given 20 p.p.m. 4-NQO in drinking water for the first 2 weeks, 25 p.p.m. for the next 2 weeks and 30 p.p.m. for the other 4 weeks. Groups 1 and 4 were fed the basal diet and the experimental diet containing 800 p.p.m. ONO-8711, respectively, during the experimental period. Groups 2 and 3 were fed the experimental diets containing 400 and 800 p.p.m., respectively starting 1 week after the cessation of 4-NQO treatment. Group 5 was fed the basal diet without the test chemical and tap water without the carcinogen throughout the experiment as an untreated control. All rats were carefully observed daily, and consumption of the drinking water containing 4-NQO or the diet mixed with the test chemical was recorded to estimate intake of the chemicals. The experiment was terminated 32 weeks after the start of the experiment, and all animals were sacrificed under ether anesthesia. At necropsy, digestive organs, including the oral cavity, were inspected to find the preneoplastic and neoplastic lesions. For histological examination, the organs except tongue were excised, fixed in 10% phosphate-buffered formalin. They were then embedded in paraffin blocks, and the sections were stained with hematoxylin and eosin for histopathology. Tongues were excised and cut longitudinally in half, one section for histopathology including immunohistochemistry, and the other one for PGE_2 assay. For PGE_2 assay the macroscopic lesions were removed, if present.

EP_1 immunohistochemistry

Immunohistochemistry of EP_1 of tongues from all the rats was done. Paraffin sections, 4 μm thick, of the 10% buffered formalin fixed tongues from all the

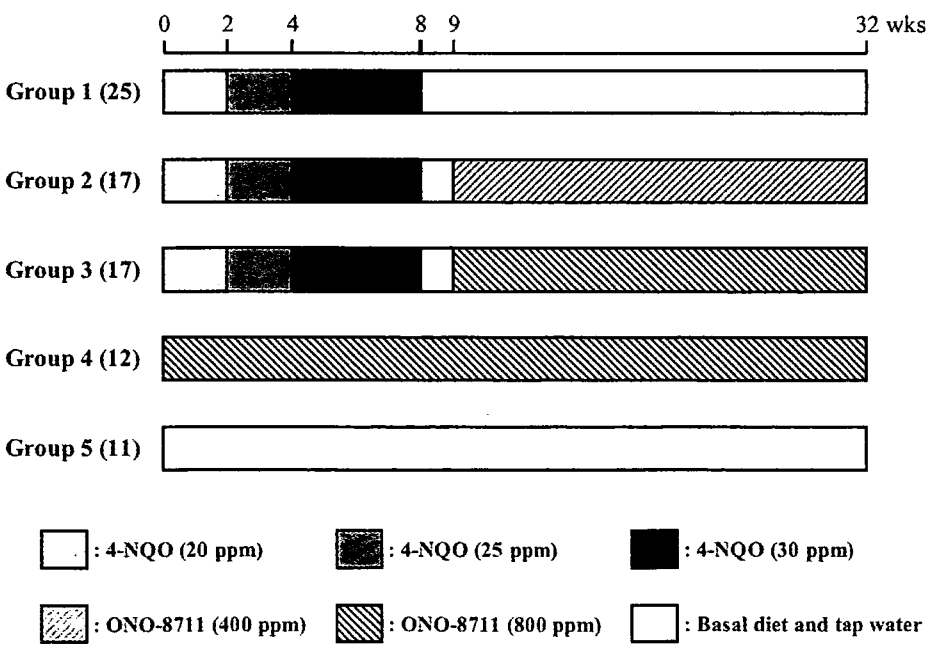

Fig. 1. Experimental protocol. Groups 1–3 were given 20 p.p.m. 4-NQO in drinking water for the first of 2 weeks, 25 p.p.m. for the next 2 weeks and 30 p.p.m. for the other 4 weeks. Rats in Groups 1 and 4 were fed the basal diet and the experimental diet containing 800 p.p.m. ONO-8711, respectively, during the study. Groups 2 and 3 were fed the experimental diets containing 400 and 800 p.p.m., respectively starting 1 week after the cessation of 4-NQO. Group 5 was an untreated control.

