Combined effects of prostaglandin E receptor subtype EP_1 and subtype EP_4 antagonists on intestinal tumorigenesis in *adenomatous polyposis coli* gene knockout mice

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Previous studies have shown that prostaglandin E_2 (PGE_2) is involved in intestinal carcinogenesis through its binding to the PGE_2 receptor subtypes EP_1 and EP_4 and activation of downstream pathways. ONO-8711 and ONO-AE2-227, prostaglandin E receptor subtype EP_1- and EP_4-selective antagonists, respectively, are known to suppress formation of intestinal polyps in *adenomatous polyposis coli* gene-deficient mice. The present study was designed to investigate the combined effects of EP_1 and EP_4 antagonists on spontaneous polyp formation in *APC1309* mice in order to determine the contribution of each receptor to intestinal tumorigenesis. *APC1309* mice were treated with 400 ppm of ONO-8711 alone, 400 ppm of ONO-AE2-227 alone or both in combination in the diet for 6 weeks. The mean area of polyps found in the intestine, calculated as the longer diameter × the shorter diameter × π, was reduced by 12%, 43% ($P<0.01$) and 56% ($P<0.01$) of the mean control value (8.8 mm^2) in the ONO-8711 alone, ONO-AE2-227 alone and combination treatment groups, respectively, suggesting clear additive effects of the combination. The same additive tendency for suppression was also observed with respect to the numbers of polyps in the intestine. Polyp size reduction was more remarkable with the EP_4 antagonist, while the number reduction was more pronounced with the EP_1 antagonist. Our results indicate that EP_1 and EP_4 may have separate intrinsic roles and, to some extent, contribute to polyp formation independently. Thus, combination treatment has potential for the chemoprevention of colon carcinogenesis. (Cancer Sci 2003; 94: 618–621)

Cyclooxygenase (COX) is a rate-limiting enzyme in the synthesis of prostaglandins (PGs), which affect cell proliferation, tumor growth, apoptosis and immune responsiveness. Two COX enzyme isoforms, known as COX-1 and COX-2, have been identified. COX-1 is constitutively expressed while COX-2 is transiently inducible by various agents such as cytokines, growth factors, and hormones, then contributing to inflammation and abnormal cell proliferation.[1)] There is evidence to suggest that COX-2 is involved in carcinogenesis in various organs, including the colon.[2–5)] A possible contribution of COX-1 to colon carcinogenesis has also been reported. Genetic disruption of the *COX-1* gene, as well as the *COX-2* gene, decreased the number of intestinal polyps by around 80% in Min mice.[6)] It should be noted that prostaglandin E_2 (PGE_2) levels are elevated in intestinal polyps, compared with surrounding normal tissue, and both COX-1 and COX-2 contributed to PGE_2 production.[6)] Moreover, we recently demonstrated that mofezolac, a COX-1 selective inhibitor, suppressed the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACFs), putative preneoplastic lesions in F344 rats, and intestinal polyp development in mice with a truncated *adenomatous polyposis coli* (*Apc*) gene at codon 1309 (*APC1309* mice), an animal model of human familial adenomatous polyposis.[7)] These findings point to a significant role for PGE_2, produced by COX-1 and COX-2, in colon carcinogenesis.

Previous studies demonstrated that 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.[8, 9)] Genetic and pharmacological studies with specific inhibitors have suggested that EP_1[10)] and EP_4[11)] are important for carcinogenesis. For example, addition of 500 ppm ONO-8711 and 300 ppm ONO-AE2-227, targeting EP_1 and EP_4, respectively, to basal diet of Min mice reduced the number of polyps by 44% and 31%, respectively. In addition, the numbers of AOM-induced colonic ACFs were also found to be reduced in both EP_1[10)] and EP_4[11)] knockout mice.

We postulated that combination treatment with EP_1 and EP_4 antagonists would decrease the number of intestinal polyps more effectively than either agent alone, assuming independent contributions to polyp formation in *Apc* gene-deficient mice. To examine this possibility, the present study was designed to investigate the combined effects of EP_1 and EP_4 antagonists on spontaneous polyp formation in *APC1309* mice and to estimate the contribution of each receptor to intestinal tumorigenesis.

Materials and Methods

Animals and chemicals. Progeny of *APC1309* mice, produced by a gene knockout method and bred by artificial insemination, were genotyped by means of the allele-specific polymerase chain reaction using tail tips.[7, 12)] The mice, at 6 weeks of age, were randomized into experimental and control groups. The animals were housed two or three to a plastic cage in a holding room controlled at 24±2°C and 55% relative humidity with a 12/12-h light-dark cycle. Water and basal diet (AIN-76A, Dyets, Inc., Bethlehem, PA) or basal diet containing test chemicals were provided *ad libitum* during the experiments. The animals were weighed weekly throughout the experiment.

