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Suppression of azoxymethane-induced colon cancer development in rats by a cyclooxygenase-1 selective inhibitor, mofezolac

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We demonstrated recently that mofezolac, a cyclooxygenase-1 (COX-1) selective inhibitor, suppresses the development of azoxymethane (AOM)-induced colonic aberrant crypt foci in F344 rats and intestinal polyps in *APC1309* mice. In the present study, we therefore investigated the effects of mofezolac on colon cancer development. Male F344 rats were injected subcutaneously with 15 mg/kg body weight of AOM in the back twice at 7-day intervals from 5 weeks of age, and fed a diet containing 600 or 1200 ppm mofezolac for 32 weeks, starting 1 day before the first dosing of AOM. Treatment with 1200 ppm mofezolac significantly reduced the incidence, multiplicity and volume of colon carcinomas to 79%, 2.15 ± 1.65 and 7.5 ± 11.8 mm³, respectively, compared with 94%, 3.19 ± 1.87 and 23.7 ± 31.2 mm³ in the AOM treatment alone. Administration of 600 ppm mofezolac showed only a slight reduction. No side effects were observed in any of the groups. These results confirm that COX-1, as well as COX-2, contributes to colon carcinogenesis and that mofezolac may be a good chemopreventive agent for human colon cancer. (*Cancer Sci* 2006; 97: 1011–1014)

The multifunctional lipid mediator PGE_2 plays important roles in colon carcinogenesis and can be detected at higher levels in colon tumors than in surrounding normal tissue.[1] PGE_2-binding membrane receptors consist of four specific subtypes, EP_1, EP_2, EP_3 and EP_4, and genetic and/or pharmacological approaches have revealed that EP_1, EP_2 and EP_4 play enhancing roles, whereas EP_3 suppresses intestinal carcinogenesis.[2–5] Thus, there is evidence that blocking prostaglandin synthesis is an effective way to prevent colon carcinogenesis.

Prostanoids, including PGE_2, are produced through conversion of arachidonic acid by the rate-limiting enzyme COX. Two enzyme isoforms of COX are known, referred to as COX-1 and COX-2. COX-1 is expressed constitutively in most organs, whereas COX-2 is transiently inducible by stimuli such as cytokines, growth factors and hormones. It is worth mentioning that COX-2 is upregulated in colon tumors, including both parenchyma and stroma. Thus, inhibiting COX-2 rather than COX-1 activity is considered best for the prevention of colon carcinogenesis. In fact, many animal studies have shown that COX-2 selective inhibitors do have anticancer properties.[6,7]

However, experimental mouse studies using genetic disruption of the *COX-1* plus *Apc* genes or the *COX-2* plus *Apc* genes have revealed that both isoforms are involved in intestinal polyp formation.[8] We have demonstrated previously that a COX-1 selective inhibitor, mofezolac, suppresses the development of AOM-induced colonic ACF, putative preneoplastic lesions in F344 rats, as well as intestinal polyp development in *APC1309* mice with a truncated *Apc* gene.[9] Furthermore, inhibiting both COX-1 and COX-2 with conventional NSAID, such as indomethacin or sulindac, has been found to reduce the number of intestinal polyps in FAP patients more effectively than by COX-2 selective inhibitors such as celecoxib and nimesulide.[10–13]

From the above observations, it is suggested that inhibiting COX-1 activity should reduce the formation of colon neoplasia. The present study was designed to test this hypothesis using mofezolac. We here document that blocking prostaglandin synthesis by inhibiting COX-1 activity does indeed reduce the development of colonic adenocarcinomas in AOM-treated F344 rats.

Materials and Methods

Animals and chemicals. Male F344/Dμ Crj rats (at 4 weeks of age) were purchased from Charles River Japan (Atsugi, Japan) and 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. AOM was purchased from Sigma Chemical Co. (St Louis, MO, USA). The COX-1 selective inhibitor mofezolac, [3,4-di(4-methoxyphenyl)-5-isoxazolyl acetic acid] was synthesized chemically at Mitsubishi Pharma Co. (Yokohama, Japan) and was well mixed with powdered basal diet (AIN-76 A; Dyets, Bethlehem, PA, USA) at doses of 600 and 1200 ppm in the diet. Mofezolac (see Fig. 1 for the chemical structure) has been used clinically to control acute pain and inflammation from surgery, injury or odontectomy.[14] Mofezolac was confirmed to be stable under the experimental conditions used in the present study.

Animal experiments. After quarantine for 1 week the animals were randomized into five groups and injected subcutaneously with AOM at a dose of 15 mg/kg body weight in the back of rats twice at 7-day intervals from 5 weeks of age (groups 1–3). Animals in groups 4 and 5 were treated with saline without AOM in a manner similar to groups 1 and 3, respectively. The number of animals per group was 53 for group 1, 34 each for groups 2 and 3, and six each for groups 4 and 5. Starting 1 day before the first dose of AOM, rats were fed a basal diet (groups 1 and 4) or an experimental diet containing 600 ppm (group 2) or 1200 ppm (groups 3 and 5) of mofezolac for 32 weeks. The doses were selected in line with the previously reported suppression of AOM-induced ACF formation in rats.[9] Body weight and food consumption were measured weekly throughout the experiment. At 37 weeks of age, complete necropsies were carried out on all surviving animals, and the liver, kidneys and spleen were

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Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; APC, adenomatous polyposis coli; COX, cyclooxygenase; FAP, familial adenomatous polyposis; mPGES, microsomal PGE_2 synthase; NSAID, non-steroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; ppm, parts per million.

