mice. Data for macroscopic incidences (percentage of mice with tumors) and multiplicities (number of tumors per mouse) of skin tumors over time are summarized in Figure 1. Skin tumors began to appear at 7 weeks after DMBA treatment in wild-type mice, and at 10 weeks in EP_3 receptor-knockout mice (Figure 1A). Multiplicities of skin tumors at week 11 were significantly different in EP_3 receptor-knockout mice and wild-type mice (0.05 ± 0.05 versus 0.46 ± 0.18, $P < 0.05$) (Figure 1A and B), but no significant differences in incidences were observed (1/19 versus 8/24, $P < 0.056$). There were no significant differences in either incidences or multiplicities of macroscopic lesions at 25 weeks after TPA treatment (Figure 1A and B).

Histological findings for skin carcinogenesis

Data for the histopathological diagnoses of skin tumors are summarized in Table II. Final incidences of skin tumors were 100% (19/19) and 95.8% (23/24), and multiplicities were 3.58 ± 0.51 and 3.17 ± 0.63 in EP_3 receptor-knockout mice and wild-type mice, respectively. The incidences and multiplicities of papillomas also did not differ between the groups. However, SCCs occurred in three wild-type mice, one with two lesions, but were not observed in EP_3 receptor-knockout mice. Instead of SCCs, keratoacanthomas were apparent in EP_3 receptor-knockout mice, but were not apparent in their wild-type counterparts (6/19 versus 0/24, $P < 0.01$).

Table II. Skin tumor incidences and multiplicities in wild-type and $EP_3^{-/-}$ mice at week 25

Mice	Papilloma		Keratoacanthoma		SCC		Total tumor	
	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
Wild-type	22/24 (91.6)	3.00 ± 0.64	0/24 (0)	0	3/24 (12.5)	0.17 ± 0.10	23/24 (95.8)	3.17 ± 0.63
$EP_3^{-/-}$	19/19 (100)	3.21 ± 0.42	6/19 (31.6)[a]	0.37 ± 0.14[b]	0/19 (0)	0	19/19 (100)	3.58 ± 0.51

[a]Significantly different from the wild-type value at $P < 0.01$.
[b]Significantly different from the wild-type value at $P < 0.05$.

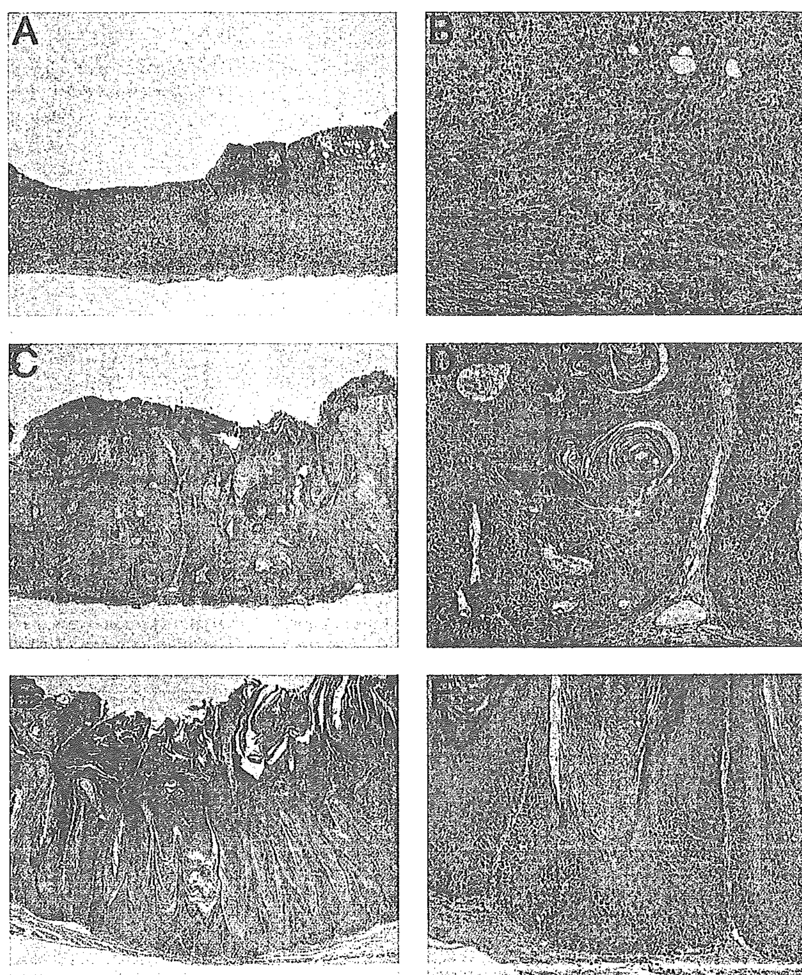

Fig. 2. Microscopical features of skin tumors observed in hematoxylin and eosin stained sections. (A–D), SCCs in wild-type mice. (E and F), keratoacanthomas in $EP_3^{-/-}$ mice. Moderately-differentiated SCC appearing as a solid mass forming shallow encrusted ulcers (**A**), and irregular masses of squamous epithelial cells and some spindle cells (**B**). A well-differentiated SCC demonstrates loss of keratin (**C**), and a number of epithelial pearls in the basal cell layer (**D**). Keratoacanthomas exhibits a bowl shape filled with mature keratin (**E**), and well-differentiated stratified squamous epithelium (**F**). Original magnification: 40× for A, C and E; 200× for B, D and F.

Microscopical features of skin tumors observed in hematoxylin and eosin stained sections are illustrated in Figure 2. One of the SCCs was moderately-differentiated and this lesion appeared as a solid mass forming shallow encrusted ulcers, with squamous epithelial cells proliferating downward and invading the adjacent subcutis and muscle (Figure 2A). This SCC was composed of irregular masses of squamous epithelial cells and some spindle cells (Figure 2B). The other three SCCs were well-differentiated and featured irregular structures of squamous cells with well defined basal cell layers (Figure 2C), and a number of epithelial pearls in the basal cell layers (Figure 2D). All keratoacanthomas exhibited a characteristic bowl shape filled with mature keratin (Figure 2E), and the walls had a buttress-like appearance. They were composed of thick, folded layers of well-differentiated stratified squamous epithelium enclosed by a prominent basal layer, and the structure of stratified squamous epithelium was similar to normal epidermis (Figure 2F).

The four SCCs and six keratoacanthomas developed at 10, 11, 17, 20 and 12, 12, 14, 17, 17, 22 weeks after the beginning of TPA treatment, respectively. SCCs were originally observed as wart-like tumors in the shape of a dome without a stem, and then grew inside mouse skin. On the other hand, keratoacanthomas arose from papilloma-like tumors in the shape of a pole or a mushroom, and then grew on the surface of mouse skin into node-like or crater-like tumors.

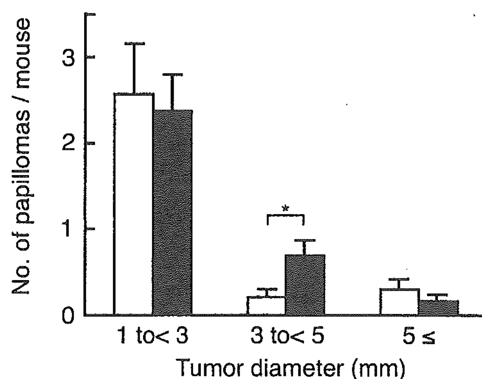

Fig. 3. Size distribution of papillomas in wild-type (open columns) and $EP_3^{-/-}$ mice (closed columns). The mean number of papillomas/mouse in each size class is given; bars, SE. *Significantly different from the corresponding wild-type value at $P < 0.05$.

The size distribution of papillomas (excluding SCCs and keratoacanthomas) is provided in Figure 3. The number of papillomas 3 to <5 mm in diameter was significantly increased in EP_3 receptor-knockout compared with wild-type mice (0.68 ± 0.19 versus 0.22 ± 0.09, $P < 0.05$).

