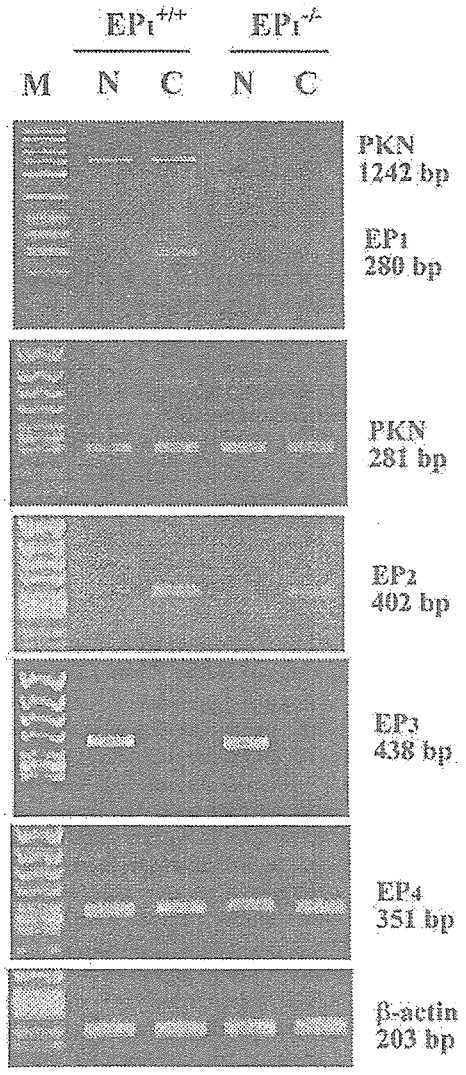

Fig. 3. EP receptors and *PKN* gene expression using RT-PCR. Lanes M, N and C indicate molecular weight control, normal mucosa and cancer of the colon, respectively. The left two lanes represent samples from $EP_1^{+/+}$ mice and the right two lanes represent samples from $EP_1^{-/-}$ mice. (Upper panel) Agarose gel indicating results for EP_1 receptor and *PKN* gene expression using primers *a* and *b*. (Second panel) Agarose gel indicating results for PKN encoding region expression using primers *c* and *d*. (Third, fourth, fifth and sixth panels) Agarose gels indicating results for EP_2, EP_3, EP_4 and β-actin gene expression, respectively.

mRNA for the PKN encoding region was at the same level in the kidneys of all $EP_1^{+/+}$ and $EP_1^{-/-}$ mice (data not shown). These results suggest that the EP_1 knockout strategy and AOM-induced colon carcinogenesis do not affect *PKN* gene stability. Thus, we conclude that colon cancer development was reduced by lack of the EP_1 receptor, not *PKN* gene instability. Next, we examined expression of other *EP* receptor mRNAs in normal mucosa and cancers of the colon in $EP_1^{+/+}$ and $EP_1^{-/-}$ mice (Figure 3, lower panels). The data clearly indicate the EP_2 receptor to be up-regulated and the EP_3 receptor down-regulated in colon cancers. The EP_4 receptor was detected in both normal mucosa and cancers of the colon. There were no differences in the results on EP receptors expression except for EP_1 between $EP_1^{-/-}$ and $EP_1^{+/+}$ mice.

Recently it was reported that the EP_2 receptor is important for intestinal polyp formation in $Apc^{\Delta 716}$ heterozygote mice (30). The authors found analogous results for *EP* receptor mRNA expression using RT-PCR, as in the present study. They, however, indicated no differences in EP_1 receptor expression between polyps and normal mucosa in both small and large intestine. The difference between our and their results may be due to the use of different samples, such as colon adenocarcinomas and intestinal polyps, respectively.

The results of this study clearly show that EP_1 receptor deficiency decreases colon cancer incidence induced by AOM from 57 to 27%. However, 27% of $EP_1^{-/-}$ mice still developed colon cancers. Interestingly, the EP_2 receptor was up-regulated and the EP_3 receptor was down-regulated in colon cancers in $EP_1^{-/-}$ mice. The EP_1 receptor mediates a PGE_2-induced elevation in free Ca^{2+} concentration in Chinese hamster ovary cells and is able to regulate Ca^{2+} channel gating via an as yet unidentified G protein (24). The EP_2 and EP_4 receptors are coupled to G_s and mediate increases in cAMP levels (25). Although alternative splicing of the EP_3 receptor gene results in several isoforms in animal species, including the mouse, rat and human (31–33), the major signaling pathway of the EP_3 receptor is inhibition of adenylate cyclase via G_i. Therefore, there may be cross-talk between second messengers after ligand binding to EP receptors and all four types of EP receptor may interact with each other. Further investigations to identify down stream coordination of these receptors are required.

In conclusion, EP_1 receptor knockout mice demonstrate significantly reduced AOM-induced colon cancer development compared with wild-type mice due to down-regulation of cell proliferation and up-regulation of apoptosis in cancer cells. Also, EP_1 receptor mRNA was found to be up-regulated in colon cancers. These results suggest that the EP_1 receptor plays a pivotal role in colon carcinogenesis and support the hypothesis that lack of the EP_1 receptor has the potential for a mechanism-based chemoprevention strategy against colon cancer development. Therefore, EP_1 receptor antagonists may be a promising chemopreventive agent against colon carcinogenesis. Further studies on the chemopreventive potential of EP_1 receptor antagonists for colon cancer in long-term *in vivo* animal models are ongoing in our laboratory.

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References

1. Thun,M.J., Namboodiri,M.M. and Heath,C.W.,Jr (1991) Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.*, 325, 1593–1596.
2. Reddy,B.S., Tokumo,K., Kulkarni,N., Aligia,C. and Kelloff,G. (1992) Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds. *Carcinogenesis*, 13, 1019–1023.
3. Singh,J., Hamid,R. and Reddy,B.S. (1997) Dietary fat and colon cancer: modulation of cyclooxygenase-2 by types and amount of dietary fat during the postinitiation stage of colon carcinogenesis. *Cancer Res.*, 57, 3465–3470.
4. Kargman,S.L., O'Neill,G.P., Vickers,P.J., Evans,J.F., Mancini,J.A. and Jothy,S. (1995) Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res.*, 55, 2556–2559.
5. Sano,H., Kawahito,Y., Wilder,R.L. *et al.* (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.*, 55, 3785–3789.

6. Kawamori,T., Rao,C.V., Seibert,K. and Reddy,B.S. (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, **58**, 409–412.
7. Jacoby,R.F., Seibert,K., Cole,C.E., Kelloff,G. and Lubet,R.A. (2000) The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. *Cancer Res.*, **60**, 5040–5044.
8. Steinbach,G., Lynch,P.M., Phillips,R.K. *et al.* (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Engl. J. Med.*, **342**, 1946–1952.
9. Tsujii,M. and DuBois,R.N. (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, **83**, 493–501.
10. Oshima,M., Dinchuk,J.E., Kargman,S.L., Oshima,H., Hancock,B., Kwong,E., Trzaskos,J.M., Evans,J.F. and Taketo,M.M. (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, **87**, 803–809.
11. Chulada,P.C., Thompson,M.B., Mahler,J.F. *et al.* (2000) Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. *Cancer Res.*, **60**, 4705–4708.
12. Yamaguchi,A., Ishida,T., Nishimura,G., Katoh,M. and Miyazaki,I. (1991) Investigation of colonic prostaglandins in carcinogenesis in the rat colon. *Dis. Colon Rectum*, **34**, 572–576.
13. Rigas,B., Goldman,I.S. and Levine,L. (1993) Altered eicosanoid levels in human colon cancer. *J. Lab. Clin. Med.*, **122**, 518–523.
14. Kawamori,T., Uchiya,N., Sugimura,T. and Wakabayashi,K. (2003) Enhancement of colon carcinogenesis by prostaglandin E2 administration. *Carcinogenesis*, **24**, 985–990.
15. Ushikubi,F., Segi,E., Sugimoto,Y. *et al.* (1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature*, **395**, 281–284.
16. Segi,E., Sugimoto,Y., Yamasaki,A. *et al.* (1998) Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem. Biophys. Res. Commun.*, **246**, 7–12.
17. Hizaki,H., Segi,E., Sugimoto,Y. *et al.* (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). *Proc. Natl Acad. Sci. USA*, **96**, 10501–10506.
18. Watanabe,K., Kawamori,T., Nakatsugi,S. *et al.* (1999) Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. *Cancer Res.*, **59**, 5093–5096.
19. Mutoh,M., Watanabe,K., Kitamura,T. *et al.* (2002) Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. *Cancer Res.*, **62**, 28–32.
20. Mukai,H. and Ono,Y. (1994) A novel protein kinase with leucine zipper-like sequences: its catalytic domain is highly homologous to that of protein kinase C. *Biochem. Biophys. Res. Commun.*, **199**, 897–904.
21. Batshake,B. and SundelinJ. (1996) The mouse genes for the EP1 prostanoid receptor and the PKN protein kinase overlap. *Biochem. Biophys. Res. Commun.*, **227**, 70–76.
22. Pozharisski,K.M. (1990) Tumours of the intestines. In Turusov,V. and Mohr,U. (eds) *Pathology of Tumours in Laboratory Animals*, IARC Scientific Publications no. 1. IARC, Lyon, pp. 159–180.
23. Tanaka,T., Takeuchi,T., Nishikawa,A., Takami,T. and Mori,H. (1989) Nucleolar organizer regions in hepatocarcinogenesis induced by N-2-fluorenylacetamide in rats: comparison with bromodeoxyuridine immunohistochemistry. *Jpn. J. Cancer Res.*, **80**, 1047–1051.
24. Watabe,A., Sugimoto,Y., Honda,A., Irie,A., Namba,T., Negishi,M., Ito,S., Narumiya,S. and Ichikawa,A. (1993) Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *J. Biol. Chem.*, **268**, 20175–20178.
25. Katsuyama,M., Nishigaki,N., Sugimoto,Y., Morimoto,K., Negishi,M., Narumiya,S. and Ichikawa,A. (1995) The mouse prostaglandin E receptor EP2 subtype: cloning, expression and northern blot analysis. *FEBS Lett.*, **372**, 151–156.
26. Sugimoto,Y., Negishi,M., Hayashi,Y., Namba,T., Honda,A., Watabe,A., Hirata,M., Narumiya,S. and Ichikawa,A. (1993) Two isoforms of the EP3 receptor with different carboxyl-terminal domains. Identical ligand binding properties and different coupling properties with Gi proteins. *J. Biol. Chem.*, **268**, 2712–2718.
27. Honda,A., Sugimoto,Y., Namba,T., Watabe,A., Irie,A., Negishi,M., Narumiya,S. and Ichikawa,A. (1993) Cloning and expression of a cDNA for mouse prostaglandin E receptor EP2 subtype. *J. Biol. Chem.*, **268**, 7759–7762.
28. Egan,M.J. and Crocker,J. (1992) Nucleolar organiser regions in pathology. *Br. J. Cancer*, **65**, 1–7.
29. Ruschoff,J., Bittinger,A., Neumann,K. and Schmitz-Moormann,P. (1990) Prognostic significance of nucleolar organizing regions (NORs) in carcinomas of the sigmoid colon and rectum. *Pathol. Res. Pract.*, **186**, 85–91.
30. Sonoshita,M., Takaku,K., Sasaki,N., Sugimoto,Y., Ushikubi,F., Narumiya,S., Oshima,M. and Taketo,M.M. (2001) Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc (Delta 716) knockout mice. *Nature Med.*, **7**, 1048–1051.
31. Kotani,M., Tanaka,I., Ogawa,Y., Usui,T., Tamura,N., Mori,K., Narumiya,S., Yoshimi,T. and Nakao,K. (1997) Structural organization of the human prostaglandin EP3 receptor subtype gene (PTGER3). *Genomics*, **40**, 425–434.
32. Namba,T., Sugimoto,Y., Negishi,M., Irie,A., Ushikubi,F., Kakizuka,A., Ito,S., Ichikawa,A. and Narumiya,S. (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature*, **365**, 166–170.
33. Negishi,M., Sugimoto,Y., Irie,A., Narumiya,S. and Ichikawa,A. (1993) Two isoforms of prostaglandin E receptor EP3 subtype. Different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *J. Biol. Chem.*, **268**, 9517–9521.