rats were mounted on salinized glass, and deparaffinized in xyline and descending strengths of ethanol. Sections were washed in 0.05 M phosphate-buffered saline (PBS, pH 7.6). Endogenous peroxidase activity and non-specific binding were blocked by incubations with 0.3% hydrogen peroxide in methanol for 5 min at room temperature. After being rinsed with PBS three times for 9 min and exposed to PBS/1% bovine serum albumin (PBA) for 5 min at room temperature to reduce non-specific binding, the slides were incubated overnight at 4°C with a rabbit polyclonal antibody against EP_1 (Code no. 101740, Cayman Chemical, Ann Arbor, MI, USA), which was diluted at 1:1500 in PBS. The slides were rinsed three times for 9 min in PBS, and incubated for 30 min in Dako Envision + peroxidase rabbit (K4003, Dako Japan, Kyoto, Japan). The slides were rinsed three times for 9 min in PBS. Then they were incubated for 1 min in 3,3'-diaminobenzidine-4HCl, and rinsed with PBS. Finally, sections were counterstained with Mayer's hematoxylin. Negative controls were prepared by substituted primary antibody with buffered saline. To compare the degree of EP_1 stainability in the lesions developed between Groups 1 and 3, the grading system (Grade 0–5) was used: Grade 0, no immunoreactivity; Grade 1, very weak immunoreactivity in 10–20% of cells; Grade 2, weak immunoreactivity in 10–20% of cells; Grade 3, weak immunoreactivity in 21–30% of cells; Grade 4, moderate immunoreactivity in 31–40% of cells; and Grade 5, marked immunoreactivity in 51–100% of cells. The EP_1 immunohistochemistry was blindly scored in the 'normal' appearing tongue squamous epithelium, severe dysplasia, squamous cell papilloma (PAP) and SCC from Groups 1 and 3.

5-Bromodeoxyurideine (BrdU)-labeling index and histopathological analysis

To assess the proliferative activity of squamous cell of the tongue, the BrdU-labeling indices of all animals were quantified. For measurement of BrdU-incorporated nuclei, all animals were given an intraperitoneal (i.p.) injection of 50 mg/kg body wt BrdU (Sigma Chemical, St Louis, MO) 1 h prior to killing. Two serial sections were made after embedding in paraffin. One section (4 μm) was used for histopathology, and the other was used for the immunohistochemical detection of BrdU incorporation using an immunohistochemical analysis kit (Dako Japan). The labeling indices were calculated by counting the BrdU-positive nuclei in at least 1500 cells at three different fields of normal or non-lesional tongue epithelium of each rat. The tongue sections for histopathological examination were stained with hematoxylin and eosin, and the tongue epithelial lesions (hyperplasia, dysplasia and neoplasia) were diagnosed according to the criteria described by Kramer *et al.* (35).

Tongue PGE_2 assay

The non-lesional tongues from Groups 1–5 were snap-frozen in liquid nitrogen, and ground in liquid nitrogen using a mortar and pestle. The powdered tongues were then placed in 1 ml of methanol, and the tissue weights were recorded. Tissues were vortexed in the methanol every 10 min for 30 min, and then spun for 10 min at 3500 r.p.m at 4°C. The supernatants were retained, 25 μl of each was dried in a CentriVap Centrifugal Concentrator (Labconco, Kansas City, MO), and re-suspended in EIA buffer (Cayman Chemical, Ann Arbor, MI) at 1:10 dilution. PGE_2 level was assayed using the PCE_2 ELISA kit (Cayman Chemical) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test. The mean values for body, liver and relative liver weights, the multiplicity of lesions, and BrdU-labeling indices were compared by the Tukey Multiple Comparisons Test. The scores of EP_1 immunohistochemistry were compared using Student's *t*-test or Welch's *t*-test. The results were considered statistically significant if the *P*-value was ≤ 0.05.