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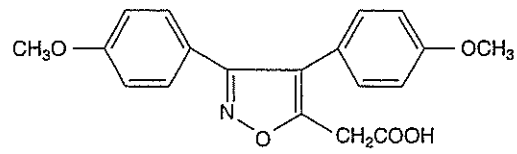

Fig. 1. The structure of mofezolac, [3,4-di(4-methoxyphenyl)-5-isoxazolyl] acetic acid.

weighed. The entire large intestine was resected, flushed with saline, opened longitudinally from the cecum to the anus, placed between two pieces of filter paper and fixed in 10% neutral buffered formalin. Location and sizes of all tumor lesions were recorded under a magnifying glass. Estimation of tumor volume was determined using the formula:

$$\text{volume} = \text{length} \times \text{width} \times \text{depth} \times \pi/6.$$

Paraffin sections of all macroscopic lesions were examined microscopically following routine processing and hematoxylin and eosin staining. In addition, for all animals in groups 1 and 3, the stomach, liver, kidney and spleen were examined microscopically in the same way. Diagnosis of intestinal tumors was carried out according to the classification of Pozharisski.[15] The experimental protocol was in accordance with the guidelines for Animal Experiments in the National Cancer Center.

Statistical analysis. Data are presented as mean ± SD values. Values for body weight, organ weight, tumor multiplicity and tumor volume were analyzed using Dunnett's multiple comparison test. Data for tumor incidence were analyzed using the Fisher's exact probability test. Results were considered to be statistically significant at $P < 0.05$ (two-tailed).

Results

During the experimental periods, one rat in the AOM-treated group was killed humanely because of decreased body weight with pale skin and two colon tumors were found. No significant difference between the groups was observed in body weight, food intake or organ weight. As tissue damage has been observed in the stomach and kidneys with NSAID treatment in humans,[10,11] the stomach and kidneys were here included for histopathological examination, but no adverse effects of mofezolac treatment were apparent. In addition, no significant microscopic changes were observed in the liver and spleen in mofezolac-treated rats.

Data for the incidence and multiplicity of colon tumors are shown in Table 1. Colon tumors were classified histopathologically into adenomas and adenocarcinomas. The incidence of colon adenocarcinomas in AOM-treated rats was 94, 88 and 79% ($P < 0.05$) in the 0, 600 and 1200 ppm mofezolac-treated groups, respectively (Table 1). The multiplicity was 3.19 ± 1.87, 2.71 ± 1.85 and 2.15 ± 1.65 ($P < 0.05$), respectively (Table 1). Thus, the incidence and multiplicity of colon adenocarcinomas in the 1200 ppm group were decreased, but values in the 600 ppm and control groups were not significantly different (Table 1). As shown in Table 2, most of the adenocarcinomas were well-differentiated, whereas 1–4% were moderately- to poorly-differentiated adenocarcinoma in the control and mofezolac-treated groups. Percentages of signet-ring cell adenocarcinomas and mucinous adenocarcinomas tended to be increased in the mofezolac-treated groups, but without statistical significance.

Data for volume of colon tumors are shown in Table 3. The mean volumes of adenocarcinomas in the 600 and 1200 ppm mofezolac-treated groups were decreased to 18.7 ± 27.8 mm^3 and 7.5 ± 11.8 mm^3 ($P < 0.05$), respectively, from 23.7 ± 31.2 mm^3 in the AOM control group (Table 3). The mean volumes of colon adenomas in the 600 and 1200 ppm mofezolac-treated groups were also lower than in the AOM control group, but not significantly (0.50 ± 1.56 mm^3 vs 0.31 ± 0.85 mm^3 and 0.22 ± 0.65 mm^3 in the 600 and 1200 ppm groups).

Azoxymethane treatment induces tumors mainly in the large intestine but several tumors were also observed in the small intestine, and mofezolac showed suppressive effects with respect to both incidence and multiplicity. The incidence of small intestinal adenocarcinoma was 32%, 9% ($P < 0.05$) and 0% ($P < 0.01$) in the 0, 600 and 1200 ppm mofezolac-treated groups, respectively. The multiplicity of adenocarcinomas in the 0 and 600 ppm groups was 0.32 ± 0.47 and 0.09 ± 0.29, respectively ($P < 0.05$). Well-differentiated adenocarcinomas were observed mainly in the control and 600 ppm mofezolac-treated groups. A few moderately-differentiated adenocarcinomas, signet-ring cell

Table 1. Effects of mofezolac treatment on the incidence and multiplicity of azoxymethane (AOM)-induced colon tumors in rats

Treatment	Effective no. animals	No. animals with tumors (%)		No. tumors per rat	
		Adenoma	Adenocarcinoma	Adenoma	Adenocarcinoma
AOM alone	53	22 (42)	50 (94)	0.62 ± 0.86	3.19 ± 1.87
AOM + Mofezolac, 600 ppm	34	17 (50)	30 (88)	0.65 ± 0.77	2.71 ± 1.85
AOM + Mofezolac, 1200 ppm	34	13 (38)	27 (79)†	0.53 ± 0.79	2.15 ± 1.65†
Saline alone	6	0	0	0	0
Mofezolac, 1200 p.p.m	6	0	0	0	0

†Significantly different from the control value at $P < 0.05$. Data are mean ± SD values.

Table 2. Effects of mofezolac on histological types of azoxymethane (AOM)-induced colon adenocarcinomas

Treatment	Effective no. animals	Total no. adenocarcinomas (%)	No. tumors per rat			
			Well-differentiated (%)	Moderately-differentiated (%)	Signet-ring cell (%)	Mucinous (%)
AOM alone	53	169 (100)	160 (95)	3 (2)	2 (1)	4 (2)
AOM + Mofezolac, 600 ppm	34	92 (100)	88 (96)	0 (0)	3 (3)	1 (1)
AOM + Mofezolac, 1200 ppm	34	73 (100)	67 (92)	0 (0)	3 (4)	3 (4)

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Table 3. Effects of mofezolac treatment on volumes of azoxymethane (AOM)-induced colon tumors in rats

Treatment	Effective no. animals	Tumor volume per rat (mm³)	
		Adenoma	Adenocarcinoma
AOM alone	53	0.50 ± 1.56	23.7 ± 31.2
AOM + Mofezolac, 600 ppm	34	0.31 ± 0.85	18.7 ± 27.8
AOM + Mofezolac, 1200 ppm	34	0.22 ± 0.65	7.5 ± 11.8†

†Significantly different from the control value at $P < 0.05$. Data are mean ± SD values.

adenocarcinomas and mucinous adenocarcinomas were also observed in the control and 600 ppm mofezolac-treated groups.