Localization of E-cadherin and β-catenin in skin tumors

Immunohistochemical analysis of paraffin-embedded specimens of skin SCCs in wild-type mice (Figure 4A–C) and keratoacanthomas in EP_3 receptor-knockout mice (Figure 4D–F) was performed to examine the localization of cell–cell adhesion molecules E-cadherin and β-catenin, using specific antibodies. Sections without primary antibody treatment were also stained as negative controls (Figure 4A and D). Reduced expression of E-cadherin in cellular membranes and abnormal localization of β-catenin in the cytoplasm were found in basal cells of SCCs (Figure 4B and C). In contrast, normal localization of E-cadherin and β-catenin were observed in basal cells of keratoacanthomas (Figure 4E and F).

Expression of PGE_2 receptors in skin tumors

Expression of PGE_2 receptors in four samples each of DMBA/TPA-treated mouse skin lesions and adjacent normal skin tissues were examined by RT–PCR. Expression of EP_1, EP_2 and EP_4 receptor mRNAs in SCCs and keratoacanthomas was very similar to that in adjacent normal skin samples. EP_3 expression in keratoacanthomas and adjacent normal skin samples from EP_3 receptor-knockout mice could not be detected, in clear contrast to the positive results obtained for all SCC and adjacent normal skin samples in wild-type mice. However, expression of EP_3 receptor mRNA in skin SCCs of wild-type mice was markedly lower than that in normal skin tissue (Figure 5A). Quantitative real-time RT–PCR analysis

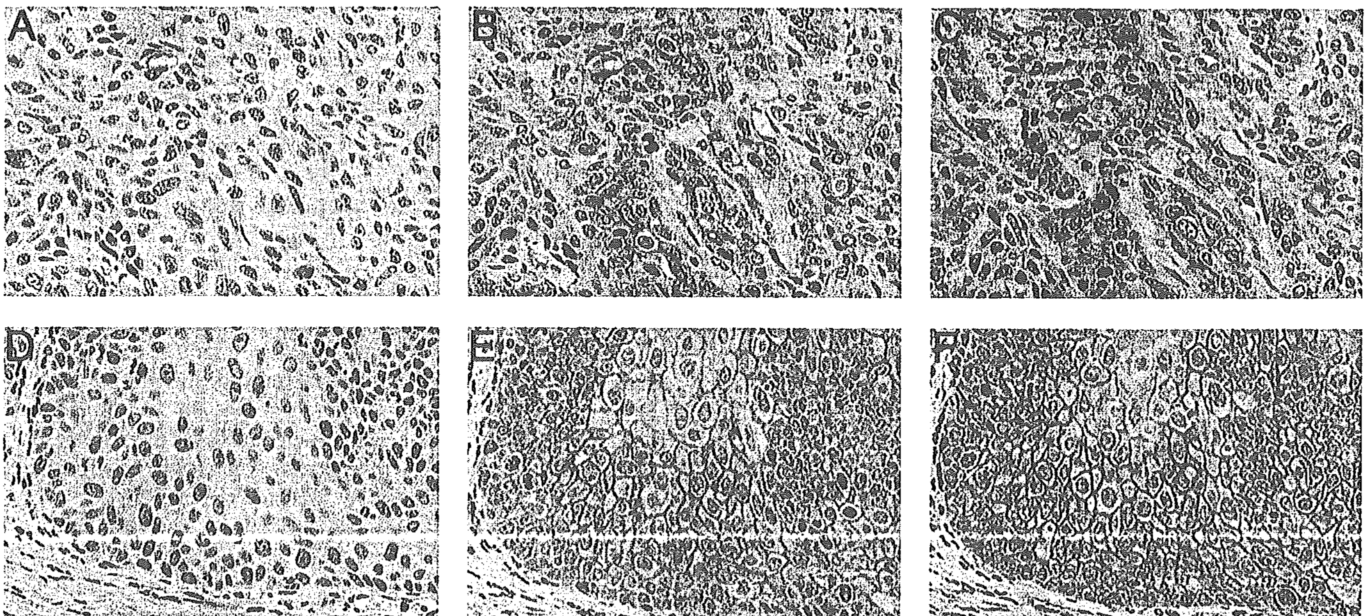

Fig. 4. Immunohistochemical staining of E-cadherin and β-catenin in a SCC in a wild-type mouse (A–C) and a keratoacanthoma in an $EP_3^{-/-}$ mouse (D–F). (**A** and **D**), negative controls without primary antibody; (**B** and **E**), E-cadherin; (**C** and **F**), β-catenin.

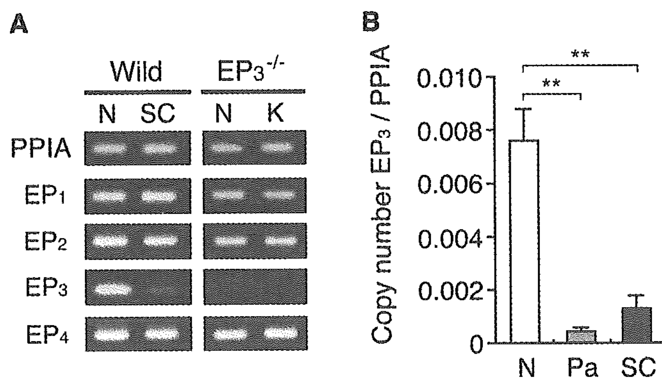

Fig. 5. Expression of PGE_2 receptors in DMBA/TPA-treated mouse skin lesions and adjacent normal skin tissues. (**A**) Expression of EP_{1-4} receptors examined by RT–PCR. *PPIA* was used as an internal control. N indicates adjacent normal skin tissue. SC and K indicate SCCs in wild-type mice and keratoacanthomas in $EP_3^{-/-}$ mice. (**B**) Expression of the EP_3 receptor examined by real-time RT–PCR. The PCR primers of mouse and rat EP_3 receptors were designed to target a sequence common to all EP_3 receptor variants expressed in the mouse. N indicates adjacent normal skin tissue ($n = 6$). Pa and SC indicate papillomas ($n = 3$) and SCCs ($n = 4$), respectively, in wild-type mice. Columns, mean; bars, ± SE; $^{**}P < 0.01$.

suggested the EP_3 receptor to be downregulated in papillomas and SCCs to 5.7 and 17.1%, respectively, of the average value of normal skin tissues (Figure 5B).

Discussion

In the present study, examination of the significance of PGE_2 receptor EP_3 for two-stage DMBA/TPA skin carcinogenesis in EP_3 receptor-knockout mice showed no overall difference in incidences and multiplicities of papillomas at week 25, but a shift from SCC to keratoacanthoma development was evident, indicating that the EP_3 receptor may promote malignant change in skin carcinogenesis.

The EP_3 receptor is widely distributed throughout the body, and its mRNA has been identified in almost all tissues in mice and rats, as well as humans (11,22,23). *In situ* hybridization analysis has revealed high level expression of EP_3 and EP_4 receptor mRNA in the dermal papilla cells of hair follicles (24). We confirmed the prostaglandin E_2 receptors EP_{1-4} to be expressed in wild-type mouse normal skin tissues by RT–PCR (Figure 5A), consistent with a previous report (25).