Results

General observation

Animals in Groups 1–5 tolerated well the oral administration of 4-NQO and/or test chemicals. There were no significant differences in the total intake of 4-NQO/rat among the three groups (Groups 1–3, data not shown). The mean daily intakes of ONO-8711 per rat in Groups 2–4 were 7.04, 13.55 and 14.86, respectively. The mean body, liver and relative liver weights of rats given the carcinogen and the diet containing a high-dose test chemical (Group 3) were lower than those of the 4-NQO alone group (Group 1) without statistical significance (data not shown). In this study, dietary administration of the test chemical caused no clinical signs of toxicity, low survival rates, poor conditions or histological changes suggesting toxicity in the liver, kidney and lung.

Incidence and multiplicity of tumors and preneoplastic tongue lesions

In this study, endophytic and exophyic tumors developed only in the oral cavity, especially the dosal site of the tongue of rats in Groups 1–3. These tumors were histologically well-differentiated SCC and PAP. Animals in Groups 4 and 5 did not have any preneoplastic or neoplastic lesions in the organs examined. The incidence and multiplicity of tongue tumors in each group is shown in Table 1. The incidence of SCC in the rats given a test chemical in diet after 4-NQO exposure (Groups 2 and 3) was significantly decreased when compared with that of Group 1 ($P < 0.05$), but did not exhibit dose-dependent efficacy. The differences of incidences of PAP were not significantly different among Groups 1, 2 and 3. The multiplicities of SCC in Groups 2 and 3 were significantly lower than in Group 1, but those of PAP were not statistically different among the groups (Table 1).

All animals in Groups 1, 2 and 3 had preneoplastic lesions (hyperplasia and dysplasia) in their tongues (Table 1). The incidences of severe dysplasia in Groups 2 and 3 were significantly lower than that of Group 1 ($P < 0.01$), without dose-dependence. The incidences of mild and moderate dysplasia in Groups 1, 2 and 3 did not differ among the groups. The multiplicities of total tongue dysplasia and severe dysplasia in Groups 2 and 3 were significantly lower than those of Group 1 (Table 1).

Immunohistochemical expression of EP_1

Negative controls that were prepared by substituted primary antibody with buffered saline did not show any immunoreactivity of EP_1. EP_1 was weakly expressed in the upper one-third part of the 'normal' appearing tongue squamous epithelium of Group 1 (Figure 2A). EP_1 was also expressed with moderate intensity in the upper half of dysplastic lesions (Figure 2C). In the tongue neoplasms (papilloma and carcinoma), the intensity of EP_1 was strong, especially in the surface part of the papilloma (Figure 2E) and in the around of keratin pearls of SCC (Figure 2G). The positive reactivity against EP_1 antibody was observed in the cell membrane and/or cytoplasm of 'normal' appearing, dysplastic, and neoplastic cells. Dietary administration with ONO-8711 resulted in the EP_1 immunohistochemical expression in the lesions induced by 4-NQO (Figure 2D, F and H), while the expression did not alter in the 'normal' appearing tongue squamous epithelium in the groups that received ONO-8711 (Figure 2B). Interestingly, severe infiltration of inflammatory cells in the stroma below the neoplasms and dysplasia (Figure 2C) was relieved by administration of ONO-8711 (Figure 2D).

The score of EP1 immunohistochemistry of the lesions in Groups 1 and 3 is illustrated in Figure 3. The scores of 'normal' appearing tongue squamous epithelium from Groups 1 and 3 were comparable. However, the values of dysplasia ($P < 0.001$), papilloma ($P < 0.01$) and carcinoma ($P < 0.001$) from Group 3 were significantly smaller than those from Group 1, respectively.