Discussion

In the present study, we provide clear evidence that a selective COX-1 inhibitor, mofezolac, suppresses AOM-induced colon tumor development, as assessed in terms of tumor incidence, multiplicity and volume in male F344 rats.

With ACF development in male F344 rats and intestinal polyp development in the *Apc*-deficient mice, administration of 200 ppm nimesulide, a COX-2 selective inhibitor, and 1200 ppm mofezolac showed a similar reduction to 52–65% of control values.[9,16] Moreover, administration of 200 or 400 ppm nimesulide reduced the incidence and multiplicity of AOM-induced colon tumors in male ICR mice.[17] However, there has been little evidence for inhibition of colon adenocarcinoma by use of COX-1 inhibitor. Thus, we used an AOM-treated rat model to investigate the effects of mofezolac on the development of colon adenocarcinomas in rats. Administration of 1200 ppm mofezolac reduced the incidence and multiplicity of the AOM-treated F344 rat colon carcinomas. Our data, obtained using a pharmacological approach, support the idea of COX-1, as well as COX-2, involvement in colon carcinogenesis, inferred on the basis of a genetic approach by Chulada *et al.*[8] Pathological observation revealed that AOM-treated F344 rats mainly developed well-differentiated adenocarcinoma, and mofezolac treatment effectively inhibited its development (Table 2). Meanwhile, mofezolac treatment did not decrease poorly-differentiated carcinomas, signet-ring cell and mucinous adenocarcinomas. In the present study, a very small number of poorly-differentiated carcinomas were developed in AOM-treated rats, such that the effect of mofezolac on poorly-differentiated carcinoma remained unclear and should be further confirmed using another animal model.

The relative contributions of COX-1 and/or COX-2 to cancer development in different organs appear complex. COX-2 is reported to be upregulated in many human tumors including breast, colon, prostrate, uterus, lung, head and neck cancers,[18–21] whereas overexpression of COX-1 has been found in human ovarian lesions, rat renal carcinomas, uterine cervical cancers, mouse lung tumors, human breast cancers and human prostate carcinomas.[18–20,22–24] Differences in the expression profiles of COX isotypes may reflect ratios of contribution to carcinogenesis. Enforced COX-1 overexpression in HeLa cells resulted in significant upregulation of cAMP-dependent PGE_2 receptors (EP_2 and EP_4), COX-2 and angiogenic factors.[22] Furthermore, analysis of the expression levels of COX-1, COX-2 and mPGES in *COX-1*$^{-/-}$ and *COX-2*$^{-/-}$ *Apc*$^{\Delta 716}$ double-knockout mice revealed that COX-1 is required from an early stage of intestinal polyp development, and that additional expression of COX-2 together with mPGES is necessary for subsequent accelerated growth of polyps.[25] In the present rat colon carcinogenesis model with AOM, high levels of COX-2 expression but not COX-1 have been reported in tumors.[26] However, it has been reported that COX-1 is the major source of PGE_2 in normal tissue and that both COX-1 and COX-2 contribute to PGE_2 production in intestinal polyp development in mice.[8] Thus, a possible mechanism for the chemopreventive action of mofezolac may be reducing PGE_2 production.

Gastrointestinal bleeding is a severe side effect of NSAIDs. However, long-term administration of the COX-1 selective inhibitor mofezolac did not induce any gastrointestinal side effects in rats under the conditions of the present study. Moreover, COX-1-deficient mice are reported to exhibit less indomethacin-induced gastric ulceration than wild-type mice.[27] Inhibiting both COX-1 and COX-2 by conventional NSAIDs is reported to be related to gastrointestinal bleeding. Mofezolac has long been applied as an anti-inflammatory drug without reports of severe side effects. However, serious adverse events such as myocardial infarction have been reported with long-term use of coxibs, the largest group of prescription drugs for selective COX-2 inhibition.[28] To establish more effective and safe chemoprevention for colon carcinogenesis, further investigation of mechanisms of action of COX-1 and COX-2 at the individual tissue level appear warranted before more clinical trials for cancer prevention are attempted.

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Suppressive effect of an inducible nitric oxide inhibitor, ONO-1714, on AOM-induced rat colon carcinogenesis

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Abstract

The expression of inducible nitric oxide synthase (iNOS) is markedly elevated in rat colon cancers induced by azoxymethane (AOM). In addition, iNOS can be detected in most adenomas and dysplastic aberrant crypt foci (ACF), suggesting that iNOS plays an important role in colon carcinogenesis. In the present study, the effect of an iNOS inhibitor, ONO-1714 ((1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride), on AOM-induced rat colon carcinogenesis was investigated. Male F344 rats were treated with 15 mg/kg body weight of AOM once a week, for 2 weeks. ONO-1714 was given to the rats at doses of 10, 20, 50, and 100 ppm in diet for 4 weeks from the day before the first carcinogen treatment. The number of AOM-induced ACF in the rats receiving 10, 20, 50 and 100 ppm ONO-1714 were 94, 73 ($P < 0.05$), 71 ($P < 0.005$), and 53% ($P < 0.0005$), respectively, of the control value. Moreover, the mean number of aberrant crypts per focus was significantly lowered in 100 ppm ONO-1714 group ($P < 0.05$). Then, the effects of long-term treatment (32 weeks) with 50 and 100 ppm ONO-1714 on AOM-induced colorectal tumor development were examined. Although incidences and multiplicities of colon tumors did not significantly differ among the groups, number of tumors developing in the middle part of colon were reduced with both 50 and 100 ppm doses ($P < 0.05$). Furthermore, colon tumor volume tended to be decreased by ONO-1714 treatment, and the number of colon tumors more than 3 mm in diameter was significantly lowered in the 100 ppm ONO-1714 group ($P < 0.01$). These results suggest that iNOS plays roles in both early and late stages of colon carcinogenesis.

