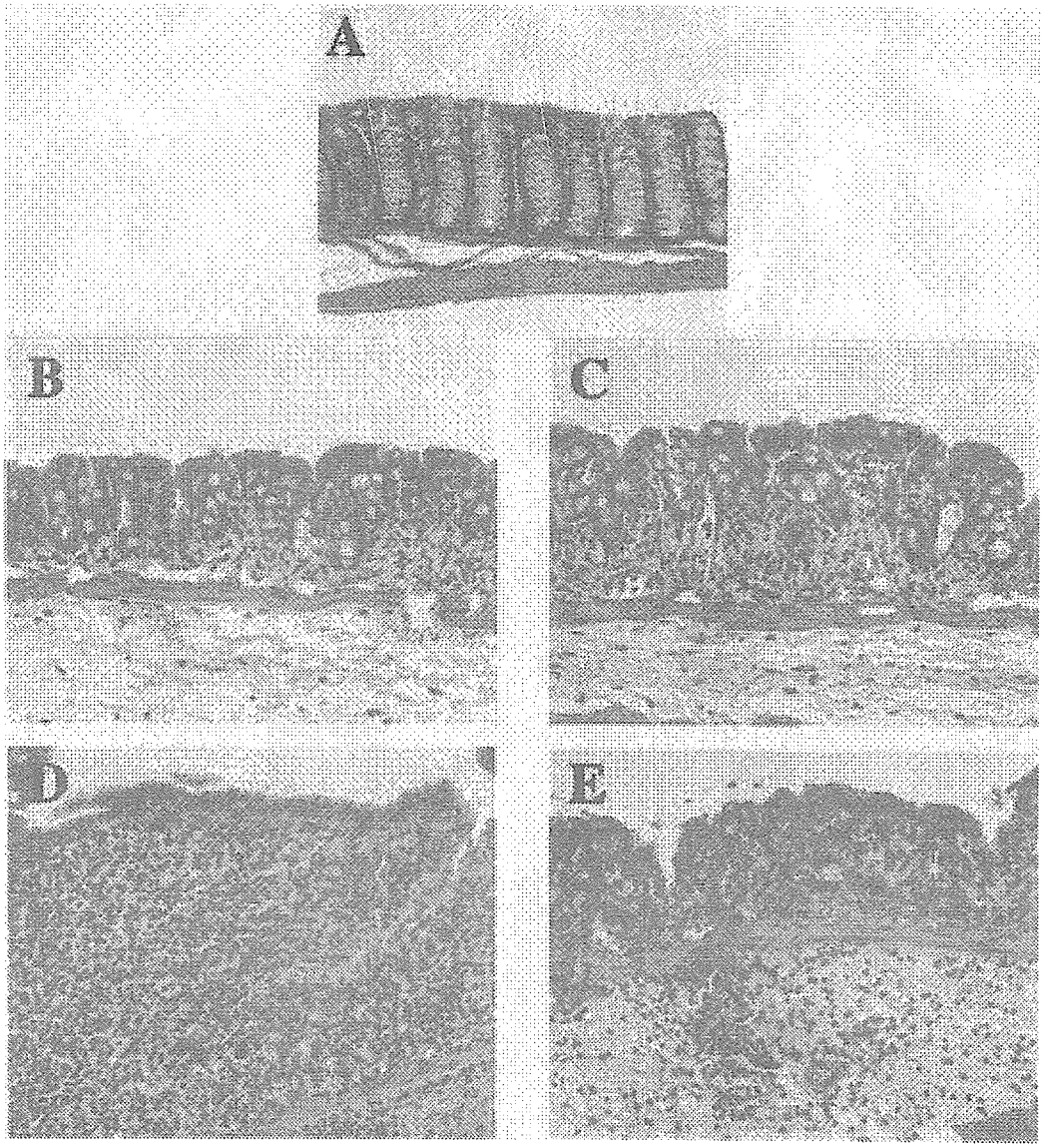

Fig. 1. Various grades of colitis. (A) Normal colon mucosa (Grade 0); (B) shortening the basal one-third of the crypts with slight inflammation and edema in the lamina propria (Grade 1); (C) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (Grade 2); (D) loss of all the crypts with severe inflammation in the lamina propria, but with the surface epithelium still remaining (Grade 3); and (E) a loss of all the crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. An exudate containing cell debris, inflammatory cells, fibrin and mucus covers the damaged mucosa (Grade 4). Hematoxylin and eosin stain. Original magnification, (A–E), 20×.