Table 1. Incidence and multiplicity of tongue neoplasms and preneoplasia of rats in each group

Group no.	Treatment (no. of rats examined)	No. of rats with tumors (no. of tumors/rat)			No. of rats with DYS (no. of DYS/rat)			
		PAP (%)	SCC (%)	Total (%)	Mild DYS (%)	Moderate DYS (%)	Severe DYS (%)	Total (%)
1	4-NQO alone (25)	6: 24 (0.24 ± 0.44[a])	16: 64 (0.88 ± 0.88)	17: 68 (1.12 ± 0.97)	20: 80 (0.80 ± 0.41)	22: 88 (0.88 ± 0.33)	20: 80 (0.91 ± 0.64)	25: 100 (2.60 ± 0.82)
2	4-NQO→400 p.p.m. ONO-8711 (17)	4: 24 (0.24 ± 0.44)	5: 29[b] (0.35 ± 0.61)	8: 47 (0.59 ± 0.71)	14: 82 (0.82 ± 0.39)	14: 82 (0.82 ± 0.39)	6: 35[b] (0.35 ± 0.49[c])	17: 100 (1.00 ± 0.79[d])
3	4-NQO→800 p.p.m. ONO-8711 (17)	3: 18 (0.18 ± 0.39)	5: 29[b] (0.29 ± 0.47[d])	7: 41 (0.47 ± 0.62[d])	15: 88 (0.88 ± 0.33)	16: 94 (0.94 ± 0.24)	4: 24[b] (0.24 ± 0.44[c])	17: 100 (1.06 ± 0.56)
4	800 p.p.m. ONO-8711 (12)	0	0	0	0	0	0	0
5	No treatment (11)	0	0	0	0	0	0	0

DYS = dysplasia; PAP = squamous cell papilloma; and SCC = squamous cell carcinoma.
[a]Mean ± SD.
[b]Significantly different from Group 1 by Fisher's exact probability test ($P < 0.05$).
[c–e]Significantly different from Group 1 by Tukey multiple comparisons test ([c]$P < 0.01$, [d]$P < 0.05$, and [e]$P < 0.001$).

BrdU-labeling index

The results of morphometric analysis of BrdU-labeling indices in the non-lesional squamous epithelium are shown in Figure 4. The mean BrdU-labeling index for the tongue epithelium exposed to 4-NQO alone (Group 1) was the highest among the groups. The value was significantly larger than that of untreated control group (Group 5) and the group treated with a test chemical alone (Group 4). Dietary administration of a test chemical after 4-NQO exposure decreased the BrdU-labeling index when compared with Group 1.

PGE_2 level of the tongue

As illustrated in Figure 5, the PGE_2 level in the tongue of rats exposed to 4-NQO alone (Group 1, $P < 0.001$) was significantly greater than untreated control (Group 5). The PGE_2 contents in the tongue of rats treated with 4-NQO and ONO-8711 at a dose of 400 p.p.m. (Group 2, $P < 0.001$) or 800 p.p.m. (Group 3, $P < 0.001$) were significantly lower than that of rats given 4-NQO alone (Group 1). The PGE_2 level of rats given 800 p.p.m. ONO-8711 alone (Group 4) was comparable with that of Group 5.

Discussion

The results in the present study demonstrated that dietary administration of the EP_1-selective antagonist ONO-8711 significantly reduced the incidence and multiplicity of 4-NQO-induced tongue malignancy without any toxicity and pathological alteration of other organs in rats. Our findings also suggest that ONO-8711 could prevent oral carcinogenesis through blocking EP_1 receptor instead of PGE_2. Although we did not observe a dose-dependent inhibition in the incidence of tongue cancer by ONO-8711 feeding, a tendency of dose-dependent suppression was found in the multiplicity of tongue malignancy. In other studies, ONO-8711 had suppressive effect on colon and breast cancer development (28,32,33,36). The results of these studies also indicated that ONO-8711 reduced the incidence/multiplicity of carcinoma or preneoplasia, and the inhibition was remarkable when rats were given the diet containing higher dose (800 or 1000 p.p.m.) of ONO-8711 (28,32,33). In this study, the decrease in the multiplicity of tongue precancerous lesion, severe dysplasia, was also remarkable in rats fed a high-dose of ONO-8711. Our findings suggesting the involvement of EP_1 in carcinogenesis in the tongue as well as colon and breast were supported by the reported in experiments with EP_1-deficient mice (28,37).

