A prostaglandin E\textsubscript{2} receptor subtype EP\textsubscript{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\textsubscript{2} (PGE\textsubscript{2}) receptor EP\textsubscript{1}-selective antagonist ONO-8711 on 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis to know whether an EP\textsubscript{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\textsubscript{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\textsubscript{1} immunohistochemical expression in the tongue lesions induced by 4-NQO. The results suggest that EP\textsubscript{1} receptor involves in oral carcinogenesis, and that an EP\textsubscript{1}-selective antagonist ONO-8711 exerts the cancer chemopreventive effects through the suppression of EP\textsubscript{1} expression, PGE\textsubscript{2} biosynthesis and cell proliferation.

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\textsubscript{2} (PGE\textsubscript{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\textsubscript{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\textsubscript{2} exerts its biological actions through binding to four specific membrane receptor subtypes known as EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3} and EP\textsubscript{4} (21,27). Genetic and pharmacological studies with specific inhibitors have suggested that EP\textsubscript{1} and EP\textsubscript{4} are...
important for carcinogenesis in organs where main malignancies are columnar cell origin (28–30). As for EP3, 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 EP1-selective antagonist, ONO-8711, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonyl-aminomethyl)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 EP1-selective antagonists might be promising candidates for chemopreventive agent (33,34).

In the present study, we investigated the involvement of EP1 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 EP1-selective antagonist, ONO-8711 on tongue carcinogenesis in rats initiated with 4-NQO. The effects of this chemical on the immunohistochemical expression of EP1 receptor, PGE2 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 EP1 in tongue carcinogenesis.

Materials and methods

Chemicals
ONO-8711, 6-[(2S,3S)-3-(4-chloro-2-methylphenylsulfonyl-aminomethyl)bicyclo[2.2.2]octan-2-yl]-5Z-hexenoic acid, an EP1-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 PGE2 assay. For PGE2 assay the macroscopic lesions were removed, if present.

EP1 immunohistochemistry
Immunohistochemistry of EP1 of tongues from all the rats was done. Paraffin sections, 4 μm thick, of the 10% buffered formalin fixed tongues from all the
rats were mounted on salinized glass, and deparaffinized in xylene 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 (BPA) 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 EP1 (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 EP1 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 EP1 immunohistochemistry was bly labeled in the ‘normal’ appearing tongue squamous epithelium, severe dysplasia, squamous cell papilloma (PAP) and SCC from Groups 1 and 3.

5-Bromodeoxyuridine 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. In this study, endophytic and exophytic 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 EP1

Negative controls that were prepared by substituted primary antibody with buffered saline did not show any immunoreactivity of EP1. EP1 was weakly expressed in the upper one-third part of the ‘normal’ appearing tongue squamous epithelium of Group 1 (Figure 2A). EP1 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 EP1 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 reaction against EP1 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 EP1 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.
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.

PGE2 level of the tongue

As illustrated in Figure 5, the PGE2 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 PGE2 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 PGE2 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 EP1-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 EP1 receptor instead of PGE2. 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 EP1 in carcinogenesis in the tongue as well as colon and breast were supported by the reported in experiments with EP1-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).
Interesting findings of our current study are that EP₁ 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 EP₁ receptor in the tongue squamous epithelium, although the presence was reported in epidermis of human and rodent (40,41). In the current study, EP₁ 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 EP₁ 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 PGE₂ 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 PGE₂ 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 PGE₂ levels, and

Fig. 2. Immunohistochemical localization of EP₁ 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 EP₁ 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 EP₁ 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 EP₁ 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 EP₁ 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 x10.
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 PGE2 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 EP1 antagonist are not clear. Although dietary administration with an EP1 receptor-specific antagonist ONO-8711 suppressed 4-NQO-induced oral carcinogenesis, the roles of EP receptors EP1–EP4 in oral carcinogenesis must be investigated in detail, since a study on intestinal polyps in APC1309 with an EP1 antagonist (ONO-8711) and an EP4 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 PGE2 signaling through its receptors are cell type dependent.
Although PGE\textsubscript{2} acts by binding to one of four different heterotrimeric G-protein coupled receptors, EP\textsubscript{1}–EP\textsubscript{4} (43), the receptors differ in the second messenger pathways activated upon PGE\textsubscript{2}-binding. The receptors can be roughly broken into two classes based on their PGE\textsubscript{2}-binding affinities: high-affinity receptors (EP\textsubscript{3} and EP\textsubscript{4}) that bind PGE\textsubscript{2} at sub-nanomolar levels and low-affinity receptors (EP\textsubscript{1} and EP\textsubscript{3}) that have dissociation constants in the low nanomolar range. While EP\textsubscript{3} and EP\textsubscript{4} receptors are coupled to adenylate cyclase activation, EP\textsubscript{1} signals are transmitted by increased intercellular Ca\textsuperscript{2+} with activation of phosphorylated protein kinase C (27). It is known that intercellular Ca\textsuperscript{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\textsuperscript{2+} to cells, slows the speed that cells mature, lowers the cell proliferation activity and finally affects tongue carcinogenesis induced by 4-NQO.

In conclusion, the present study demonstrated that the EP\textsubscript{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.

References


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