(Cat. No. 160110, Aurora, OH). CRF-1 (Oriental Yeast, Tokyo, Japan) was used as the basal diet throughout the study.

Experimental procedure

After they were brought, the mice were acclimated for 1 week with tap water and a pelleted basal diet, CRF-1, *ad libitum*. The experimental groups in each strain of mice included the AOM and DSS group, the AOM alone group, the DSS alone group and the untreated control group. The experimental protocol in the current study was slightly modified from our original protocol (8). We chose 1% as the dose level of DSS since this dose has been shown to exert sufficient tumor-promoting effects (32). In addition, the duration (4 days) of DSS exposure in drinking water was shortened based on our preliminary investigation, in which 4 days of exposure to DSS was found to enhance AOM-initiated colon carcinogenesis in ICR mice of either sex. All mice were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were maintained under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C).

Histopathological analysis

At the end of the experiment (Week 18), all the mice were killed by an ether overdose. At autopsy, their large bowel was flushed with saline and excised. After measuring the length of the large bowel (from the ileocecal junction to the anal verge), it was cut open longitudinally along the main axis and washed with saline. The large bowel was then carefully inspected for the presence of pathological lesions and fixed in 10% buffered formalin for at least 24 h. Paraffin-embedded sections of the large bowel were then made by routine procedures. Any histopathological alterations in the colon were examined on hematoxylin and eosin-stained sections. Colitis was recorded and scored according to the following morphological criteria described by Cooper *et al.* (33): Grade 0 (Figure 1A), normal colonic mucosa; Grade 1 (Figure 1B), shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa; Grade 2 (Figure 1C), loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3 (Figure 1D), loss of all crypts with severe inflammation in the mucosa, but with the surface epithelium still remaining; and Grade 4 (Figure 1E), loss

of all crypts and the surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. Intestinal neoplasms were diagnosed according to the criteria described by Pozharisski (34).

Immunohistochemistry

Nitrotyrosine immunohistochemistry was carried out on 4-μm-thick paraffin-embedded sections from the colons in all four strains of mice administered 1% DSS alone as previously described (8,35). The deparaffinized sections were incubated overnight with a primary rabbit polyclonal anti-nitrotyrosine (diluted 1:1500, CHEMICON International, CA) or with a control solution. Control sections included buffer alone or non-specific purified rabbit secondary antibody and avidin–biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The color was developed using 3,3′-diaminobenzidine-4HCl as the chromogen. The stained sections were examined for the localization and intensity of immunoreactivity by microscopy (Olympus AX70, Olympus Optical, Tokyo, Japan). To the degree of nitrotyrosine stainability, the following grading system (Grade 0–4) was applied: Grade 0, no immunoreactivity and no positive cells; Grade 1, weak immunoreactivity and <10% positive cells; Grade 2, mild immunoreactivity and 10–30% positive cells; Grade 3, moderate immunoreactivity and 31–60% positive cells; and Grade 4, strong immunoreactivity and 61–100% positive cells with extensive immunoreactivity (36).

Statistical analysis

Where applicable, the data were analyzed using one-way ANOVA with either Bonferroni correction or Fisher's exact probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), with $P < 0.05$ as the criterion of significance.