Keywords: Colon cancer; iNOS inhibitor; Rat; Azoxymethane; ONO-1714

Chronic infection and inflammation release many cytokines [1] and activate nuclear factor κB (NF-κB) [2], resulting in the expression of NF-κB-regulated, inflammatory-related genes, such as inducible nitric oxide synthase (iNOS) [3]. Resultant overproduction of nitric oxide (NO) contributes to multistage carcinogenesis by inducing DNA mutations and tissue damage [4–6]. Increased expression of iNOS in human cancers, including examples in the colon, stomach, esophagus, lung, pancreas, and prostate, has been described [7–14]. The expression of inducible nitric oxide synthase (iNOS) is markedly elevated in rat colon cancers induced by azoxymethane (AOM) [15]. In addition, iNOS can be detected in most adenomas and dysplastic aberrant crypt foci (ACF), while it is hardly detectable in normal colon mucosa [16]. Thus these previous findings suggest that iNOS plays an important role in colon carcinogenesis.

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We have also shown that iNOS expression can be markedly elevated by transfection of K-*ras* mutant cDNA into IEC-6 rat intestinal epithelial cells in the presence of interleukin-1β (IL-1β) or lipopolysaccharide (LPS) [17] and growth of tumors formed in nude mice by s.c. injection of the K-*ras* mutant-transfected cells was suppressed by feeding diets containing NOS inhibitors, L-N^G-nitroarginine methyl ester (L-NAME) and ONO-1714 ((1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride) [17].

Since K-*ras* mutations are frequently observed in hyperplastic ACF and large tumors in AOM-treated rats [16], as well as in human lesions [18], K-*ras* activation might also contribute to enhancement of their cell proliferation. Indeed, it has been reported that K-*ras* mutations and/or activation increase expression of cyclin D1 and cyclooxygenase-2 (COX-2) in AOM-induced rat colon tumors [19], and K-*ras*-enhanced iNOS expression could conceivably also play a role.

Previously, we have shown that a NOS inhibitor, L-NAME, suppressed ACF formation in the colon of rats treated with AOM [20]. Suppressive effects of iNOS-selective inhibitors, *S*,*S*′-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea (PBIT) and L-N^6-(1-iminoethyl)lysine tetrazole-amide (SC-51), on ACF formation in the rat colon have also been reported [21,22]. However, there has hitherto been no report concerning effects on colon cancer development. It has been reported that ONO-1714 is 10-fold more selective for human iNOS than for human endothelial NOS, 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 [23,24]. In addition, ONO-1714 is effective even when orally administrated [17]. Therefore in the present study, we examined the influence of ONO-1714, an iNOS-selective inhibitor, on rat colon carcinogenesis induced by AOM, and noted suppressive effects on ACF formation, as well as tumor size.

Materials and methods

Chemicals

ONO-1714 was chemically synthesized at Ono Pharmaceutical (Osaka, Japan). AOM was synthesized at the Nard Institute (Amagasaki, Japan).

Animals

Male F344 rats, purchased from Charles River Japan (Atsugi, Japan) at 6 weeks of age, were used. They were housed in plastic cages in an air-conditioned room with a 12-h light-dark cycle and provided with diet (AIN-76A, Dyets Inc., Bethlehem, PA) and water ad libitum. Body weights and food intake were measured weekly.

Short-term experiment for ACF analysis

Forty five rats were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight, once a week for 2 weeks, and 9 animals each were given a basal diet or diet containing ONO-1714 at doses of 10, 20, 50 or 100 ppm from the day before the first carcinogen treatment until the end of the experiment. The doses were chosen from the results of our previous study in which 50 and 100 ppm ONO-1714 in diet suppressed growth of K-*ras* mutant-transfected rat IEC-6 cells in nude mice [17]. As negative controls, six rats were treated subcutaneously with saline only, and 3 animals each were given a basal diet or diet containing ONO-1714 at a dose of 100 ppm, respectively. Four weeks after the first carcinogen treatment, the rats were sacrificed and their colons were removed, fixed flat between sheets of filter paper in buffered 10% formalin, and stained with 0.2% methylene blue in saline, using the method of Bird [25]. The number of ACF per colon, the number of aberrant crypts (ACs) in each focus, and the location of each focus were determined by microscopy at a magnification of 40×. To categorize the distribution of ACF, we defined the rectum as the segment 2 cm proximal to the anus and divided the remaining colon into three segments of about 6 cm length, the distal colon, the middle colon, and the proximal colon, as described previously [26].

Long-term experiment for analysis of colon cancer

A total of 90 rats were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight once a week for 2 weeks, while 45 rats were similarly given injections of saline without any carcinogen as vehicle-controls. One third of each group was given basal diet or diet containing ONO-1714 at doses of 50 or 100 ppm from the day before the first carcinogen treatment until the end of the experiment. The animals were sacrificed 32 weeks after the first carcinogen treatment and the number, size, and location of all intestinal tumors more than 1 mm in diameter, detected without a microscope, were determined. The volume of the tumors was also assessed as previously described [27]. All tumor samples were fixed in 10% formalin–PBS and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination and tumors were classified according to established criteria [28,29]. The experimental protocol was according to the guidelines for Animal Experiments in the National Cancer Center.

Statistical analysis

Significant differences in the incidences of tumors as well as histological findings were analyzed by the χ^2 test. Group means were compared among the groups using

one-way ANOVA followed by Dunnett's test. A P value of less than 0.05 was regarded as significant. Dose-dependency on tumor volumes per rat was tested using coefficients for linear contrast.

Results

Suppression of ACF formation by ONO-1714 in short-term experiment

The final body weights (g) of the AOM alone, AOM + 10, 20, 50, and 100 ppm ONO-1714, saline alone and saline + 100 ppm ONO-1714 groups were 218.3 ± 2.0 (SE), 215.8 ± 2.0, 207.2 ± 2.3, 207.2 ± 2.2, 204.0 ± 2.8, 230.6 ± 4.7, and 211.6 ± 4.9, respectively. The average food consumption values (g/day/rat) for these groups were 11.8, 11.7, 11.1, 11.1, 11.0, 13.1, and 11.4, respectively. The food consumption did not differ among 20, 50, and 100 ppm ONO-1714 groups. The body weights and food consumption in the 100 ppm ONO-1714 groups were 6.5 and 7.0% lower in AOM-treated groups, and 8.2 and 13% lower in saline-treated groups, respectively, suggesting a link between the two and there were no apparent toxic effects observed during the experiments.

