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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

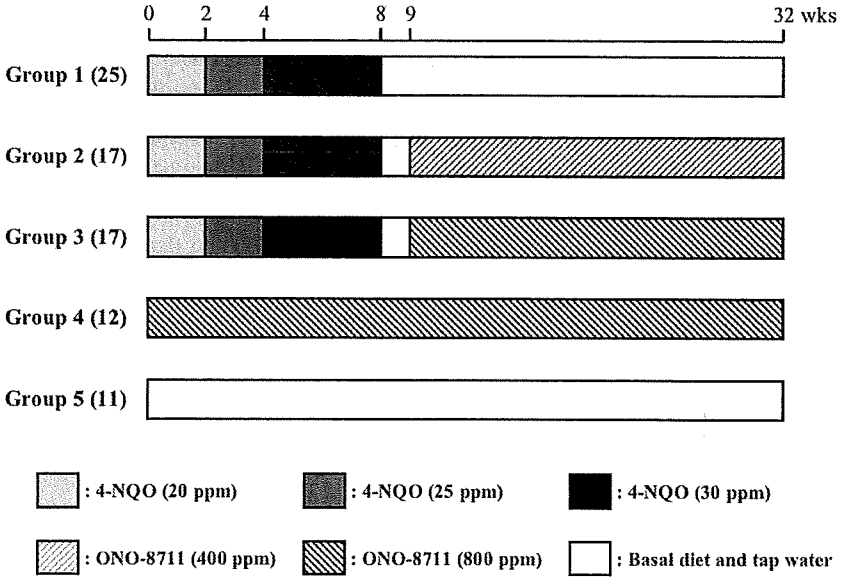

Fig. 1. Experimental protocol. Groups 1–3 were given 20 p.p.m. 4-NQO in drinking water for the first of 2 weeks, 25 p.p.m. for the next 2 weeks and 30 p.p.m. for the other 4 weeks. Rats in Groups 1 and 4 were fed the basal diet and the experimental diet containing 800 p.p.m. ONO-8711, respectively, during 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 I. 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).

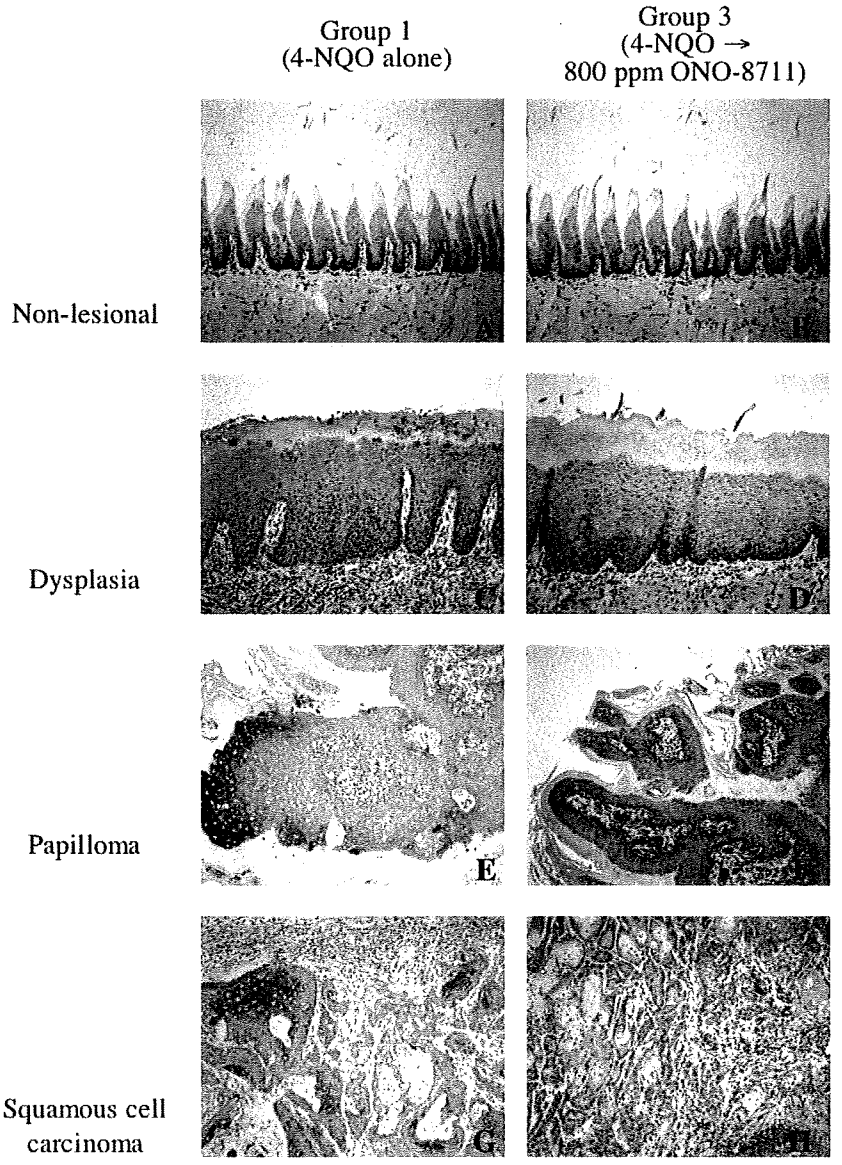

Fig. 2. Immunohistochemical localization of EP_1 in the 'normal' appearing tongue squamous epithelium, dysplasia and neoplasms from Groups 1 (**A, C, E,** and **G**) and 3 (**B, D, F,** and **H**). In the 'normal' appearing squamous epithelium, weak expression of EP_1 is seen in the upper one-third of 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

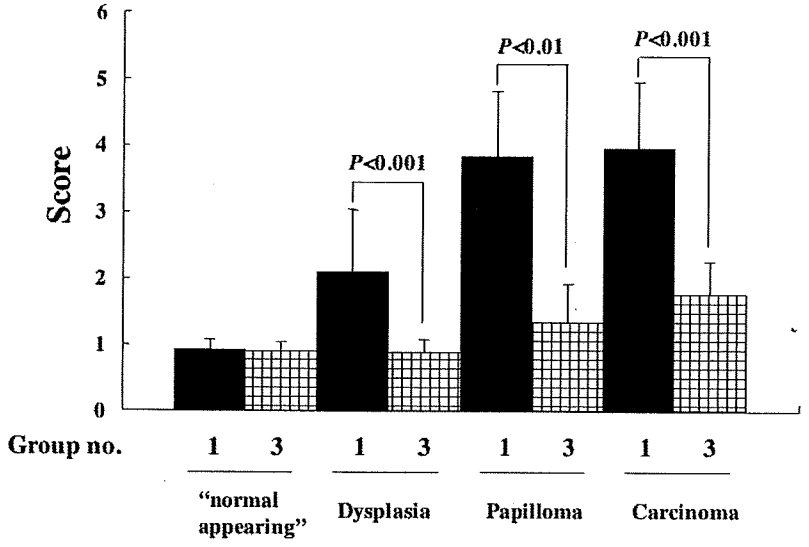

Fig. 3. Scores of EP_1 immunohistochemical stainability. The degree of EP_1 stainability in the lesions developed was compared between Groups 1 and 3 using the grading system (Grade 0–5, see the Materials and methods section). The scores were determined in the 'normal' appearing tongue squamous epeithelium ($n = 25$ 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).