Results

General observation

The intake of DSS-containing tap water did not significantly differ among the four strains of mice (data not shown). Mice that received AOM and 1% DSS or 1% DSS alone demonstrated bloody stools either during DSS administration or soon after the cessation of DSS exposure. The degree of this symptom varied among the strains: Balb/c and C3H/HeN mice showed severe symptoms while C57BL/6N and DBA/2N mice showed mild symptoms. The mean body weight and colon length of the mice are summarized in Table I. The mean body weight of the Balb/c mice, which received AOM/DSS, was significantly lower than that of the C3H/HeN mice ($P < 0.01$) and C57BL/6N mice ($P < 0.01$), which were given AOM and DSS. A significant difference on the mean body weight was found between the AOM/DSS group and the untreated group ($P < 0.001$) in Balb/c mice. As listed in Table I, the mean lengths of the colon in the Balb/c mice ($P < 0.001$) and C3H/HeN mice ($P < 0.001$) that were treated with AOM/DSS were statistically longer than in the C57BL/6N mice. A significant difference ($P < 0.001$) was also observed between the C57BL/6N and DBA/2N mice that were exposed to AOM/DSS. The C57BL/6N mice given AOM alone has a significantly shorter colon than the Balb/c ($P < 0.01$) and DBA/2N mice ($P < 0.01$) treated with AOM alone. As for the untreated group, the colon length of the C57BL/6N mice was significantly shorter than that of the Balb/c ($P < 0.01$) and DBA/2N mice ($P < 0.01$).

Incidence and multiplicity of large bowel neoplasms

Macroscopically, colonic neoplasms developed with a different incidence and multiplicity for each strain of mice that received AOM and 1% DSS. Flat, nodular, polypoid or caterpillar-like tumors were mainly located in the middle and/or distal colon if any tumors existed (Figure 2). Histopathologically, they were tubular adenoma (Figure 3A) or adenocarcinoma (Figure 3B). Dysplastic lesions were also observed in the colonic mucosa surrounding the tumors. None of the strains of mice given AOM alone, 1% DSS alone or tap water had any colonic tumors.

Table I. Body and relative liver weights and lengths of colon in each strain of mice

Strain	Treatment (no. of mice examined)	Body weight (g)	Length of colon (cm)
Balb/c	AOM→1% DSS (10)	25.1 ± 3.8[a,b,c,d]	12.7 ± 1.0[e]
	AOM (4)	30.9 ± 0.8	14.0 ± 1.0[f]
	1% DSS (5)	34.1 ± 2.0	13.0 ± 0.6
	None (5)	32.4 ± 1.1	13.7 ± 0.5[g]
C3H/HeN	AOM→1% DSS (7)	30.2 ± 0.6	12.7 ± 1.3[e]
	AOM (5)	32.6 ± 2.2	12.5 ± 0.6
	1% DSS (5)	32.2 ± 1.2	13.1 ± 1.1
	None (3)	31.8 ± 1.1	11.9 ± 0.6
C57BL/6N	AOM→1% DSS (10)	29.3 ± 1.9	11.1 ± 0.6[h]
	AOM (5)	31.3 ± 2.0	11.7 ± 0.5[i]
	1% DSS (5)	32.0 ± 1.7	12.8 ± 0.9
	None (5)	33.0 ± 4.7	11.6 ± 1.0[j]
DBA/2N	AOM→1% DSS (10)	28.3 ± 2.3	13.2 ± 1.0
	AOM (5)	28.9 ± 1.3	14.1 ± 0.9
	1% DSS (5)	30.5 ± 0.6	14.0 ± 0.8
	None (5)	30.7 ± 1.4	13.6 ± 1.7

[a]Mean ± SD.
[b]Significantly different from untreated Balb/c mice ($P < 0.001$).
[c]Significantly different from C3H/HeN mice which received AOM/DSS ($P < 0.01$).
[d]Significantly different from C57BL/6N mice which received AOM/DSS ($P < 0.01$).
[e]Significantly different from C57BL/6N mice which received AOM/DSS ($P < 0.001$).
[f]Significantly different from C57BL/6N mice which received AOM alone ($P < 0.01$).
[g]Significantly different from untreated C57BL/6N mice ($P < 0.01$).
[h]Significantly different from DBA/2N mice which received AOM/DSS ($P < 0.001$).
[i]Significantly different from DBA/2N mice which received AOM alone ($P < 0.01$).
[j]Significantly different from untreated DBA/2N mice ($P < 0.01$).