TPA treatment is known to increase epidermal PGE_2 production in mice skin (10), and PGE_2 promotes mast cell activation and IL-6 production through the EP_3 receptor (26–28). Furthermore, acute cutaneous inflammation induced by arachidonic acid is markedly attenuated in EP_3 receptor-knockout mice (29). Thus, PGE_2/EP_3 signaling is considered to be a major pathway of acute inflammation in mouse skin. In the present study, macroscopic observation demonstrated that formation of tumors was delayed for 3 weeks in EP_3 receptor-knockout mice, and multiplicity was significantly lower at week 11 than in wild-type mice. DMBA/TPA two-stage skin tumorigenesis using *IL-6*-knockout C57BL/6 mice also features a delay in tumor formation compared with wild-type mice (30) so that the delay of 3 weeks in formation of tumors in EP_3 receptor-knockout mice may have been caused by attenuation of acute inflammation due to deficiency of the EP_3 receptor. Therefore, it is important to determine whether the temporal delay in tumor formation in EP_3 receptor-knockout mice consequently influenced formation of keratoacanthomas. Examination of the time points of occurrence of the individual keratoacanthomas and SCCs, however, suggested no clear difference in the developing period between two types of tumor. Therefore, it is considered that the lack of SCCs in EP_3-knockout mice was not simply due to a delay, but rather to a block in malignant development because of the EP_3 deficiency. Indeed, characteristic differences in generation of keratoacanthomas and SCCs were observed as macroscopic findings, described in the Results. These might reflect variation, especially regarding cell invasiveness, in the early promotion stage occurring in wild-type but not EP_3 receptor-knockout mice. Clearly, a number of molecules downstream of the EP_3 receptor could play important roles in early stages of skin carcinogenesis; however, which are actually critical for SCC formation have yet to be clarified.

The present histological assessment suggested that tumor development in wild-type and EP_3 receptor-knockout mice distinctly differs (Figure 2). The structural differences apparent between SCCs and keratoacanthomas would be expected to correlate with several factors including cell–cell adhesion, cell invasiveness and cell polarity, and this was confirmed by our immunohistochemical findings for adhesion molecules, E-cadherin and β-catenin. Chu *et al.* (31,32) earlier reported E-cadherin and catenin (α-, β- and γ-) to be normally localized in cell membranes of keratoacanthomas, whereas abnormal cytoplasmic localization or loss of expression are characteristic of SCCs and our results are in agreement.

There was no significant difference in incidences and multiplicities of tumor formation at the end point of the experiment as shown in Figure 1 and Table II. Although formation of tumors was delayed for 3 weeks in EP_3 receptor-knockout mice (Figure 1), papillomas of 3 to <5 mm in diameter were increased (Figure 3), suggesting elevated proliferation of tumor cells. Indeed, our previous study has shown that downregulation of EP_3 receptor expression in colon cancer might be associated with increased multiplicity of lesions 2–5 mm in diameter. Furthermore, the EP_3 receptor-selective agonist, ONO-AE-248 decreases cell proliferation in the HCA-7 human colon adenocarcinoma cell line (15). Konger *et al.* (33) reported that growth stimulation of human kerationocyte cells occurs via an EP_2 and/or EP_4 receptor-adenylate cyclase coupled response, while cell growth was inhibited by EP_3 receptor agonist sulprostone. Our studies, together with recent reports, suggest that the EP_3 receptor plays a role in suppression of skin epithelial cell proliferation and inhibits tumor growth.

The mouse EP_3 receptor has three isoforms, $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$ generated by alternative splicing from the single EP_3 receptor gene. The major signaling pathway for the $EP_{3\alpha}$ and $EP_{3\beta}$ receptors is inhibition of adenylate cyclase via G_i (34,35). In contrast, the EP_2 and EP_4 receptors are coupled to G_s and stimulate cAMP production by adenylate cyclase (11). Konger *et al.* (36) reported that inhibition of EP_2 receptor expression by its anti-sense construct in a HaCat immortalized human keratinocyte cell line, which expresses the EP_2 receptor predominantly and trace amounts of EP_3 and EP_4 receptors, is associated with decreased expression of paxillin, a critical component for focal adhesion assembly. Inhibition of EP_2 receptor expression decreased EP_2 agonist-induced cAMP production in HaCat cells, and endowed extensive deep invasion capacity in a 3D organ culture model of normal skin. Our findings provide indirect evidence that attenuation of cAMP production by PGE_2 via EP_3 receptor in keratinocyte cells

might enhance neoplastic progression. It has been reported that the EP_3 selective agonist ONO-AE-248 blocks the rise in intracellular cAMP induced by forskolin, an activator of adenylate cyclase, in CHO cells transfected with $EP_{3\alpha}$ receptor (37). Additional studies are now needed to investigate interactions between the EP_3 and other EP receptors in skin carcinogenesis.

In conclusion, our present data suggest that the PGE_2 receptor EP_3 may play a role in neoplastic progression in skin carcinogenesis.

Acknowledgements

We thank Professor Hiroyuki Tsuda (Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences) for his comments on histology of skin tumors. We are also grateful to Mr Naoaki Uchiya and Miss Yurika Teramoto for excellent technical assistance. This work was supported in part by Grants-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control and for Research on Advanced Medical Technology, from the Ministry of Health, Labor and Welfare of Japan. Y.S. was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research during performance of this work.

Conflict of Interest Statement: None declared.