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Suppression of azoxymethane-induced colon cancer development in rats by a prostaglandin E receptor EP_1-selective antagonist

Naoko Niho,[1,4] Michihiro Mutoh,[1] Tomohiro Kitamura,[1] Mami Takahashi,[1] Hidetaka Sato,[2] Hiroshi Yamamoto,[3] Takayuki Maruyama,[3] Shuichi Ohuchida,[3] Takashi Sugimura[1] and Keiji Wakabayashi[1]

[1]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, [2]Department of Biological Safety Research, Japan Food Research Laboratories, 3 Bunkyo, 2-chome, Chitose-shi, Hokkaido 066-0052, and [3]Minase Research Institute, Ono Pharmaceutical Co., 3-1-1 Sakurai, Shimamoto-cho, Mishima-gun, Osaka 618-8585 Japan

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Prostaglandin E_2 is involved in colon carcinogenesis through its binding to the PGE_2 receptor subtypes EP_1, EP_2, EP_3 and EP_4. We have demonstrated that administration of ONO-8711, an EP_1-selective antagonist, suppresses development of AOM-induced ACF in C57BL/6 mice and F344 rats. ONO-8711 also reduced the numbers of intestinal polyps in Min mice. In the present study, we investigated the long-term effects of ONO-8711 on colon cancer development in rats treated with AOM. Male F344 rats were injected subcutaneously with AOM (15 mg/kg body weight) once a week for the first 2 weeks to develop colon cancer. Administration of 400 or 800 p.p.m. ONO-8711 in their diets for 32 weeks reduced the incidence, multiplicity and volume of colon carcinomas. The incidence of colon adenocarcinomas in AOM-treated rats was 97, 83 and 76% ($P < 0.05$) in the 0, 400 and 800 p.p.m. of ONO-8711 groups, respectively. The multiplicity of adenocarcinomas was also decreased significantly, being 3.31 ± 0.33, 2.34 ± 0.27 ($P < 0.05$) and 2.06 ± 0.34 ($P < 0.01$) with 0, 400 and 800 p.p.m. of ONO-8711, respectively. Moreover, treatment with 800 p.p.m. ONO-8711 reduced the mean volume of adenocarcinomas to 49% ($P < 0.05$) of the value for the AOM treatment alone. Furthermore, the BrdU labeling index was decreased significantly in colon cancer cells by 800 p.p.m. ONO-8711. These results confirm that EP_1 is involved in colon carcinogenesis and that EP_1-selective antagonists might be promising candidates for colon cancer chemopreventive agents. (*Cancer Sci* 2005; 96: 260–264)

Colon cancer is one of the most frequent cancers in the world.[1] Epidemiological and experimental studies have indicated that aspirin and other NSAIDs can reduce the development of colon cancer,[1–5] the suggested mechanism being inhibition of COX activity, which catalyzes the synthesis of prostanoids. There are two isoforms of COX, the constitutively expressed COX-1 and the inducible COX-2. Both contribute to PGE_2 production and colon carcinogenesis, and PGE_2 levels are known to be elevated in human and rodent colon tumors.[6–9]

Prostanoids such as PGD_2, PGE_2, PGF_2, PGI_2 and TXA_2 exert their biological actions through binding to specific membrane receptors, which include DP for PGD_2, FP for PGF_2, IP for PGI_2, TP for TXA_2 and EP_1 to EP_4 for PGE_2.[10–12] The PGE_2 receptors are transmembrane G protein-coupled receptors and it has been established that EP_1 signals are transmitted by increased intracellular Ca^{2+} concentrations, with activation of phosphorylated PKC.[10] However, the species of G protein coupling to EP_1 remains unidentified. EP_2 and EP_4 receptors couple to Gs and increase cAMP synthesis by adenylate cyclase, and the EP_3 receptor couples to Gi and decreases cAMP synthesis by inhibition of adenylate cyclase.[13]

Our previous study using EP_{1-4}, DP, FP, IP and TP receptor knockout mice showed that deficiency of either EP_1 or EP_4 receptors decreases formation of AOM-induced colon ACF, putative preneoplastic lesions.[14,15] It has also been reported that the numbers of intestinal polyps in EP_2-deficient $Apc^{\Delta 716}$ mice are lower than in their $Apc^{\Delta 716}$ counterparts.[16] In contrast, enhancement of AOM-induced colon cancer development has been observed in EP_3 receptor knockout mice.[17] Thus, it is considered that EP_1, EP_2 and EP_4 receptors are involved in enhancement of colon carcinogenesis, while the EP_3 receptor acts against tumor development.

In addition to genetic approaches, pharmacological research has been carried out to examine the roles of EP_1 and EP_4. Administration of ONO-8711, 6-[(2*S*,3*S*)-3-(4-chloro-2-methylphenylsulfonylaminomethyl)-bicyclo[2.2.2]octan-2-yl]-5*Z*-hexenoic acid, an EP_1-selective antagonist, reduced AOM-induced ACF formation in C57BL/6 mice and F344 rats.[14,18] Furthermore, treatment with ONO-8713, 4-{2-[*N*-isobutyl-*N*-(2-furylsulfonyl)amino]-5-trifluoromethylphenoxymethyl}cinnamic acid, another EP_1 antagonist, and ONO-AE2-227, 2-{2-[2-(1-naphthyl)propanoylamino]phenyl}methylbenzoic acid, an EP_4 antagonist, similarly suppressed AOM-induced ACF formation in C57BL/6 mice.[15,19] Administration of ONO-8711 and ONO-AE2-227 to Min mice, an animal model for human FAP, also reduced the number of intestinal polyps.[14,15] EP receptors transmit their signals by independent pathways, and combination treatment with ONO-8711 and ONO-AE2-227 caused additional reduction in intestinal polyp formation in mice with a truncated *adenomatous polyposis coli* (*Apc*) gene at codon 1309 (Apc^{1309} mice).[20]

Although evidence has thus accumulated that EP_1 is involved in colon carcinogenesis, significant effects of EP_1 antagonists on yields of actual colon cancers have yet to be reported. The present study was therefore designed to determine the suppressive influence of an EP_1-selective antagonist, ONO-8711, on AOM-induced colon tumor development in male F344 rats. Administration of ONO-8711 reduced the colon cancer incidence, multiplicity and volume, along with cell proliferation in rat colon tumor cells. On the basis of these results, possible mechanisms of ONO-8711 in colon cancer suppression are discussed.

[4]To whom correspondence should be addressed. E-mail: nniho@gan2.ncc.go.jp

Abbreviations: ABC, avidin biotin complex; ACF, aberrant crypt foci; AOM, azoxymethane; Apc, adenomatous polyposis coli; BrdU, 5-bromo-2′-deoxyuridine; cAMP, cyclic AMP; COX, cyclooxygenase; FAP, familial adenomatous polyposis; Gi, inhibitory G protein; Gs, stimulatory G protein; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PKC, protein kinase C; TX, thromboxane.

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Materials and Methods

Animals and chemicals. Male F344 rats, at 4 weeks of age, were purchased from Charles River Japan (Atsugi, Japan) and acclimated to laboratory conditions for 1 week. Two or three animals were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room air-conditioned at 24 ± 2°C and 55% humidity, on a 12 : 12 h light:dark cycle. AOM and BrdU were purchased from Sigma Chemical Co. (St Louis, MO, USA). The selective PGE receptor EP_1 antagonist ONO-8711, 6-([2*S*,3*S*]-3-[4-chloro-2-methylphenylsulfonylaminomethyl]-bicyclo{2.2.2}octan-2-yl)-5*Z*-hexenoic acid, was synthesized chemically at Ono Pharmaceutical Co. (Osaka, Japan) and well mixed with powdered basal diet AIN-76A (Dyets, Bethlehem, PA, USA) at concentrations of 400 and 800 p.p.m. The doses were selected based on the results of our previous study, in which 400 or 800 p.p.m. ONO-8711 in the diet suppressed intestinal polyp formation in *Apc*^{1309} mice and PhIP-induced breast cancer development.[20,21]

Animal experiments. The rats were divided into five groups as shown in Fig. 1. Groups 1–3 (36 animals per group) were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight once a week for 2 weeks. From the day of the first treatment with AOM, these animals were fed the following diets for 32 weeks: group 1, the basal diet; group 2, 400 p.p.m. ONO-8711; group 3, 800 p.p.m. ONO-8711. Groups 4 and 5, six animals per group, were the corresponding controls to groups 1 and 3, respectively, and were injected with saline without AOM followed by the basal diet or 800 p.p.m. of ONO-8711. Food and water were available ad libitum. The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured weekly. At 37 weeks of age, all animals were sacrificed under anesthesia, 1 h after i.p. injection of BrdU in saline solution (50 mg/kg body weight) and complete autopsies were carried out. The liver, kidney and spleen were removed and weighed. Each intestinal tract was removed, opened longitudinally and fixed flat between sheets of filter paper in 10% neutral buffered formalin. The number, size and location of all intestinal tumors were determined. Estimation of tumor volume (V) was determined using the formula $V = L \times W \times D \times \pi/6$, where L is length, W is width and D is depth of colon tumor.[22] Tissues were processed in paraffin, and sections were stained with hematoxylin and eosin for histopathological examination. Diagnosis of intestinal tumors was carried out according to the classification of Pozharisski.[23] The experimental protocol followed the guidelines for Animal Experiments in the National Cancer Center.

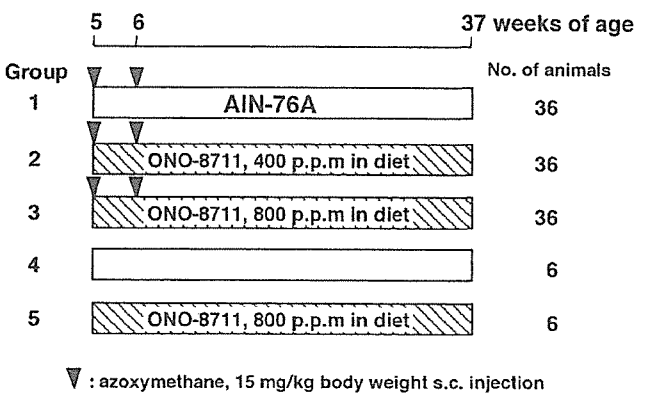

Fig. 1. Experimental protocol. The animals were male F344 rats at 5 weeks of age at the commencement. They were treated subcutaneously with AOM (15 mg/kg body weight) once a week for 2 weeks and from the day of the first treatment were fed a basal diet or a diet containing 400 or 800 p.p.m. ONO-8711 for 32 weeks.

BrdU labeling index. Two serial sections, including colon adenocarcinoma tissues and normal colonic epithelium, were prepared. One section was used for determination of BrdU labeling indexes and the other for apoptotic indexes. Cell proliferation in the colon carcinomas and normal colonic epithelium were examined by immunohistochemical detection of BrdU incorporation using the ABC method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and a mouse monoclonal antibody to BrdU (1 : 300; Dako, Kyoto, Japan). For determination of BrdU labeling indexes, three representative fields in each section of a colon carcinoma and surrounding normal-appearing tissue were selected under light-microscope examination at a magnification of ×400. In each section, one carcinoma per rat was used for counting. The number of BrdU-positive nuclei in a minimum of 1500 cells was counted and the result was expressed as a percentage value.

Apoptotic index. Sections were stained with the aid of an ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. For analysis of apoptosis, well-defined and darkly stained apoptotic cells and bodies in the colon carcinomas were counted in the same way as for BrdU-positive cells. The percentage of positive cells (apoptotic index) was determined by calculating the positive cell number : total cell number × 100.

Statistical analysis. Data are presented as mean ± SE values. Data for body weight, organ weight, tumor multiplicity, tumor volume, BrdU labeling and apoptotic index were compared by Student's *t*-test or Welch's *t*-test. Data for tumor incidences were analyzed by Fisher's exact probability test. Differences were considered to be statistically significant with $P < 0.05$ (two-tailed).

Results

Administration of ONO-8711 at doses of 400 and 800 p.p.m. in the diet for 32 weeks did not affect food intake or clinical signs of AOM-treated animals. The mean body weights at 37 weeks of age were 355.0 ± 3.3 g in the AOM control group, 350.5 ± 3.0 g in the AOM + 400 p.p.m. ONO-8711 group, 340.4 ± 3.8 g in the AOM + 800 p.p.m. ONO-8711 group, 354.3 ± 5.9 g in the saline control group and 342.7 ± 10.5 g in the saline + 800 p.p.m. ONO-8711 group. In AOM- and saline-treated rats fed the diet containing ONO-8711, there were no gross changes in body weight or any organ weight that would point to any toxicity of ONO-8711.

Data for incidences and multiplicities of intestinal tumors are summarized in Table 1. More were well-differentiated tubular adenocarcinomas and the main sites of development were the distal and middle colons. The incidence of colon adenocarcinomas in AOM-treated rats was 97, 83 and 76% ($P < 0.05$) in the 0, 400 and 800 p.p.m. ONO-8711-treated groups, respectively (Table 1). The multiplicity of colon adenocarcinomas also decreased dose-dependently, from 3.31 ± 0.33 for the AOM-treated control group to 2.34 ± 0.27 ($P < 0.05$), and to 2.06 ± 0.34 ($P < 0.01$) by 400 and 800 p.p.m. ONO-8711 treatments, respectively (Table 1). The incidence and multiplicity of colon adenomas in the groups treated with 800 p.p.m. ONO-8711 were increased slightly, but not significantly. The percentage of well-differentiated adenocarcinomas was also increased slightly in the 800 p.p.m. group (Table 2). On the other hand, suppression of adenocarninoma volume was observed by ONO-8711 administration (Fig. 2). The reduction of adenocarcinomas was from 26.3 ± 5.8 mm^3 for the AOM control group to 14.1 ± 4.5 mm^3 at 400 p.p.m. and to 13.0 ± 3.1 mm^3 ($P < 0.05$)

Table 1. Effects of ONO-8711 treatment on the incidences and multiplicities of AOM-induced intestinal tumors in rats

Treatment	Effective no. of animals	No. of animals with tumors (%)			No. of tumors per rat (mean ± SE)		
		Small intestine (carcinoma)	Large intestine		Small intestine (carcinoma)	Large intestine	
			Adenoma	Carcinoma		Adenoma	Carcinoma
AOM alone	35	10 (29)	15 (43)	34 (97)	0.29 ± 0.08	0.66 ± 0.15	3.31 ± 0.33
AOM + ONO-8711, 400 p.p.m.	35	3 (9)	16 (46)	29 (83)	0.09 ± 0.05*	0.66 ± 0.15	2.34 ± 0.27*
AOM + ONO-8711, 800 p.p.m.	33	6 (18)	16 (48)	25 (76)*	0.21 ± 0.08	0.85 ± 0.19	2.06 ± 0.34**
Saline alone	6	0	0	0	0	0	0
ONO-8711, 800 p.p.m.	6	0	0	0	0	0	0

*Significantly different from the control value at $P < 0.05$. **Significantly different from the control value at $P < 0.01$.

Table 2. Effects of ONO-8711 on histological types of AOM-induced colon carcinomas

Treatment	Effective no. of animals	Total no. of carcinomas (%)	No. of carcinomas diagnosed			
			Well-differentiated (%)	Moderately-differentiated (%)	Signet-ring cell (%)	Mucinous (%)
AOM alone	35	116 (100)	108 (93)	3 (3)	1 (1)	4 (3)
AOM + ONO-8711, 400 p.p.m.	35	82 (100)	77 (94)	2 (2)	3 (4)	0 (0)
AOM + ONO-8711, 800 p.p.m.	33	68 (100)	65 (96)	0 (0)	2 (3)	1 (1)

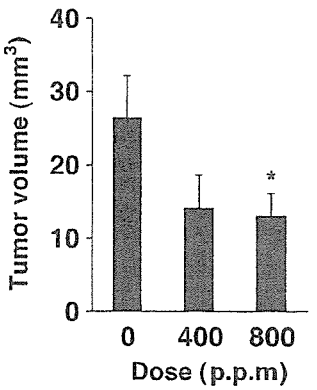

Fig. 2. Effects of ONO-8711 on the mean volumes of adenocarcinomas of the AOM-treated rat colon. Bars show AOM alone (control group), AOM + 400 p.p.m. ONO-8711 and AOM + 800 p.p.m. ONO-8711. *Significantly different from the control value at $P < 0.05$.

at 800 p.p.m. The mean volumes of colon adenomas in the 400 and 800 p.p.m. ONO-8711-treated groups also tended to be reduced, but they were not big enough to evaluate a significant difference from that in the control group (0.48 ± 0.26 mm^3 vs 0.30 ± 0.13 mm^3 and 0.14 ± 0.04 mm^3 in the 400 and 800 p.p.m. groups, respectively). In the small intestine, the incidence and multiplicity of adenocarcinomas were not significantly lower in rats treated with ONO-8711, and no dose-dependency was observed: the incidences were 29, 9 and 18% in the 0, 400 and 800 p.p.m. of ONO-8711-treated groups, respectively, and the multiplicities were 0.29 ± 0.08, 0.09 ± 0.05 ($P < 0.05$) and 0.21 ± 0.08 (Table 1).