The animal models in chemically induced oral carcinogenesis used widely are the hamsters buccal pouch with 7,12-dimethylbenz[*a*]anthracene (21,38) and rats or mice with 4-NQO (3,25,39). Generally, carcinogenic dosage of 4-NQO in drinking water is used at 20 p.p.m. for ~8 weeks (3). The dosage could produce ~30–50% tongue SCC in ~24 weeks after the 4-NQO exposure has been stopped. In this study, we slightly modified this experimental protocol to produce more aggressive tongue tumors: rats were given 4-NQO in drinking water for 8 weeks at dose levels of 20 p.p.m. (for the initial 2 weeks), 25 p.p.m. (for the subsequent 2 weeks) and 30 p.p.m. (for the additional 4 weeks). However, the incidence of tumor, their histology and aggressiveness were almost similar to those of our previous studies (3).

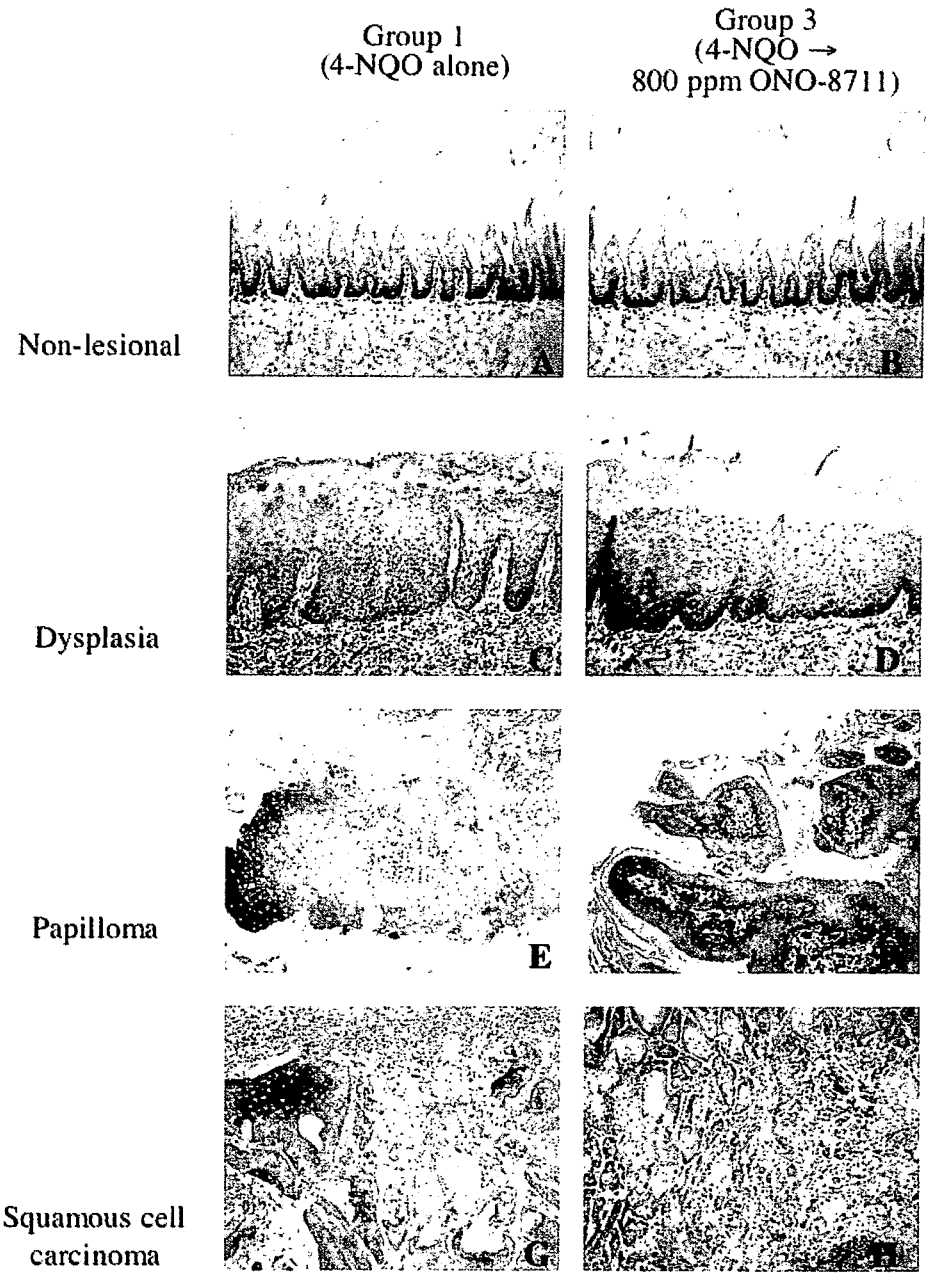

Fig. 2. Immunohistochemical localization of EP_1 in the 'normal' appearing tongue squamous epithelium, dysplasia and neoplasms from Groups 1 (**A, C, E,** and **G**) and 3 (**B, D, F,** and **H**). In the 'normal' appearing squamous epithelium, weak expression of EP_1 is seen in the upper one-third of the tongue epithelium of a rat exposed to 4-NQO alone (A). The expression is similar to that in a rat given 4-NQO and 800 p.p.m. ONO-8711. While EP_1 expression with moderate intensity is present in a dysplastic lesion from a rat treated with 4-NQO alone (C), treatment with ONO-8711 (800 p.p.m.) decreases this expression (D). Strong expression of EP_1 is seen in the surface part of a papilloma from a rat given 4-NQO alone (E), and feeding with ONO-8711 reduced the expression (F). Similarly, the