References

1. Asano,T., Shoda,J., Ueda,T., Kawamoto,T., Todoroki,T., Shimonishi,M., Tanabe,T., Sugimoto,Y., Ichikawa,A., Mutoh,M., Tanaka,N. and Miwa,M. (2002) Expressions of cyclooxygenase-2 and prostaglandin E-receptors in carcinoma of the gallbladder: crucial role of arachidonate metabolism in tumor growth and progression. *Clin. Cancer Res.*, **8**, 1157–1167.
2. Fujita,T., Matsui,M., Takaku,K., Uetake,H., Ichikawa,W., Taketo,M.M. and Sugihara,K. (1998) Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas. *Cancer Res.*, **58**, 4823–4826.
3. Murata,H., Kawano,S., Tsuji,S., Tsuji,M., Sawaoka,H., Kimura,Y., Shiozaki,H. and Hori,M. (1999) Cyclooxygenase-2 overexpression enhances lymphatic invasion and metastasis in human gastric carcinoma. *Am. J. Gastroenterol.*, **94**, 451–455.
4. Denkert,C., Kobel,M., Pest,S., Koch,I., Berger,S., Schwabe,M., Siegert,A., Reles,A., Klosterhalfen,B. and Hauptmann,S. (2002) Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. *Am. J. Pathol.*, **160**, 893–903.
5. Ristimaki,A., Sivula,A., Lundin,J., Lundin,M., Salminen,T., Haglund,C., Joensuu,H. and Isola,J. (2002) Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res.*, **62**, 632–635.
6. Fischer,S.M., Lo,H.H., Gordon,G.B., Seibert,K., Kelloff,G., Lubet,R.A. and Conti,C.J. (1999) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, and indomethacin against ultraviolet light-induced skin carcinogenesis. *Mol. Carcinog.*, **25**, 231–240.
7. Prescott,S.M. and Fitzpatrick,F.A. (2000) Cyclooxygenase-2 and carcinogenesis. *Biochim. Biophys. Acta*, **1470**, M69–M78.
8. Müller-Decker,K., Neufang,G., Berger,I., Neumann,M., Marks,F., and Fürstenberger,G. (2002) Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. *Proc. Natl Acad. Sci. USA*, **99**, 12483–12488.
9. Chang,S.H., Liu,C.H., Conway,R., Han,D.K., Nithipatikom,K., Trifan,O.C., Lane,T.F. and Hla,T. (2004) Role of prostaglandin E_2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. *Proc. Natl Acad. Sci. USA*, **101**, 591–596.
10. Fürstenberger,G., Gross,M. and Marks,F. (1989) Eicosanoids and multistage carcinogenesis in NMRI mouse skin: role of prostaglandins E and F in conversion (first stage of tumor promotion) and promotion (second stage of tumor promotion). *Carcinogenesis*, **10**, 91–96.
11. Narumiya,S., Sugimoto,Y. and Ushikubi,F. (1999) Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.*, **79**, 1193–1226.
12. Watanabe,K., Kawamori,T., Nakatsugi,S. *et al.* (1999) Role of the prostaglandin E receptor subtype EP_1 in colon carcinogenesis. *Cancer Res.*, **59**, 5093–5096.
13. 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.
14. 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 EP_2 in $Apc^{Delta\ 716}$ knockout mice. *Nat. Med.*, **7**, 1048–1051.
15. Shoji,Y., Takahashi,M., Kitamura,T. *et al.* (2004) Downregulation of prostaglandin E receptor subtype EP_3 during colon cancer development. *Gut*, **53**, 1151–1158.
16. Armuth,V. and Berenblum,I. (1976) Phorbol as a possible systemic promoting agent for skin carcinogenesis. *Z. Krebsforsch Klin. Onkol. Cancer Res. Clin. Oncol.*, **85**, 79–82.
17. Ushikubi,F., Segi,E., Sugimoto,Y. *et al.* (1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP_3. *Nature*, **395**, 281–284.
18. Morikawa,T., Wanibuchi,H., Morimura,K., Ogawa,M. and Fukushima,S. (2000) Promotion of skin carcinogenesis by dimethylarsinic acid in Keratin (K6)/ODC transgenic mice. *Jpn. J. Cancer Res.*, **91**, 579–581.
19. Takahashi,M., Fukuda,K., Ohata,T., Sugimura,T. and Wakabayashi,K. (1997) Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. *Cancer Res.*, **57**, 1233–1237.
20. Takahashi,M., Fukuda,K., Sugimura,T. and Wakabayashi,K. (1998) Beta-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Res.*, **58**, 42–46.
21. Weisinger,G., Gavish,M., Mazurika,C. and Zinder,O. (1999) Transcription of actin, cyclophilin and glyceraldehyde phosphate dehydrogenase genes: tissue- and treatment-specificity. *Biochim. Biophys. Acta*, **1446**, 225–232.
22. Sugimoto,Y., Namba,T., Honda,A., Hayashi,Y., Negishi,M., Ichikawa,A. and Narumiya,S. (1992) Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_3 subtype. *J. Biol. Chem.*, **267**, 6463–6466.
23. Kotani,M., Tanaka,I., Ogawa,Y., Usui,T., Mori,K., Ichikawa,A., Narumiya,S., Yoshimi,T. and Nakao,K. (1995) Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol.*, **48**, 869–879.
24. Torii,E., Segi,E., Sugimoto,Y., Takahashi,K., Kabashima,K., Ikai,K. and Ichikawa,A. (2002) Expression of prostaglandin E_2 receptor subtypes in mouse hair follicles. *Biochem. Biophys. Res. Commun.*, **290**, 696–700.
25. Muller-Decker,K., Leder,C., Neumann,M., Neufang,G., Bayerl,C., Schweizer,J., Marks,F. and Furstenberger,G. (2003) Expression of cyclooxygenase isozymes during morphogenesis and cycling of pelage hair follicles in mouse skin: precocious onset of the first catagen phase and alopecia upon cyclooxygenase-2 overexpression. *J. Invest. Dermatol.*, **121**, 661–668.
26. Rao,T.S., Currie,J.L., Shaffer,A.F. and Isakson,P.C. (1993) Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation. *Inflammation*, **17**, 723–741.
27. Beetz,A., Messer,G., Oppel,T., van Beuningen,D., Peter,R.U. and Kind,P. (1997) Induction of interleukin 6 by ionizing radiation in a human epithelial cell line: control by corticosteroids. *Int. J. Radiat. Biol.*, **72**, 33–43.
28. Nguyen,M., Solle,M., Audoly,L.P., Tilley,S.L., Stock,J.L., McNeish,J.D., Coffman,T.M., Dombrowicz,D. and Koller,B.H. (2002) Receptors and signaling mechanisms required for prostaglandin E2-mediated regulation of mast cell degranulation and IL-6 production. *J. Immunol.*, **169**, 4586–4593.
29. Goulet,J.L., Pace,A.J., Key,M.L. *et al.* (2004) E-prostanoid-3 receptors mediate the proinflammatory actions of prostaglandin E_2 in acute cutaneous inflammation. *J. Immunol.*, **173**, 1321–1326.
30. Suganuma,M., Okabe,S., Kurusu,M., Iida,N., Ohshima,S., Saeki,Y., Kishimoto,T. and Fujiki,H. (2002) Discrete roles of cytokines, TNF-alpha, IL-1, IL-6 in tumor promotion and cell transformation. *Int. J. Oncol.*, **20**, 131–136.
31. Papadavid,E., Pignatelli,M., Zakynthinos,S., Krausz,T. and Chu,A.C. (2001) The potential role of abnormal E-cadherin and alpha-, beta- and gamma-catenin immunoreactivity in the determination of the biological behaviour of keratoacanthoma. *Br. J. Dermatol.*, **145**, 582–589.
32. Papadavid,E., Pignatelli,M., Zakynthinos,S., Krausz,T. and Chu,A.C. (2002) Abnormal immunoreactivity of the E-cadherin/catenin

(alpha-, beta-, and gamma-) complex in premalignant and malignant non-melanocytic skin tumours. *J. Pathol.*, **196**, 154–162.

33. Konger,R.L., Malaviya,R. and Pentland,A.P., (1998) Growth regulation of primary human keratinocytes by prostaglandin E receptor EP2 and EP3 subtypes. *Biochim. Biophys. Acta*, **1401**, 221–234.
34. Sugimoto,Y., Negishi,M., Hayashi,Y., Namba,T., Honda,A., Watabe,A., Hirata,M., Narumiya,S. and Ichikawa,A. (1993) Two isoforms of the EP_3 receptor with different carboxyl-terminal domains. Identical ligand binding properties and different coupling properties with Gi proteins. *J. Biol. Chem.*, **268**, 2712–2718.
35. Irie,A., Sugimoto,Y., Namba,T., Harazono,A., Honda,A., Watabe,A., Negishi,M., Narumiya,S. and Ichikawa,A. (1993) Third isoform of the prostaglandin-E-receptor EP_3 subtype with different C-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *Eur. J. Biochem.*, **217**, 313–318.
36. Konger,R.L., Scott,G.A., Landt,Y., Ladenson,J.H. and Pentland,A.P. (2002) Loss of the EP_2 prostaglandin E_2 receptor in immortalized human keratinocytes results in increased invasiveness and decreased paxillin expression. *Am. J. Pathol.*, **161**, 2065–2078.
37. Zacharowski,K., Olbrich,A., Piper,J., Hafner,G., Kondo,K. and Thiemermann,C. (1999) Selective activation of the prostanoid EP_3 receptor reduces myocardial infarct size in rodents. *Arterioscler. Thromb. Vasc. Biol.*, **19**, 2141–2147.

Received February 16, 2005; revised July 16, 2005; accepted July 22, 2005

Int. J. Cancer: **118,** 25–34 (2006)
© 2005 Wiley-Liss, Inc.

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.
© *2005 Wiley-Liss, Inc.*

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.

Grant sponsor: Ministry of Health, Labour and Welfare of Japan; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant number: 15592007; Grant sponsor: Kanazawa Medical University; Grant number: C2004-4; Grant sponsor: High-Technology Center of Kanazawa Medical University; Grant number: H2004-6.
*Correspondence to: Fax: +81-76-826-6926.
E-mail: takutt@kanazawa-med.ac.jp
Received 9 February 2005; Accepted after revision 26 April 2005
DOI 10.1002/ijc.21282
Published online 27 July 2005 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer
UICC global cancer control

P-169

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]