Data for BrdU-positive cells (BrdU labeling indexes) and apoptotic cells (apoptotic indexes) in the normal colon epithelium and colon carcinomas are summarized in Table 3. The mean BrdU labeling index for carcinomas in the AOM-treated control group was higher than in the other groups. Administration of AOM + 400 and 800 p.p.m. ONO-8711 reduced the BrdU labeling index to 59 and 54% ($P < 0.05$) of the AOM-treated control value, respectively. On the other hand, AOM + 400 and 800 p.p.m. ONO-8711 treatment did not show any effect on BrdU labeling indexes in normal mucosa of rats. The apoptotic indexes for carcinomas in the groups of AOM + 400 and 800 p.p.m. ONO-8711 treatment were 103 and 118% of the AOM control value, respectively (Table 3).

Discussion

The present study demonstrated administration of the EP_1-selective antagonist ONO-8711 to significantly reduce the incidence and multiplicity of AOM-induced rat colon carcinomas, without any toxic effects in terms of body and organ weights. A decrease in the mean volume of adenocarcinomas was observed, and the BrdU labeling index for colon carcinoma was also reduced by treatment with ONO-8711. These results support our previous short-term experiment that treatment with 800 p.p.m. ONO-8711 in the diet reduced the proliferative activity of the colon ACF in rats.[18]

Table 3. Effects of ONO-8711 on cell proliferation and apoptosis in the colon

Treatment	No. of rats tested	BrdU labeling index (mean ± SE)		Apoptotic index (mean ± SE)
		Normal mucosa	Carcinoma	Carcinoma
AOM alone	4	3.5 ± 0.1	9.0 ± 1.2	0.39 ± 0.06
AOM + ONO-8711, 400 p.p.m.	4	3.8 ± 0.1	5.3 ± 1.1	0.40 ± 0.16
AOM + ONO-8711, 800 p.p.m.	4	3.3 ± 0.2	4.9 ± 0.8*	0.46 ± 0.05

*Significantly different from the control value at $P < 0.05$.

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In our previous study, 800 p.p.m. ONO-8711 administration inhibited ACF development (31% reduction),[18] and a 38% reduction was observed in the number of tumors in the present study. The suppressive effect of 800 p.p.m. ONO-8711 was observed not only in the number of ACF, but also in their growth,[18] as mentioned. For instance, the numbers of ACF with one crypt, two crypts, three crypts and greater than four crypts were decreased by 11, 32, 51 and 55%, respectively.[18] This is in line with the decreased carcinoma volume and BrdU labeling index in the colon carcinoma in the present study. Our previous report[18] also shows that the BrdU labeling index in the normal colonic epithelium in AOM-treated rats given 800 p.p.m. ONO-8711 for 5 weeks from 5 weeks of age were almost the same as those in the AOM-treated control rats. The present study confirmed the previous findings. Although the incidence and multiplicity of colon adenomas and the percentages of well-differentiated adenocarcinomas in the 800 p.p.m. ONO-8711 group were slightly increased, it may not be stating it too strongly that ONO-8711 could block progression of colon carcinogenesis. Substances that prevent early stages of carcinogenesis or delay the carcinogenic process are considered to be good candidates as chemopreventive agents. Therefore, EP_1 antagonists, including ONO-8711, deserve consideration in this respect.

The tumor suppressive mechanisms of ONO-8711 need to be revealed in more detail. EP_1 signals are transmitted by increased intracellular Ca^{2+} concentrations, with activation of PKC.[10] As noted above, the physiological activities of the EP_2 and EP_4 receptors and EP_3 receptor through activation and inhibition of cAMP synthesis being consistent with their respective effects on colon tumor development.[13] In contrast, the effect of the EP_1-selective antagonist on PKC activity is uncertain. It has been reported decreased PKCα and δ and increased $PKC\beta_{II}$ expression levels were observed in colon tumors compared with surrounding normal epithelial cells.[24] PKCα and PKCδ inhibit cell proliferation and promote differentiation of many cell types *in vitro*,[25–28] and $PKC\beta_{II}$ is linked to enhancement of cell proliferation and suppression of apoptosis.[29] In our preliminary experiments, the expression levels of phosphorylated PKCα and δ in the colon tumors were not affected by 800 p.p.m. ONO-8711 treatment (data not shown). However, the roles of PKC in colon cancer formation appear complex, and further studies are needed to investigate the relationship between EP_1 receptor signaling and downstream targets, like PKC isozymes, in colon carcinogenesis.

In conclusion, the present study demonstrated that the EP_1-selective antagonist ONO-8711 has the potential to suppress AOM-induced development of colon cancers in rats. ONO-8711 may be a promising candidate chemopreventive agent for colon cancer.

Acknowledgments

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References

1 Stewart BW, Kleihues P, eds. *World Cancer Report*. Lyon: IARC Press, 2003; 12–17.
2 Thun MJ, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991; **325**: 1593–6.
3 Rao CV, Rivenson A, Simi B, Zang E, Kelloff G, Steele V, Reddy BS. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Res* 1995; **55**: 1464–7.
4 Smalley WE, DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv Pharmacol* 1997; **39**: 1–20.
5 Chan AT. Aspirin, non-steroidal anti-inflammatory drugs and colorectal neoplasia: future challenges in chemoprevention. *Cancer Causes Control* 2003; **14**: 413–18.
6 Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 1998; **38**: 97–120.
7 Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O, Langenbach R. Genetic disruption of *Ptgs-1*, as well as *Ptgs-2*, reduces intestinal tumorigenesis in Min mice. *Cancer Res* 2000; **60**: 4705–8.
8 Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 1993; **122**: 518–23.
9 Pugh S, Thomas GA. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E_2. *Gut* 1994; **35**: 675–8.
10 Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994; **46**: 205–29.
11 Suda M, Tanaka K, Natsui K, Usui T, Tanaka I, Fukushima M, Shigeno C, Konishi J, Narumiya S, Ichikawa A, Nakao N. Prostaglandin E receptor subtypes in mouse osteoblastic cell line. *Endocrinology* 1996; **137**: 1698–705.
12 Ushikubi F, Hirata M, Narumiya S. Molecular biology of prostanoid receptors; an overview. *J Lipid Mediat Cell Signal* 1995; **12**: 343–59.
13 Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 1999; **79**: 1193–226.
14 Watanabe K, Kawamori T, Nakatsugi S, Ohta T, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Ushikubi F, Narumiya S, Sugimura T, Wakabayashi K. Role of the prostaglandin E receptor subtype EP_1 in colon carcinogenesis. *Cancer Res* 1999; **59**: 5093–6.
15 Mutoh M, Watanabe K, Kitamura T, Shoji Y, Takahashi M, Kawamori T, Tani K, Kobayashi M, Maruyama T, Kobayashi K, Ohuchida S, Sugimoto Y, Narumiya S, Sugimura T, Wakabayashi K. Involvement of prostaglandin E receptor subtype EP_4 in colon carcinogenesis. *Cancer Res* 2002; **62**: 28–32.
16 Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM. Acceleration of intestinal polyposis through prostaglandin receptor EP_2 in $Apc^{\Delta 716}$ knockout mice. *Nature Med* 2001; **7**: 1048–51.
17 Shoji Y, Takahashi M, Kitamura T, Watanabe K, Kawamori T, Maruyama T, Sugimoto Y, Negishi M, Narumiya S, Sugimura T, Wakabayashi K. Downregulation of prostaglandin E receptor subtype EP_3 during colon cancer development. *Gut* 2004; **53**: 1151–8.
18 Kawamori T, Uchiya N, Kitamura T, Ohuchida S, Yamamoto H, Maruyama T, Sugimura T, Wakabayashi K. Evaluation of a selective prostaglandin E receptor EP_1 antagonist for potential properties in colon carcinogenesis. *Anticancer Res* 2001; **21**: 3865–9.
19 Watanabe K, Kawamori T, Nakatsugi S *et al*. Inhibitory effect of a prostaglandin E receptor subtype EP_1 selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice. *Cancer Lett* 2000; **156**: 57–61.
20 Kitamura T, Itoh M, Noda T, Tani K, Kobayashi M, Maruyama T, Kobayashi K, Ohuchida S, Sugimura T, Wakabayashi K. Combined effects of prostaglandin E receptor subtype EP_1 and subtype EP_4 antagonists on intestinal tumorigenesis in *adenomatous polyposis coli* gene knockout mice. *Cancer Sci* 2000; **94**: 618–21.
21 Kawamori T, Uchiya N, Nakatsugi S, Watanabe K, Ohuchida S, Yamamoto H, Maruyama T, Kondo K, Sugimura T, Wakabayashi K. Chemopreventive effects of ONO-8711, a selective prostaglandin E receptor EP_1 antagonist, on breast cancer development. *Carcinogenesis* 2001; **22**: 2001–4.
22 Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS. Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. *Cancer Res* 2002; **62**: 165–70.
23 Pozharisski KM. Tumors of the intestines. In: Turusov VS, ed. *Pathology of Tumours in Laboratory Animals*. Lyon: IARC Scientific Publications, 1990; 119–40.
24 Verstovsek G, Byrd A, Frey MR, Petrelli NJ, Black JD. Colonocyte differentiation is associated with increased expression and altered distribution of protein kinase C isozymes. *Gastroenterology* 1998; **115**: 75–85.
25 Diaz-Meco MT, Berra E, Municio MM, Sanz L, Lozano J, Dominguez I, Diaz-Golpe V, Lain de Lera MT, Alcami J, Paya CV *et al*. A dominant negative protein kinase C ζ subspecies blocks NF-κB activation. *Mol Cell Biol* 1993; **13**: 4770–5.
26 Frey MR, Saxon ML, Zhao X, Rollins A, Evans SS, Black JD. Protein kinase C isozyme-mediated cell cycle arrest involves induction of $p21^{waf1/Cip1}$ and

$p27^{kip1}$ and hypophosphorylation of the retinoblastoma protein in intestinal epithelial cells. *J Biol Chem* 1997; **272**: 9424–35.
27 Borner C, Ueffing M, Jaken S, Parker PJ, Weinstein IB. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. Possible molecular mechanisms. *J Biol Chem* 1995; **270**: 78–86.
28 Scaglione-Sewell B, Abraham C, Bissonnette M, Skarosi SF, Hart J, Davidson NO, Wali RK, Davis BH, Sitrin M, Brasitus TA. Decreased PKCα expression increases cellular proliferation, decreases differentiation, and enhances the transformed phenotype of CaCo-2 cells. *Cancer Res* 1998; **58**: 1074–81.
29 Davidson LA, Brown RE, Chang WC, Morris JS, Wang N, Carroll RJ, Turner ND, Lupton JR, Chapkin RS. Morphodensitometric analysis of protein kinase C β_{II} expression in rat colon: modulation by diet and relation to *in situ* cell proliferation and apoptosis. *Carcinogenesis* 2000; **21**: 1513–19.

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Concurrent suppression of hyperlipidemia and intestinal polyp formation by NO-1886, increasing lipoprotein lipase activity in Min mice

Naoko Niho, Michihiro Mutoh, Mami Takahashi, Kazuhiko Tsutsumi, Takashi Sugimura, and Keiji Wakabayashi

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Concurrent suppression of hyperlipidemia and intestinal polyp formation by NO-1886, increasing lipoprotein lipase activity in Min mice

Naoko Niho*, Michihiro Mutoh*, Mami Takahashi*, Kazuhiko Tsutsumi†, Takashi Sugimura*, and Keiji Wakabayashi*‡

*Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; and †Research and Development, Otsuka Pharmaceutical Factory, Inc., 115 Muya-cho, Naruto, Tokushima 772-8601, Japan

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We have previously reported a hyperlipidemic state in two strains of *Apc*-deficient mice, Min and *Apc*^{1309}, associated with low expression levels of lipoprotein lipase (LPL) in the liver and small intestine, and enforced induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR)α and PPARγ agonists clearly suppressed hyperlipidemia and intestinal polyp formation in these mice. Meanwhile, a compound, NO-1886, has been shown to increase LPL mRNA and protein levels but not to possess PPARα and PPARγ agonistic activity. In this study, therefore, the effects of NO-1886 on hyperlipidemia and intestinal polyp formation were investigated in Min mice. Administration of 400 and 800 ppm NO-1886 in the diet for 13 weeks from 7 weeks of age caused a reduction of serum triglycerides to 39% and 31% of the untreated value, respectively, and the values for very low-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were improved almost to the wild-type level with a corresponding elevation of the LPL mRNA. Moreover, total numbers of intestinal polyps in the groups receiving NO-1886 at 400 and 800 ppm were decreased to 48% and 42% of the control value, respectively. We also found that NO-1886 suppressed cyclooxygenase-2 transcriptional promoter activity in a reporter gene assay and reduced cyclooxygenase-2 mRNA levels in the small intestine of Min mice. These results indicate that suppression of serum lipid levels by increasing LPL activity may contribute to a reduction of intestinal polyp formation with *Apc*-deficiency, and NO-1886 and its derivatives could be useful as chemopreventive agents for colon cancer.

Colon cancer is one of the most frequent cancers in developed countries, and many epidemiological studies have suggested a correlation with obesity and hyperlipidemia (1, 2). Recently, we reported an age-dependent hyperlipidemic state in *Apc*-deficient Min and *Apc*^{1309} mice, animal models of human familial adenomatous polyposis (3, 4). The mRNA levels for lipoprotein lipase (LPL), which catalyzes hydrolysis of triglycerides, were shown to be down-regulated in the livers and small intestines of these *Apc*-deficient animals compared with their wild-type counterparts. We also demonstrated that treatment with a peroxisome proliferator-activated receptor α (PPARα) agonist, bezafibrate, and a PPARγ agonist, pioglitazone, concomitantly suppressed hyperlipidemia and intestinal polyp formation in the mice with induction of LPL mRNA (3, 4). Thus, LPL expression levels may correlate with intestinal polyp development in *Apc*-deficient mice.