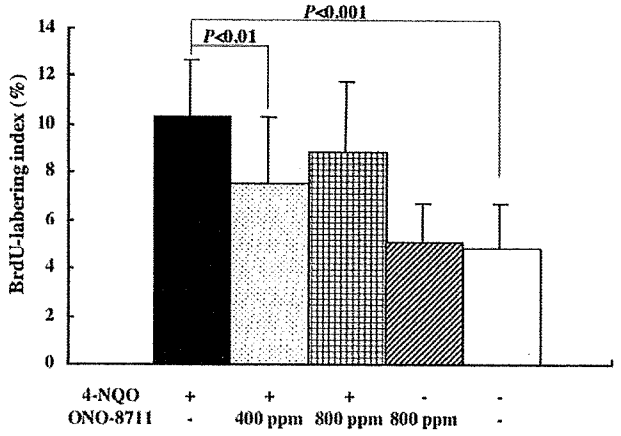

Fig. 4. BrdU-labeling index. The mean BrdU-labeling index in the non-lesional tongue epithelium of Group 1 that was given 4-NQO alone was significantly greater than Group 5 (untreated control) ($P < 0.001$). The mean BrdU-labeling indices of Groups 2 (4-NQO→400 p.p.m. ONO-8711) and 3 (4-NQO→800 p.p.m.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

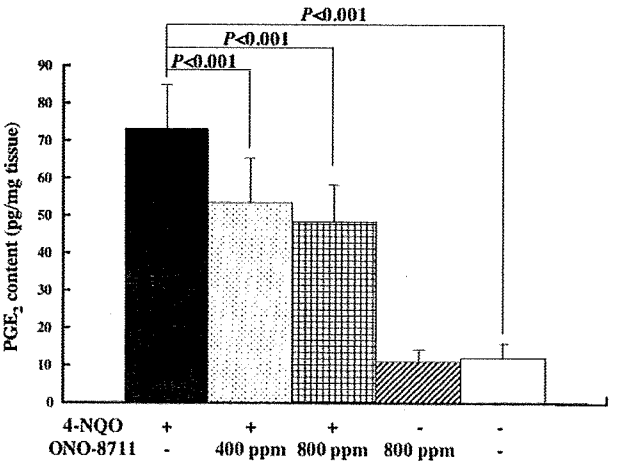

Fig. 5. PGE_2 levels in the tongues. The mean PGE_2 content in the non-tumorous tongue tissue of Group 1 that was given 4-NQO alone was significantly greater than Group 5 (untreated control) ($P < 0.001$). The mean PGE_2 levels of Groups 2 (4-NQO→400 p.p.m. ONO-8711) and 3 (4-NQO→800 p.p.m. ONO-8711)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

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Ursodeoxycholic Acid versus Sulfasalazine in Colitis-Related Colon Carcinogenesis in Mice

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Abstract **Purpose:** Inflammation influences carcinogenesis. In the current study, we investigated whether ursodeoxycholic acid (UDCA) can inhibit colitis-related mouse colon carcinogenesis and compared it with the effects of sulfasalazine.

Experimental Design: Male CD-1 mice were given a single i.p. injection of azoxymethane followed by 1-week oral exposure of 1% dextran sodium sulfate in drinking water. They are then maintained on a basal diet mixed with UDCA (0.016%, 0.08%, or 0.4%) or sulfasalazine (0.05%) for 17 weeks. At week 20, the tumor-inhibitory effects of both chemicals were assessed by counting the incidence and multiplicity of colonic neoplasms. The immunohistochemical expression of the proliferating cell nuclear antigen labeling index in colonic epithelial malignancies was also assessed. Finally, at week 5, the mRNA expressions for cyclooxygenase-2, inducible nitric oxide synthase, peroxisome proliferator-activated receptor-γ, and tumor necrosis factor-α were measured in nontumorous mucosa.

Results: Feeding the mice with UDCA at all doses significantly inhibited the multiplicity of colonic adenocarcinoma. The treatment also significantly lowered the proliferating cell nuclear antigen labeling index in the colonic malignancies. UDCA feeding reduced the expression of inducible nitric oxide synthase and tumor necrosis factor-α mRNA in the colonic mucosa, while not significantly affecting the expression of cyclooxygenase-2 mRNA and peroxisome proliferator-activated receptor-γ mRNA. Sulfasalazine caused a nonsignificant reduction in the incidence and multiplicity of colonic neoplasia and did not affect these mRNA expression.

Conclusions: Our findings suggest that UDCA rather than sulfasalazine could serve as an effective suppressing agent in colitis-related colon cancer development in mice.

Colorectal cancer is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (1). Ulcerative colitis patients are 10 times more likely to develop colorectal dysplasia and adenocarcinoma than the general population (2). The precise mechanisms involved in IBD-related carcinogenesis are largely unclear, but chronic inflammation influences the development of IBD-related carcinogenesis (3).

Epidemiologic studies suggest that environmental and dietary factors contribute to the development of colorectal cancer (4). High fats generate secondary bile acids that are known to enhance cell proliferation and promote colorectal cancer development (5). In humans, high levels of serum and fecal secondary bile acids are found in patients with colitis (6) and colorectal neoplasia (7). On the other hand, ursodeoxycholic acid (UDCA) reduces the colonic concentration of the secondary bile acids (8) and suppresses colon carcinogenesis (9–11) in animal studies.

Primary prevention of colorectal cancer in IBD has recently received more attention. A number of therapeutic agents using IBD may have chemopreventive effects and offer some hope for primary chemoprevention of colorectal cancer in IBD (12–14). Aminosalicylates, including sulfasalazine, are the most commonly prescribed anti-inflammatory agents for IBD. The intake of sulfasalazine, especially as a long-term maintenance treatment in chronic IBD, was found to be associated with a decreased risk of colorectal cancer (15). UDCA is also reported to have colon cancer chemopreventive effects in preclinical studies (9–11). A recent report has suggested that UDCA decreases the risk for developing dysplasia in ulcerative colitis patients with primary sclerosing cholangitis (16, 17).

For understanding the pathogenesis of IBD and IBD-related colorectal cancer, several animal models have been established. Most common is a mouse model with dextran sodium sulfate (DSS; ref. 18). The modifying effects of certain xenobiotics on colorectal cancer–related colon carcinogenesis have been reported (19) using this model. The model uses DSS, with or without carcinogen, and also requires a long period and repeated administration of DSS to induce colitis and colitis-related

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colorectal cancer. We therefore developed a novel colitis-related mouse colorectal cancer model initiated with a colon carcinogen azoxymethane and promoted by DSS. In this new model, colorectal cancer develops within a short-term period (20), and the expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) increased the colonic neoplasia (20) and human IBD (21, 22). In addition, peroxisome proliferator-activated receptors (PPAR) play important roles in both the lipid metabolism and inflammation (23). PPAR ligands attenuate the colonic inflammation in the mouse model of colitis (24), and the protein levels of PPARγ are decreased in cryptal cells from the ulcerative colitis patients (25). Using this animal model, we showed that dietary administration of COX-2 inhibitor and PPAR ligands can suppress colitis-related colonic carcinogenesis through suppression of COX-2 and iNOS expression (24).

To determine the chemoprevention ability of UDCA in colitis-related colon carcinogenesis in the present study, we examined the effect of UDCA on our mouse colon carcinogenesis model and compared with sulfasalazine used for patients with IBD.

Materials and Methods

Animals, chemicals, and diets. Male Crj:CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo, Japan) ages 5 weeks were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to Institutional Animal Care Guidelines. All animals were housed in plastic cages (five or six mice per cage) with free access to drinking water and a pelleted basal diet and CRF-1 (Oriental Yeast Co. Ltd., Tokyo, Japan). The mice were under controlled conditions of humidity (50 ± 10%), light (12/12-h light/dark cycle), and temperature (23 ± 2°C). They were quarantined for the first 7 days then separated randomly by body weight into experimental and control groups. A colonic carcinogen azoxymethane and sulfasalazine were purchased from Sigma-Chemical Co. (St. Louis, MO), and DSS with a molecular weight of 36,000 to 50,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH). The DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v). UDCA was obtained from Mitsubishi Pharma Corp. (Osaka, Japan). Experimental diets were prepared by mixing chemopreventive agents with a modified CRF-1 control diet. The compound was mixed at concentrations of 0.016%, 0.08%, and 0.4% with the powdered basal diet CRF-1. The highest dose (0.4%) was a selected to investigate the effects of UDCA on colon polyp formation as used by Narisawa et al. (10). The dose of sulfasalazine was chosen from the results of a study conducted by Suzuki et al. (26), in which a diet, including sulfasalazine, suppressed colorectal carcinogenesis.