expression of EP_1 immunoreactivity is strong in the surrounding of keratin pearls of a SCCs from a rat treated with 4-NQO alone. This expression is lowered by the treatment with ONO-8711 (800 p.p.m.) in diet. All photographs were taken at a magnification of ×10.

Interesting findings of our current study are that EP_1 expression was present in the tongue squamous epithelium and various lesions induced by 4-NQO. This is the first report that shows the presence of EP_1 receptor in the tongue squamous epithelium, although the presence was reported in epidermis of human and rodent (40,41). In the current study, EP_1 expression was immunohistochemically observed in the upper-third of normal tongue squamous epithelium. The expression and intensity was increased with disease progression (squamous cell dysplasia, papilloma and carcinoma). The expression pattern is similar to that of COX-2 that was observed in our previous study (22). In the current study, the expression and intensity of EP_1 in the tongue lesions was decreased when rats were fed the diet containing ONO-8711 (Figures 3 and 4). Also, feeding with ONO-8711 reduced PGE_2 biosynthesis (Figure 5) in the tongue tissues. We observed in this study that inflammatory cell (mainly neutrophils) infiltration surrounding the lesions was decreased in rats treated with 4-NQO and ONO-8711 (Groups 2 and 3, data not shown). This may be related to decrease in PGE_2 levels by ONO-8711 treatment. Similar findings were reported in the mouse skin tumorigenesis (41). Thus, it may be possible that ONO-8711 has anti-inflammatory action, by which decreases PGE_2 levels, and

