

Role of the Prostaglandin E Receptor Subtype EP₁ in Colon Carcinogenesis¹

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Abstract

Although the cyclooxygenase pathway of the arachidonic acid cascade has been suggested to play an important role in colon carcinogenesis, the molecular species of prostanoids and receptors involved have not been fully elucidated yet. We examined the development of aberrant crypt foci (ACFs), putative preneoplastic lesions of the colon, in two lines of knockout mice, each deficient in prostaglandin E receptors, EP₁ and EP₃, by treatment with the colon carcinogen, azoxymethane. Formation of ACFs was decreased only in the EP₁-knockout mice to ~60% of the level in wild-type mice. Administration of 250, 500, or 1000 ppm of a novel selective EP₁ antagonist, ONO-8711, in the diet to azoxymethane-treated C57BL/6J mice also resulted in a dose-dependent reduction of ACF formation. Moreover, when Min mice, having a nonsense mutation in the *adenomatous polyposis coli* gene, were given 500 ppm ONO-8711 in the diet, the number of intestinal polyps was significantly reduced to 57% of that in the basal diet group. These results strongly suggest that prostaglandin E₂ contributes to colon carcinogenesis to some extent through its action at the EP₁ receptor. Thus, EP₁ antagonists may be good candidates as chemopreventive agents for colon cancer.

Introduction

Colon cancer is one of the most common malignancies in humans. Epidemiological studies have revealed a significant decrease in the death rates from colon cancer in individuals who have taken aspirin, a NSAID,³ for prolonged periods (1). Various NSAIDs also inhibit chemically induced colon carcinogenesis in rodents (2, 3), and the NSAID sulindac reduced the number and size of intestinal polyps in patients with familial adenomatous polyposis (4, 5). The common mechanism of action of NSAIDs is the inhibition of COX, which catalyzes the synthesis of prostanoids such as PGs and TXs.

Two isoforms of COX, referred to as COX-1 and COX-2, have been identified. COX-1 is expressed constitutively and participates in various physiological functions, whereas COX-2 is inducible and contributes to pathological processes such as inflammation and abnormal cell proliferation (6). The abundance of COX-2 is increased in colon carcinomas of humans and rodents (7, 8), and the number and size of intestinal polyps in the offspring of crosses between COX-2 gene knockout mice and *Apc* knockout mice are markedly decreased

relative to those apparent in the parental animals (9). These observations suggest that COX-2 and, by inference, the prostanoids formed by the action of this isozyme play an important role in colon carcinogenesis. However, whether prostanoids actually contribute to this process and, if so, the identity of the specific prostanoid responsible remain unknown.

The prostanoids PGD₂, PGE₂, PGF_{2α}, PGI₂, and TXA₂ exert their biological actions through binding to specific receptors with seven transmembrane domains. These receptors include DP for PGD₂, FP for PGF_{2α}, IP for PGI₂, TP for TXA₂, and the four subtypes EP₁ to EP₄ for PGE₂ (10, 11). The recent development of mice lacking the genes encoding these receptors (12–15) facilitated the clarification of the types of prostanoid ligands and receptors involved in the development of colon cancer.

Several reports have demonstrated increased levels of PGE₂ in human colon cancer tissue compared with surrounding normal mucosa (16). Moreover, it has been suggested that PGE₂ inhibits programmed cell death and enhances the tumorigenic potential of colonic epithelial cells (17). Among four subtype receptors, EP₁ to EP₄ for PGE₂, it was only possible to use EP₁- and EP₃-knockout mice for the experiments because the numbers of EP₂- and EP₄-knockout mice available are very limited because of failure of fertilization or death in the neonatal period (15, 18). In the present study, we therefore examined the development of ACFs, putative preneoplastic lesions of the colon (19), in two lines of mice lacking EP₁ or EP₃ receptors for PGE₂ (14). The results indicated a requirement for the EP₁ receptor in ACF induction by the colon carcinogen, AOM. To confirm these results, a newly developed selective EP₁ antagonist, ONO-8711, was tested for chemopreventive effects on development of AOM-induced ACF in mice and of intestinal polyps in Min mice containing a nonsense mutation of the *Apc* gene.