Experimental procedure. A total of 130 male ICR mice were divided into eight experimental and control groups. The mice in groups 1 to 5 were given a single i.p. injection of azoxymethane (10 mg/kg body weight). Starting 1 week after the injection, they were administered 1% DSS in drinking water for 7 days. The mice in group 1 were maintained on the basal diet throughout the study. Mice in groups 2 to 4 were given a diet, including UDCA (0.016% in group 2, 0.08% in group 3, and 0.4% in group 4), starting 1 week after the stop of DSS administration. Group 5 was fed a diet mixed with 0.05% sulfasalazine, starting 1 week after the stop of DSS administration. Animals in groups 6 and 7 were given a diet containing 0.4% UDCA alone and 0.05% sulfasalazine alone, respectively. Group 8 was untreated. To assess the relative quantification of gene expression in colonic mucosa at week 5, samples of colon of mice (n = 5 per group) were washed with PBS, and they were quickly frozen in liquid nitrogen and stored at −80°C. All the remaining animals were sacrificed at week 20. At sacrifice, all organs were removed, and the large bowels were flushed with PBS, excised, measured in length (from ileocecal junction to the anal verge), cut open longitudinally along the main axis, and then washed with PBS. The large bowels were macroscopically inspected, and whole colon was processed for paraffin embedding. The large bowels were macroscopically inspected, cut, and fixed in 10% buffered formalin for at least 24 h. A histologic examination was then done on paraffin-embedded sections after H&E staining. Colonic neoplasms were diagnosed according to the description by Ward (27).

Immunohistochemistry of proliferating cell nuclear antigen. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was done on 4-μm-thick paraffin-embedded sections from colonic adenocarcinomas of mice in groups 1 to 5 at week 20 as previous described (24). Briefly, the sections were incubated overnight at 4°C with a primary antibody, anti-PCNA mouse monoclonal antibody (1:50 dilution; PC10; Dako, Glostrup, Denmark). To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Inc., Burlingame, CA) was applied for 1 h. Horseradish peroxidase activity was visualized through treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck Ltd., Tokyo, Japan). For control sections, incubation with the primary antibodies was omitted. Intensity and localization of immunoreactivity against the primary antibody were examined on all colonic adenocarcinomas using a microscope (Olympus BX41, Olympus Optical Co. Ltd., Tokyo, Japan). For the determination of PCNA-incorporated nuclei, a PCNA immunohistochemistry was done according to the method described by Watanabe et al. (28). The PCNA indices were determined by counting the number of positive cells among at least 200 cells in a lesion and were indicated as percentages.

RNA extraction and synthesis of cDNA. The extraction of total RNA from frozen colonic mucosa was done using an RNeasy Mini kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's directions. The concentration of total RNA was measured using a spectrophotometer. The A_{260}/A_{280} ratio of RNA was 1.8 to 2.0. Oligo (dT)20 primer and SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen/Life Technologies, Paisley, United Kingdom) was used to synthesize cDNA according to the manufacturer's protocol and stored at −30°C until analyzed.

Relative quantification of gene expression by LightCycler reverse transcription-PCR. Reverse transcription-PCR analysis showed comparable levels of glyceraldehyde-3-phosphate dehydrogenase mRNA in the non-lesional colonic mucosa of five mice from each group at week 5 after DSS administration. All real-time experiments were conducted using a LightCycler FastStart DNA Master HybProbe kit (Roche, Mannheim, Germany). Oligonucleotide primers and hybridization probes are purchased from Nihon Gene Research Labs, Inc. (Sendai, Japan) and listed in Table 1. PCR reactions contained 15 μL of master mix and 5 μL of template cDNA. The final reaction mixture contained glyceraldehyde-3-phosphate dehydrogenase, iNOS, tumor necrosis factor-α (TNF-α), 3 mmol/L $MgCl_2$, 0.5 μmol/L of the primers (forward and reverse), 0.2 μmol/L of the Fluorescein probe, 0.4 μmol/L of the LCRed probe, and 1× LightCycler FastStart DNA Master HybProbe. It also contained PPARγ, 3 mmol/L $MgCl_2$, 0.3 μmol/L of the primers (forward and reverse), 0.2 μmol/L of the probes (Fluorescein and LCRed), 1× LightCycler FastStart DNA Master HybProbe, and COX-2 based on the manufacturer's recommendations. The contents were placed in a glass capillary, capped, briefly centrifuged, and placed in the LightCycler. The denaturing process for all genes was one cycle at 95°C for 10 min. The amplification conditions were as follows: glyceraldehyde-3-phosphate dehydrogenase, 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 9 s; iNOS, 40 cycles of 95°C for 10 s, 62°C for 15 s, and 72°C for 9 s; TNF-α, 40 cycles of 95°C for 10 s, 61°C for 15 s, and 72°C for 7 s; and PPARγ and COX-2, 45 cycles of 95°C for 10 s, 62°C for 15 s, and 72°C for 7 s. The amplification protocol was followed by a cooling period of one cycle at 40°C for 30 s. Data collection was done during extension and monitored through the F2/1

Table 1. Primers and probes used for real-time PCR

Gene	Primer or probe	Sequence (5′-3′)
GAPDH	Forward primer	TGAACGGGAAGCTCACTGG
	Reverse primer	TCCACCACCCTGTTGCTGTA
	Donor probe	CTGAGGACCAGGTTGTCTCCTGCGA
	Acceptor probe	TTCAACAGCAACTCCCACTCTTCCACC
iNOS	Forward primer	GCAAACCCAAGGTCTACGTT
	Reverse primer	GGAAAAGACTGCACCGAAGA
	Donor probe	TGGTAGCCACATCCCGAGCCAT
	Acceptor probe	CGCACATCTCCGCAAATGTAGAGG
TNF-α	Forward primer	CCACGTCGTAGCAAACCAC
	Reverse primer	TGGGTGAGGAGCACGTAGT
	Donor probe	TGGTGCCAGCCGATGGGTTGTAC
	Acceptor probe	TTGTCTACTCCCAGGTTCTCTTCAAGG
PPARγ	Forward primer	GAGTTCCTCAAAAACCTGCG
	Reverse primer	TGTCTTGGATGTCCTCGATG
	Donor probe	CAATGCACTGGAATTAGATGACAGTGACTTGGCTA
	Acceptor probe	ATTTATAGCTGTCATTATTCTCAGTGGAGACCGCCC
COX-2	Forward primer	CCATCTGTTCTCCTCAATAC
	Reverse primer	TTTGGTAGGCTGTGGAT
	Donor probe	ACCTTTGGAGGCGAAGTGGGTTTTAAGATC
	Acceptor probe	TCAATACTGCCTCAATTCAGTCTCTCATCTGCA

NOTE: Donor probes were labeled at the 3′ end with fluorescein. Acceptor probes were labeled at the 5′ end with LightCyler Red 640.

channel of the instrument. Data analyses were conducted using the second derivative maximum method of the LightCycler software.

Statistical analysis. All measurements were compared using Tukey's or Bonferroni's multiple comparison post test or Fisher's exact probability test. Differences were considered statistically significant at $P < 0.05$.

Results

General observation. Bloody stool was observed in a few mice that received 1% DSS, and body weight gains were slightly decreased during the course of the treatment. However, thereafter, no clinical symptoms were noted. Mean weights, mean liver weights, relative liver weights, and mean length of large bowel in all groups at the end of the study are shown in Table 2. The mean body weights, liver weights, and relative liver weights did not significantly differ among the groups. The mean length of the large bowels in groups 2 to 5 was lower than in group 1, but the differences did not reach statistical significance. Histologically, there were no pathologic alterations suggesting toxicity of UDCA and sulfasalazine in the liver, kidneys, lung, or heart. Food consumption (g/d per mice) did not significantly differ among the groups (data not shown).