LPL is the major enzyme responsible for the hydrolysis of triglyceride-rich lipoproteins such as chylomicrons and very low-density lipoprotein (VLDL). LPL mRNA is expressed ubiquitously in the body but especially in adipose tissue and skeletal muscle, where it is synthesized then transferred to the surface of endothelial cells, to become bound to membrane-anchored heparan sulfate proteoglycans (5, 6). There are reports on association of hyperlipidemia with lowered or lack of LPL (7, 8). However, there have been no reports directly addressing links between LPL and colon carcinogenesis.

There has been great interest in development of an LPL-selective inducer for effective control of hypertriglyceridemia and low levels of high-density lipoprotein cholesterol (HDL-C) in serum. Several diethyl benzyl phosphonate derivatives were examined and one example, NO-1886, has been reported to increase LPL mRNA and protein levels, resulting in a reduction of plasma triglycerides and an increase in HDL-C levels in rats (9). NO-1886 also improves obesity in rats through induction of fatty acid oxidation-related enzymes, such as long-chain acyl-CoA dehydrogenase and acetyl-CoA acyltransferase (10). Moreover, NO-1886 reduces high-cholesterol diet-induced atherosclerotic lesions in rat coronary arteries (9).

It is well known that PPARα and PPARγ agonists improve hypertriglyceridemia and hypercholesterolemia through induction of lipid metabolism-related genes such as LPL (11). Moreover, these agonists have been documented to show antiproliferative and proapoptotic effects in various types of cancer cells, including colon cancer cells (12). Using a reporter gene assay, NO-1886 was revealed not to possess PPARα and PPARγ agonistic activity, unlike bezafibrate and pioglitazone (10). Thus, the LPL selective activator, NO-1886, may be a very essential agent for determining the relationship between hyperlipidemia due to LPL depression and colon carcinogenesis. In this study, we therefore examined the effects of 400 and 800 ppm NO-1886 in the diet on both hyperlipidemia and intestinal polyp formation in Min mice and demonstrated concomitant suppression of both. Taking account of the fact that NO-1886 also suppressed cyclooxygenase-2 (COX-2) mRNA expression, possible mechanisms of its action in *Apc*-deficient mice and the usage of NO-1886 or its derivatives as possible candidates of colon cancer prevention are also discussed.

Materials and Methods

Animals and Chemicals. Female C57BL/6-*Apc*$^{Min/+}$ mice (Min mice) were purchased from The Jackson Laboratory at 5 weeks of age and genotyped by the method reported in ref. 13. We used female animals for experimental convenience because there are no significant differences in the numbers of intestinal polyps and serum lipid levels between males and females (3, 4, 14). Heterozygotes of the Min strain and wild-type (C57BL/6J) mice were acclimated to laboratory conditions for 2 weeks. Three to five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room air-conditioned at 24 ± 2°C and 55% humidity on a 12-h light/dark cycle. The LPL selective inducer NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl]benzylphosphonate, was chemically synthesized at Otsuka Pharmaceutical Factory (9). Its structure is shown in Fig. 1. NO-1886 was well mixed at concentrations of

Abbreviations: COX, cyclooxygenase; HDL, high-density lipoprotein; HDL-C, HDL cholesterol; iNOS, inducible NO; LDL, low-density lipoprotein; LDL-C, LDL cholesterol; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; VLDL, very low-density lipoprotein; VLDL-C, VLDL cholesterol.

‡To whom correspondence should be addressed. E-mail: kwakabay@gan2.res.ncc.go.jp.

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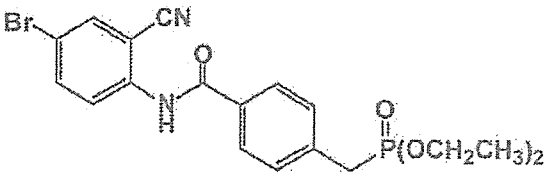

Fig. 1. Structure of NO-1886.

400 and 800 ppm with AIN-76A powdered basal diet (CLEA Japan, Tokyo). The chemical was confirmed to be stable under the experimental conditions used in this study.

Animal Experiments. To investigate the effects of NO-1886 on both hyperlipidemia and intestinal polyp formation, 10–13 female Min mice at 7 weeks of age were given 0 (control), 400, or 800 ppm NO-1886 in the diet for 13 weeks. Food and water were available ad libitum. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. At the kill time points, animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. Serum levels of triglycerides and total cholesterol were measured as reported in ref. 3. In addition, the levels of the major lipoprotein classes for cholesterol, VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), were measured by HPLC (15). The experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

The intestinal tract was removed, filled with 10% buffered formalin, and separated into the small intestine, cecum, and colon. The small intestine was divided into the proximal segment (≈4 cm in length), and then the proximal (middle) and distal halves of the remainder. All segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The numbers and sizes of polyps and their distributions in the intestine were assessed with a stereoscopic microscope (3).

RT-PCR Analysis. Tissue samples from the normal parts of liver and small intestine of mice ($n = 3$ each) were rapidly deep-frozen in liquid nitrogen and stored at −80°C. Cells of a human colon adenocarcinoma cell line, DLD-1, were purchased from the Health Science Research Resources Bank (Osaka) and cultured according to the supplier's instructions. Total RNA was isolated from tissues by using Isogen (Nippon Gene, Tokyo), treated with DNase (Invitrogen) and applied at 3-μg aliquots in a final volume of 20 μl for synthesis of cDNA by using an Omniscript RT Kit (Qiagen, Hilden, Germany) and an oligo(dT) primer. The mixture was incubated for 10 min at 32°C, then 50 min at 42°C, and immediately cooled on ice. As an internal control to confirm the integrity of the isolated mRNA, β-actin was used (3). PCR was performed with specific primers for mouse LPL (5′-primer-GGATCCGTGGCCGCAGCAGACGCAGGAAGA, 3′-primer-GAATTCCATCCAGTTGATGAATCTGGCCAC) (16), COX-1 (5′-GTCATCAAGGAGTCCCGAG, 3′-CCAGTTTCTTCAGTGAGGC), COX-2 (5′-CACACTCTATCACTGGCACC, 3′-CTCTCTGCTCTGGTCAATGG), inducible nitric oxide (iNOS) (5′-CTTGGAGCGAGTTGTGGATTG, 3′-CAGGAAGTAGGTGAGGGC), PPARγ (5′-TGAGACCAACAGCCTGACG, 3′-GATGTCAAAGGAATGCGAGTGG), PPARα (5′-TCTTACCTGTGAACACGACCTG, 3′-AGCAGTGGAAGAATCGGACC). PCR amplification of 1 μl of cDNA was carried out in a final volume of 10 μl with an PTC-200 DNA Engine (MJ Research, Waltham, MA), by using a HotStarTaq (Qiagen). Cycling conditions were as follows: 94°C for 20 sec, annealing temperature (60–64°C) for 30 sec, 72°C for 80 sec, and 25–35 cycles after an initial step of 95°C for 15 min. A final elongation step of 72°C for 10 min completed the PCR. The products were then electrophoresed in 2% agarose gels.

Reporter Gene Assay for COX-2 Promoter-Dependent Transcriptional Activity. Stable transfectants containing pB2-Gal-BSD and pCOX-2/B2-Gal-BSD in the genome DNA of DLD-1 cells were prepared as described in ref. 17. Cells were seeded at a density of 2×10^4 cells per well in 96-well plates and precultured for 24 h. They were then cotreated with type α TGF (TGFα) (100 ng/ml) and/or NO-1886 (2.5, 5, and 10 μM), and the total β-gal activities of cells in each well were determined by colorimetric assay with *o*-nitrophenyl-β-D-gal as described in ref. 17. The background β-gal activity of DLD-1 cells was determined with a control nontreated culture of DLD-1/B2-Gal-BSD cells, and the value was set as 0. The basal β-gal activity of nontreated DLD-1/COX2-B2-Gal-BSD was set as 100%. The percentage of β-gal activity with each treatment was then calculated by using data from triplicate wells. The values for β-gal activity were normalized for total protein amount. All assays were carried out in triplicate, and the experiment was repeated at least twice.

Statistical Analysis. The results were expressed as mean ± SE values, and statistical analysis was performed with Student's *t* test. Differences were considered to be statistically significant with $P < 0.05$.

Results

Improvement of Serum Lipid Levels in Min Mice by NO-1886. Consistent with our previous reports (3, 4), a hyperlipidemic state was observed in the Min mice fed the basal diet at 20 weeks of age. Namely, serum levels of triglycerides at 20 weeks of age were dramatically increased to almost 30 times the wild-type value (Fig. 2*A*). Total cholesterol levels in Min mice were also increased 1.7-fold (Fig. 2*B*). Moreover, VLDL cholesterol

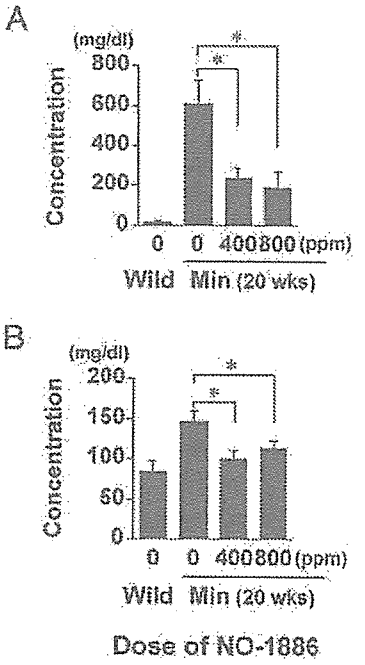

Fig. 2. Suppression of serum lipid levels in Min mice by NO-1886. Values for serum levels of triglyceride (*A*) and total cholesterol (*B*) in female Min mice given diet containing NO-1886 at doses of 0 ($n = 7$), 400 ($n = 8$), and 800 ppm ($n = 10$) for 13 weeks and wild-type mice ($n = 6$) are shown. Data are means; bars are SE. *, $P < 0.05$; **, $P < 0.01$.

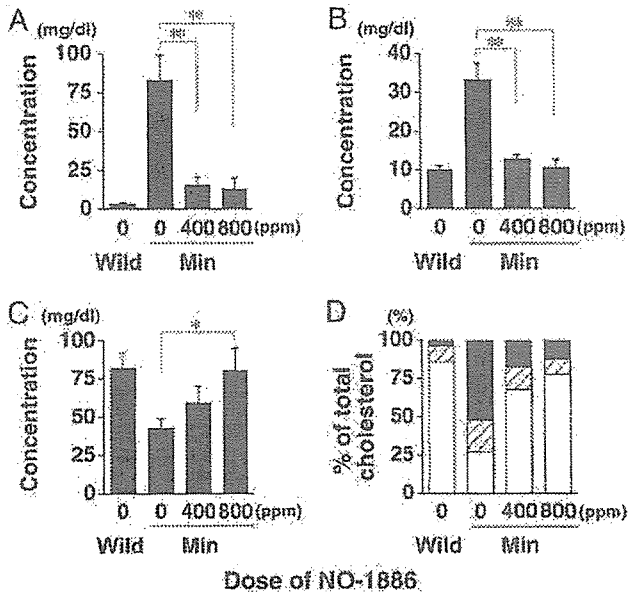

Fig. 3. Serum levels of cholesterol lipoproteins in female Min mice treated with NO-1886. Shown are lipoprotein classes in female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks and wild-type mice. (*A*) VLDL cholesterol. (*B*) LDL cholesterol. (*C*) HDL cholesterol. (*D*) The proportions of cholesterol lipoproteins. Open box, HDL cholesterol; crosshatched box, LDL cholesterol; filled box, VLDL cholesterol). Data are means; bars are SE ($n = 5$). *, $P < 0.05$; **, $P < 0.01$.

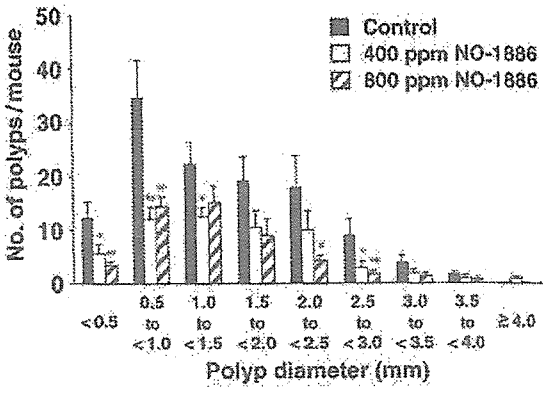

Fig. 4. Effects of NO-1886 on the size distribution of intestinal polyps in Min mice. Min mice were fed a basal diet (filled box) or a diet containing 400 ppm (open box) or 800 ppm (hatched box) NO-1886 for 13 weeks. The number of polyps per mouse in each size class is given as a mean value; bars are SE. *, $P < 0.05$; **, $P < 0.01$.

(VLDL-C) levels in the basal diet group of Min mice were 24-fold higher than in their wild-type counterparts (Fig. 3*A*). LDL cholesterol (LDL-C) levels were increased 3.3-fold, whereas HDL-C was decreased to 50% of the wild-type value (Fig. 3 *B* and *C*). The proportions of HDL-C, LDL-C, and VLDL-C in the total cholesterol in Min mice (27%, 21%, and 52%, respectively) were almost opposite to those in wild-type mice (86%, 10%, and 4%, respectively) (Fig. 3*D*).

Administration of 400 and 800 ppm NO-1886 did not affect body weights or clinical signs of Min mice throughout the experimental period. Amounts of daily food intake were not different among groups, and daily intakes of NO-1886 in the 400- and 800-ppm groups of Min mice were 1.2–1.5 mg per mouse per day and 2.8–3.1 mg per mouse per day, respectively. In addition, there were no changes observed in any organ weights that could be attributable to toxicity. Administration of 400 and 800 ppm NO-1886 clearly decreased serum levels of triglycerides to 39% and 31% of the untreated control value, respectively (Fig. 2*A*). The levels of total cholesterol were also decreased to 69% and 77% of the untreated control value (Fig. 2*B*). Furthermore, levels of both triglyceride-rich lipoproteins, VLDL-C and LDL-C, were dramatically suppressed by NO-1886 treatment. The levels of VLDL-C in the groups treated with NO-1886 at 400 and 800 ppm were reduced to 19% and 15% of the untreated control value, and the levels of LDL-C were to 39% and 32% of the untreated control value, respectively (Fig. 3 *A* and *B*). In contrast, HDL-C levels were increased to the wild-type value at 800 ppm (Fig. 3*C*). Overall, administration of NO-1886 improved the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice (Fig. 3*D*).