The incidence (percent of mice with tumors) of colonic neoplasms is summarized in Figure 4A. The incidence of colonic neoplasms in the Balb/c mice (100%) was significantly higher than in the C3H/HeN mice (29%, $P = 0.0034$) and the DBA/2N mice (20%, $P = 0.0004$). A statistically significant difference ($P = 0.0115$) was also noted between the C57BL/6N (80%) and the DBA/2N mice. The order of the incidence of colonic adenoma was Balb/c mice (90%) > C57BL/6N mice (70%) > C3H/HeN mice (29%) > DBA/2N mice (20%). The incidence of adenoma in Balb/c mice was statistically greater than in C3H/HeN mice ($P = 0.0175$) and DBA/2N mice ($P = 0.0027$), and the difference between C57BL/6N mice and DBA/2N mice was statistically significant ($P = 0.0349$). The incidence of colonic adenocarcinoma was 100% in the Balb/c mice and 50% in the C57BL/6N mice and a statistically significant difference ($P = 0.0163$) was found between these two strains of mice. However, this malignancy was not found in the C3H/HeN and DBA/2N mice. As shown in Figure 4B, the multiplicity of colonic neoplasms (/mouse) was 11.4 ± 5.9 in Balb/c mice, 0.7 ± 1.5 in C3H/HeN mice, 2.5 ± 2.1 in C57BL/6N mice and 0.2 ± 0.4 in DBA/2N mice. The value for the Balb/c mice was significantly higher ($P < 0.001$) than that of other strains of mice. The order of the multiplicity of adenoma was Balb/c mice (3.7 ± 3.3) > C57BL/6N mice (1.5 ± 1.3) > C3H/HeN mice (0.7 ± 1.5) > DBA/2N mice (0.2 ± 0.4). The value for multiplicity of adenoma in the Balb/c mice was statistically greater than in the C3H/HeN