The data for ACF formation in the groups treated with AOM are summarized in Table 1. ACF were found in the colons of all animals treated with AOM. These ACF were mainly located in the distal and middle colon, a few in the rectum and very few in the proximal colon. With administration of 10, 20, 50, and 100 ppm of ONO-1714 to AOM-treated rats, the number of AOM-induced ACF per colon were decreased in a dose-dependent manner to 94, 73, 71, and 53%, respectively, of the control value, 237.4 ± 19.3 (SE). Total number of aberrant crypts (ACs) per colon, and number of ACF consisting of ⩾4 ACs per focus were also decreased by ONO-1714 treatment in a dose-dependent manner. Significant differences were observed for these parameters with 20, 50, and 100 ppm ONO-1714 treatment. Furthermore, treatment with 100 ppm ONO-1714 significantly decreased the mean number of ACs per focus ($P < 0.05$). No ACF were observed in the colons of the saline-injected groups given neither the basal diet nor 100 ppm ONO-1714.

Effects of ONO-1714 on colon tumor development in long-term experiment

The final body weights (g) of the AOM alone, AOM + 50, and 100 ppm ONO-1714, saline alone, and saline + 50, and 100 ppm ONO-1714 groups were 371.0 ± 3.2 (SE), 355.2 ± 4.6, 338.4 ± 3.3, 375.2 ± 7.7, 357.8 ± 5.9, and 354.3 ± 4.2, respectively. The body weights in the 100 ppm ONO-1714 groups with and without AOM were 8.8 and 5.6% lower than the respective control values, this being considered due to the lowered food consumption. During the experiment, one animal in the AOM + 50 ppm ONO-1714 group suffered accidental mortality at week 5 and was excluded from the tumor analysis. Two animals in the AOM group (at weeks 24 and 28), 2 in the AOM + 50 ppm ONO-1714 group (at weeks 28 and 31), and 2 in the AOM + 100 ppm ONO-1714 group (at weeks 28 and 30), which died of tumor development before the termination were, however, included.

The incidences and multiplicities of intestinal tumors (adenomas and carcinomas) at week 32 are summarized in Tables 2 and 3. Colorectal tumor incidences did not significantly differ among the AOM alone, AOM + 50 ppm ONO-1714, and AOM + 100 ppm ONO-1714 groups, being 92, 83, and 87%, respectively. Multiplicities of colorectal tumors were slightly lower in the groups treated with ONO-1714, but the values were not statistically different: being 3.07 ± 0.34 (SE) in the AOM alone group, 2.41 ± 0.36 in the AOM + 50 ppm ONO-1714, and 2.27 ± 0.32 in the AOM + 100 ppm ONO-1714 group. There was no statistically significant variation in the incidences and multiplicities of small intestine and cecum tumors.

Colorectal tumors were located mainly in the distal and middle colon in the AOM alone group, as shown in Fig. 1. Interestingly, number of tumors developing in the middle part of colon were significantly lowered in the 50 and 100 ppm ONO-1714 groups compared with the control ($P < 0.05$) and those in the proximal colon also tended to be decreased, while those in the distal colon and rectum were not altered.

Table 1
Suppression of AOM-induced ACF formation in rat colon by treatment with ONO-1714 for 4 weeks

Dose of ONO-1714 in diet (ppm)	Incidence of rats with ACF	No. of ACF/colon[a] (% of the control)	No. of ACs[a] (% of the control)	Mean no. of ACs/focus[a]	No. of ACF with ⩾ 4 ACs[a] (% of the control)
0	9/9	237.4 ± 19.3 (100)	515.2 ± 47.6 (100)	2.15 ± 0.05	25.2 ± 4.07 (100)
10	9/9	223.9 ± 16.1 (94.3)	458.7 ± 34.8 (89.0)	2.05 ± 0.04	18.0 ± 2.2 (71.4)
20	9/9	174.1 ± 14.9[b] (73.3)	360.4 ± 34.2[b] (70.0)	2.06 ± 0.03	14.9 ± 2.3[b] (59.0)
50	9/9	169.2 ± 7.4[c] (71.3)	342.2 ± 18.1[c] (66.4)	2.02 ± 0.05	10.8 ± 1.7[c] (42.7)
100	9/9	124.7 ± 12.9[d] (52.5)	251.4 ± 28.7[d] (48.8)	1.99 ± 0.05[b]	8.9 ± 1.8[c] (35.2)

[a] Data presented are mean ± SE values.
[b,c,d] Significantly different from the corresponding control values at $P < 0.05$, $P < 0.005$, and $P < 0.0005$, respectively.

Table 2
Effects of ONO-1714 treatment on the incidence of intestinal tumors induced by AOM at week 32

Dose of ONO-1714 in diet (ppm)	Effective no. of animals	No. of animals with tumors in each site (%)				
		Small intestine	Cecum	Colon and rectum		
				Total[a]	Adenoma	Carcinoma
0	30	5 (17)	2 (7)	27 (92)	9 (30)	27 (92)
50	29	8 (28)	4 (14)	24 (83)	7 (24)	24 (83)
100	30	4 (13)	3 (10)	26 (87)	3 (10)	26 (87)

[a] The total represents animals with adenomas and/or carcinomas.

Table 3
Effects of ONO-1714 treatment on the multiplicities of intestinal tumors induced by AOM at week 32

Dose of ONO-1714 in diet (ppm)	Effective no. of animals	No. of tumors per rat[a]				
		Small intestine	Cecum	Colon and rectum		
				Total[b]	Adenoma	Carcinoma
0	30	0.30 ± 0.15	0.07 ± 0.05	3.07 ± 0.34	0.43 ± 0.14	2.63 ± 0.28
50	29	0.31 ± 0.10	0.14 ± 0.06	2.41 ± 0.36	0.28 ± 0.10	2.12 ± 0.33
100	30	0.17 ± 0.08	0.10 ± 0.06	2.27 ± 0.32	0.10 ± 0.06	2.17 ± 0.30

[a] Data presented are mean ± SE values.
[b] The total represents animals with adenomas and/or carcinomas.