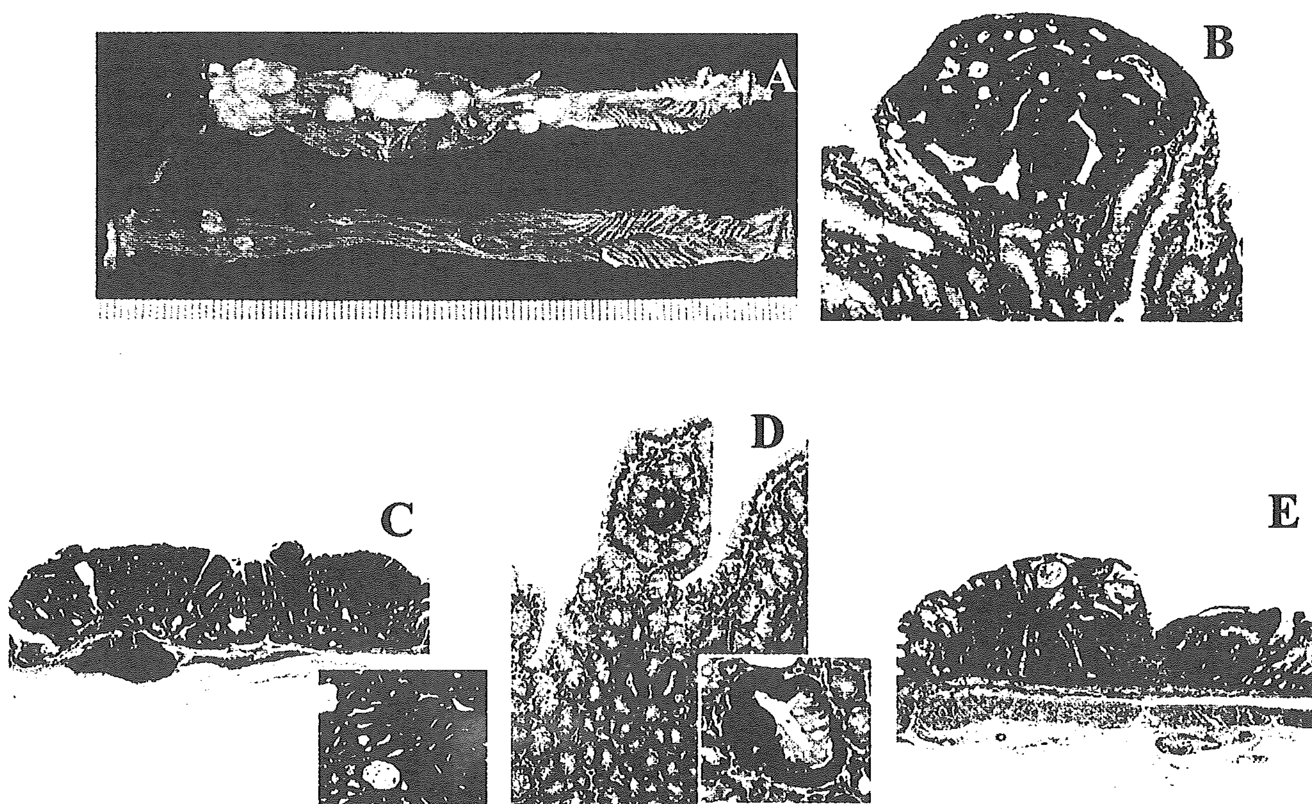

FIGURE 1 – Macroscopic view of the large bowel (*a*) and histopathology of the colonic lesions (*b–e*) of male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*) Male $Apc^{Min/+}$ mice given 2% DSS had multiple colonic tumors (upper), while male $Apc^{Min/+}$ mice given tap had a few colonic tumors (lower); (*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′-TCT-CCTTGG CTGGCCTTTCTA-3′; McatR, 5′-GTCACACAGCCC-TGTCAAGA-3′) and exon 1 of the k-*ras* gene (MrasF, 5′-GCC-TGCTGAAAATGACTGAG-3′; MrasR, 5′-CTTTACAAGCGC-ACGCAGAC-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

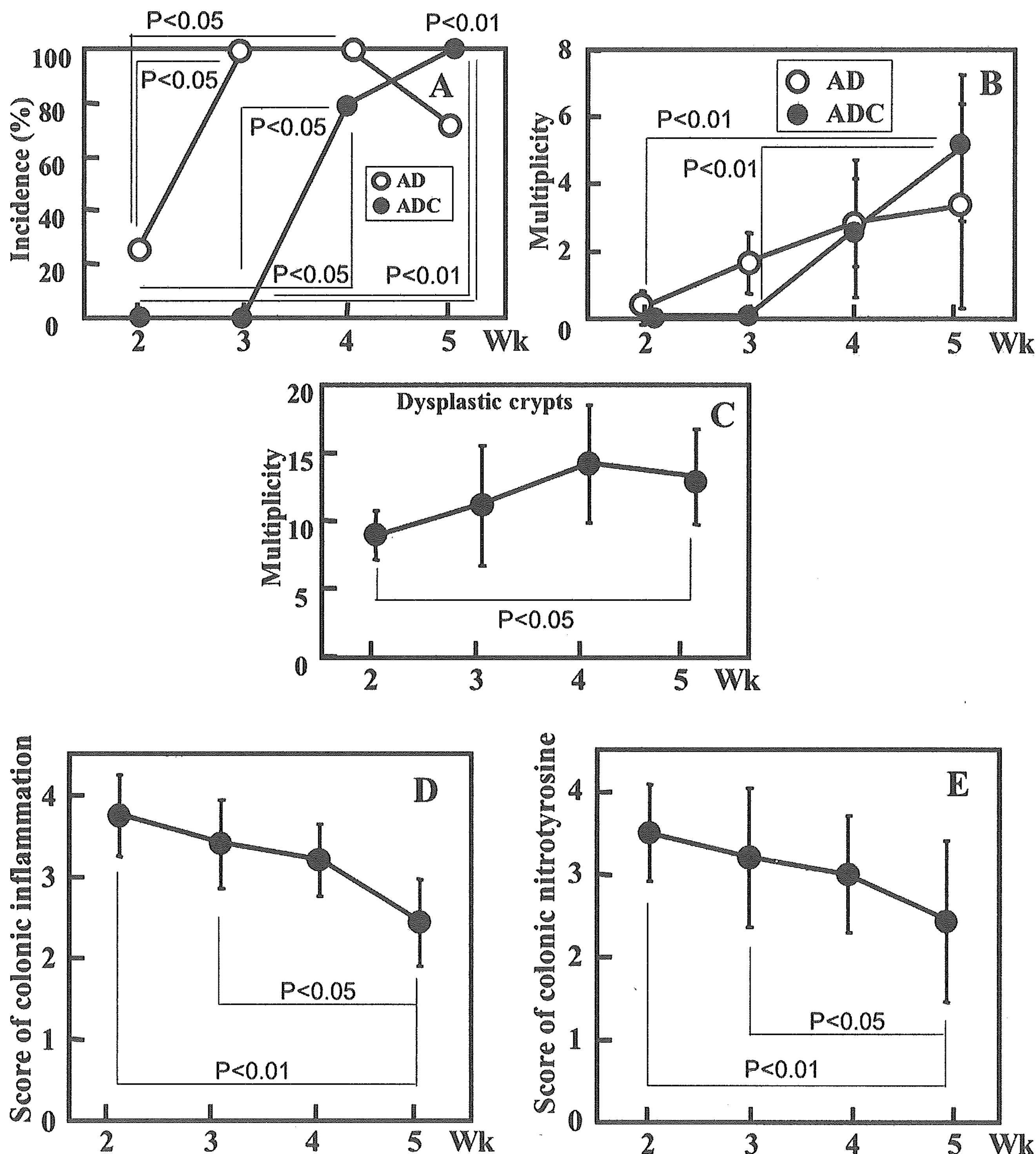

FIGURE 2 – Time-course observation of colonic lesions in female $Apc^{Min/+}$ mice treated with 2% DSS. The incidence (*a*) and multiplicity (*b*) of colonic neoplasms and the multiplicity (*c*) of colonic dysplastic crypt were gradually increased with time. Scores of colonic inflammation (*d*) and nitrotyrosine-positivity (*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-inmmunohistochemistry (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-