Suppression of Intestinal Polyp Formation in Min Mice by NO-1886. Table 1 summarizes data for the number and distribution of intestinal polyps in the basal diet and NO-1886-treated groups. Almost all polyps developed in the small intestine, with only a few in the colon (Table 1). The total number of polyps were significantly decreased by administration of 400- and 800-ppm NO-1886 to 48% and 42% of the untreated control value, respectively, with reduction in the proximal, middle, and distal parts by 63%, 57%, and 45% with 400 ppm, and by 74%, 63%, and 49% with 800 ppm. Treatment with NO-1886 also significantly decreased the numbers of colon polyps.

Fig. 4 shows the size distributions of intestinal polyps in the basal diet and NO-1886-treated groups. The main polyp sizes observed in the basal diet groups were 0.5–3.0 mm in diameter. Administration of NO-1886 reduced the numbers of polyps of all sizes.

Down-Regulation of COX-2 Transcriptional Activity by NO-1886. Expression of enzymes associated with inflammation has been reported to be increased in colon carcinogenesis (18). To cast light on mechanisms of the effect of NO-1886 on colon carcinogenesis, we investigated expression levels of mRNAs for COX-1, COX-2, and iNOS in DLD-1 human colon cancer cells by RT-PCR. As shown in Fig. 5*A*, the TGFα-stimulated mRNA levels for COX-2 were reduced to nonstimulated mRNA levels by NO-1886 in DLD-1 cells. On the other hand,

Table 1. Suppression of intestinal polyp development in Min mice by NO-1886

Dose, ppm	No. of mice	Small intestine			Colon	Total
		Proximal	Middle	Distal		
0	7	23.1 ± 4.2	37.1 ± 11.1	60.4 ± 12.2	1.0 ± 0.2	121.7 ± 26.0
400	8	8.5 ± 1.4 (37)*	16.1 ± 3.8 (43)	33.0 ± 6.5 (55)	0.4 ± 0.2 (38)†	58.0 ± 10.8 (48)†
800	10	5.9 ± 1.0 (26)*	13.8 ± 2.6 (37)†	30.5 ± 5.0 (51)†	0.3 ± 0.2 (30)†	50.5 ± 7.8 (42)*

Data are means ± SE of the number of polyps per mouse. Numbers in parentheses are percentages of the control value.
*Significantly different from the basal diet group at $P < 0.01$.
†Significantly different from the basal diet group at $P < 0.05$.

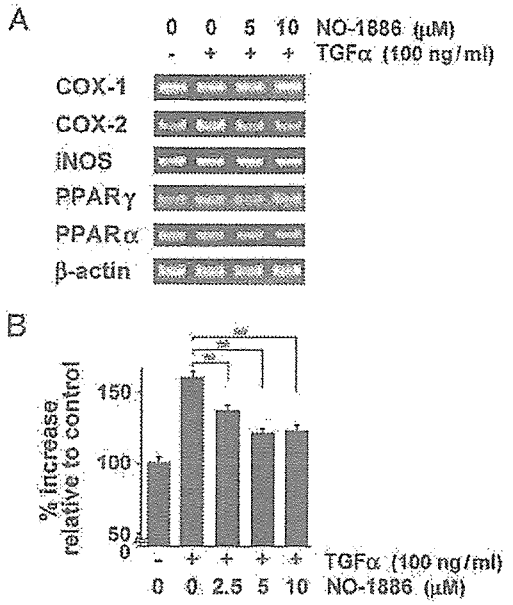

Fig. 5. Suppression of COX-2 mRNA level and COX-2 transcriptional activity in a human colon cancer cell line by NO-1886. (*A*) RT-PCR analysis of mRNA expression levels for COX-1, COX-2, iNOS, PPARγ, and PPARα in DLD-1 cells treated with the indicated dose of TGFα and/or NO-1886. (*B*) Reporter gene assay for COX-2 promoter-dependent transcriptional activity in DLD-1 cells. All assays were carried out in triplicate, and data are representative of at least two separate experiments. Data are means; bars are SD. **, $P < 0.01$.

there was no obvious variation in the mRNA levels for COX-1 and iNOS (Fig. 5*A*). In addition, we confirmed that NO-1886 did not change the mRNA levels for PPARγ and PPARα in DLD-1 cells (Fig. 5*A*). Fig. 5*B* shows the results for β-gal reporter gene assay in DLD-1 cells. Treatment of cells with 100 ng/ml TGFα for 48 h increased COX-2 transcriptional levels to 1.6-fold of the control value, whereas NO-1886 at 5 and 10 μM suppressed TGFα-stimulated COX-2 transcriptional activity to 1.2-fold of the control value. No significant decrease of cell viability was observed after 48 h culture with NO-1886 at these concentrations.

Expression of LPL and COX-2 mRNAs in Liver and Small Intestine in Min Mice Assessed by RT-PCR. LPL mRNA levels in the liver and the small intestine in the Min mice at 20 weeks of age were very low but were markedly increased by the treatment with NO-1886 dose-dependently (Fig. 6). Consistent with the *in vitro* data in Fig. 5, administration of NO-1886 reduced mRNA levels of COX-2 in normal parts of small intestine of Min mice at 20 weeks of age (Fig. 6*B*).

Discussion

This study provided clear evidence that administration of the LPL selective inducer NO-1886, which increases LPL mRNA and protein levels, suppresses both hyperlipidemia and intestinal polyp formation in Min mice. Decrease in serum triglycerides, VLDL-C and LDL-C, and an increase in HDL-C were demonstrated, with elevation of LPL mRNA level and suppression of COX-2 expression. NO-1886 is shown not to have a potential of PPARα and PPARγ agonists (10). It is therefore speculated that LPL activity itself may play an important role in the intestinal polyp formation in *Apc*-deficient mice.

We earlier reported dramatically increased serum levels of triglycerides and markedly low levels of liver and small intestine LPL mRNA in Min mice compared with their wild-type counterparts (3, 4). This data provided concrete evidence that the expression levels of LPL, which catalyzes hydrolysis of triglycerides, correlate with hypertriglyceridemia in Min mice. At present, it still cannot be stated with certainty whether hyperlipidemia is a leading cause of intestinal polyp formation. Colon tumors induced by 1,2-dimethylhydrazine in rats, however, are not linked to serum lipid levels (19). As hyperlipidemia and polyp formation could be related to *Apc*-deficiency independently, we now address whether low LPL activity and high serum lipid levels could promote intestinal polyp formation in these mice.

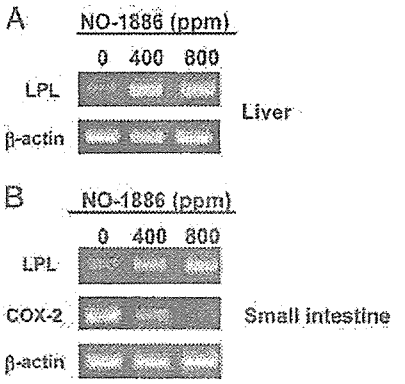

Fig. 6. Changes of mRNA levels for LPL and COX-2 in the liver and small intestine of female Min mice. (*A*) RT-PCR analysis of LPL mRNA expression in the livers of female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks. (*B*) RT-PCR analysis of LPL and COX-2 mRNA expression in normal parts of the small intestine of female Min mice given diets containing NO-1886 at doses of 0, 400, and 800 ppm for 13 weeks. Data are representative of three mice of each group.

It has been reported that inflammation-associated enzymes such as COX-2 and iNOS are overexpressed in colon carcinogenesis (18). Treatment with NO-1886 reduced COX-2 expression levels in a reporter gene assay as well as normal parts of the small intestine of Min mice. Immunohistochemically, expression of COX-2 is reported to be observed in normal parts of the small intestine in Min mice (20). Moreover, it is well known that expression of COX-2 is markedly elevated in colon cancers of humans and AOM-treated rats and in intestinal polyps of *Apc*-deficient mice (21–23), playing an important role in cancer cell proliferation and angiogenesis (24). Therefore, down-regulation of COX-2 by NO-1886 is clearly one possible mechanism underlying suppression of intestinal polyp development.

There is evidence that prostaglandin (PG)E_2, produced by COX-1 and COX-2, is a potent inhibitor of LPL expression in macrophages (25). PGE_2 levels are also known to be elevated in both human and rodent colon tumors (26, 27). These findings support the speculation that the role of LPL in intestinal polyp development may be associated with the arachidonic cascade.

In conclusion, this study indicates that the LPL selective inducer, NO-1886, has potential benefits for treatment of both hyperlipidemia and intestinal polyp development. LPL may be a good target for chemoprevention. Thus, NO-1886 and its derivatives are suggested to be promising candidate chemopreventive agents for colon cancer. It is very important to now clarify LPL functions and their significance for cancer development.

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1. Le Marchand, L., Wilkens, L. R., Kolonel, L. N., Hankin, J. H. & Lyu, L. C. (1997) *Cancer Res.* **57**, 4787–4794.
2. Bruce, W. R., Wolever, T. M. & Giacca, A. (2000) *Nutr. Cancer* **37**, 19–26.
3. Niho, N., Takahashi, M., Kitamura, T., Shoji, Y., Itoh, M., Noda, T., Sugimura, T. & Wakabayashi, K. (2003) *Cancer Res.* **63**, 6090–6095.
4. Niho, N., Takahashi, M., Shoji, Y., Takeuchi, Y., Matsubara, S., Sugimura, T. & Wakabayashi, K. (2003) *Cancer Sci.* **94**, 960–964.
5. Semenkovich, C. F., Chen, S. H., Wims, M., Luo, C. C., Li, W. H. & Chan, L. (1989) *J. Lipid Res.* **30**, 423–431.
6. Goldberg, I. J. (1996) *J. Lipid Res.* **37**, 693–707.
7. Gehrisch, S. (1999) *Curr. Atheroscler. Rep.* **1**, 70–78.
8. Mead, J. R., Irvine, S. A. & Ramji, D. P. (2002) *J. Mol. Med.* **80**, 753–769.
9. Tsutsumi, K., Inoue, Y., Shima, A., Iwasaki, K., Kawamura, M. & Murase, T. (1993) *J. Clin. Invest.* **92**, 411–417.
10. Doi, M., Kondo, Y. & Tsutsumi, K. (2003) *Metabolism* **52**, 1547–1550.
11. Schoonjans, K., Staels, B. & Auwerx, J. (1996) *Biochim. Biophys. Acta* **1302**, 93–109.
12. Rosen, E. D. & Spiegelman, B. M. (2001) *J. Biol. Chem.* **276**, 37731–37734.
13. Moser, A. R., Pitot, H. C. & Dove, W. F. (1990) *Science* **247**, 322–324.
14. Mutoh, M., Watanabe, K., Kitamura, T., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., *et al.* (2002) *Cancer Res.* **62**, 28–32.
15. Usui, S., Hara, Y., Hosaki, S. & Okazaki, M. (2002) *J. Lipid Res.* **43**, 805–814.
16. Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B. & Auwerx, J. (1996) *EMBO J.* **15**, 5336–5348.
17. Mutoh, M., Takahashi, M., Fukuda, K., Matsushima-Hibiya, Y., Mutoh, H., Sugimura, T. & Wakabayashi, K. (2000) *Carcinogenesis* **21**, 959–963.
18. Bing, R. J., Miyataka, M., Rich, K. A., Hanson, N., Wang, X., Slosser, H. D. & Shi, S. R. (2001) *Clin. Cancer Res.* **7**, 3385–3392.
19. Barton, T. P., Cruse, J. P. & Lewin, M. R. (1987) *Br. J. Cancer* **56**, 451–454.
20. Hull, M. A., Booth, J. K., Tisbury, A., Scott, N., Bonifer, C., Markham, A. F. & Coletta, P. L. (1999) *Br. J. Cancer* **79**, 1399–1405.
21. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. & Hla, T. (1995) *Cancer Res.* **55**, 3785–3789.
22. DuBois, R. N., Radhika, A., Reddy, B. S. & Entingh, A. J. (1996) *Gastroenterology* **110**, 1259–1262.
23. Williams, C. S., Luongo, C., Radhika, A., Zhang, T., Lamps, L. W., Nanney, L. B., Beauchamp, R. D. & DuBois, R. N. (1996) *Gastroenterology* **111**, 1134–1140.
24. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. & DuBois, R. N. (1998) *Cell* **93**, 705–716.
25. Desanctis, J. B., Varesio, L. & Radzioch, D. (1994) *Immunology* **81**, 605–610.
26. Pugh, S. & Thomas, G. A. (1994) *Gut* **35**, 675–678.
27. Chulada, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H. F., Morham, S. G., Smithies, O. & Langenbach, R. (2000) *Cancer Res.* **60**, 4705–4708.