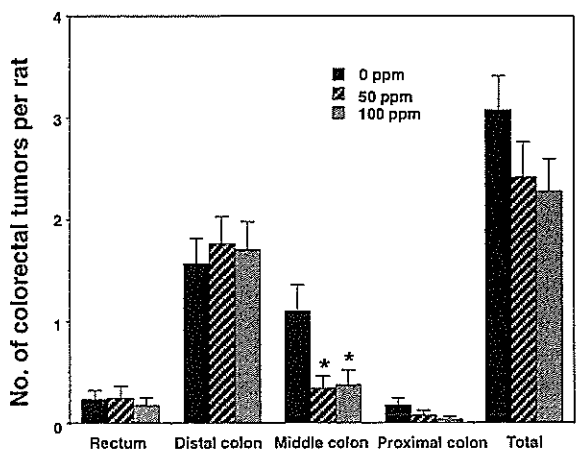

Fig. 1. Effects of ONO-1714 treatment on the location of AOM-induced colorectal tumors. The data for the AOM + 0 ppm ONO-1714 (■), AOM + 50 ppm ONO-1714 (▨) and AOM + 100 ppm ONO-1714 (▩) groups are mean ± SE values. *Significantly different from the respective control at $P < 0.05$.

Colorectal tumor volumes per rat tended to be decreased by ONO-1714 treatment when tested using coefficients for linear contrast ($P < 0.05$), being 70.8 ± 18.5 (SE) mm^3 in the AOM alone group, 47.0 ± 13.8 mm^3 in the AOM + 50 ppm ONO-1714 group, and 30.3 ± 11.0 mm^3 in the AOM + 100 ppm ONO-1714 group, although there was no statistical significance. The number of colorectal tumors ≥3 mm in diameter was almost the same as that <3 mm, at 1.57 ± 0.25 (SE), and 1.50 ± 0.21, respectively, in the AOM alone group. The number of colorectal tumors more than 3 mm in diameter were lowered by 50 and 100 ppm ONO-1714 treatment to 64 and 46% of the control value, respectively, and a significant difference was observed in the value for the 100 ppm ONO-1714 group ($P < 0.01$), while number of tumors less than 3 mm in diameter did not differ from the control value (Fig. 2A). Especially, the suppressive effect of ONO-1714 on tumor development more than 3 mm in diameter was evident in the middle colon, being 21 and 19% of the control value in 50 and 100 ppm ONO-1714 groups, respectively ($P < 0.01$) (Fig. 2D), and tumor development more than 3 mm in diameter in the rectum and proximal colon also tended to be decreased by ONO-1714 treatment (Figs. 2B and E). Interestingly, number of tumors less than 3 mm in diameter in the middle colon were also decreased to about half by 50 and 100 ppm ONO-1714 treatment, although there was no statistical significance. On the other hand, in the distal colon, the number of tumors more than 3 mm in diameter was slightly decreased by 100 ppm ONO-1714 treatment and that less than 3 mm was slightly increased (Figs. 2C).

Table 4 shows the results of histological examination of AOM-induced colorectal carcinomas. In all groups, most were well-differentiated adenocarcinomas. Signet-ring cell carcinomas were rare and observed mostly in the proximal colon. Compared to the AOM alone group, ONO-1714-treated groups had lower incidences of signet-ring cell carcinomas, although this did not reach statistical significance. The proportions of tumors demonstrating invasion of submucosa (sm) were also slightly, but not significantly, lower in the ONO-1714-treated groups.

Discussion

The present study demonstrated that an iNOS inhibitor, ONO-1714, can effectively decrease development of

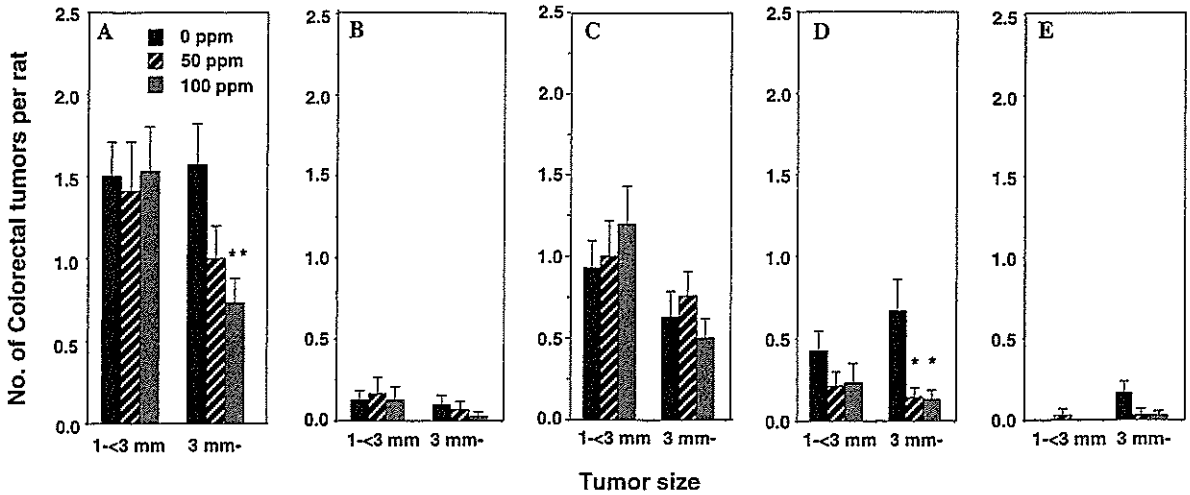

Fig. 2. Effect of ONO-1714 treatment on the size of colorectal tumors. The number of each size of colorectal tumors per rat in the whole colorectum (A), rectum (B), distal colon (C), middle colon (D), and proximal colon (E) for the AOM + 0 ppm ONO-1714 (■), AOM + 50 ppm ONO-1714 (▨) and AOM + 100 ppm ONO-1714 (▩) groups are mean ± SE values. *,**Significantly different from the respective control at $P < 0.05$ and $P < 0.01$, respectively.