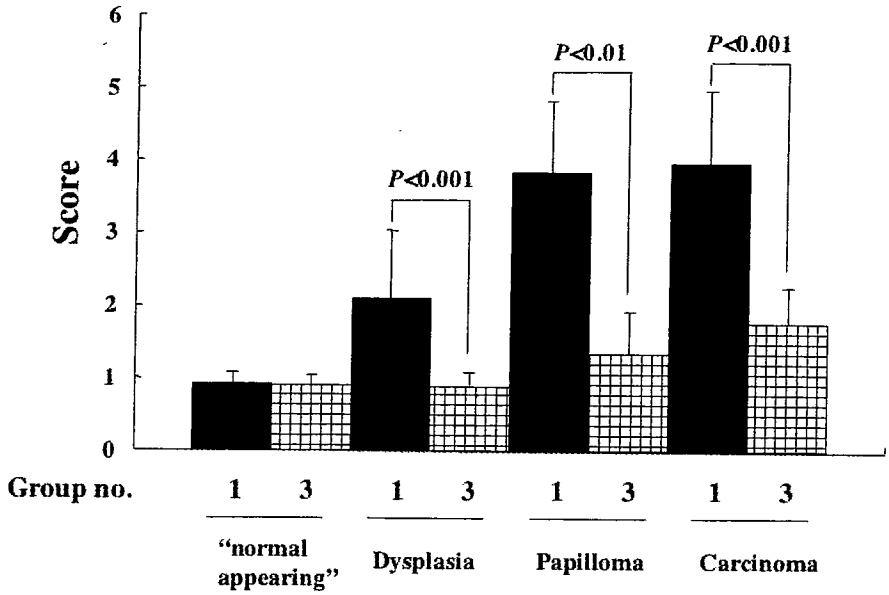

Fig. 3. Scores of EP_1 immunohistochemical stainability. The degree of EP_1 stainability in the lesions developed was compared between Groups 1 and 3 using the grading system (Grade 0–5, see the Materials and methods section). The scores were determined in the 'normal' appearing tongue squamous epeithelium (n = 25 from Group 1 and n = 17 from Group 3), severe dysplasia (n = 20 from Group 1 and n = 4 from Group 3), PAP (n = 6 from Group 1 and n = 3 from Group 3), and SCC (n = 22 from Group 1 and n = 4 from Group 3).

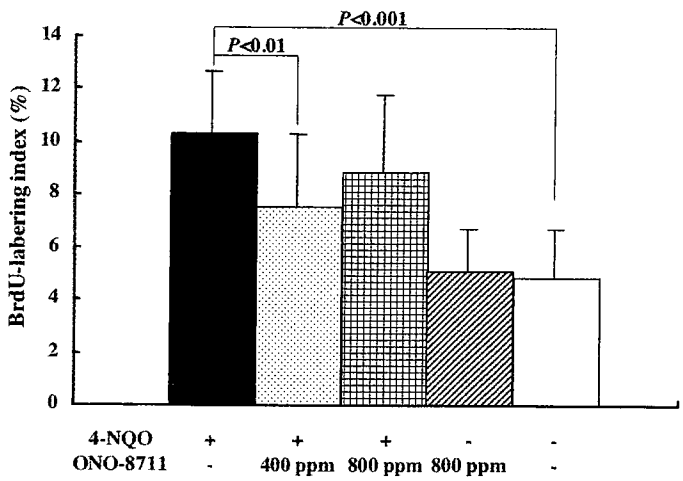

Fig. 4. BrdU-labeling index. The mean BrdU-labeling index in the non-lesional tongue epithelium of Group 1 that was given 4-NQO alone was significantly greater than Group 5 (untreated control) (P < 0.001). The mean BrdU-labeling indices of Groups 2 (4-NQO→400 p.p.m. ONO-8711) and 3 (4-NQO→800 p.p.m. ONO-8711) were lower than Group 1, and the difference between Groups 1 and 2 was statistically significant (P < 0.01).