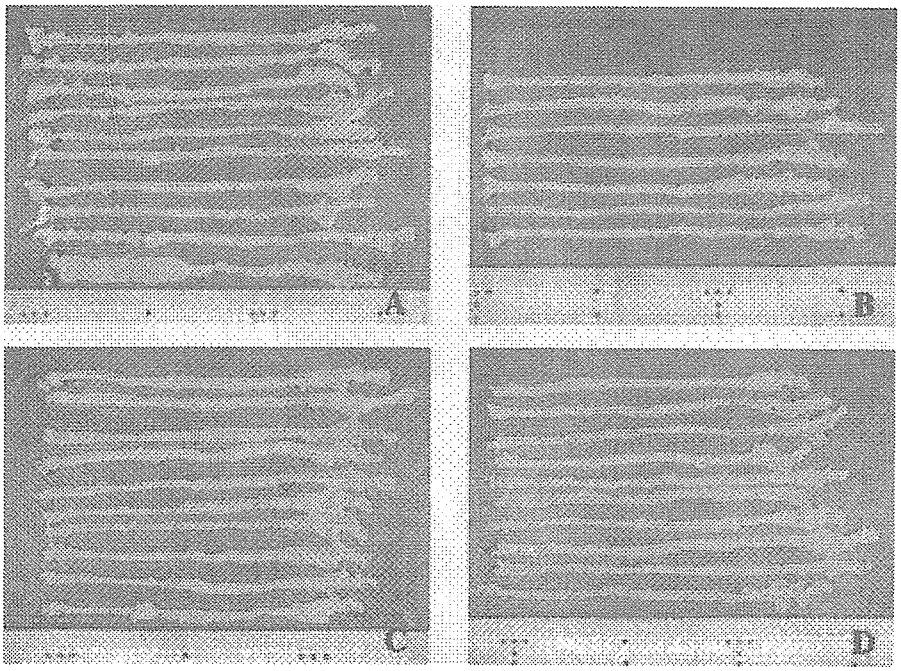

Fig. 2. Macroscopic view of the large bowel treated with AOM and 1% DSS. (A) Numerous colon tumors (2–21 tumors per mouse) develop in all Balb/c mice. (B) One or four colonic tumors are seen in two out of seven C3H/HeN mice. (C) One to five colonic tumors are found in 8 out of 10 C57BL/6N mice. (D) One colonic tumor is present in 2 out of 10 DBA/2N mice.

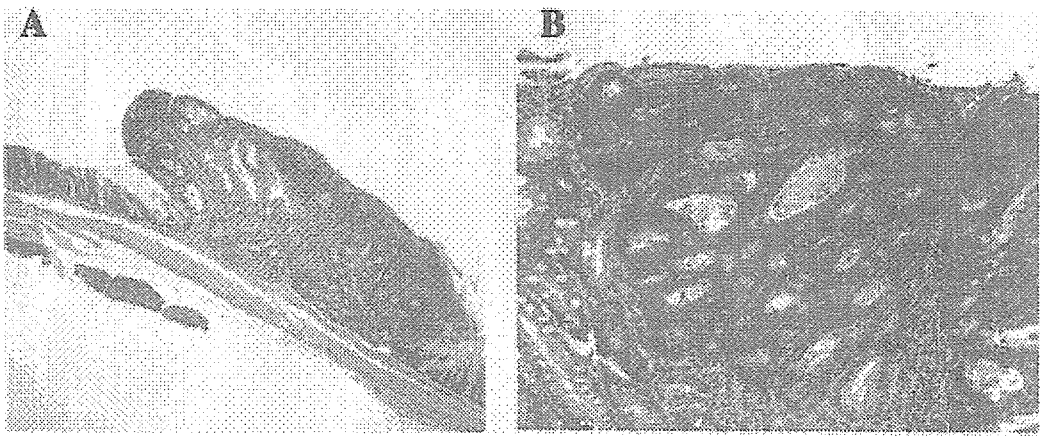

Fig. 3. Histopathology of colonic neoplasms in male Balb/c mice treated with AOM and 1% DSS. (A) Tubular adenoma and (B) moderately-differentiated adenocarcinoma. Hematoxylin and eosin stain. Original magnification, A, 2× and B, 20×.

mice ($P < 0.05$) and DBA/2N mice ($P < 0.01$). The multiplicity of adenocarcinoma in the Balb/c mice (7.7 ± 4.3) was the greatest among the four strains and it was significantly larger than that in the C3H/HeN mice (1.0 ± 1.2, $P < 0.001$).

The scores of inflammation and nitrotyrosine

As shown in Figure 5, the inflammation scores of each strain of mice initiated with AOM and followed by DSS exposure were 1.2 ± 1.1 in Balb/c, 2.3 ± 1.3 in C3H/HeN, 0.4 ± 0.7 in C57BL/6N and 0.6 ± 0.7 in DBA/2N, respectively. The score of C3H/HeN was significantly greater than that for C57BL/6N ($P<0.01$) and DBA/2N ($P<0.01$). As for the mice that received 1% DSS alone, the inflammation score of the C3H/HeN mice (1.4 ± 0.5) was the highest among the strains (1.0 ± 1.2 in Balb/c mice and 0.2 ± 0.4 in DBA/2N mice). C57BL/6N mice given 1% DSS alone had quite a low score of inflammation. The mice treated with AOM alone and the untreated mice demonstrated extremely weak inflammation in the colon.