Table 4
Effects of ONO-1714 treatment on histological types and depth of invasion of AOM-induced colorectal carcinomas at week 32

Dose of ONO-1714 in diet (ppm)	Total no. of carcinomas	No. of carcinomas of each histological type (%)				Depth of invasion (%)		
		Well-differentiated	Moderately-differentiated	Signet-ring cell	Mucinous	m[a]	sm[b]	pm[c]
0	79	72 (93)	0	5 (6)	1 (1)	67 (86)	11 (14)	0
50	62	61 (98)	0	1 (2)	0	57 (92)	5 (8)	0
100	65	64 (99)	0	1 (1)	0	61 (94)	4 (6)	0

[a] Mucosa and muscularis mucosae.
[b] Submucosa.
[c] Muscularis propria.

preneoplastic lesions, ACF, especially large ACF, providing further support for the concept that iNOS plays important roles in the early stage of colon carcinogenesis. Although the colon tumor incidence was not decreased by ONO-1714 treatment, number of colon tumors larger than 3 mm in diameter were lowered, indicating a suppressive effect on growth. Thus, we can conclude that ONO-1714 also impacts on late stages of colon carcinogenesis, even if it not appreciably suppressing malignant tumor development.

Furthermore, ONO-1714 significantly reduced the number of tumors in the middle colon, where relatively large carcinomas often develop. In our previous studies, treatment with docosahexaenoic acid (DHA) also decreased tumors in the middle colon more effectively than in other parts of colorectum [30]. DHA has similarly been reported to down-regulate iNOS expression in colon cancer cells [31]. Further examination of differences in tumor properties in the middle and distal colon appears warranted.

Although iNOS expression was not detected immunohistochemically in most hyperplastic ACF [16], NOS inhibitors, including iNOS-selective examples, clearly decreased ACF formation in the present and previous studies [20–22]. It is possible that very low levels are present in ACF, many of which possess K-*ras* mutations, and iNOS inhibitors may be more effective on such a low activity state of iNOS. Further mutations such as in the *β-catenin* gene may elevate iNOS expression to a detectable degree [16], and once iNOS expression is elevated, it may be difficult to inhibit its activity completely by iNOS inhibitors. Moreover, in these studies, treatment with iNOS inhibitors was overlapped with AOM treatment. Therefore, it is possible that suppression of ACF formation might be in part due to inhibition of the initiation step, namely DNA alkylation by AOM metabolites or metabolic activation of AOM [32].

It has been reported that angiogenesis is necessary to supply oxygen and nutrients to solid tumors more than 1–2 mm^3 [33] but NO enhances their vascular permeability, partly through activation of matrix metalloproteinases [34]. Thus, suppression of development of tumors more than 3 mm in diameter in ONO-1714-treated groups may be associated with inhibition of angiogenesis by the iNOS inhibitor. NO also enhances activity and expression of COX-2 in several cell lines [35–38] and co-expression of iNOS and COX-2 has been reported for human cancers of the colon [6,38,39], esophagus [9],

stomach [40], pancreas [12], and ovary [41], endometrium [42], and brain [43]. Overexpression of COX-2 promotes angiogenesis through prostanoid-mediated increase in vascular endothelial growth factor (VEGF) [38,40,43]. In the AOM-induced rat colon carcinogenesis model, COX-2 expression is also increased in well-differentiated carcinoma cells of large tumors [16]. It has been reported that iNOS inhibitors, 1400W and SC-51, reduce not only iNOS activity but also COX-2 activity [22,38]. Therefore, reduction of NO-mediated COX-2 activation may be one of the mechanisms underlying suppressive effects of ONO-1714 on colon carcinogenesis.

Our previous studies indicated K-*ras* activating mutations to be frequent in hyperplastic ACF and large tumors [16]. The present study showed that iNOS may contribute to development of preneoplastic lesions, ACF, and expansion of tumor masses in later stage of colon carcinogenesis. These observations in vivo agree with our previous finding that a K-*ras* activating mutation enhances iNOS expression mediated by IL-1β or LPS in cell culture [17]. It should be borne in mind that other cancers with frequent K-*ras* mutations, such as lung and pancreatic examples [44], also show increased iNOS expression [10–13]. Thus, it is suggested that NO production by iNOS is generally involved in tumor-promoting effects of activated K-*ras*, and iNOS-selective inhibitors should be considered as possible candidate agents for prevention of all cancers featuring K-ras activation.

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Possible Involvement of Hyperlipidemia in Increasing Risk of Colorectal Tumor Development in Human Familial Adenomatous Polyposis

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Background: Familial adenomatous polyposis (FAP) results from germline *adenomatous polyposis coli* (*APC*) gene mutations and many affected patients die from colorectal cancers which arise from colorectal polyps. We previously reported that two strains of *Apc* gene-deficient mice developing multiple intestinal polyps exhibit a hyperlipidemic state. The triglyceride (TG) levels were ~10-fold higher than the levels observed in wild-type mice.
Methods: To examine whether a positive relationship might exist between hyperlipidemia and colorectal tumor development in FAP patients, as with *Apc* gene-deficient mice, a pilot experiment was performed using readily available clinical data such as ages, serum lipid levels, number of colorectal polyps and cancer development in 28 FAP patients from the National Cancer Center Hospital, Japan.
Results: The overall prevalence of hyperlipidemia in FAP cases was 58%. Average TG levels in the 40–60 year age groups of FAP patients were ≥150 mg/dl (the defined threshold level of hyperlipidemia). Moreover, there was a tendency for higher serum TG levels in patients who developed colorectal cancer, as compared with those without colorectal cancer.
Conclusions: These results show that a hyperlipidemic state occurs in FAP patients. Although it is weaker than that in *Apc* gene-deficient mice, it may be linked to colon tumor development. These data warrant further studies for wider poplulations of FAP patients.

Key words: APC gene – colorectal cancer – familial adenomatous polyposis – hyperlipidemia

INTRODUCTION

Familial adenomatous polyposis (FAP) is characterized by the appearance of hundreds or thousands of adenomatous polyps in the colon and rectum. The polyps are caused by germline mutations of the *adenomatous polyposis coli* (*APC*) gene located on chromosome 5q21. Patients face increased mortality due to inevitable colorectal cancer developing from intestinal polyps. Thus, prophylactic colectomy is performed usually before 25 years of age (1). In such individuals at extremely elevated risk of colorectal cancer, it is mandatory that any promoting factors be elucidated and appropriate preventive measures be devised.