ONO-8711, {6-[(2*S*,3*S*)-3-(4-chloro-2-methylphenylsulfonylaminomethyl)-bicyclo[2.2.2]octan-2-yl]-5*Z*-hexenoic acid}, and ONO-AE2-227, 2-[2-{2-(1-naphthyl)propanoylamino}phenyl]methylbenzoic acid, were chemically synthesized at Ono Pharmaceutical Co. Their structures are shown in Fig. 1. The K_i

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values for ONO-8711 in Chinese hamster ovary cell lines, stably expressing each type of prostanoid receptor,[13] were 1.7, 0.6, 67 and 7.6 n*M* for mouse EP_1, human EP_1, mouse EP_3 and human TP receptors,[10] respectively, while those for the other receptors examined, including mouse DP, mouse EP_2, mouse EP_4, mouse FP and human IP receptors were higher than 1000 n*M*.[10] The K_i values for ONO-AE2-227 were 2.7 and 21 n*M* for mouse EP_4 and EP_3 receptors,[11] respectively, while those for the other receptors examined, including mouse EP_1, EP_2, DP, FP, IP and TP receptors were more than 1000 times higher than that for the mouse EP_4 receptor.[11] Experimental diets were prepared weekly by thoroughly mixing each compound with the basal diet. ONO-8711 and ONO-AE2-227 proved to be stable for at least 4 weeks at room temperature when added to the basal diet. The experimental protocol was according to the Guideline for Animal Experiments in the National Cancer Center.

Experimental protocol. *APC1309* heterozygous male mice, starting at 6 weeks of age, were fed a control diet or experimental diet containing 400 ppm of ONO-8711 and/or 400 ppm of ONO-AE2-227 throughout the experiment. For comparison, wild-type animals received the same treatments. The numbers of animals per group were 10 and 5 for the knockout and wild-type animals, respectively. After 6 weeks of treatment, complete necropsies were performed on all animals, and the liver, kidneys and spleen were weighed. The entire intestinal tract was resected, filled with 10% neutral buffered formalin, and divided into the small intestine, cecum and large 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 under ×5 magnification with a stereoscopic microscope, detectable polyps being more than 0.2 mm in diameter.[7] Paraffin sections of intestinal polyps were examined microscopically following H&E staining.

Statistical analysis. Statistical analysis was performed with Welch's *t* test vs. the basal diet group. Results were considered statistically significant with $P<0.05$.

Results

Body weights and food intake were not influenced by the treatment with EP_1- and/or EP_4-selective antagonists regardless of the genotype. No adverse effects of the treatments were apparent in terms of necropsy findings or organ weights.

Intestinal polyps were detected in all *APC1309* mice but in none of the wild-type animals. Histopathological analysis revealed no differences in the morphology of polyps, identified as adenomas, between *APC1309* mice fed the basal diet and those given test chemicals.

Fig. 2 shows the number of polyps for each longer diameter class. Administration of the EP_1-selective antagonist, ONO-8711 (400 ppm) alone, resulted in fewer polyps in almost all diameter classes, with a significant decrease in the 4.0- to 4.9-mm diameter class, compared with control values. The single treatment with the EP_4-selective antagonist, ONO-AE2-227 (400 ppm), decreased the numbers of polyps measuring more than 2.0 mm in diameter, but the number of those measuring less than 1.0 mm was significantly increased, compared with

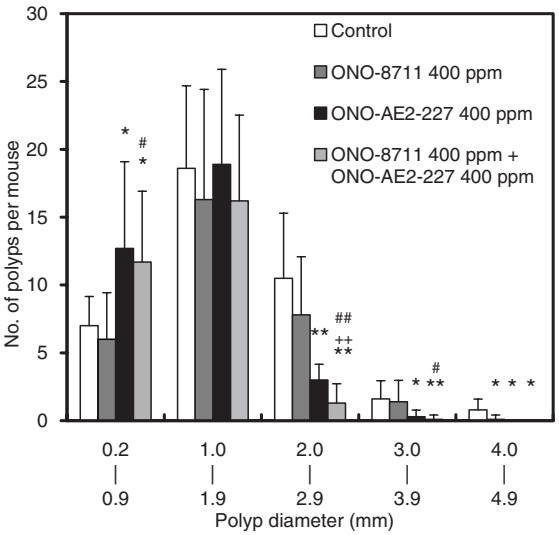

Fig. 2. Effects of ONO-8711 and ONO-AE2-227 on the size distributions of intestinal polyps in male *APC1309* mice. *APC1309* mice were fed basal diet or diet containing 400 ppm ONO-8711 alone, 400 ppm ONO-AE2-227 alone, or 400 ppm ONO-8711 plus 400 ppm ONO-AE2-227 for 6 weeks. Polyps were grouped at intervals of 0.5 mm according to the length of the longer diameter. The number of polyps per mouse in each size group is presented as the mean ±SD. ∗ $P<0.05$, ∗∗ $P<0.01$ vs. the basal diet group, # $P<0.05$, ## $P<0.01$ vs. the 400 ppm ONO-8711 alone group, and ++ $P<0.01$ vs. the 400 ppm ONO-AE2-227 alone group.