Materials and Methods

Animals. Male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and male Min mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. The mouse genes encoding EP₁ or EP₃ receptors were disrupted by gene knockout methods using homologous recombination, as reported previously (14). The generated chimeric mice were back-crossed with C57BL/6 mice, and the resulting wild-type and homozygous mutant male mice of these F₂ progeny were used at 7 (EP₁) and 12 (EP₃) weeks of age. Genotypes of these knockout mice were confirmed by PCR according to the method described previously (15). The animals were housed in plastic cages at 24 ± 2°C and 55% humidity with a 12-h light-dark cycle. Water and basal diet (AIN-76A; Bio-Serv, Frenchtown, NJ) or experimental diets prepared once every 2 weeks were given *ad libitum*. Body weights were measured weekly.

AOM-induced ACF Development in Prostanoid Receptor-Knockout Mice. EP₁- and EP₃-deficient homozygous mice (EP₁^{-/-} and EP₃^{-/-}) and wild-type mice received AOM (Sigma Chemical Co., St. Louis, MO) at a dose of 10 mg/kg body weight i.p. once a week for 3 weeks. The numbers of knockout mice treated with AOM were 9 for EP₁ and 10 for EP₃, and those of

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³ The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; TX, thromboxane; ACF, aberrant crypt focus; AOM, azoxymethane; *Apc*, *adenomatous polyposis coli*; AC, aberrant crypt.

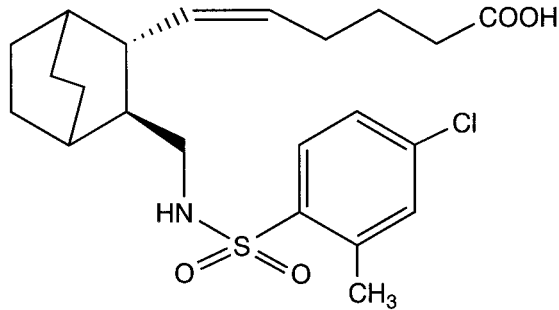


Fig. 1. Structure of ONO-8711.

 Table 1 Effect of EP₁ receptor deficiency on AOM-induced ACF formation in the mouse colon

Mice	Mice with ACFs	ACFs/colon ^a	Mean of ACs/focus ^a
Wild type	10/10	11.8 ± 1.3	1.7 ± 0.1
EP ₁ ^{-/-}	9/9	7.6 ± 1.1 ^b	1.6 ± 0.1

^a Data are means ± SE.

^b *P* < 0.05 versus wild type.

wild-type mice in each experiment were 10 and 7, respectively. All mice were sacrificed at 5 weeks after the first dosing of AOM. After laparotomy, the entire colon was resected and filled with 10% neutral buffered formalin and then opened longitudinally from the anus to the cecum. Each colon was then fixed flat between sheets of filter paper in 10% neutral buffered formalin, stained with 0.2% methylene blue in saline, and scored under a light microscope for the number of ACFs per colon and mean number of ACs per focus, according to the procedure of Bird (19).

A Selective EP₁ Antagonist, ONO-8711. A selective EP₁ antagonist, ONO-8711 {6-[(2*S*,3*S*)-3-(4-chloro-2-methylphenylsulfonylamino)methyl]-bicyclo[2.2.2]octan-2-yl]-5*Z*-hexenoic acid} was chemically synthesized at Ono Pharmaceutical Co., Ltd. The structure of ONO-8711 is shown in Fig. 1. The *K_i* values of this compound in Chinese Hamster Ovary cell lines, stably expressing each type of the prostanoid receptor (20), were 1.7 and 0.6 nM for mouse and human EP₁ receptors, respectively, and 67 nM for mouse EP₃ receptor and 76 nM for human TP receptor. Its *K_i* values for the other receptors including mouse DP, mouse EP₂, mouse EP₄, mouse FP, and human IP receptors were >1000 nM. Analysis of its agonistic and antagonistic actions showed that this compound acted as a competitive antagonist at EP₁ receptors; it inhibited the PGE₂-induced increase in cytosolic Ca²⁺ concentration with median inhibitory concentrations of 0.21 and 0.05 μM for the mouse and human receptors, respectively. ONO-8711 was stable for at least 8 weeks at room temperature when added to the basal diet. The chemical synthesis and detailed biological activities of ONO-8711 will be described elsewhere.