There are several mouse models for FAP with different germline *Apc* mutation sites such as codons 716, 850, 1309 and 1638 (2–4). We previously reported that two strains of *Apc* gene-deficient mice, Min (*Apc* gene mutation at codon 850) and Apc^{1309} (mutated at codon 1309) mice, show particularly large numbers of intestinal polyps and a hyperlipidemic state (5,6). In these mice, serum triglyceride (TG) levels increase with age, to ~500–800 mg/dl when 12–15 weeks old, associated with low mRNA expression levels of lipoprotein lipase (LPL) in the liver and small intestine. These TG levels are ~10-fold higher than the 70 mg/dl typically observed in wild-type mice. Serum total cholesterol (TC) levels are slightly elevated. Moreover, we have reported that a peroxisome proliferator-activated receptor (PPAR) α agonist, bezafibrate, and a PPARγ agonist, pioglitazone,

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Abbreviations: BMI, body mass index; ChE, cholinesterase; COX, cyclooxygenase; FAP, familial adenomatous polyposis; LPL, lipoprotein lipase; NSAIDs, non-steroidal anti-inflammatory drugs; PPAR, peroxisome proliferator-activated receptor; TC, total cholesterol; TG, triglyceride

concomitantly suppress hyperlipidemia and intestinal polyp formation in the mice, with induction of LPL mRNA. Indeed, an LPL inducer, NO-1886, also suppresses hyperlipidemia and intestinal polyp formation in the mice (7).

In the present study, we performed a pilot study to examine whether hyperlipidemia might also be a complication in FAP patients, similar to FAP model mice, and possible associations with colon tumor development are discussed.

PATIENTS AND METHODS

FAP Patients

Twenty-eight Japanese FAP patients, presenting at the National Cancer Center Hospital from 1999 to 2004 for follow-up of their health conditions, were reviewed. FAP was diagnosed by observing >100 intestinal polyps and all 28 patients underwent prophylactic colectomy. In addition, pathological studies were performed on these polyps. Germline mutations of the *APC* gene were investigated in 24 of the 28 cases, but the other 4 patients did not give written informed consent. The following data were collected: age; sex; body mass index (BMI = kg/m^2); history of previous surgery; serum levels of albumin, total protein, cholinesterase (ChE), TC and TG; and presence or absence of colon and gastric tumors. Patients who did not give written informed consent for collecting samples and clinical information were excluded. The use of each individual's material was approved by the ethics review committee of the National Cancer Center.

Detection of Hyperlipidemia in FAP Patients

The criteria for hyperlipidemia (hypertriglyceridemia and/or hypercholesterolemia) were based on the Japan Atherosclerosis Society Guideline (8): a fasting serum TG level ≥150 mg/dl and a fasting serum TC level ≥220 mg/dl. Decisions were made using data from more than two blood samples collected independently, with at least a month's interval. Values obtained within a month of receiving abdominal surgery were disregarded. The data for TG levels at different ages were those from 2002.

Detection of Rectal Polyps in FAP Patients

Rectal polyps were examined by front-viewing endoscopy and recorded as digital photographs. The numbers were counted and the rectal polyps were classified into two types as follows: confluent (jammed together) and scattered (isolated from each other). The extent of rectal polyp development was further classified into five groups: no polyps/field of view (−), 1–5 polyps/field of view (±), 6–10 polyps/field of view (+), 11–20 polyps/field of view (++) and >20 polyps/field of view (+++).

Identification of Germline Mutations in the *APC* Gene

Germline *APC* gene mutations were analysed in colon cancer samples or genomic DNA and cDNA samples prepared from the peripheral lymphocytes. Mutations were first screened by a protein truncation test (9) followed by confirmation by direct sequencing of the PCR-amplified genomic sequences.

RESULTS

The Overall Prevalence of Hyperlipidemia in FAP Patients

Data for history of colorectal cancer development and numbers of rectal polyps in the 28 FAP patients presenting at the National Cancer Center Hospital from 1999 to 2004 are summarized in Table 1. Serum lipid levels for the patients are given in Table 2. Twenty-seven FAP patients had undergone prophylactic colectomy and none received medical treatment and/or nutritional management for hyperlipidemia. Serum lipid data for Patients 7 and 8 were not informative enough and were excluded from the analysis.

Table 1 provides general data for ages and genders, the positions of *APC* germline mutation, existing rectal polyps in 2004 and past history of colorectal cancer. In Table 2, the data for minimum, maximum and average TG levels, and also the maximum levels of TC are shown. Patients in a hyperlipidemic state with serum TG levels ≥150 mg/dl and/or TC levels ≥220 mg/dl, determined at least two times independently, are underlined. In addition, frequencies of blood examination and number of occasions on which TG was ≥150 mg/dl are shown. The overall prevalence of hyperlipidemia in the patients was 57.7% (15/26): the prevalence of hypertriglyceridemia (150–429 mg/dl) was 73.3% (11/15) and the prevalence of hypercholesterolemia (220–296 mg/dl) was 53.3% (8/15). Differences in other clinical data such as BMI, serum albumin levels, serum total protein levels and serum ChE levels were not observed between patients with and without hyperlipidemia (data not shown).

Change of Serum TG and TC Levels during Aging

Regarding the changes in average serum TG levels during aging, age-dependent increases of TG levels were observed in patients in their thirties and maximum levels were observed in those in their sixties (Fig. 1A). Reported average serum TG levels in each 10 year cohort of the Japanese are highest in those in their forties [129 mg/dl, total number = 12,839, ref. (10)]. Meanwhile, the average serum TC levels in FAP patients were <220 mg/dl except for two FAP patients in their seventies (253 mg/dl, Fig. 1B). Reported average serum TC levels in each 10 year cohort of the Japanese are highest in those in their sixties [211 mg/dl, ref. (10)]. Sex difference is apparent in the serum lipid levels: males tend to have high TG levels (150 mg/dl for men in their forties) and females to have TC levels (218 mg/dl for women in their fifties and sixties). In the