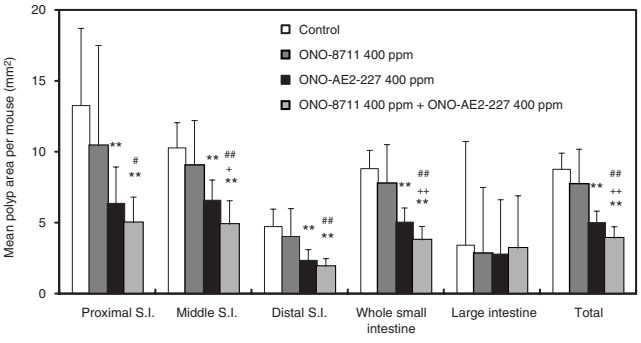

Fig. 3. Effects of ONO-8711 and ONO-AE2-227 on the mean areas of intestinal polyps in male *APC1309* mice. *APC1309* mice were fed basal diet or diet containing 400 ppm ONO-8711 alone, 400 ppm ONO-AE2-227 alone, or 400 ppm ONO-8711 plus 400 ppm ONO-AE2-227 for 6 weeks. The area of each polyp was calculated using the formula (longer diameter×shorter diameter×π). The mean polyp area was calculated for each animal, and then group means were analyzed statistically. The mean polyp area per mouse is presented as the mean±SD. ∗∗ $P<0.01$ vs. the basal diet group, # $P<0.05$, ## $P<0.01$ vs. the 400 ppm ONO-8711 alone group, and + $P<0.05$, ++ $P<0.01$ vs. the 400 ppm ONO-AE2-227 alone group. S.I. means small intestine.

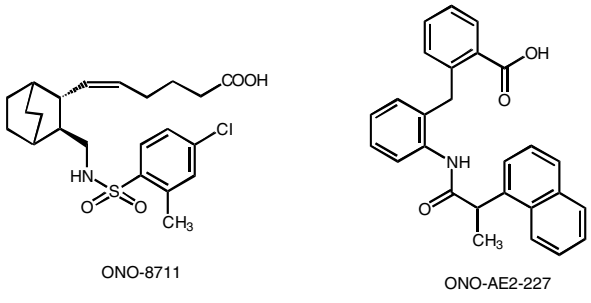

Fig. 1. The structures of ONO-8711, {6-[(2*S*,3*S*)-3-(4-chloro-2-methylphenylsulfonylaminomethyl)-bicyclo[2.2.2]octan-2-yl]-5*Z*-hexenoic acid}, and ONO-AE2-227, 2-[2-{2-(1-naphthyl)propanoylamino}phenyl]methylbenzoic acid.

control values. Thus, polyp size reduction was significant with ONO-AE2-227. A combination of ONO-8711 with ONO-AE2-227 decreased the numbers of polyps measuring more than 2.0 mm in diameter, compared with those in animals treated with each antagonist alone. The combination treatment also resulted in an increased number of polyps measuring less than 1.0 mm in diameter, and the effect was almost equal to that seen with ONO-AE2-227 alone.

In order to analyze the effects of combination treatment with EP_1 and EP_4 antagonists on polyp growth, the area of each polyp was calculated using the formula: area=(long diameter×short diameter×π). Fig. 3 shows values for the intestinal segment in each group. The mean polyp areas in the ONO-8711 alone, ONO-AE2-227 alone and combination treatment groups were 88%, 57% and 44% of the control value (mean, 8.8 mm^2), respectively. Suppressive effects were noted in proximal, middle and distal small intestinal segments, but not in the large intestine. The sum of percentage inhibition by ONO-8711 (12%) and ONO-AE2-227 (43%) alone was 55% and almost equal to that with the combination treatment (56%), suggesting a clear additive effect in the combination. The numbers of intestinal polyps in the ONO-8711 alone and ONO-AE2-227 alone groups were 82% and 91% of the control value (mean, 38.4 polyps per mouse), respectively. On the other hand, that in the combination treatment group was 76% of the control value, although the inter-group differences were not statistically significant. Similar suppressive patterns were observed in the proximal and middle small intestine segments, but not in the large intestine. The sum of percent inhibitions by ONO-8711 (18%) and ONO-AE2-227 (9%) alone was 27% and almost equal to that of combination treatment (24%), suggesting a tendency for an additive effect of the combination.