Incidence and multiplicity of colonic neoplasm. Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of mice in groups 1 to 5. Histopathologically, dysplasia, adenoma, and adenocarcinoma (Fig. 1A, C, E, G, and I) developed in the azoxymethane/DSS–treated mice. The mice in groups 6 to 8 did not exhibit large bowel neoplasms in any organs examined, including the colon. The incidences and multiplicity of colon neoplasma are summarized in Table 3. Group 1 (azoxymethane/DSS) induced a 65% incidence of colon tumors with a multiplicity of 3.7 ± 6.0. The incidences of colon tumors in group 2 (azoxymethane/DSS/0.016% UDCA), group 3 (azoxymethane/DSS/0.08% UDCA), and group 4 (azoxymethane/DSS/0.4% UDCA) were lower than group 1. The differences, however, were not statistically

Table 2. Body, liver, and relative liver weights

Group no.	Treatment	No. mice	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Length of large bowel (cm)
1	AOM/DSS	20	49.8 ± 5.6	2.98 ± 0.50	5.98 ± 0.68	14.1 ± 1.0
2	AOM/DSS/0.016% UDCA	20	44.7 ± 4.2	2.78 ± 0.40	6.21 ± 0.64	14.2 ± 1.1
3	AOM/DSS/0.08% UDCA	20	47.3 ± 6.4	2.72 ± 0.28	5.79 ± 0.46	14.0 ± 1.2
4	AOM/DSS/0.4% UDCA	20	48.9 ± 2.6	2.63 ± 0.42	5.38 ± 0.71	14.6 ± 1.1
5	AOM/DSS/0.05% SASP	20	45.0 ± 4.3	2.75 ± 0.23	6.14 ± 0.59	13.4 ± 1.2
6	0.4% UDCA	5	42.0 ± 1.8	2.35 ± 0.19	5.60 ± 0.36	13.8 ± 0.6
7	0.05% SASP	5	45.5 ± 3.4	2.80 ± 0.30	6.17 ± 0.54	14.9 ± 0.6
8	None	5	43.6 ± 3.8	2.67 ± 0.43	6.13 ± 0.95	13.4 ± 1.1

NOTE: Values are expressed as mean ± SD.
Abbreviations: AOM, azoxymethane; SASP, sulfasalazine.

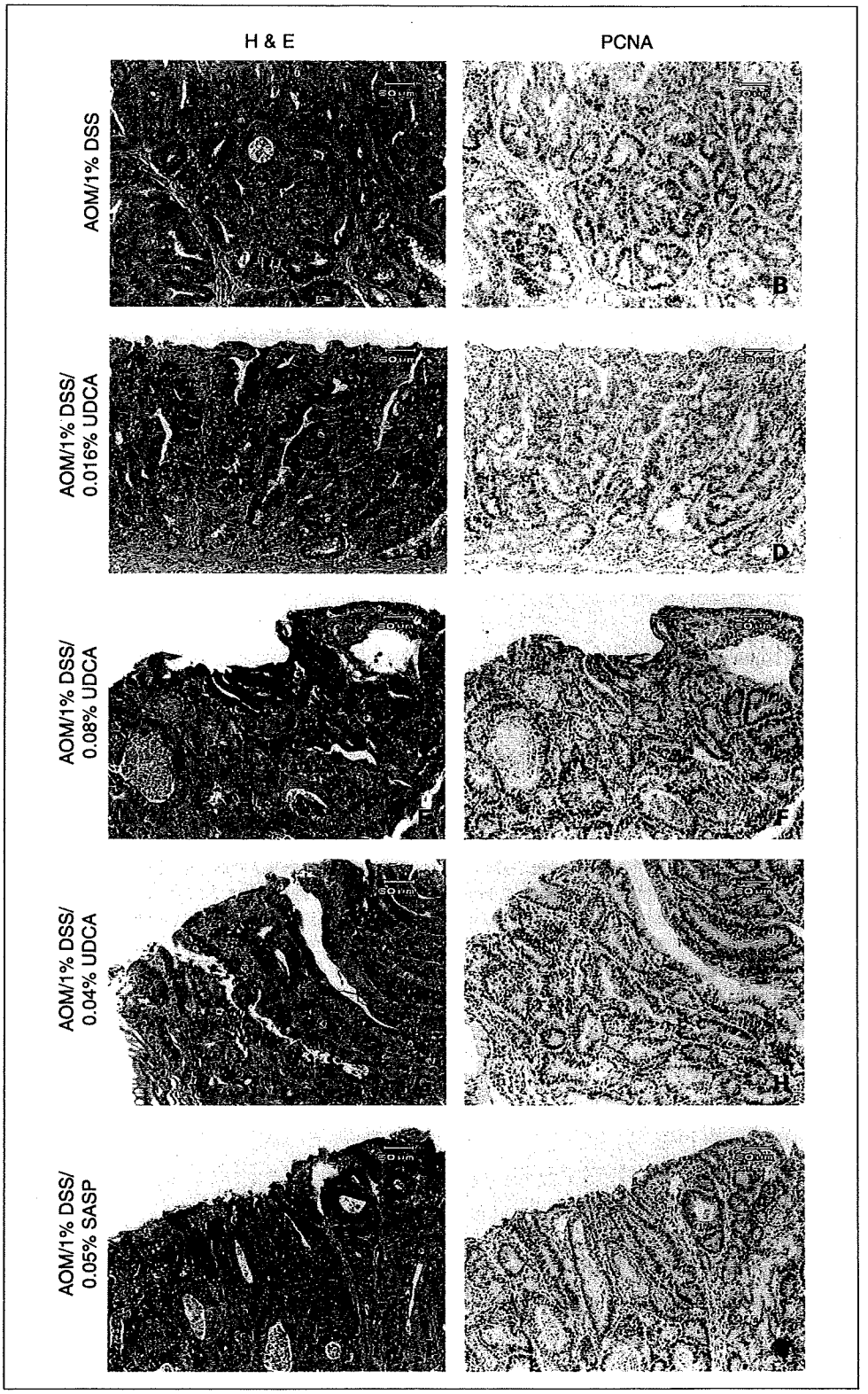

Fig. 1. Histopathology of adenocarcinomas and their corresponding immunohistochemistry of PCNA. An adenocarcinoma from a mouse of group 1 (*A* and *B*), 2 (*C* and *D*), 3 (*E* and *F*), 4 (*G* and *H*), or 5 (*I* and *J*). H&E stain (*A, C, E, G,* and *I*) and PCNA immunohistochemistry (*B, D, F, H,* and *J*). Bar, 60 μm.

significant. The multiplicities of colon tumors and adenocarcinomas in group 2 ($P < 0.05$), group 3 ($P < 0.05$), and group 4 ($P < 0.05$) were significantly lower than that of group 1. Finally, whereas the multiplicities of colon tumors and adenocarcinomas in group 5 (azoxymethane/DSS/0.05% sulfasalazine) were smaller than group 1, the differences did not reach to statistical significance.

Immunohistochemistry for PCNA in colonic adenocarcinoma. As summarized in Table 4, the PCNA labeling indices of colonic adenocarcinomas developed in group 2 ($P < 0.01$; Fig. 1D), group 3 ($P < 0.001$; Fig. 1F), and group 4 ($P < 0.001$; Fig. 1H) were significantly smaller than group 1 (Fig. 1B). Dietary sulfasalazine (group 5) did not significantly affect the PCNA

Table 3. Incidence and multiplicity of colonic neoplasia

Group no.	Treatment	No. mice	Incidence (no. mice with neoplasms)			Multiplicity (no. tumors/mice)*		
			Total	Adenoma	Adenocarcinoma	Total	Adenoma	Adenocarcinoma
1	AOM/DSS	20	13/20 (65%)	12/20 (60%)	9/20 (45%)	3.7 ± 6.0	1.5 ± 1.8	2.2 ± 4.5
2	AOM/DSS/0.016% UDCA	20	8/20 (40%)	6/20 (30%)	5/20 (25%)	0.9 ± 1.5†	0.6 ± 1.1	0.3 ± 0.4†
3	AOM/DSS/0.08% UDCA	20	9/20 (45%)	5/20 (25%)	5/20 (25%)	0.6 ± 0.8†	0.4 ± 0.7	0.3 ± 0.4†
4	AOM/DSS/0.4% UDCA	20	8/20 (40%)	8/20 (40%)	4/20 (20%)	1.0 ± 1.5†	0.7 ± 0.9	0.3 ± 0.7†
5	AOM/DSS/0.05% SASP	20	11/20 (55%)	9/20 (45%)	8/20 (40%)	1.6 ± 1.9	0.9 ± 1.1	0.7 ± 1.0
6	0.4% UDCA	5	0/4 (0%)	0/4 (0%)	0/4 (0%)	0	0	0
7	0.05% SASP	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0
8	None	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0

*Values are expressed as mean ± SD.
†$P < 0.05$, significantly different from group 1 by Tukey's multiple comparison post-test.

labeling index in the colonic adenocarcinoma (Fig. 1J) of the mice in comparison with the azoxymethane/DSS group (Fig. 1B).