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Dextran sodium sulfate strongly promotes colorectal carcinogenesis in $Apc^{Min/+}$ mice: Inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms

Takuji Tanaka[1*], Hiroyuki Kohno[1], Rikako Suzuki[1], Kazuya Hata[2], Shigeyuki Sugie[1], Naoko Niho[3], Katsuhisa Sakano[3], Mami Takahashi[3] and Keiji Wakabayashi[3]

[1]*Department of Oncologic Pathology, Kanazawa Medical University, Uchinada, Ishikawa, Japan*
[2]*BMR Laboratories, Sunplanet Co., Ltd., Kamiishidu, Gifu Japan*
[3]*Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan*

The mouse model for familial adenomatous polyposis, $Apc^{Min/+}$ mouse, contains a truncating mutation in the *Apc* gene and spontaneously develops numerous adenomas in the small intestine but few in the large bowel. Our study investigated whether dextran sodium sulfate (DSS) treatment promotes the development of colonic neoplasms in $Apc^{Min/+}$ mice. $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes were exposed to 2% dextran sodium sulfate in drinking water for 7 days, followed by no further treatment for 4 weeks. Immunohistochemistry for cyclooxygenase-2, inducible nitric oxide synthase, β-catenin, p53, and nitrotyrosine, and mutations of β-*catenin* and K-*ras* and loss of wild-type allele of the *Apc* gene in the colonic lesions were examined. Sequential observation of female $Apc^{Min/+}$ mice that received DSS was also performed up to week 5. At week 5, numerous colonic neoplasms developed in male and female $Apc^{Min/+}$ mice but did not develop in $Apc^{+/+}$ mice. Adenocarcinomas developed in $Apc^{Min/+}$ mice that received DSS showed loss of heterozygosity of *Apc* and no mutations in the β-*catenin* and K-*ras* genes. The treatment also significantly increased the number of small intestinal polyps. Sequential observation revealed increase in the incidences of colonic neoplasms and dysplastic crypts in female $Apc^{Min/+}$ mice given DSS. DSS treatment increased inflammation scores, associated with high intensity staining of β-catenin, cyclooxygenase-2, inducible nitric oxide synthase and nitrotyrosine. Interestingly, strong nuclear staining of p53 was specifically observed in colonic lesions of $Apc^{Min/+}$ mice treated with DSS. Our results suggest a strong promotion effect of DSS in the intestinal carcinogenesis of $Apc^{Min/+}$ mice. The findings also suggest that strong oxidative/nitrosative stress caused by DSS-induced inflammation may contribute to the colonic neoplasms development.
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Key words: $Apc^{Min/+}$; mice; dextran sodium sulfate; colon carcinogenesis; p53; nitrotyrosine

Carcinogenesis and inflammation are pathological consequences of injury and repair at the cellular and molecular levels[1,2] and are influenced by several life style factors, including dietary factors.[3] Recent studies suggest inflammation in enhancing the risk of various types of cancer[2] including colon cancer.[4] In fact, individuals suffering with inflammatory bowel disease (IBD) are at high risk of developing colon cancer.[5,6] We recently proposed a novel mouse colon carcinogenesis model and demonstrated the powerful tumor-promoting effects of dextran sodium sulfate (DSS), which can induce colonic mucosal inflammation, resembling the histopathology of one of the IBD ulcerative colitis (UC),[7] on colon carcinogenesis initiated with azoxymethane (AOM),[8–10] 1,2-dimetylhydrazine (DMH)[11] or heterocyclic amines (HCAs)[12] in mice. Thus, inflammation/inflammatory stimuli induced by a short-term (for a week) treatment with 2% DSS in drinking water after initiation with a low-dose of carcinogens is effective for rapid induction of colon neoplasms possessing β-catenin gene mutations in mice.[11,12] Similarly, Cooper *et al.*[13] found that inflammation plays an important role in the dysplasia-cancer sequence in the colon. They also reported the development of colon cancer in 60-day-old $Apc^{Min/+}$ mice that received 4% DSS alone.[14] In addition, Barbour *et al.*[15] suggested that a relationship between chronic inflammation and small intestinal tumorigenesis in $Apc^{Min/+}$ mice.

Cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) play an important role in colon tumor growth and progression. COX catalyzes the committed step in the conversion of arachidonic acid to protumorigenic eicosanoids, such as prostaglandin E_2, which are involved in the maintenance of tumor integrity.[16] COX-2 is frequently undetectable in normal tissues but is induced by cytokines, growth factors, reactive oxygen species and tumor promoters.[17] Gene expression of COX-2 is upregulated in 80–85% of human colonic adenocarcinomas,[18] in colonic tumors induced by AOM in rodents[19] and in 80–85% of $Apc^{Min/+}$ mouse adenomas.[20] Nitric oxide (NO) is endogenously produced by a family of enzymes. NO is reported to cause mutagenesis[21] and DNA deamination,[22] and is implicated in the inflammatory responses and in the production of vascular endothelial growth factor.[23] Several studies also report that iNOS is up-regulated in human cancers, including colon cancer[24,25] and in AOM-induced colon tumors in rodents.[26] In addition, one study reported that iNOS inhibitors suppress the development of AOM-induced aberrant crypt foci in rats.[27] Although the role of iNOS plus NO and related radical species in intestinal polyposis is still controversial,[28,29] NO/iNOS may be involved in intestinal tumorigenesis.[30–33] The interaction between iNOS and p53 as a crucial pathway in inflammatory-mediated carcinogenesis is also suggested.[34] An increased cancer risk occurs in the tissues undergoing chronic inflammation.[35] Thus, NO is a candidate free radical, and the *p53* tumor suppressor gene is a candidate molecular target.[36]

Familial adenomatous polyposis (FAP) is an inherited form of human colon cancer characterized by the development of 100–1,000 adenomas in the large intestine.[37] If not removed, these benign epithelial neoplasms inevitably progress to carcinomas.[37] FAP can be caused by germline mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene.[38] Min mice were a germline mutation in the *Apc* gene and develop multiple polyps in the intestine.[39] *Apc*-deficient mice including Min mice are considered to be good models of FAP and have been used for investigating the influence of environmental factors, such as dietary factors, carcinogens, chemopreventive agents and other xenobiotics.[40] However, unfortunately, unlike human FAP, most of the neo-

Abbreviations: AOM, azoxymethane; APC, adenomatous polyposis coli; COX, cyclooxygenase; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; H&E, hematoxylin and eosin; HCAs, heterocyclic amines; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; LOH, loss of heterozygosity; NO, nitric oxide; UC, ulcerative colitis.

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***Correspondence to:** Fax: +81-76-826-6926.
E-mail: takutt@kanazawa-med.ac.jp

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plasms occur predominantly in the small intestine of these genetically altered mice. Yamada *et al.*[41] recently reported that a number of adenomatous lesions together with a few tumors are present in the colon of old $Apc^{Min/+}$ mice. The finding suggests the presence of precursor cryptal lesions for colonic epithelial malignancies and the possibility of progression of the lesions to epithelial neoplasms under appropriate experimental conditions. Mutations of several genes, including *Apc*, β-*catenin*, K-*ras*, *DCC*, *p53* and alterations proteins' expression, such as COX-2, β-catenin, iNOS and Wnt/Apc/β-catenin signaling, play important roles in both chemically induced colon carcinogenesis and human cancer development.[33] Thus, colon carcinogenesis is characterized by a succession of molecular changes involving basic cellular process such as cell proliferation, cell signaling and DNA integrity, but it is poorly understood what sifts the balance between them, causing a cryptal cell to lose its normal phenotype. Such knowledge could be crucial for the first step in fighting colon cancer development.

In our study, we investigated whether acute inflammation induced by DSS enhances small and large intestinal carcinogenesis in $Apc^{Min/+}$ mice. Mutational analysis of β-*catenin* and K-*ras* genes and immunohistochemical analysis of Apc, β-catenin, COX-2, iNOS and p53 expression were also performed in the colonic neoplasms. The immunohistochemistry of nitrotyrosine, a good marker for oxidative stress caused by inflammation,[42] was performed on the colonic mucosa of mice given DSS. In addition, sequential pathological alteration of the large intestines of female $Apc^{Min/+}$ mice exposed to DSS was investigated to test our hypothesis that inflammation induced by DSS promotes the growth of the early colonic cryptal lesions, dysplastic aberrant crypt foci[43] or adenomatous lesions[41] and the treatment resulted in the high frequency of colonic neoplasms in the short-term (5 weeks).

Material and methods

Animals, chemicals and diets

Male and female C57BL/6J $Apc^{Min/+}$ and $Apc^{+/+}$ mice aged 3 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were genotyped to identify carriers of the *Min* allele of *Apc* with a PCR assay as described.[44] They were housed in plastic cages (4 or 5 mice/cage) under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2°C). Drinking water and a pelleted basal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan) were available *ad libitum*. They were quarantined for 7 days after arrival and then randomized by body weights into experimental and control groups. DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH).

Experimental procedure

Forty-seven $Apc^{Min/+}$ mice (16 males and 31 females) and 50 $Apc^{+/+}$ mice (29 males and 21 females) were used. Animals of the experimental groups were given 2% (w/v) DSS in drinking water for 1 week, starting 4 weeks of age. The control group (9 male and 10 female $Apc^{Min/+}$ mice, and 17 male and 11 female $Apc^{+/+}$ mice) were given the tap water without DSS throughout the experiment. Among them, 14 female $Apc^{Min/+}$ mice exposed to 2% DSS were sequentially sacrificed at weeks 2 (4 mice), 3 (5 mice) and 4 (5 mice) to monitor the pathological alterations in the large intestine. All the remaining animals were sacrificed at week 5. At sacrifice, all organs were removed, and the small and large intestines were cut open along their longitudinal axis, and fixed flat in 10% buffered formalin for 24 hr at room temperature after macroscopic inspection. Longitudinal sections of the large intestine were made, and then processes for histopathological examination were performed by routine procedures. Small intestine was divided into 3 equal segments (proximal, middle and distal parts), the number and distribution were determined under a dissecting microscope Nikon SMZ1000 (Nikon Co., Tokyo, Japan). After counting, cross sections of the small intestine were made at 2 mm intervals and processed for histopathological evaluation of the polyps by routine procedures. Histological examination was performed on hematoxylin and eosin (H&E)-stained sections. On H&E-stained sections, histological alterations, such as mucosal dysplasia and colonic tumors, were examined. Colonic mucosal dysplasia was diagnosed according to the criteria described by Paulsen *et al.*[43] Colonic tumors were diagnosed according to the description by Ward.[45]

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 with or without mucosal ulceration was graded according to the following morphological criteria described by Cooper *et al.*:[46] grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 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 on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry

Immunohistochemical analyses for β-catenin, COX-2, iNOS, p53 and nitrotyrosine were carried out with 4 μm-thick paraffin-embedded sections as previously described[8,9,47] or a report by Mollersen *et al.*[48] As the primary antibodies, anti-β-catenin mouse monoclonal antibody (diluted 1:1,000, Transduction Laboratories, Lexington, KY), anti-COX-2 mouse monoclonal antibody (diluted 1:200, Transduction Laboratories), anti-iNOS mouse monoclonal antibody (diluted 1:250, Transduction Laboratories), anti-p53 rabbit polyclonal antibodies (CM5, diluted 1:100, Novocastra Laboratories, Ltd., Newcastle, UK) and rabbit polyclonal anti-nitrotyrosine (diluted 1:500, Upstate Biotechnology, Lake Placid, NY) were used. 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 p53 and nitrotyrosine immunohistochemistry, normal rabbit serum was used to block background staining. Nonspecific binding was blocked by incubating the slides with a blocking solution (0.1 M PBS containing 0.1% triton X-100 and 2% normal goat serum) for nitrotyrosine. Staining was performed using a LSAB KIT or DAKO EnVision kit (DAKO, Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). At the last step, the sections were counterstained with hematoxylin. As a negative control, omission of the primary antibody was used. To quantitate the degree of nitrotyrosine stainability, the grading system (Grade 0–4) was used according to the following criteria described by Zingarelli *et al.*[49]: Grade 0, no immunoreactivity; Grades 1–3, increasing degrees of intermediate immunoreactivity and Grade 4, extensive immunoreactivity.

Apc allelic loss analysis

Seventeen tissues (14 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received 2% DSS, and 5 tissues (2 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received tap water without DSS were selected at random for *Apc* allelic loss analysis. They were digested overnight at 50°C in 20 μl of lysis buffer containing 500 μg/ml proteinase K, 10 mmol/liter Tris-HCl (pH 8.0), 50 mmol/liter KCl, 0.45% NP40 and 0.45% Tween 20. The proteinase K was heat inactivated (10 min at 95°C). The tubes were centrifuged for 5 min, and the supernatant was transferred to new tubes. Loss of heterozygosity (LOH) of the *Apc* gene was checked using PCR with mismatched primers, as described previously.[50]

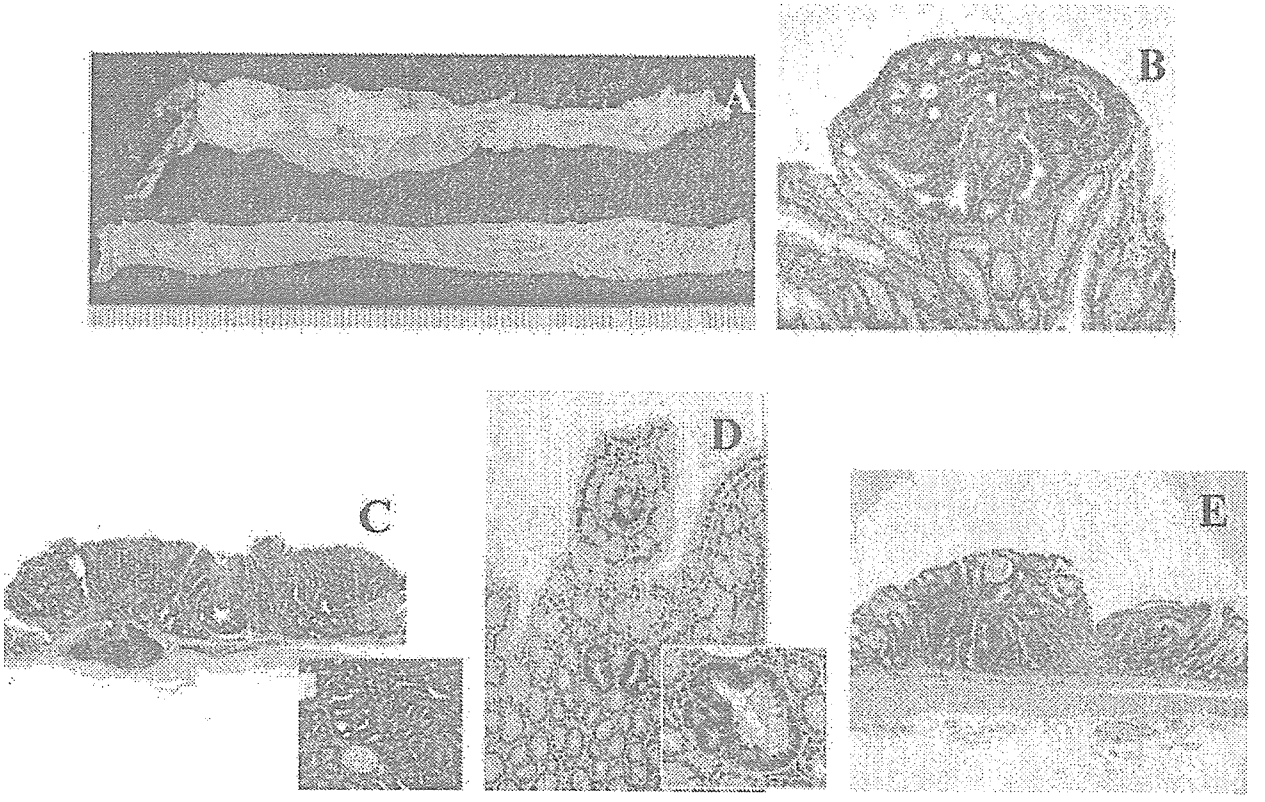

FIGURE 1 – Macroscopic view of the large bowel (*a*) and histopathology of the colonic lesions (*b–e*) of male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*) Male $Apc^{Min/+}$ mice given 2% DSS had multiple colonic tumors (upper), while male $Apc^{Min/+}$ mice given tap had a few colonic tumors (lower); (*b*) A polypoid tumor is diagnosed as tubular adenoma compressing surrounding crypts; (*c*) A nodular tumor is diagnosed as well-differentiated tubular adenocarcinoma (insert: cancer cells with tubular pattern); (*d*) Three dysplastic crypts with hyperchromatic nuclei (insert: a dysplastic crypt with bud formation) are noted in the colonic mucosa; and (*e*) Colonic mucosal ulcer with regenerative hyperplasia is seen in the colonic mucosa. H&E stain, original magnification: (*b*), (*d*), ×100; (*c*), ×10; (*e*) ×20; (*c*, insert), ×100; and (*d*, insert), ×200.