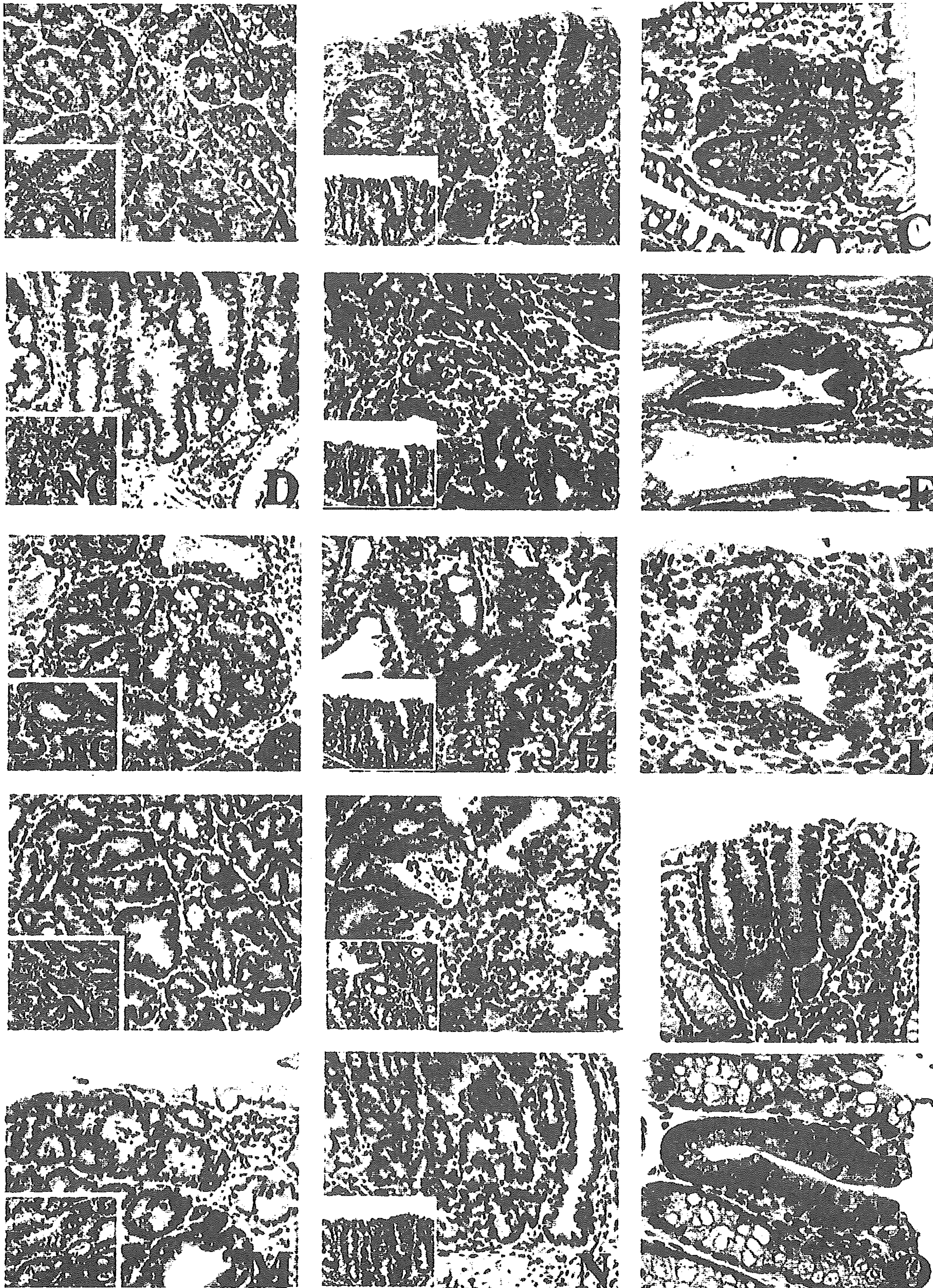

Figure 3 – Immunohistochemistry of the colonic lesions developed in male *Apc*$^{Min/+}$ mice treated with 2% DSS. (*a*)–(*c*), β-catenin immunohistochemistry; (*d*)–(*f*), COX-2 immunohistochemistry; (*g*)–(*i*), iNOS immunohistochemistry; (*j*)–(*l*), nitrotyrosine immunohistochemistry and (*m*)–(*o*), 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.

imental condition. Our results also support the findings of our previous works,[8,9,12] suggesting the importance of inflammatory stimuli as a promotion event after the initiation events (genetic alterations) in colon carcinogenesis. There were no differences between males and females in the effects of DSS on large and small intestinal carcinogenesis of $Apc^{Min/+}$, and the histopathology of colonic lesions including neoplasms was similar in both sexes.

As for the development of small intestinal polyps, treatment with DSS significantly increased their number and size, especially at the distal portion of the small intestine. Macrophages engulfing DSS particles were observed in the large intestine and surrounding lymph nodes of mice 1 day after DSS exposure, and then found in the jejunum and ileum 7 days after DSS treatment.[51] In our study, mild mucosal inflammation was observed in the distal portion of the small intestine of mice given 2% DSS. Thus, DSS could also influence the formation of small intestinal polyp in $Apc^{Min/+}$ mice. The Min mouse has been regarded as a human FAP model in spite of the fact that the polyps (adenomas) develop in the small intestine. Although the biological pathways in human colon and Min intestine are assumed to be similar, our model described here could be applied for investigation of the genesis, pathophysiology and chemoprevention of human FAP and/or inflammation-related colon tumorigenesis.

In our study, sequential observation on the pathological alteration in the large intestines of female $Apc^{Min/+}$ mice after 1-week exposure to 2% DSS revealed that the frequencies of dysplastic crypts and colonic neoplasms (adenoma and adenocarcinoma) gradually increased over time (Fig. 2*a*,*b*), indicating that dysplastic crypts[43] or adenomatous lesions[41] are precursor lesions for colon carcinoma and DSS treatment could promote their growth. The findings support an earlier report by Cooper *et al.*,[14] but their incidence of colonic cancer was low: 22% in Min mice exposed to 1-cycle of DSS (administration 4% DSS for 4 days and H_2O for 17 days) and 40% in Min mice exposed to 2-cycle of DSS. The discrepancy existing in these 2 studies may be due to the differences in the treatment period and the dose and molecular weight of DSS. In the present study, the incidence of colonic adenocarcinoma was 80% at week 4 and 100% at week 5 (Fig. 2*a*). When compared to our previous study on the effects of DSS on chemically induced colon carcinogenesis,[9] where we observed 40% and 100% incidences of colonic epithelial malignancy at week 4 and week 6, respectively, in male ICR mice, it is likely that deletion of the *Apc* gene plays an important role in colitis-associated carcinogenesis, as suggested by Cooper *et al.*[14]

In our study, we investigated the immunohistochemical expression of β-catenin, COX-2, iNOS and p53, in the colonic lesions developed in *ApcMin*/+ mice that received 2% DSS. The results on immunohistochemistry against these antibodies except for p53 expression in the lesions were similar to those observed in our previous studies, where the lesions were induced by AOM[8,9], HCAs[12] or DMH[11] followed by DSS in ICR mice, suggesting the similarity of histopathology and immunohistochemistry, and biological nature of the lesions observed in ICR mice given a colonic carcinogen and DSS and $Apc^{Min/+}$ mice treated with DSS. Increased immunohistochemical expression of COX-2 and iNOS in the colonic tumors of either $Apc^{Min/+}$ mice that received 2% DSS was confirmed by reverse transcription-polymerase chain reaction (data not shown). The findings of nitrotyrosine immunohistochemistry in the current study are also in accordance with those in our previous study[9] and suggest that oxidative/nitrosative stress strongly promotes the development of colonic neoplasms in $Apc^{Min/+}$ mice. iNOS has been shown to be the only isoform involved in stimulating tumor growth, probably through an increase in vascular endothelial growth factor production.[52] Moreover, NO regulates COX-2 expression.[53] Our results on the immunohistochemistry of iNOS and COX-2 indicate that the inflammatory response, the interaction between NO synthase and COX pathways may stand at the center of the pathophysiological basis of inflammation-related colon carcinogenesis in $Apc^{Min/+}$ mice treated with DSS, as are the cases of inflammatory diseases,[54] and chemically induced colon carcinogenesis.[33]