Discussion

In the present study, we investigated the effects of the combination of EP_1 and EP_4 antagonists on polyp formation in *APC1309* mice in order to estimate the degree of contribution of each receptor to intestinal tumorigenesis. The major finding was that the combination treatment with ONO-8711, an EP_1-selective antagonist, and ONO-AE2-227, an EP_4-selective antagonist, had an additive effect compared to treatment with each antagonist alone, with respect to the mean area and number of polyps, especially in the small intestine. Our results indicate that EP_1 and EP_4 may thus have separate roles, independently contributing to polyp formation. It is known that PGE_2 activates adenylate cyclase and then increases cAMP, via a stimulatory G protein, through binding to the EP_4 receptor.[14] It is reported that cAMP can inhibit or stimulate cell growth, depending on the cell type.[15] Cyclic AMP-dependent stimulation of cell proliferation has been suggested for a human prostate cancer cell line, PC-3 cells,[16] fibroblasts from mouse embryos, 3T3 cells,[17] and rat thyroid derived FRTL-5 cells.[18] In the PC-3 cells, induction of the immediate early gene c-*fos* by arachidonic acid is mediated by PGE_2, which activates the PKA pathway via the EP_2/EP_4 receptors.[16] However, there is no direct evidence suggesting that the cAMP-PKA system play a positive role in the growth of intestine epithelial cells in *APC1309* mice. The EP_1 receptor is a transmembrane G protein-coupled receptor, but its signal transduction mechanism is not known in detail. EP_1 signals are known to be transmitted by increased intracellular Ca^{2+} concentrations, with activation of protein kinase C (PKC).[8, 9] Further studies are needed to investigate events downstream of the EP_1 and EP_4 receptor signaling pathways, the cell types on which the receptors reside, and any links between EP_1 and EP_4 receptors in the intestine of *Apc* gene-deficient mice.

It should be noted that the suppressive effects of ONO-8711 and ONO-AE2-227 on the number of polyps in *APC1309* mice were relatively weak compared with those observed in Min mice.[10, 11] In the latter, total numbers of polyps in the basal diet groups were 59.3[10] and 61.4[11] per mouse, while in the present study, that in *APC1309* mice was 38.4 per mouse. When polyp size distributions in the two kinds of *Apc* gene knockout mice were compared, the proportion of polyps measuring more than 2.0 mm in diameter was higher in *APC1309* mice than that in Min mice (data not shown). From these observations, it is suggested that the growth of polyps is much faster in *APC1309* than in Min mice, while the increase of polyp numbers is more pronounced in the Min case. These differences may be related to the variation in responses to ONO-8711 and ONO-AE2-227. Differences in experimental design in *APC1309* and Min mice may also have affected the number of polyps in these *Apc* gene-deficient mice.

In two previous separate studies in Min mice, ONO-8711[10] and ONO-AE2-227[11] decreased the numbers of polyps to 56% and 69% of the control value, respectively, but only the latter antagonist had a major effect on the growth of polyps. The same was basically the case in the present study. Polyp size-reducing effects were thus clearly more evident with the EP_4 antagonist, while the number reduction was more pronounced with the EP_1 antagonist. Combination treatment with the two antagonists reduced both the number and size of polyps, as previously observed in the case of treatment with a COX-2-selective inhibitor, nimesulide, in *APC1309* and Min mice,[7, 19] suggesting possible differential roles of these two receptors in intestinal tumorigenesis and that simultaneous blocking of EP_1 and EP_4 may mimic the inhibitory effect of PGE_2 synthesis inhibitor.

The fact that the combination of the two antagonists did not completely suppress polyp development suggests the presence of other pathways involved in intestinal tumorigenesis. The exact roles of each of the four prostaglandin E receptor subtypes remain unknown. Previous studies revealed that homozygous deletion of the *EP_2* gene decreased the number and size of intestinal polyps in *$Apc^{\Delta 716}$* mice, another mutant strain with a truncated *Apc* gene.[20] Other reports of intestinal polyps in *Apc* gene knockout mice indicated that increased angiogenesis[21] and a reduced apoptosis:mitosis ratio[22] are important mechanisms regulating growth of polyps. Further genetic and pharmacological studies are necessary to determine the precise mechanisms involved in intestinal tumorigenesis. Several selective COX-2 inhibitors have already been developed with fewer gastrointestinal side effects or renal dysfunction on clinical application. However, COX inhibition inevitably results in reduced synthesis of PGs and therefore some undesirable drop in PG levels may cause adverse effects. Identification of the principal PGE receptor subtype(s) involved in tumorigenesis would allow development of an optimal drug with less adverse effects. The combination treatment with EP_1 and EP_4 antagonists may be an example of this approach.

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