Briefly, the amplification of the Apc^{Min} allele resulted in a 155 bp PCR product with 1 *Hind*III site, whereas the 155 bp product from the Apc^{+} allele contained 2 *Hind*III sites. *Hind*III digestion of PCR-amplified DNA from $Apc^{Min/+}$ heterozygous tissue resulted in a 123 bp product from the Apc^{+} allele and a 144 bp product from the Apc^{Min} allele. Therefore, PCR products from tissue with LOH displayed only 1 band (144 bp) from the Apc^{Min} allele. Samples were assayed at least twice, independently.

DNA sequencing and mutation analysis of β-catenin and K-Ras genes

A total of 17 tissues (14 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received 2% DSS were subjected to analysis of β-*catenin* and K-*ras*. Also, a total of 5 tissues (2 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received tap water without DSS were subjected to analysis of these genes. PCR was performed in β-*catenin* and K-*ras* genes and the statuses were determined by direct sequencing. Exon 3 of the β-*catenin* gene (McatF, 5′-TCTCCTTGG CTGGCCTTTCTA-3′; McatR, 5′-GTCACACAGCCCTGTCAAGA-3′) and exon 1 of the k-*ras* gene (MrasF, 5′-GCCTGCTGAAAATGACTGAG-3′; MrasR, 5′-CTTTACAAGCGCACGCAGAC-3′) were amplified by PCR. Primers were included in the following PCR reaction mixture, which contained in a total volume of 20 μl: 20 μM of each primer, 200 μM of each deoxynucleotide triphosphate, 1 unit of *Taq* polymerase in 1 × PCR buffer (Promega. Madison, WI) and template DNA. The mixture was heated at 94°C for 5 min and subjected to 30 cycles of denaturation (94°C, 45 sec), annealing (57°C, 45 sec) and extension (72°C, 1 min) using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The products were sequenced directly after gel-purification in both directions using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's recommendations. Reactions were analyzed on an ABI Prism 3100 DNA Sequencer (Applied Biosystems).

Statistical analysis

Statistical significance of differences was evaluated by one-way ANOVA with Bonferroni correction or Fisher's exact probability test. Values were considered significantly different when $p < 0.05$.

Results

Pathological findings

$Apc^{Min/+}$ mice, but not $Apc^{+/+}$ mice, of both sexes exposed to 2 % DSS had bloody stools during DSS exposure. Other animals were healthy during the study. At week 5, macroscopically, a number of nodular, polypoid or caterpillar-like colonic tumors (Fig. 1*a*) were observed mainly in the middle and distal colon of male and female $Apc^{Min/+}$ mice treated with 2% DSS, but few in those treated with tap water. Microscopically, they were tubular adenoma (Fig. 1*b*) or well-/moderately-differentiated tubular adenocarcinoma (Fig. 1*c*). Similarly, dysplastic crypts (Fig. 1*d*) were frequently observed in all $Apc^{Min/+}$ mice of both sexes. Also, mucosal ulcer was noted in mice given 2% DSS in drinking water (Fig. 1*e*).

TABLE I – INCIDENCE AND MULTIPLICITY OF LARGE INTESTINAL TUMORS AND DYSPLASTIC CRYPTS AT WEEK 5

Genotype	Sex	Total: incidence (multiplicity[1])		Colonic tumors: incidence (multiplicity) AD[2]		ADC[2]		Dysplastic crypts	
		2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water
$Apc^{min/+}$	Male	7/7, 100%[3] (9.43±3.31[4])	3/9, 33% (0.44±0.73)	7/7, 100%[4] (3.86±2.19[4])	2/9, 22% (0.22±0.44)	7/7, 100%[4] (5.57±2.37[4])	2/9, 22% (0.22±0.44)	7/7, 100% (18.86±2.18[4])	9/9, 100% (6.56±1.67)
	Female	7/7, 100%[5] (8.29±5.02)[5]	3/10, 30% (0.50±0.97)	5/7, 71% (3.29±3.04[6])	2/10, 20% (0.30±0.67)	7/7,100% (5.00±2.16[5])	2/10, 20% (0.20±0.42)	7/7, 100% (13.29±3.45[6])	10/10, 100% (7.70±4.14)
$Apc^{+/+}$	Male	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)
	Female	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)

[1]No. of tumors/mouse, Mean ± SD.–[2]AD, adenoma; and ADC, adenocarcinoma.–[3]Significantly different from $Apc^{Min/+}$ males received tap water by one-way ANOVA with Fisher's exact probability test ($P < 0.05$).–[4]Significantly different from $Apc^{Min/+}$ males received tap water by Fisher's exact probability test or one-way ANOVA with Bonferroni correction ($P < 0.01$).–[5]Significantly different from $Apc^{Min/+}$ females received tap water by Fisher's exact probability test or one-way ANOVA with Bonferroni correction ($P < 0.01$).–[6]Significantly different from $Apc^{Min/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).

The incidences and multiplicities of colonic neoplasms (adenomas and adenocarcinomas) and total colonic tumors are summarized in Table I. The incidences of total tumors and adenocarcinomas in $Apc^{Min/+}$ mice of both sexes given 2% DSS were significantly greater than those given tap water alone (total tumors: males, 100% *vs.* 33%, $p < 0.05$ and females, 100% vs. 30%, $p < 0.01$; adenocarcinoma: males, 100% *vs.* 22%, $p < 0.01$ and females, 100% *vs.* 20%, $p < 0.01$). Treatment with 2% DSS significantly increased the incidence of colonic adenomas in male $Apc^{Min/+}$ mice when compared to that of male $Apc^{Min/+}$ given tap water alone ($p < 0.01$). As for dysplastic foci (Table I), the frequencies in *ApcMin/+* mice of both sexes given 2% DSS were significantly greater than those given tap water alone ($p < 0.01$ for males and $p < 0.05$ for females).

Time-course observation of colonic tumors in female $Apc^{Min/+}$ mice revealed that the initial tumor (histologically tubular adenoma) developed at week 2 (Fig. 2*a*). The incidence of colonic adenomas reached 100% at week 3 and that of adenocarcinomas did at week 5, respectively (Fig. 2*a*), and their multiplicitis gradually increased up to week 5 (Fig. 2*b*). As for the frequency of dysplastic foci, there was no further increase in dysplastic crypts from week 4 to week 5 (Fig. 2*c*). The value at week 5 was significantly larger than that at week 2 ($p < 0.05$).

As summarized in Table II, a number of small intestinal polyps (histologically tubular adenoma) developed in all $Apc^{Min/+}$ mice with both sexes treated with or without 2% DSS, but not in $Apc^{+/+}$ mice with both sexes treated with or without 2% DSS. Their frequencies in $Apc^{Min/+}$ males and females given 2% DSS were significantly greater than in those given tap water alone ($p < 0.05$ for males and $p < 0.05$ for females). Considering the distribution of the polyps, significant increases in number were found at the distal region ($p < 0.01$ for males and $p < 0.01$ for females) in $Apc^{Min/+}$ mice of both sexes treated with 2% DSS when compared to those in $Apc^{Min/+}$ mice that received tap water. At the middle regions of small intestine the numbers of polyps were significantly decreased ($p < 0.05$ for males and $p < 0.01$ for females) in 2% DSS treated *ApcMin/+* mice of both sexes. Also, 2% DSS treatment increased the size (by 18%) of polyps in the small intestine. On the other hand, we could not find any polyps or tumors in the small intestine of wild type mice.

Score for inflammation in the iintestine

Table III summarizes data on colonic inflammation scores at week 5. The values in $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes treated with 2% DSS were significantly larger than those given tap water alone ($p < 0.01$). No significant differences on the degrees of colonic mucosal inflammation were noted between mice of 2 genotypes, $Apc^{Min/+}$ and $Apc^{+/+}$. Scoring of inflammation in the time-course study indicated that the value decreased after the cessation of 2% DSS (Fig. 2*d*). DSS exposure also produced small intestinal inflammation in both $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes: the inflammation scores in $Apc^{Min/+}$ mice were relatively greater than those in $Apc^{+/+}$ mice (data not shown). The scores of $Apc^{Min/+}$ mice that received 2% DSS were high in order of the distal (1.29±0.76 for males and 1.14±0.69 for females), middle (0.57±0.79 for males and 0.43±0.79 for females) and proximal (0.43±0.53 for males and 0.29±0.49 for females) parts.

Immunohistochemistry of β-catenin, COX-2, iNOS, p53 and nitrotyrosine

The immunoreactivities against β-catenin, COX-2, iNOS and nitrotyrosine were found in all colonic lesions including neoplasms and dysplastic crypts (Fig. 3) in the large intestine of $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes that received 2% DSS. Their intensity in the normal mucosa and the lesions induced in mice given tap water was relatively weaker than that in $Apc^{Min/+}$ mice treated with 2% DSS. p53 was positive in the nuclei of the colonic lesions developed in $Apc^{Min/+}$, while negative in those in $Apc^{+/+}$ mice. The immunoreactivity against 3 antibodies (β-catenin, COX-2 and iNOS) was also observed in the small intestinal polyps (tubular adenomas) in $Apc^{Min/+}$ mice of both sexes: the intensity in mice given tap water was lower than those treated with DSS.

β-Catenin staining in adenoma cells showed positive in their cell membrane and/or a few nuclei (Fig. 3*a*) in $Apc^{Min/+}$ mice treated with 2% DSS. Strong β-catenin expression was observed in the nucleus and cytoplasm of adenocarcinoma cells (Fig. 3*b*) in $Apc^{Min/+}$ mice given 2% DSS. The intensity of β-catenin staining in adenoma cells was relatively weak when compared to carcinoma cells. β-Catenin immunoreactivity was also observed in the cell membrane, cytoplasm, and a few nuclei of dysplastic cells (Fig. 3*c*). Nonlesional cryptal cells showed weak positivity of bgr;-catenin in their cell membrane. In addition, a positive reaction against β-catenin antibody was noted in the vascular endothelium, infiltrated inflammatory cells and ganglion cells in Auerbach's plexus.

Strong COX-2 immunoreactivity was present in adenoma (Fig. 3*d*) and adenocarcinoma cells (Fig. 3*e*) in their cytoplasm in $Apc^{Min/+}$ mice treated with 2% DSS. Dysplastic cells (Fig. 3*f*) showed relatively strong positivity for COX-2 when compared to neoplastic cells. Nonlesional cryptal cells at the lower part of crypts were weakly positive for COX-2, while strongly positive reaction of COX-2 was seen in the endothelium of small blood vessels and inflammatory cells infiltrated in the lamina propria. Smooth muscle cells and fibroblasts in inflamed large bowel showed weak reaction of COX-2.

iNOS-immunohistochemistry showed strong immunoreactivity in the cytoplasm of adenoma (Fig. 3*g*) and adenocarcinoma cells (Fig. 3*h*) in $Apc^{Min/+}$ mice given 2% DSS: the intensity was greater in carcinoma cells when compared to adenoma