In the current study, we also screened for mutations of β-*catenin* and K-*ras* in colon tumors developed in male $Apc^{Min/+}$ mice. In contrast with previous reports,[12,33,55] we did not detect the mutations of these genes in any of the colonic adenocarcinomas examined. However, our results are not surprising. Suzui *et al.*[56] reported that adenocarcinomas developed in $Apc^{Min/+}$ mice treated with AOM did not have β-*catenin* gene mutations. In our study, cytoplasmic and/or nuclear accumulation of β-catenin protein was detected in the colonic neoplasms, but β-*catenin* gene mutations were not present. In the FAP patients, mutations of *APC* are common, but mutations of β-*catenin* were rare.[57,58] In addition, β-*catenin* germline mutations were not found in FAP patients with germline *APC* mutations.[57] Thus, concerning the β-*catenin* mutation, the colon tumors developed in the current animal model may imitate the colon carcinogenesis as in the FAP patients, that is, by a second hit in the *APC* gene such as loss of Apc^+ allele or somatic mutations in the *Apc* gene. Immunohistochemical staining with an antibody for the C-terminal of *Apc* showed the loss of wild-type *Apc* in colonic tumors in $Apc^{Min/+}$ mice (data not shown). As for the mutation of K-*ras*, no mutations were found in the colonic adenocarcinomas examined in the current study. Our results on K-*ras* mutations are in accordance with IBD-related colon carcinogenesis[59] and suggest that activation of the K-*ras* gene is not essential for the development and growth of colonic neoplasms in our model.

p53 gene mutation occurs in the late stage of human colon carcinogenesis.[33,59] In our study, p53 immunohistochemistry revealed positive reaction in the nuclei of neoplastic cells in $Apc^{Min/+}$ mice treated with DSS, although we did not examine its mutation in our study. The accumulation of p53 shown in our study is interesting and may be important for colon cancer development in *Apc*-deficient mice, since an increased *p53* mutation load in the inflamed colon tissue from UC patients being a high-risk for colon cancer[60] and a potential mechanism link between NO and p53 in UC and sporadic colon cancer[61] were reported. In addition, COX-2, iNOS and p53 are suggested to be fundamental "play-makers" of the angiogenesis processes.[62]

Taken together, our results suggest that a novel $Apc^{Min/+}$ mouse model with DSS may provide new insight into the genesis and chemoprevention of colon cancer development in FAP patients. In our model, a single allele *Apc* gene followed by appropriate promotional stimuli is sufficient for the development and growth of colonic neoplasms in $Apc^{Min/+}$ mice, and COX-2, iNOS, p53, oxidative/nitrosative stress and interactions of these may play important roles in colon carcinogenesis in $Apc^{Min/+}$ mice given DSS. Our model can be applied for investigating the pathogenesis in carcinogenesis of IBD, since the Wnt/β-catenin signaling pathway may be involved in carcinogenesis of UC.[59,63] Our ongoing microarray analysis will provide new information of the mechanism(s) for the effects of DSS on large and small intestinal tumorigenesis in $Apc^{Min/+}$ mice.

Acknowledgements

Dr. R. Suzuki is a Research Fellow of the Japan Society for the Promotion of Science, 6 Ichiban-cho, Chiyoda-ku, Tokyo 102-8471, Japan.

References

1. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.
2. Ohshima H, Tatemichi M, Sawa T. Chemical basis of inflammation-induced carcinogenesis. Arch Biochem Biophys 2003;417:3–11.
3. Szilagyi A. Altered colonic environment, a possible predisposition to colorectal cancer and colonic inflammatory bowel disease: rationale of dietary manipulation with emphasis on disaccharides. Can J Gastroenterol 1998;12:133–46.

4. Seril DN, Liao J, Yang GY, Yang CS. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis 2003;24:353–62.
5. Munkholm P. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. Aliment Pharmacol Ther 2003;18:1–5.
6. van der Woude CJ, Kleibeuker JH, Jansen PL, Moshage H. Chronic inflammation, apoptosis and (pre-)malignant lesions in the gastro-intestinal tract. Apoptosis 2004;9:123–30.
7. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;98:694–702.
8. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci. 2003;94:965–73.
9. Suzuki R, Kohno H, Sugie S, Tanaka T. Sequential observations on the occurrence of preneoplastic and neoplastic lesions in the mouse colon treated with azoxymethane and dextran sodium sulfate. Cancer Sci. 2004;95:721–7.
10. Suzuki R, Kohno H, Sugie S, Tanaka T. Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. Histol Histopathol 2005;20:483–92.
11. Kohno H, Suzuki R, Sugie S, Tanaka T. β-*Catenin* mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. Cancer Sci 2005; 96:69–76.
12. Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, Wakabayashi K. Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and dextran sodium sulfate in male ICR mice possess bgr;-catenin gene mutations and increase immunorectivity for bgr;-catenin, cyclooxygenase-2, and inducible nitric oxide synthase. Carcinogenesis 2005;26: 229–38.
13. Cooper HS, Murthy S, Kido K, Yoshitake H, Flanigan A. Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis associated neoplasia in the human: study of histopathology, beta-catenin and p53 expression and the role of inflammation. Carcinogenesis 2000;21:757–68.
14. Cooper HS, Everley L, Chang W-C, Pfeeiffer G, Lee B, Murthy S, Clapper ML. The role of mutant Apc in the development of dysplasia and cancer in the mouse model of dextran sulfate sodium–induced colitis. Gastroenterology 2001;121:1407–16.
15. Barbour KW, Davis T, White A, Baumann H, Berger FG. Haptoglobin, inflammation, and tumorigenesis in the MIN mouse. Redox Rep 2001;6:366–8.
16. Hansen-Petrik MB, McEntee MF, Jull B, Shi H, Zemel MB, Whelan J. Prostaglandin E2 protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in $Apc^{Min/-}$ mice. Cancer Res 2002;62:403–8.
17. Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 2000;1470:M69–78.
18. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–8.
19. DuBois RN, Radhika A, Reddy BS, Entingh AJ. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology 1996;110:1295–62.
20. Hull MA, Booth JK, Tisbury A, Scott N, Bonifer C, Markham AF, Coletta PL. Cyclooxygenase 2 is up-regulated and localized to macrophages in the intestine of Min mice. Br J Cancer 1999;79:1399–405.
21. Arroyo PL, Hatch-Pigott V, Mower HF, Cooney RV. Mutagenicity of nitric oxide and its inhibition by antioxidants. Mutat Res 1992;281:193–202.
22. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science (Washington, DC) 1991;254:1001–3.
23. Xiong M, Elson G, Legarda D, Leibovich SJ. Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway. Am J Pathol 1998;153:587–98.
24. Kojima M, Morisaki T, Tsukahara Y, Uchiyama A, Matsunari Y, Mibu R, Tanaka M. Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. J Surg Oncol 1999;70:222–9.
25. Bing RJ, Miyataka M, Rich KA, Hanson N, Wang X, Slosser HD, Shi SR. Nitric oxide, prostanoids, cyclooxygenase, and angiogenesis in colon and breast cancer. Clin Cancer Res 2001;7:3385–92.
26. Takahashi M, Fukuda K, Ohata T, Sugimura T, Wakabayashi K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. Cancer Res 1997;57:1233–7.
27. Rao CV, Kawamori T, Hamid R, Reddy BS. Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. Carcinogenesis 1999;20:641–4.
28. Ahn B, Ohshima H. Suppression of intestinal polyposis in $Apc^{Min/+}$ mice by inhibiting nitric oxide production. Cancer Res. 2001;61: 8357–60.
29. Scott DJ, Hull MA, Cartwright EJ, Lam WK, Tisbury A, Poulsom R, Markham AF, Bonifer C, Coletta PL. Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the $Apc^{Min/+}$ mouse. Gastroenterology 2001;121:889–99.
30. Mabley JG, Pacher P, Bai P, Wallace R, Goonesekera S, Virag L, Southan GJ, Szabo C. Suppression of intestinal polyposis in $Apc^{min/+}$ mice by targeting the nitric oxide or poly(ADP-ribose) pathways. Mutat Res 2004;548:107–16.
31. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol 2001;2:149–56.
32. Hofseth LJ, Hussain SP, Wogan GN, Harris CC. Nitric oxide in cancer and chemoprevention. Free Radic Biol Med 2003;34: 955–68.
33. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci 2004;95:475–80.
34. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer 2003;3:276–85.
35. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. Proc Natl Acad Sci U S A 1995;92:5258–65.
36. Hussain SP, Hofseth LJ, Harris CC. Tumor suppressor genes: at the crossroads of molecular carcinogenesis, molecular epidemiology and human risk assessment. Lung Cancer 2001;34:S7–S15.
37. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
38. Powell SM, Petersen GM, Krush AJ, Booker S, Jen J, Giardiello FM, Hamilton SR, Vogelstein B, Kinzler KW. Molecular diagnosis of familial adenomatous polyposis. N Engl J Med 1993; 329:1982–7.
39. Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, Dove WF. ApcMin: a mouse model for intestinal and mammary tumorigenesis. Eur J Cancer 1995;31A:1061–4.
40. Paulsen JE. Modulation by dietary factors in murine FAP models. Toxicol Lett 2000;112–113:403–9.
41. Yamada Y, Hata K, Hirose Y, Hara A, Sugie S, Kuno T, Yoshimi N, Tanaka T, Mori H. Microadenomatous lesions involving loss of *Apc* heterozygosity in the colon of adult $Apc^{Min/+}$ mice. Cancer Res 2002; 62:6367–70.
42. Murata M, Kawanishi S. Oxidative DNA damage induced by nitrotyrosine, a biomarker of inflammation. Biochem Biophys Res Commun 2004;316:123–8.
43. Paulsen JE, Steffensen I-L, Loberg EM, Husoy T, Namork E, Alexander J. Qualitative and quantitative relationship between dysplastic aberrant crypt foci and tumorigenesis in the *Min*/+ mouse colon. Cancer Res 2001;61:5010–5.
44. Steffensen I-L, Paulsen JE, Eide TJ, Alexander J. 2-Amino-1-methyl-6- phenylimidazo[4,5-*b*]pyridine increases the numbers of tumors, cystic crypts and aberrant crypt foci in multiple intestinal neoplasia mice. Carcinogenesis (Lond.) 1997;18:1049–54.
45. Ward JM. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. Lab Invest 1974;30:505–13.
46. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 1993;69:238–49.
47. Kohno H, Suzuki R, Noguchi R, Hosokawa M, Miyashita K, Tanaka T. Dietary conjugated linolenic acid inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Jpn J Cancer Res 2002;93:133–42.
48. Mollersen L, Paulsen JE, Olstorn HB, Knutsen HK, Alexander J. Dietary retinoic acid supplementation stimulates intestinal tumour formation and growth in multiple intestinal neoplasia (Min)/+ mice. Carcinogenesis 2004;25:149–53.
49. Zingarelli B, Szabo C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. Gut 1999;45:199–209.
50. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of *Apc*+ in intestinal adenomas from Min mice. Cancer Res 1994;54:5947–52.
51. Kitajima S, Takuma S, Morimoto M. Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. J Vet Med Sci 1999;61:67–70.
52. Konopka TE, Barker JE, Bamford TL, Guida E, Anderson RL, Stewart AG. Nitric oxide synthase II gene disruption: implications for tumor growth and vascular endothelial growth factor production. Cancer Res 2001;61:3182–7.
53. Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci U S A 1993;90:7240–4.