Effects of ONO-8711 on AOM-induced ACF Development in C57BL/6J Mice and Intestinal Polyps in Min Mice. C57BL/6J male mice, 6 weeks of age, received i.p. injections of AOM or the vehicle (saline), as described above for the experiments for prostanoid receptor-knockout mice. The mice in the EP₁ antagonist-treated group were fed diets containing 250, 500, and 1000 ppm ONO-8711 starting the day before the first treatment of AOM until the

end of the experiment at week 5. In addition, diets containing the antagonist were given to mice from 2 days after the last treatment of AOM to the day of sacrifice at week 5 (post-AOM treatment). ACF in the colon of mice were assessed as described above.

Min mice were fed a diet containing 500 ppm ONO-8711 or the basal diet from 6 weeks of age throughout the experiment for 7 weeks. All animals were sacrificed at 13 weeks of age. After laparotomy, the entire intestinal tract was resected, filled with 10% neutral buffered formalin, and divided into four sections: the colon and three segments of small intestine. The small intestine was divided into the duodenum (~4 cm in length; proximal) and the proximal (middle) and distal halves of the remainder (distal). These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% neutral buffered formalin. The numbers and sizes of polyps as well as their distribution in the intestine were determined with a stereoscopic microscope.

Statistical Analysis. Statistical analysis of the data on ACF and polyp formation was performed with Student's *t* test. The results were considered statistically significant at *P* < 0.05.

Results

ACF Development in EP₁- and EP₃-Knockout Mice. ACFs were detected in the colons of all mice treated with AOM but not in the colons of animals treated with vehicle (saline). The ACFs were located mostly in the distal colon, with fewer present in the middle colon and rectum. The data on ACFs for EP₁^{-/-} mice and the wild-type mice are shown in Table 1. The number of ACFs per colon in EP₁^{-/-} mice was reduced significantly, by ~40%, relative to that for wild-type animals. The mean number of ACs per focus in EP₁^{-/-} mice did not differ from that in the wild-type mice. In contrast, there were no differences in the number of ACFs per colon and the mean number of ACs per focus between EP₃^{-/-} mice and their wild-type counterparts (data not shown). The mean body weights of the AOM-treated EP₁^{-/-} and EP₃^{-/-} mice remained virtually identical to those of the AOM-treated wild-type animals 5 weeks after the first AOM injection. No abnormal signs were observed in the treated animals during the course of the experiment, and no difference in organ (liver, kidneys, or spleen) weights was apparent among the groups.

Effect of an EP₁ Antagonist on AOM-induced ACF Development in C57BL/6J Mice. Administration of the diets containing 250, 500, and 1000 ppm of a selective EP₁ antagonist, ONO-8711, to AOM-treated mice throughout the 5-week experimental period reduced the number of ACFs per colon in a dose-dependent manner (Table 2). In contrast, the mean number of ACs per focus in AOM-treated mice was not affected by administration of ONO-8711. Administration of ONO-8711 after AOM treatment also reduced the number of ACFs per colon to almost the same extent as that apparent in animals who received the antagonist throughout the experimental period (Table 2). No ACFs were detected in five vehicle-injected mice fed a basal diet or in three vehicle-injected mice fed with the diet containing 1000 ppm of ONO-8711 throughout the experiment. Administration of the diets containing 250, 500, or 1000 ppm of ONO-