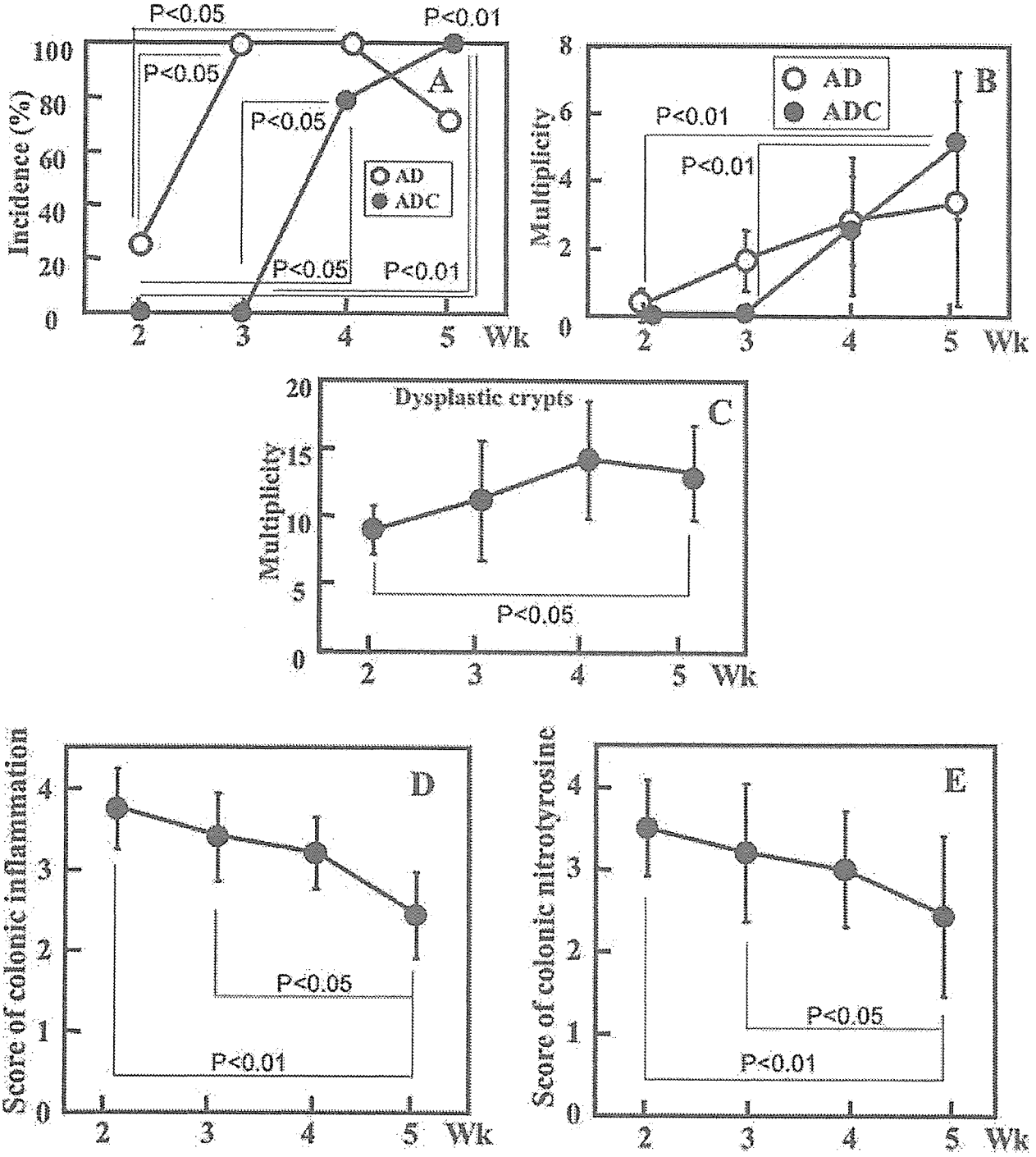

FIGURE 2 – Time-course observation of colonic lesions in female $Apc^{Min/+}$ mice treated with 2% DSS. The incidence (*a*) and multiplicity (*b*) of colonic neoplasms and the multiplicity (*c*) of colonic dysplastic crypt were gradually increased with time. Scores of colonic inflammation (*d*) and nitrotyrosine-positivity (*e*) gradually decreased after the cessation of DSS treatment with time. AD and ADC refer to adenoma and adenocarcinoma, respectively. Data represent mean±SD (n = 4 mice at week 2, 5 mice at week 3, 5 mice at week 4, and 8 mice at week 5). Statistical significance of differences was evaluated by Fisher's exact probability test (*a*) or one-way ANOVA with Bonferroni correction (*b–e*). Statistical significances of the squared correlation coefficients were found for the multiplicity of adenoma ($r = 0.9817$, $p < 0.05$), inflammation score ($r = -0.9618$, $p < 0.05$), and nitrotyrosine positive score ($r = -0.9764$, $p < 0.05$).

cells. Also, dysplastic cells (Fig. 3*i*) were positive for iNOS in their cytoplasm and the intensity was relatively greater than neoplastic cells. The faint positive reaction was found in the cytoplasm of nonlesional cryptal cells. Immunohistochemical iNOS expression was strong in the endothelial cells of small blood vessels and inflammatory cells in the lamina propria. COX-2- and iNOS-stained inflammatory cells were also frequently observed in the mucosa.

TABLE II – INCIDENCE AND MULTIPLICITY OF SMALL INTESTINAL POLYPS AT WEEK 5

Genotype	Sex	Incidence (multiplicity) of small intestinal polyps at							
		Proximal region		Middle region		Distal region		Total	
		2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water
$Apc^{Min/+}$	Male	7/7, 100% (9.4±2.4[1])	9/9, 100% (9.1±2.1)	7/7, 100% (11.6±2.6[2])	9/9, 100% (16.0±3.7)	7/7, 100% (42.9±10.4[3])	9/9, 100% (24.7±5.8)	7/7, 100% (64.3±13.3[2])	9/9, 100% (49.8±9.8)
	Female	7/7, 100% (7.2±2.4)	10/10, 100% (8.5±2.3)	7/7, 100% (8.7±2.3[4])	10/10, 100% (14.1±3.0)	7/7,100% (35.3±4.8[4])	10/10, 100% (20.0±5.1)	7/7, 100% (51.2±5.4[5])	10/10, 100% (42.6±9.3)
$Apc^{+/+}$	Male	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)
	Female	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)

[1]The number of polyps per mouse (Mean±SD).–[2]Significantly different from $Apc^{Min/+}$ males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).–[3]Significantly different from $Apc^{Min/+}$ males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).–[4]Significantly different from $Apc^{Min/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).–[5]Significantly different from $Apc^{Min/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).

TABLE III – SCORES OF INFLAMMATION AND NITROTYROSINE IMMUNOHISTOCHEMISTRY OF COLONIC MUCOSA AT WEEK 5

Genotype	Sex	Score of inflammation (number of mice examined)		Score of nitrotyrosine-immmunohistochemistry (number of mice examined)	
		2% DSS	Tap water	2% DSS	Tap water
$Apc^{Min/+}$	Male	2.86±0.69[1,2] (7)	0.22±0.44 (9)	2.71±0.95[2] (7)	0.11±0.33 (9)
	Female	2.14±0.69[3] (7)	0.20±0.42 (10)	2.14±0.69[3] (7)	0.10±0.32 (10)
$Apc^{+/+}$	Male	2.33±0.65[4] (12)	0.24±0.44 (17)	2.25±1.06[4] (12)	0.12 ± 0.33 (17)
	Female	2.10±0.74[5] (10)	0.18±0.41 (11)	2.14±0.69[5] (10)	0.09±0.30 (11)

[1]Mean ± SD.–[2]Significantly different from $Apc^{Min/+}$ males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).–[3]Significantly different from $Apc^{Min/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).–[4]Significantly different from $Apc^{+/+}$ males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).–[5]Significantly different from $Apc^{+/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.001$).

Immunoreactivity of nitrotyrosine was noted in the cryptal cells with or without disruption, infiltrated mononuclear inflammatory cells, and endothelial cells of the small vessels in the colonic mucosa and submucosa in $Apc^{Min/+}$ and $Apc^{+/+}$ mice that received 2% DSS. Among them, the stainability was strong in the infiltrated mononuclear inflammatory cells. Adenoma cells (Fig. 3*j*), adenocarcinoma cells (Fig. 3*k*) and dysplastic cryptal cells (Fig. 3*l*) also showed moderately positive immunoreactivity of nitrotyrosine in their cytoplasm. The intensity in the colonic lesions in $Apc^{Min/+}$ mice given 2% DSS was strong when compared to that observed in $Apc^{+/+}$ mice given tap water alone. As summarized in Table III, scores of nitrotyrosine-immunoreactivity in the colonic mucosa of $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes given 2% DSS were significantly greater than those given tap water alone ($p < 0.001$). The score in the time-course observation indicated that the value decreased after the cessation of 2% DSS (Fig. 2*e*), as was the value of inflammation (Fig. 2*d*).

P53 immunoreactivity was observed in the nuclei of neoplastic cells (adenoma and adenocarcinoma cells) with a variety of stainability, which developed in the colon of $Apc^{Min/+}$ mice treated with DSS (Fig. 3*m,n*) but not in those given tap water alone. Also, the nuclei of dysplastic crypts were positive for p53 antibody (Fig. 3*o*). Surrounding the mucosal ulcer, some nuclei of regenerative hyperplastic crypts in the colon were weakly positive for p53 antibody in the colon of $Apc^{Min/+}$ mice treated with DSS (date not shown). No stainability of p53 was observed in the small intestinal polyps (data not shown) in $Apc^{Min/+}$ mice treated with or without DSS.

Apc allelic loss in colonic neoplasms

One hundred percent (14 of 14) of adenocarcinomas and 0% (0 of 3) of histologically normal colonic mucosa from male $Apc^{Min/+}$ mice that received 2% DSS showed LOH of *Apc*. In male $Apc^{Min/+}$ mice that received tap water alone, 100% (2 of 2) of adenocarcinomas showed LOH of *Apc* and 0% (0 of 3) of histologically normal colonic mucosa was negative for LOH.

Mutation of β-catenin and K-Ras genes

β-*Catenin* and K-*ras* mutations were not detected in any of the colonic adenocarcinomas examined.

Discussion

In our study, we investigated the influences of the inflammation induced by 1-week exposure of 2% DSS in the drinking water on intestinal carcinogenesis in $Apc^{Min/+}$ mice and found that the treatment resulted in a much higher incidence and multiplicity of large intestinal neoplasms in $Apc^{Min/+}$ mice up to 5 weeks. Also, the treatment significantly increased the number of small intestinal polyps (tubular adenomas) at the distal regions. Thus, we developed an $Apc^{Min/+}$ mouse model with multiple colonic neoplasms, which develop within 4 weeks after 1-week exposure DSS, in addition to the increase in the number of small intestinal polyps. Regardless of the types of gene and gender, all mice treated with 2% DSS had intestinal mucosal inflammation with various degrees. However DSS treatment did not induce preneoplastic and neoplastic lesions in the large bowel wild-type ($Apc^{+/+}$) mice of either sex. This report describing rapid development of a number of colonic neoplasms in $Apc^{Min/+}$ mice within a short-term period (5 weeks) support an earlier work by Cooper *et al.*,[14] who found that treatment with 2 cycles of 4% DSS results in 40% incidence of colon cancer with a multiplicity of 0.67±0.27 in female Min mice at 42 days. Our findings suggest that the development of colonic dysplastic crypts and/or neoplasms in the short-term (up to 5 weeks) needs both the gene (*Apc*) mutation and subsequent inflammatory stimuli, but not either alone under the current exper-

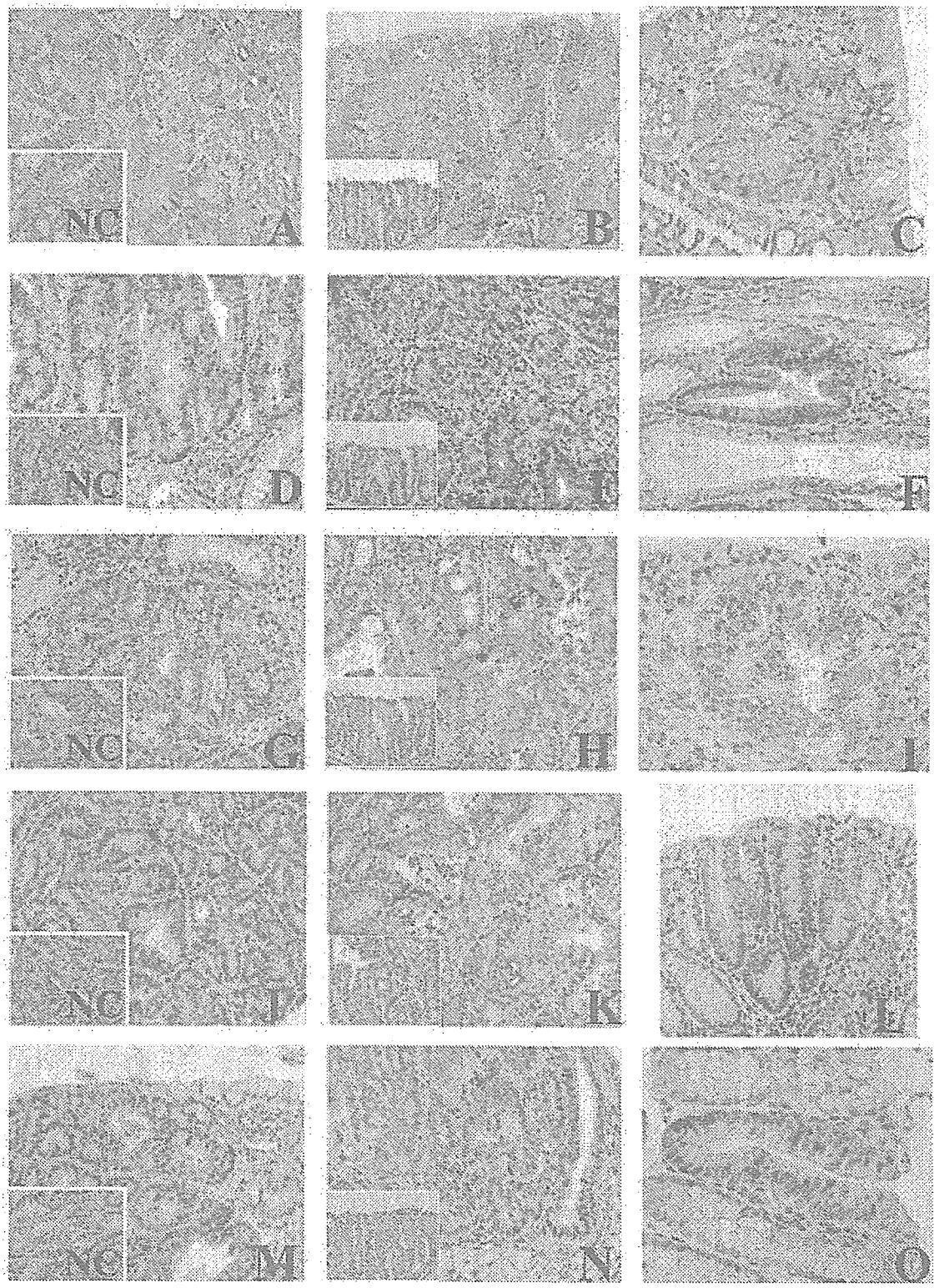

FIGURE 3 – Immunohistochemistry of the colonic lesions developed in male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*)–(*c*), β-catenin immunohistochemistry; (*d*)–(*f*), COX-2 immunohistochemistry; (*g*)–(*i*), iNOS immunohistochemistry; (*j*)–(*l*), nitrotyrosine immunohistochemistry and (*m*)–(*o*), p53 immunohistochemistry. Adenomas (*a*, *d*, *g*, *i* and *m*), adenocarcinomas (*b*, *e*, *h*, *k* and *n*), and dysplastic crypts (*c*, *f*, *i*, *l* and *o*) show positive reaction with a variety of intensity against β-catenin, COX-2, iNOS, nitrotyrosine and p53 antibodies. Inserts of *a*, *d*, *g*, *j* and *m* are negative controls (NC) immunostained without antibodies show negative reactions. Inserts of *b*, *e*, *h*, *k* and *n* are immunohistochemistry of adenocarcinomas developed in $Apc^{Min/+}$ mice given tap water. Original magnification: (*a*), (*b*), (*d*), (*e*), (*g*), (*h*), (*j*), (*k*), (*l*), (*m*) and (*n*), ×100; (*c*), (*f*), (*i*) and (*o*), ×200; inserts, ×200.