Validation of selected genes by real-time quantitative reverse transcription-PCR. As shown in Table 5, the mRNA expression of COX-2, iNOS, and TNF-α in group 8 (nontreated group) were very low in the colonic mucosa, but the expression dramatically increased when the mice were given azoxymethane/DSS (group 1). PPARγ mRNA expression in mice treated with azoxymethane (groups 1–5) was higher than non-azoxymethane mice (groups 6–8), but the values were not statistically different among the groups.

The mRNA expression of iNOS in the colonic tissue of group 2 (azoxymethane/DSS/0.016% UDCA, $P < 0.001$), group 3 (azoxymethane/DSS/0.08% UDCA, $P < 0.001$), and group 4 (azoxymethane/DSS/0.4% UDCA, $P < 0.01$) was significantly lower than that of group 1 (azoxymethane/DSS). The expression of TNF-α mRNA was significantly lower in the group 2 ($P < 0.001$), group 3 ($P < 0.001$), and group 4 ($P < 0.01$) in comparison with group 1. In contrast, there were no differences in the expression of COX-2 and PPARγ in the colonic mucosa among the groups. The differences in expression of COX-2, iNOS, PPARγ, and TNF-α mRNA between group 1 (azoxymethane/DSS) and group 5 (azoxymethane/DSS/sulfasalazine) were not statistical significant.

Discussion

The results of the present study clearly indicated that UDCA effectively inhibits azoxymethane/DSS–induced colitis-related colonic carcinogenesis without any adverse effects in mice. The inhibitory effect of UDCA was closely similar at all dose levels. This correlates with the findings of other reports, in which of carcinogen-induced colon carcinogenesis by feeding with UDCA was not dose dependent (9–11). The suppressive effect of UDCA on the development of colonic adenocarcinoma closely correlated with the inhibition of the PCNA labeling index of colonic adenocarcinomas and the suppression of iNOS and TNF-α mRNA levels in the nontumorous colonic mucosa.

In both experimental animal and human studies, secondary bile acids, such as DCA, have been shown to enhance colonic epithelial cell proliferation, to be cytotoxic to colonic epithelial cells (29), and to be moderately mutagenic (30). In contrast, UDCA inhibited colorectal cancer and adenoma formation in azoxymethane-induced rat carcinogenesis models (11). Furthermore, recent reports suggested that UDCA decreases the risk for developing dysplasia and cancer in ulcerative colitis patients with primary sclerosing cholangitis (16, 17). More recently, Loddenkemper et al. (31) found in their colitis-associated model that the development of murine colorectal tumors can be inhibited by oral treatment with UDCA. Although they noted that colonic tumors were histologically mucinous adenocarcinomas or squamous carcinomas, we observed well/moderately differentiated tubular adenocarcinomas in the present study. These findings may suggest that dietary UDCA is able to suppress the development of various histologic types of colon carcinoma.

Sulfasalazine is commonly used for the treatment of IBD, such as ulcerative colitis and Chron's disease (32). Published evidence indicates that sulfasalazine prevents the development of dysplasia and colorectal cancer in patients with IBD (33). Recently, Suzuki et al. (26) reported that treatment with sulfasalazine resulted in a reduction of tumorous lesions with high-grade dysplasia in female CBA/J mice initiated with azoxymethane and promoted by three cycle administration of DSS. In this study, the suppressive effects of sulfasalazine given in the promotion/progression phase on the development of colorectal cancer were relatively weak. The discrepancy between their results and ours may be due to the differences in the treatment period of sulfasalazine and/or the strains of mice used.

Table 4. PCNA indices in colonic adenocarcinomas

Group no.	Treatment	PCNA labeling index (%)*
1	AOM + 1% DSS	61.4 ± 11.0 (16)
2	AOM + 1% DSS/0.016% UDCA	39.8 ± 9.3 (5)†
3	AOM + 1% DSS/0.08% UDCA	38.2 ± 6.1 (5)‡
4	AOM + 1% DSS/0.4% UDCA	37.1 ± 7.7 (6)‡
5	AOM + 1% DSS/0.05% SASP	52.6 ± 10.0 (14)

*Values in column are expressed as mean ± SD. Numbers in parentheses are numbers of lesions examined.
†$P < 0.01$, significantly different from group 1 by Tukey's multiple comparison post-test.
‡$P < 0.001$, significantly different from group 1 by Tukey's multiple comparison post-test.

Table 5. mRNA expression of COX-2, iNOS, PPARγ, and TNF-α in colonic mucosa at week 5

Group no.	Treatment (no. mice examined)	mRNA/GAPDH mRNA ratio (×10⁴)			
		COX-2	iNOS	PPARγ	TNF-α
1	AOM + 1% DSS (5)	1.03 ± 0.46*	1.41 ± 0.69†	5.13 ± 1.00	7.98 ± 3.38†
2	AOM + 1% DSS/0.016% UDCA (5)	1.25 ± 0.40	0.15 ± 0.22‡	4.83 ± 0.87	2.60 ± 0.79‡
3	AOM + 1% DSS/0.08% UDCA (5)	0.70 ± 0.36	0.26 ± 0.21‡	6.50 ± 2.29	2.57 ± 1.06‡
4	AOM + 1% DSS/0.4% UDCA (5)	1.06 ± 0.40	0.52 ± 0.40§	4.55 ± 1.08	3.38 ± 2.18§
5	AOM + 1% DSS/0.05% SASP (5)	1.07 ± 0.89	1.19 ± 0.51	5.34 ± 0.75	5.00 ± 2.38
6	0.4% UDCA (5)	0.04 ± 0.01	0.02 ± 0.01	2.51 ± 1.08	1.00 ± 0.48
7	0.05% SASP (5)	0.08 ± 0.07	0.03 ± 0.02	2.49 ± 0.43	0.65 ± 0.32
8	None (5)	0.07 ± 0.06	0.06 ± 0.06	2.90 ± 0.83	0.79 ± 0.72

NOTE: Values are expressed as mean ± SD.
*$P < 0.05$, significantly different from group 8 by Bonferroni's multiple comparison post-test.
†$P < 0.01$, significantly different from group 8 by Bonferroni's multiple comparison post-test.
‡$P < 0.001$, significantly different from group 1 by Bonferroni's multiple comparison post-test.
§$P < 0.01$, significantly different from group 1 by Bonferroni's multiple comparison post-test.

The development of colonic neoplasia is influenced by alterations in the balance of cell renewal and cell death that regulate normal cellular homeostasis in the colon (34). UDCA inhibits the proliferation of colon cancer cell lines *in vitro* (9), while also decreasing the size and number of colon tumors induced by *N*-methylnitrosourea (10) or azoxymethane (11) in rats. In addition, Wali et al. (35) reported that UDCA inhibits cyclin D1 expression. This is an important positive cell cycle regulator (36) in aberrant crypt foci and non-aberrant crypt foci crypts in rats treated with azoxymethane in conjunction with their inhibition of crypt cell hyperplasia and tumors. In the present study, UDCA feeding significantly lowered the PCNA labeling index in the adenocarcinomas, thus suggesting that dietary UDCA therefore suppresses the abnormal proliferative activity of preneoplastic and neoplastic cells, thereby inhibiting carcinogenesis.