Nitrotyrosine immunoreactivity was mainly observed in the neoplastic cells, cryptal cells, blood endothelial cells and mononuclear cells, which infiltrated the colonic mucosa (Figure 6). The stainability was relatively weak for infiltrative mononuclear cells in comparison with the cryptal cells and endothelial cells (Figure 6). As shown in Figure 7, the nitrotyrosine immunohistochemistry findings for the Balb/c mice (3.6 ± 0.5) treated with AOM and DSS were significantly higher than those for C3H/HeN (1.7 ± 0.8, $P<0.001$) and DBA/2N mice (1.6 ± 0.5, $P<0.001$). The score of nitrotyrosine-positivity in C57BL/6N mice (3.4 ± 0.5) was

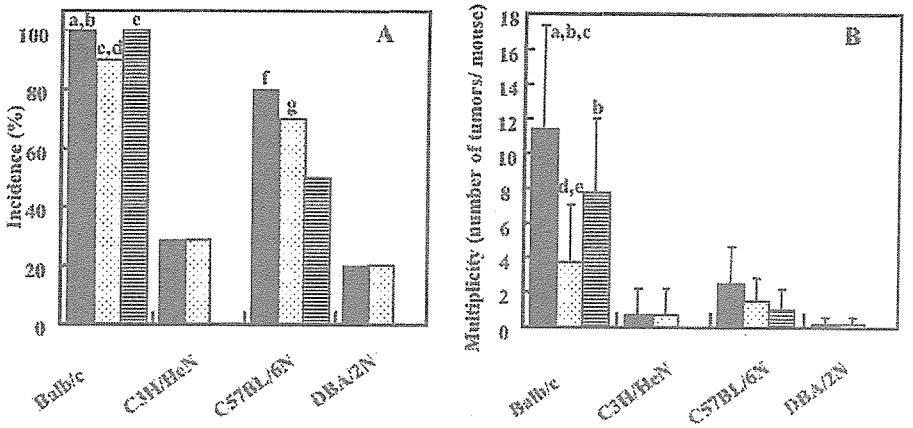

Fig. 4. Incidence and multiplicity of colonic tumors. (A) Incidence of colonic tumors. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN (P=0.0034); b, significantly different from DBA/2N (P = 0.0004); c, significantly different from C57BL/6N (P=0.0175); d, significantly different from DBA/2N (P=0.0027); e, significantly different from C57BL/6N (P=0.0163); f, significantly different from DBA/2N (P=0.0115); and g, significantly different from DBA/2N (P=0.0349). (B) Multiplicity of colonic tumors. Values are the mean ± SD. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN (P<0.001); b, significantly different from C57BL/6N (P<0.001); c, significantly different from DBA/2N (P<0.001); d, significantly different from C3H/HeN (P<0.05); and e, significantly different from DBA/2N (P<0.01).

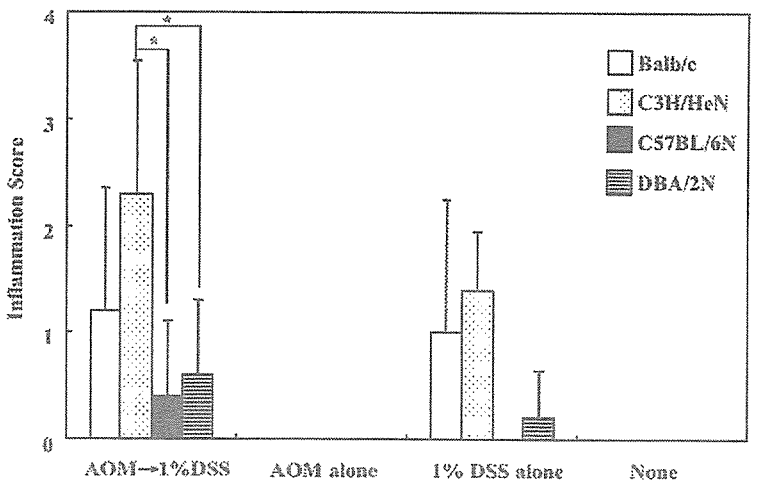

Fig. 5. Inflammation score in the colon for four strains of mice. Values are the mean ± SD. white column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *P<0.01.