54. Perez-Sala D, Lamas S. Regulation of cyclooxygenase-2 expression by nitric oxide in cells. Antioxid Redox Signal 2001;3: 231–48.
55. Takahashi M, Nakatsugi S, Sugimura T, Wakabayashi K. Frequent mutations of the β-*catenin* gene in mouse colon tumors induced by azoxymethane. Carcinogenesis 2000;21:1117–20.
56. Suzui M, Okuno M, Tanaka T, Nakagama H, Moriwaki H. Enhanced colon carcinogenesis induced by azoxymethane in min mice occurs via a mechanism independent of β-*catenin* mutation. Cancer Lett 2002;183:31–41.
57. Muller O, Nimmrich I, Finke U, Friedl W, Hoffmann I. A β-catenin mutation in a sporadic colorectal tumor of the RER phenotype and absence of β-catenin germline mutations in FAP patients. Genes Chromosomes Cancer 1998;22:37–41.
58. Miyoshi Y, Ando H, Nagase H, Nishisho I, Horii A, Miki Y, Mori T, Utsunomiya J, Baba S, Petersen G, Hamilton S, Kinzler K et al. Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients. Proc Natl Acad Sci. U S A. 1992;89:4452–6.
59. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol 2004;287:G7–17.
60. Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, Shields PG, Ham AJ, Swenberg JA, Marrogi AJ, Harris CC. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res 2000; 50:3333–7.
61. Goodman JE, Hofseth LJ, Hussain SP, Harris CC. Nitric oxide and p53 in cancer-prone chronic inflammation and oxyradical overload disease. Environ Mol Mutagen 2004;44:3–9.
62. Chiarugi V, Magnelli L, Gallo O. Cox-2, iNOS and p53 as playmakers of tumor angiogenesis (review). Int J Mol Med 1998;2: 715–9.
63. Uthoff SM, Eichenberger MR, Lewis RK, Fox MP, Hamilton CJ, McAuliffe TL, Grimes HL, Galandiuk S. Identification of candidate genes in ulcerative colitis and Crohn's disease using cDNA array technology. Int J Oncol 2001;19:803–10.

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

(Received January 6, 2005/Revised March 10, 2005/Accepted March 14, 2005/Online publication May 16, 2005)

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 $Apc^{\Delta716}$ mice are lower than in their $Apc^{\Delta716}$ 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.

© Japanese Cancer Association
doi: 10.1111/j.1349-7006.2005.00047.x

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)-5Z-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.

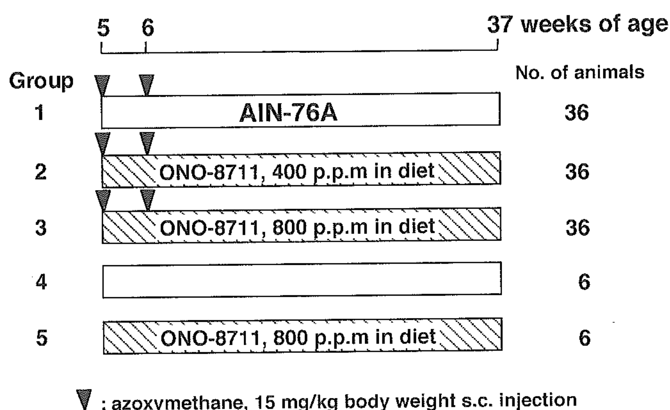

Fig. 1. Experimental protocol. The animals were male F344 rats at 5 weeks of age at the commencement. They were treated subcutaneously with AOM (15 mg/kg body weight) once a week for 2 weeks and from the day of the first treatment were fed a basal diet or a diet containing 400 or 800 p.p.m. ONO-8711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

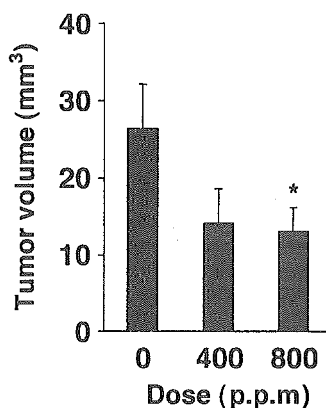

Fig. 2. Effects of ONO-8711 on the mean volumes of adenocarcinomas of the AOM-treated rat colon. Bars show AOM alone (control group), AOM + 400 p.p.m. ONO-8711 and AOM + 800 p.p.m. ONO-8711. *Significantly different from the control value at $P < 0.05$.

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 $P < 0.05$.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00047.x

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

This work was supported by Grants-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, and for the Research on Advanced Medical Technology from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-Aid for the Sagawa Foundation for Promotion of Cancer Research.

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.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00047.x