Table 2 Inhibition of AOM-induced ACF development in the colons of C57BL/6J mice by ONO-8711

Treatment	Intake of ONO-8711 (mg/kg/day)	Mice with ACFs	ACFs/colon ^a	Mean of ACs/focus ^a
Basal diet		10/10	16.3 ± 1.2	1.4 ± 0.1
Mice fed ONO-8711 during and post-AOM				
ONO-8711 (250 ppm)	37	10/10	12.7 ± 1.0 (22) ^{b,c}	1.4 ± 0.1
ONO-8711 (500 ppm)	71	10/10	11.3 ± 1.4 (31) ^c	1.3 ± 0.1
ONO-8711 (1000 ppm)	141	10/10	10.6 ± 1.2 (35) ^d	1.3 ± 0.0
Mice fed ONO-8711 post-AOM				
ONO-8711 (250 ppm)	35	10/10	13.4 ± 1.7	1.4 ± 0.1
ONO-8711 (500 ppm)	72	10/10	12.5 ± 1.7	1.3 ± 0.1
ONO-8711 (1000 ppm)	137	10/10	11.3 ± 0.9 (31) ^d	1.2 ± 0.0

^a Data are means ± SE.

^b Numbers in parentheses indicate percentage of inhibition compared with the basal diet group.

^{c,d} Versus basal diet: ^c *P* < 0.05; ^d *P* < 0.01.

8711 did not affect body or organ weight in either the AOM- or vehicle-injected groups. Thus, our pharmacological approach confirmed a role for the EP₁ receptor in ACF development.

Effect of an EP₁ Antagonist on Intestinal Polyp Development in Min Mice. Most polyps were located in the small intestine, with only a few apparent in the colons of the basal diet and ONO-8711 groups. Administration of ONO-8711 at a dose of 500 ppm in the diet for 7 weeks reduced the total number of polyps to 56% of the value for the basal diet group; the number of polyps in the middle and distal parts of small intestine were reduced to 61 and 54%, respectively, of the basal diet values (Table 3). Furthermore, ONO-8711 reduced the number of polyps in each size group (Fig. 2). Histological analysis revealed no differences in polyps between mice fed the basal diet and those exposed to ONO-8711. Administration of ONO-8711 did not affect body weight, food intake, or behavior of Min mice.

Discussion

In the present study, examination of the effects of EP₁ and EP₃ receptors on AOM-induced ACF formation in mice provided evidence of an involvement of the PGE receptor subtype EP₁, but not EP₃, in colon carcinogenesis. The involvement of the EP₁ receptor in colon carcinogenesis was confirmed using a novel EP₁ antagonist, ONO-8711, in terms of ACF development. This antagonist was also effective in decreasing ACF formation, even when administered after AOM treatment. This indicates that the reduction of ACF formation by the antagonist was mediated not at the level of AOM metabolism but rather through postinitiation processes. Moreover, ONO-8711 clearly suppressed intestinal polyp formation in Min mice.

It has been reported that the number of AOM-induced ACFs per colon is reduced by 34–53%, relative to control values, by administration of traditional NSAIDs (sulindac and piroxicam) and COX-2-selective inhibitors (nimesulide and celecoxib) in rodents (21–23). The observed suppression potential is comparable to that of the EP₁ antagonist, ONO-8711, shown in the present study. Inhibition rates for intestinal polyp formation in Min mice or *Apc*^{Δ716} knockout mice with a traditional NSAID (sulindac) and the COX-2-selective inhibitors (nimesulide and MF-tricyclic) are reported to be 48–70% (9, 24, 25), again almost similar to that for ONO-8711. From the above observations, it is suggested that the EP₁ receptor plays some role in colon carcinogenesis. Regarding prostanoid receptors, eight lines of knockout mice have been developed. Among these, EP₁- and EP₃-knockout mice were used in the present study. In addition to the EP₂- and EP₄-knockout mice mentioned above, we were not able to study FP-knockout mice because of their limited availability as a result of delivery problems (12). Experiments with IP-, TP-, and DP-knockout mice will be carried out soon, but for the present, the question of whether EP₂, EP₄, FP, IP, TP, and DP receptors contribute to colon carcinogenesis remains open.