statistically higher than those in C3H/HeN (P < 0.001) and DBA/2N (P<0.001) mice. In mice that received 1% DSS alone, the scores in Balb/c (2.8 ± 0.8) and C57BL/6N (2.4 ± 1.1) mice were higher than those in C3H/HeN (1.6 ± 0.5) and DBA/2N mice (1.4 ± 0.5); however, no significant differences were observed among the strains. As for the mice given AOM alone, the scores of nitrotyrosine in the Balb/c mice and C57BL/6N mice were 0.5 ± 0.6 and 0.2 ± 0.4, respectively. C3H/HeN mice and DBA/2N mice treated with AOM alone showed either no or faint stainability of nitrotyrosine. The degree of nitrotyrosine stainability in untreated mice was almost null.

Discussion

The present investigation demonstrated the different susceptibilities of the four strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice to colon tumorigenesis induced by the combination treatments with AOM and DSS. Apparently, Balb/c mice were extremely sensitive to AOM/DSS-induced colon carcinogenesis in the present experimental condition. The sensitivity of Balb/c mice observed in the present study was almost similar to those found in ICR mice (8,32,35). Colonic adenocarcinoma also developed in C57BL/6N, but the incidence was lower than in Balb/c. In contrast, the susceptibility of C3H/HeN and DBA/2N to the administration of AOM and DSS was quite low and only a few colonic adenomas developed in both the strains of mice.

Regarding the sensitivity of the mice to AOM initiation, the Balb/CJ mice were reported to have a remarkable susceptibility to the formation of distal colon tumors after treatment with AOM (26), whereas C3H, C57BL/6J, and DBA/2 mice were found to have a low incidence of colonic tumors by AOM initiation (25,26,29). Strain differences in the susceptibility to DSS have also been demonstrated: Balb/c, C3H/HeJ and C57BL/6J are relatively susceptible to DSS, whereas DBA/2J mice are virtually resistant based on the frequency of ulceration or the histological score of inflammation in the colon