Inflammation-caused oxidative/nitrosative cellular damage is suspected to contribute to the development of IBD-associated colorectal neoplasms. Expression and activity of iNOS is increased in the colonic mucosa in patients with IBD (37) and colonic adenomas (38) in murine noncancerous colonic mucosa (39, 40). Numerous iNOS-positive and nitrotyrosine-positive inflammatory cells are observed in the nontumorous tissues of mice treated with DSS (40). Rao et al. (41) showed that an iNOS-selective inhibitor suppressed azoxymethane-induced colonic aberrant crypt foci development and their iNOS activity. UDCA also inhibited the induction of iNOS in the human intestinal adenocarcinoma cell lines and the colonic epithelium of rats exposed to lipopolysaccharide (42). In the current study, UDCA significantly reduced iNOS mRNA expression in the colonic mucosa at week 5. Therefore, the suppression of iNOS expression and/or activity may thus be related to the chemopreventive effects of UDCA.

TNF-α is a potent pro-inflammatory cytokine involved in the pathogenesis of IBD, which plays a crucial role in the progression inflammatory responses in the colon (43). In fact, the plasma and colonic TNF-α levels are elevated in patients with IBD (44, 45), and the colonic expression of TNF-α mRNA is also enhanced in DSS-induced colitis in mice (46). Although the results from the study on treatment with anti-TNF-α monoclonal antibody in DSS-treated mice suggested a more complex role for TNF-α in colonic inflammation (47), Myers et al. (48) reported that antisense oligonucleotide specific for murine TNF-α could prevent DSS-induced colitis. In the current study, we found that dietary UDCA thus reduces the expression of TNF-α mRNA in colonic mucosa of mice treated with azoxymethane and DSS. Based on our findings, the suppression of the iNOS and TNF-α expression and/or the activity by UDCA may thus explain its chemopreventive ability in colitis-related mouse carcinogenesis induced by azoxymethane/DSS. Taken together, UDCA rather than sulfasalazine could be a valuable approach for the chemoprevention of human IBD-related colorectal cancer.

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A specific inducible nitric oxide synthase inhibitor, ONO-1714 attenuates inflammation-related large bowel carcinogenesis in male $Apc^{Min/+}$ mice

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It is generally assumed that inflammation influences carcinogenesis. We previously reported that dextran sodium sulfate (DSS) strongly enhances colon carcinogenesis in the $Apc^{Min/+}$ mice and the over-expression of inducible nitric oxide synthase (iNOS) contributes to this enhancement. In the current study, we investigated the effect of a selective iNOS inhibitor, ONO-1714 on colitis-related colon carcinogenesis in the $Apc^{Min/+}$ mouse treated with DSS. Male C57BL/6J $Apc^{Min/+}$ and $Apc^{+/+}$ mice were exposed to 1% DSS in their drinking water for 7 days. ONO-1714 was given to the mice at a dose level of 50 or 100 ppm in diet for 5 weeks (during the administration of DSS). The tumor inhibitory effects by ONO-1714 were assessed at week 5 by counting the incidence and multiplicity of colonic neoplasms. Additionally, we assessed serum lipid levels and colonic mRNA expression for cyclooxygenase (COX)-2, iNOS, tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Feeding with ONO-1714 significantly inhibited the occurrence of colonic adenocarcinoma in a dose-dependent manner in the $Apc^{Min/+}$ mice. In addition, the treatment with ONO-1714 significantly lowered the serum triglyceride levels and mRNA expression levels of COX-2, TNFα and IL-1β of colonic mucosa in the DSS-treated $Apc^{Min/+}$ mice. Neither ONO-1714 nor DSS affected the colonic pathology in the $Apc^{+/+}$ mice. Our findings may suggest that ONO-1714 could therefore serve as an effective agent for suppression of colitis-related colon cancer development in the $Apc^{Min/+}$ mice.

Key words: colitis-related carcinogenesis; chemoprevention; dextran sodium sulfate; iNOS; $Apc^{Min/+}$ mice

The link between carcinogenesis and chronic inflammation has been recognized for certain types of cancer, including colorectal cancer (CRC).[1] CRC is one of the known serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC).[1,2] For understanding the pathogenesis of IBD and IBD-related CRC, we and others have been studying the colitis-associated carcinogenesis process using the dextran sodium sulfate (DSS) model in mice.[3–7] In our previous studies, inflammation/inflammatory stimuli induced by DSS treatment after the initiation with a low-dose of colonic carcinogen is effective for the rapid induction of colonic neoplasms that possess β-*catenin* gene mutations in ICR mice.[4,5] Furthermore, we recently reported that DSS treatment results in intestinal mucosa inflammation and numerous colorectal neoplasms in $Apc^{Min/+}$ mice, which demonstrates a germline mutation in the adenomatous polyposis coli (APC) gene.[8] Cooper *et al.*[7] also found a relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis in the $Apc^{Min/+}$ mice. Therefore, DSS models with or without carcinogen can be useful for investigating the IBD-associated colorectal carcinogenesis.

Nitric oxide (NO) is a mediator of physiological processes in the gastrointestinal tract, including mucosal protection, the regulation of blood flow and the regulation of motility.[9] On the other hand, the overproduction of NO contributes to tissue damage, colon tumor growth and DNA deamination.[1,10] Increased NO production as well as the expression of inducible nitric oxide synthase (iNOS) in intestinal mucosa is known to be associated with the disease activity of IBD.[11] In colon carcinogenesis, an increased expression of iNOS was up-regulated in human colon adenomas and adenocarcinomas,[1,10] and azoxymethane (AOM)-induced rat large intestinal aberrant crypt foci (ACF), and tumors as well.[12] We also observed that the immunohistochemical expression of iNOS increased in inflamed colonic mucosa and colonic adenocarcinoma in the mice that received AOM and/or DSS.[3,8] In addition, the oxidative/nitrosative stress caused by DSS exposure contributes to the development of a high incidence of colonic adenocarcinomas in mice.[13] Therefore, NO and iNOS may play a certain role in the experimental inflammation-related colon carcinogenesis and the development of human UC-associated cancer.[1]

According to these data, treatment with several selective iNOS inhibitors may serve as a novel experimental approach to colitis and/or colitis-associated carcinogenesis.[10] Among these iNOS inhibitors, ONO-1714, being 10-fold more selective for human iNOS than for human endothelial NOS, is very potent with an ID_{50} value of 0.010 mg/kg s.c. and lowly toxic with a maximum tolerated dose of 30 mg/kg i.v. in mice.[14,15] These biological natures of ONO-1714 and its effectiveness even when orally administered[16] thus led us to investigate the modifying effects of ONO-1714 on colon carcinogenesis in the $Apc^{Min/+}$ mice receiving DSS.

Material and methods

Animals, chemicals and diets

Male C57BL/6J $Apc^{Min/+}$ and $Apc^{+/+}$ were purchased from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and they were genotyped by the method reported previously.[17] They were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, quarantined for the first 7 days, and then randomized by body weight into ex-

Abbreviations: Alb, albumin; A/G, albumin:globlin ratio; ACF, aberrant crypt foci; ALT, alanine aminotransferase; ALP, alkaline phosphatase; APC, adenomatous polyposis coli; AST, aspartate aminotransferase; AOM, azoxymethane; BUN, blood urea nitrogen; CRC, colorectal cancer; COX, cyclooxygenase; DSS, dextran sodium sulfate; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; Glu, glucose; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; IL, interleukin; LDH, lactate dehydrogenase; nNOS, neural nitric oxide synthase; NO, nitric oxide; RT-PCR, reverse transcriptase-polymerase chain reaction; T-Bil, total bilirubin; T-Cho, total cholesterol; TP, total protein; TG, triglyceride; TNF, tumor necrosis factor; UC, ulcerative colitis.