Inhibition of COX-1 by traditional NSAIDs such as indomethacin, sulindac, and piroxicam is accompanied by gastrointestinal side effects that limit the long-term application of these drugs as chemopre-

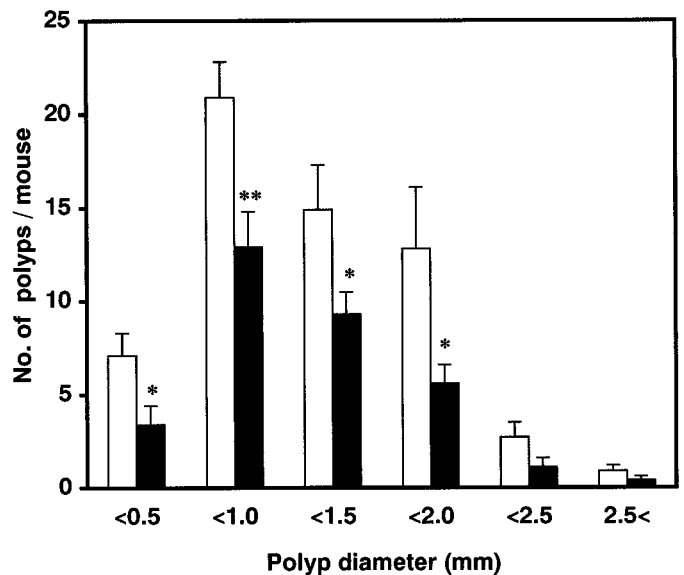


Fig. 2. Effect of ONO-8711 on the size distribution of intestinal polyps in Min mice. Mice were fed the basal diet (open columns) or a diet containing 500 ppm of ONO-8711 (filled columns) for 7 weeks. Polyps were grouped at intervals of 0.5 mm according to their diameters. The number of polyps per mouse in each group is presented as the mean; bars, SE. *, *P* < 0.05, versus basal diet group; **, *P* < 0.01 versus basal diet group.

ventive agents. Such adverse effects may be avoided by drugs that selectively target COX-2. Several types of prostanoids are produced as a result of COX activity in a variety of cells in response to various physiological or pathological stimuli (26). In light of the present results, selective EP₁-receptor antagonists may prove particularly beneficial as chemopreventive agents for colon cancer with toxicities even lower than those of COX-2-selective inhibitors.

In conclusion, the data obtained in the present study strongly suggest that PGE₂ mediates carcinogenic changes by acting at the EP₁ receptor in the colon. To confirm this involvement of EP₁, long-term colon carcinogenesis experiments with EP₁-knockout mice and the EP₁ antagonist are being conducted in our laboratory. Moreover, to extend our understanding, cross-breeding of EP₁ gene knockout and *Apc* gene knockout mice, and the expression of EP₁ receptor in the colon need to be examined.

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Table 3 Inhibition of intestinal polyp development by ONO-8711 in Min mice^a

Polyp location	No. of polyps per mouse ^b	
	Basal diet	ONO-8711
Proximal small intestine	2.3 ± 0.9	1.4 ± 0.3
Middle small intestine	13.2 ± 1.7	8.1 ± 1.4 ^c
Distal small intestine	42.9 ± 6.2	23.1 ± 2.9 ^d
Colon	0.9 ± 0.2	0.8 ± 0.2
Total	59.3 ± 7.3	33.4 ± 4.3 ^d

^a Mice were fed the basal diet or a diet containing 500 ppm of ONO-8711 for 7 weeks.

^b Data are means ± SE.

^{c,d} Versus basal diet group: ^c *P* < 0.05; ^d *P* < 0.01.

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