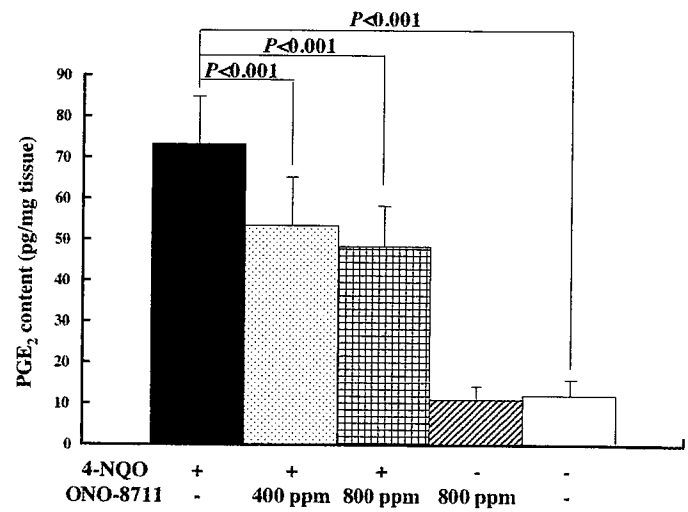

Fig. 5. PGE_2 levels in the tongues. The mean PGE_2 content in the non-tumorous tongue tissue of Group 1 that was given 4-NQO alone was significantly greater than Group 5 (untreated control) (P < 0.001). The mean PGE_2 levels of Groups 2 (4-NQO→400 p.p.m. ONO-8711) and 3 (4-NQO→800 p.p.m. ONO-8711) were significantly smaller than Group 1 (P < 0.001 for each comparison).

can inhibit 4-NQO-induced rat tongue carcinogenesis, as is the case of NSAIDs and COX-2 inhibitors.

In the current study, dietary administration with ONO-8711 after 4-NQO exposure decreased BrdU-labeling indices in the non-lesional tongue squamous epithelium when compared with 4-NQO exposure alone. This may indicate that ONO-8711 in the diet is able to reduce the cell proliferative activity in the target organs (32,33) via inhibition of PGE_2 biosynthesis. Since the inhibitory effect on cell proliferation activity without side effects is important for the ideal chemopreventive chemicals (42), ONO-8711 could be used for a chemopreventive against cancer development in the oral cavity in addition to colon and breast.

The exact mechanisms involved in the suppression of tumor development by EP_1 antagonist are not clear. Although dietary administration with an EP_1 receptor-specific antagonist ONO-8711 suppressed 4-NQO-induced oral carcinogenesis, the roles of EP receptors EP_1–EP_4 in oral carcinogenesis must be investigated in detail, since a study on intestinal polyps in *APC1309* with an EP_1 antagonist (ONO-8711) and an EP_4 antagonist (ONO-AE2-227) indicated that reducing effect on polyp size was more remarkable with ONO-AE2-227, while reduction in the polyp number was more pronounced with ONO-8711 (36). Also, the effects of PCE_2 signaling through its receptors are cell type dependent.

Although PGE_2 acts by binding to one of four different heterotrimeric G-protein coupled receptors, EP_1–EP_4 (43), the receptors differ in the second messenger pathways activated upon PGE_2-binding. The receptors can be roughly broken into two classes based on their PGE_2-binding affinities: high-affinity receptors (EP_3 and EP_4) that bind PGE_2 at sub-nanomolar levels and low-affinity receptors (EP_1 and EP_2) that have dissociation constants in the low nanomolar range. While EP_2 and EP_4 receptors are coupled to adenylate cyclase activation, EP_1 signals are transmitted by increased intercellular Ca^{2+} with activation of phosphorylated protein kinase C (27). It is known that intercellular Ca^{2+} with activation of phosphorylated protein kinase C and phospholipase C is needed for the maturation of spinous-granular layer in the squamous epithelium (44). Turnover of phosphatidylinositol is also increased in this layer (44). Therefore, we can speculate that ONO-8711 treatment decreases the inflow of Ca^{2+} to cells, slows the speed that cells maturate, lowers the cell proliferation activity and finally affects tongue carcinogenesis induced by 4-NQO.

In conclusion, the present study demonstrated that the EP_1 antagonist ONO-8711 had an inhibitory effect on 4-NQO-induced oral carcinogenesis in rats, and such a modifying effect might be related partly to the suppression of cell proliferation. Our findings suggest that ONO-8711 is one of the promising candidate chemopreventive agents for oral cancer.

Conflict of Interest Statement: None declared.

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Received July 12, 2006; revised August 25, 2006; accepted September 11, 2006