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TABLE I – BODY WEIGHTS, LIVER WEIGHTS, RELATIVE LIVER WEIGHTS AND LENGTHS OF LARGE BOWEL

Group no.	Treatment (no. of mice)	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100 g body wt.)	Length of large bowel (cm)
$Apc^{Min/+}$	1% DSS (12)	20.4 ± 4.4[1]	1.01 ± 0.24	4.95 ± 0.52	8.25 ± 0.42
	1% DSS + 50 ppm ONO-1714 (10)	20.8 ± 4.2	0.88 ± 0.27	4.27 ± 1.09	8.79 ± 0.63[2]
	1% DSS + 100 ppm ONO-1714 (10)	19.2 ± 3.1	0.84 ± 0.18	4.36 ± 0.58	9.22 ± 0.34[3]
$Apc^{+/+}$	1% DSS (5)	26.8 ± 2.1	1.19 ± 0.36	4.48 ± 1.45	10.06 ± 0.29[3]
	1% DSS + 50 ppm ONO-1714 (5)	24.0 ± 2.1	1.09 ± 0.30	4.49 ± 1.01	10.25 ± 0.31
	1% DSS + 100 ppm ONO-1714 (5)	23.5 ± 2.1	1.19 ± 0.25	5.09 ± 1.15	10.16 ± 0.46

[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 $^3p < 0.001$).

perimental and control groups. All animals were housed in plastic cages (4 or 5 mice/cage) with free access to drinking water and a powdered basal diet AIN-76A (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity [(50 ± 10)%], light (12/12 hr light/dark cycle) and temperature [(23 ± 2)°C]. DSS with a molecular weight of 36,000–50,000 (Cat No. 160110) was purchased from MP Biochemicals, LLC (Aurora, OH, USA). DSS was dissolved in tap water at a concentration of 2% (w/v). ONO-1714 was chemically synthesized at Ono Pharmaceutical (Osaka, Japan). The experimental diets were prepared by mixing ONO-1714 (50 and 100 ppm) with modified AIN-76A diet every week.

Experimental procedures

Forty-seven male $Apc^{Min/+}$ and 30 male $Apc^{+/+}$ mice were divided into experimental and control groups as shown in the Tables. The animals of the experimental groups were administered 1% (w/v) DSS in drinking water for 1 week from 6 weeks of age. They were also given a basal diet (modified AIN-76A) or a diet containing ONO-1714 at a dose of 50 or 100 ppm for 5 weeks from 6 weeks of age. The doses were determined based on the results of previous studies.[16,18] Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. The body weights and food consumption were measured weekly. At sacrifice (week 5), the animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. They were starved overnight prior to blood collection. Clinical chemistry measured included triglyceride (TG), total cholesterol (T-Cho), glucose (Glu), total protein (TP), albumin (Alb), albumin:globlin ratio (A/G), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatinine and blood urea nitrogen (BUN). To assess relative quantification of gene expression in colonic mucosa, samples of colonic mucosa of mice ($n = 5$ per group) were washed with phosphate buffer saline, quickly frozen in liquid nitrogen, and stored at −80°C. The intestinal tract of the remaining mice was removed, filled with 10% phosphate-buffed formalin, and then divided into 4 sections; the colon and 3 segments of small intestine; the proximal (~4 cm in length from the pylorus ring of stomach), and the middle and distal halves of the remainder. These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% phosphate-buffered formalin. Polyp numbers and sizes, and their distributions in the small intestine were determined under a dissecting microscope Nikon SMZ1000 (Nikon, Tokyo, Japan), as described.[19] A histological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. Intestinal neoplasms were diagnosed according to the description by Ward.[20]

Scoring of inflammation in the intestinal mucosa

Mucosal inflammation with or without ulceration in the entire intestine was analyzed on H&E-stained sections. Small and large intestinal inflammation was graded according to the following morphological criteria described by Cooper *et al.*[21]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium and Grade 4, presence of mucosal ulcer with severe inflammation (neutrophil, lymphocyte and plasma cell infiltration) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made based on the entire intestine with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry

Immunohistochemistry for COX-2 and iNOS was performed on 4-μm-thick paraffin-embedded sections from colons of $Apc^{Min/+}$ mice in each group as previously described.[3,8] As primary antibodies, anti-COX-2 mouse monoclonal antibody (1:200 dilution, Transduction Laboratories, Lexington, KY) and anti-iNOS mouse monoclonal antibody (1:250 dilution, Transduction Laboratories) were used. To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. In the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections. In the control sections, incubation with the primary antibodies was omitted.

RNA extraction and synthesis of cDNA

The extraction of total RNA from frozen colonic mucosa was done using the RNeasy® Mini Kit (Qiagen, CA) following the manufacturer's directions. The concentration of total RNA was measured with a spectrophotometer and the A_{260}/A_{280} ratio of RNA was 1.8–2.0. cDNA was synthesized by oligo (dT)20 primer and SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's protocol, and then it was stored at −30°C until analyzed.

Relative quantification of gene expression by LightCycler RT-PCR

All real-time experiments were carried out with a LightCycler® FastStart DNA Master HybProbe kit (Roche, Mannheim, Germany). Oligonucleotide primers and hybridization probes are purchased from Nihon Gene Research Lab's (Sendai, Japan). PCR was performed with specific primers for mouse cyclooxygenase (COX)-2 (forward primer; 5′-CCATCTGTTCTCCTCAATAC-3′, reverse primer; 5′-TTTGGTAGGCTG TGGAT-3′), iNOS (forward primer; 5′-GCAAACCCAAGGTCTACGTT-3′, reverse primer; 5′-GGAAAAGACTGCACCGAAGA-3′), interleukin (IL)-1β (forward primer; 5′-CTGTGGCAGCTACCTGTGTC-3′, reverse primer; 5′-GTTCATCTCGGAGCC TGTAG-3′), tumor necrosis factor (TNF)-α (forward primer; 5′-CCACGTCGTAGC AAACCAC-3′, reverse primer; 5′-TGGGTGAGGAGCACGTAGT-3′) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (forward primer; 5′-TGAACGGGAAGCTCACTGG-3′, reverse primer; 5′-TCCACCACCCTGTTG CTGTA-3′). PCR reactions contained 15 μl of master mix and 5 μl of template cDNA. The final reaction mix-

ture contained: for GAPDH, iNOS, IL-1β and TNF-α, 3 mM $MgCl_2$, 0.5 μM of the primers (Forward and Reverse), 0.2 μM of the Fluorescein probe, 0.4 μM of the and LCRed probe and 1X LightCycler (FastStart DNA Master HybProbe); and for cyclooxygenase (COX)-2, 3 mM $MgCl_2$, 0.2 μM of the primers (Forward and Reverse), 0.2 μM of the probes (Fluorescein and LCRed) and 1X LightCycler® FastStart DNA Master HybProbe based on the manufacturer's recommendations. The contents were placed in a glass capillary, capped, briefly centrifuged and placed in the LightCycler. The denaturing condition for all genes was 1 cycle at 95°C for 10 min. The amplification conditions were as follows: GAPDH, 40 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 9 sec; iNOS, 40 cycles of 95°C for 10 sec, 62°C for 15 sec and 72°C for 9 sec; IL-1β and TNF-α, 40 cycles of 95°C for 10 sec, 61°C for 15 sec and 72°C for 7 sec; and COX-2, 45 cycles of 95°C for 10 sec, 62°C for 15 sec and 72°C for 7 sec. The amplification protocol was followed by a cooling period of 1 cycle at 40°C for 30 sec. All data collection were performed during extension and the data were monitored through the F2/1 channel of the instrument. Data analyses were carried out using the second derivative maximum method of the LightCycler software program.

Statistical analysis

All measurements were compared by Tukey's multiple comparison post test, Bonferroni's multiple comparison post test or Fisher's exact probability test. Differences were considered to be statistically significant at $p < 0.05$.

Results

General observation

During the study, animals tolerated the oral administration of DSS and ONO-1714, and no clinical signs of toxicity were present in any groups. The intake of DSS and food consumption (g/day/mice) did not significantly differ among the groups (data not shown). The body weights, liver weights, relative liver weights and lengths of large bowel at the end of the study are given in Table I. The mean body weights, liver weights and relative liver weights did not significantly differ among the groups. The mean length of large bowel in the groups of $Apc^{Min/+}$ mice treated with ONO-1714 at 50 and 100 ppm doses were significantly longer than that of the DSS alone group ($p < 0.05$ and $p < 0.01$, respectively). Histologically, there were no pathological alterations suggesting the toxicity of ONO-1714 in the liver, kidneys, lung and heart in mice.

Pathological findings

Macroscopically, nodular and polypoid colonic tumors were observed in the cecum and colon of $Apc^{Min/+}$ mice that received DSS, but not in the $Apc^{+/+}$ mice. Histopathologically, colonic proliferative lesions, including dysplastic crypts, adenomas and adenocarcinomas were found in the cecum and colon of $Apc^{Min/+}$ mice treated with DSS. The incidences and multiplicities of large bowel neoplasma are shown in Table II. The incidences of large bowel tumors in the $Apc^{Min/+}$ mice did not significantly differ among the DSS alone, DSS + 50 ppm ONO-1714 and DSS + 100 ppm ONO-1714 groups. However, the multiplicities of large bowel tumors in the groups of $Apc^{Min/+}$ mice that received DSS and ONO-1714 at dose levels of 50 and 100 ppm were significantly lower than that of the DSS alone groups ($p < 0.05$ and $p < 0.05$, respectively). Although the multiplicities of adenomas did not significantly differ among the groups, the multiplicities of the adenocarcinomas significantly decreased by feeding with ONO-1714 to 49% (50 ppm, $p < 0.001$) and 58% (100 ppm, $p < 0.001$) of the value of DSS alone group. The number of small intestinal polyps (adenomas) of the DSS and ONO-1714-treated groups were lower than that of the DSS alone group, but the differences among the groups were not statistically significant (Table III).

TABLE II - EFFECT OF ONO-1714 ON THE DEVELOPMENT OF LARGE BOWEL TUMORS IN THE I $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Geno-type	Treatment (no. of mice)	Colon			Cecum			Large bowel (total)		
		AD[1] (incidence)	ADC[2] (incidence)	Total (incidence)	AD (incidence)	ADC (incidence)	Total (incidence)	AD (incidence)	ADC (incidence)	Total(incidence)
$Apc^{Min/+}$	1% DSS (12)	3.25 ± 2.30[3] (100%)	4.58 ± 2.43 (100%)	7.83 ± 4.32 (100%)	5.00 ± 2.80 (100%)	11.50 ± 5.60 (100%)	16.50 ± 6.97 (100%)	8.25 ± 3.65 (100%)	16.08 ± 5.76 (100%)	24.33 ± 8.68 (100%)
	1% DSS + 50 ppm ONO-1714 (10)	3.10 ± 1.52 (100%)	1.40 ± 0.84[4] (90%)	4.50 ± 1.96 (100%)	5.00 ± 1.83 (100%)	6.80 ± 2.57[5] (100%)	11.80 ± 3.29 (100%)	8.10 ± 2.77 (100%)	8.20 ± 3.12[3] (100%)	16.30 ± 4.19[5] (100%)
	1% DSS + 100 ppm ONO-1714 (10)	3.70 ± 1.42 (90%)	1.40 ± 0.97[4] (100%)	5.10 ± 1.97 (100%)	5.70 ± 2.16 (100%)	5.30 ± 2.06[5] (100%)	11.00 ± 3.83 (100%)	9.40 ± 2.76 (100%)	6.70 ± 2.67[3] (100%)	16.10 ± 5.02[5] (100%)
$Apc^{+/+}$	1% DSS (5)	0	0	0	0	0	0	0	0	0
	1% DSS DSS + 50 ppm ONO-1714 (5)	0	0	0	0	0	0	0	0	0
	1% DSS DSS + 50 ppm ONO-1714 (5)	0	0	0	0	0	0	0	0	0

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

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 ([2]$p < 0.05$ and [3]$p < 0.01$).

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 $Apc^{min/+}$

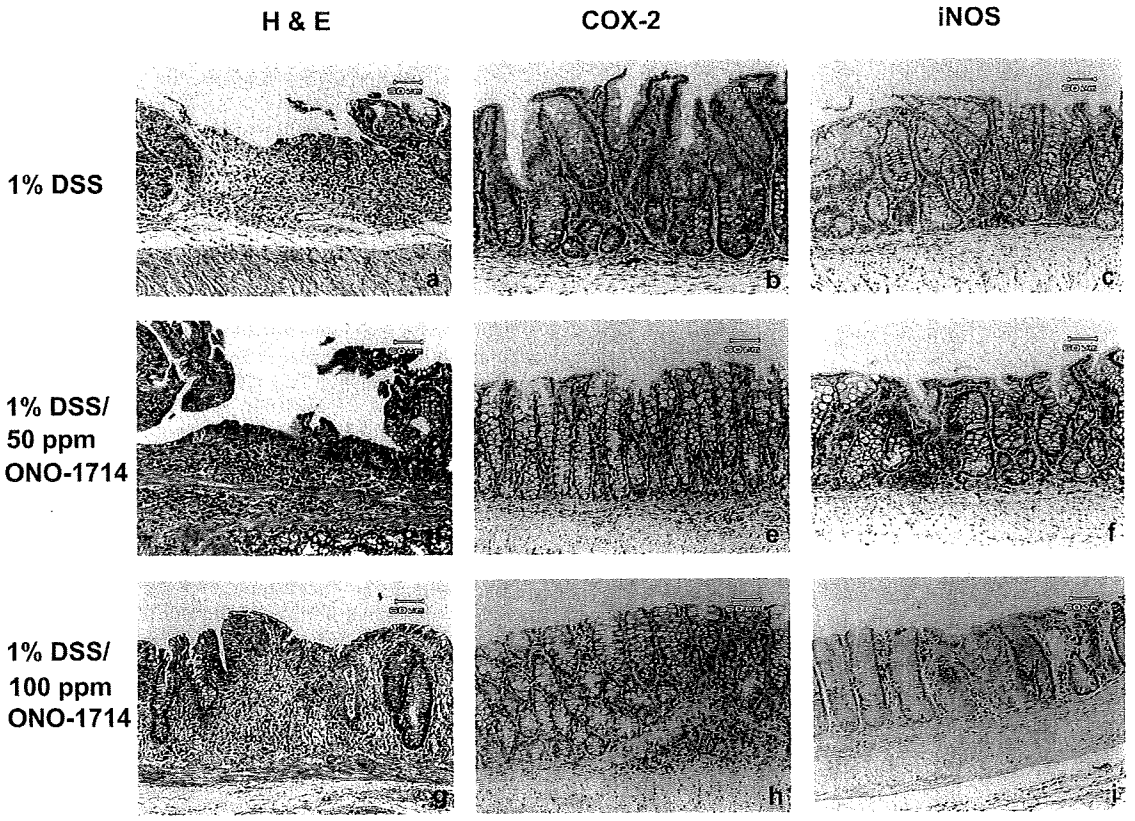

FIGURE 1 – Histopathology and immunohistochemistry of COX-2 and iNOS of colonic mucosa of $Apc^{Min/+}$ mice treated with 1% DSS or 1% DSS/ONO-1714. Histopathological observation revealed mucosal inflammation scored as Grade 3 in 1% DSS group (*a*), 1% DSS/50 ppm ONO-1714 group (*d*) and 1% DSS/100 ppm 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 ([2]$p < 0.001$).–[3–5]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Bonferroni's multiple comparison post test ([3]$p < 0.001$, [4]$p < 0.01$ and [5]$p < 0.05$).

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