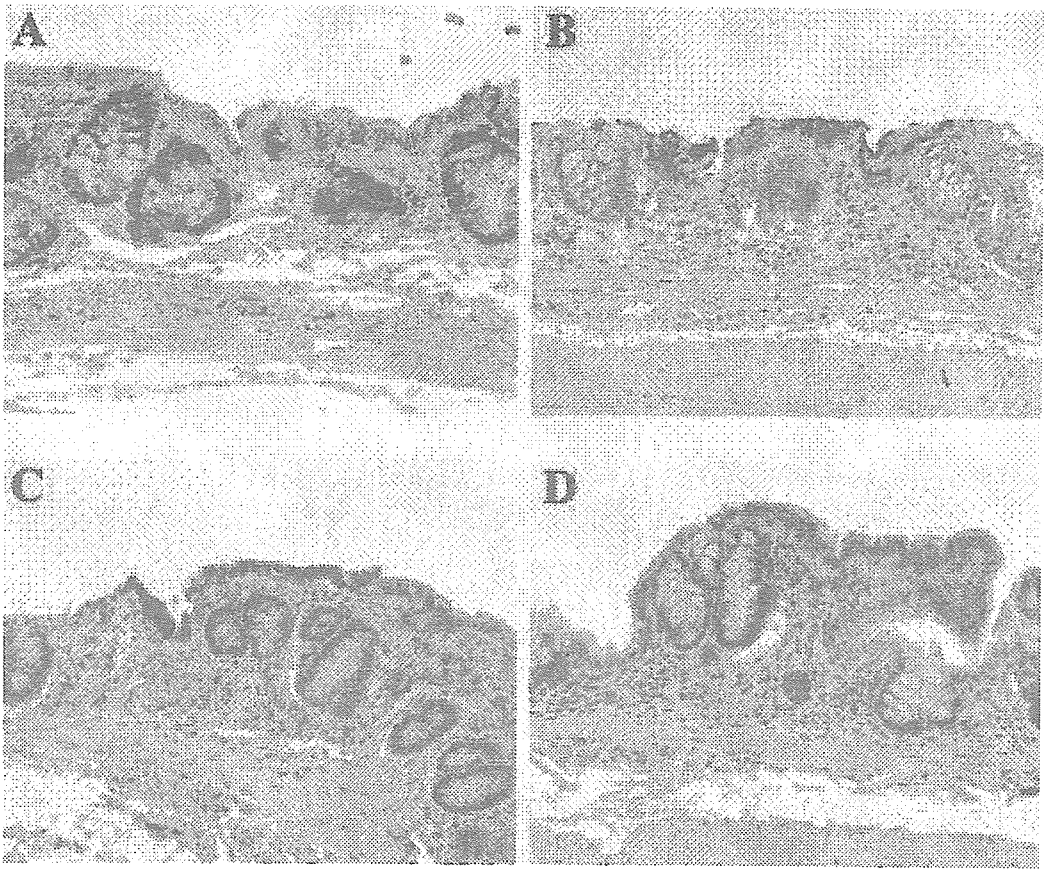

Fig. 6. Nitrotyrosine immunohistochemistry of the colon from four strains of mice given 1% DSS. (A) Balb/c; (B) C3H/HeN; (C) C57BL/6N; and (D) DBA/2N.Original magnification, (A–D), 20×.

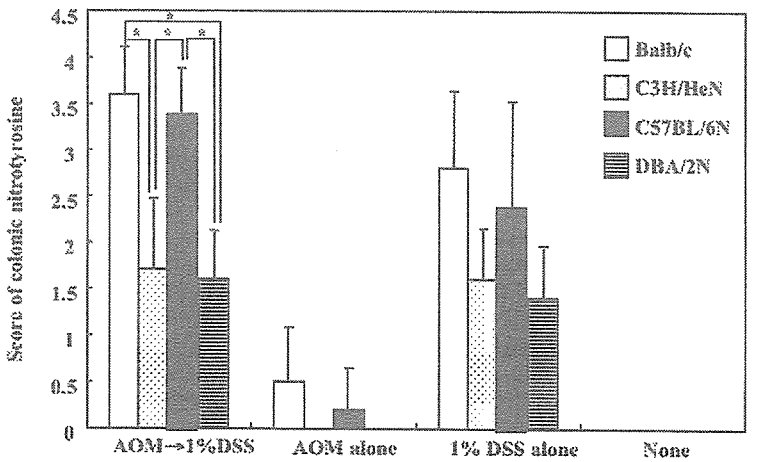

Fig. 7. Score for nitrotyrosine immunohistochemistry. Values are the mean ± SD. White column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *$P<0.001$.

(27,28). In the current study, the sensitivities of the four strains to DSS were somewhat dissimilar to those of previous studies (27,28). The inflammation score of colonic mucosa revealed a severe and moderate inflammation to be present in the C3H/HeN and Balb/c mice treated with both AOM and DSS, respectively, while C57BL/6N and DBA/2N mice had only a relatively weak inflammation. In the case of the receptivity of C57BL mice to lipopolysaccharide (LPS), C57BL/10ScCr mice were resistant to LPS, whereas C57BL/10ScSn mice responded to LPS (37). Similarly, C3H/HeJ and C3H/HeN are LPS-responder and LPS-non-responder mice, respectively (38,39). As a result, the discrepancy in the response of DSS in mice might be due to differences in the substrains. In the current study, the highest incidence of colonic tumors was

found in Balb/c. C57BL/6N had the second highest incidence among the strains tested. On the other hand, C3H/HeN and DBA/2N had only a few benign colonic tumors (adenomas). The shortening of colon length in the mice that received DSS is one of the biological markers of severity of colonic inflammation (8–10,32,35). When comparing the colon length in mice treated with AOM and DSS with that in untreated mice, the order of the shortening rate of the colon length of mice was Balb/c (7%) > C57BL/6N (4%) > DBA/2N (3%) > C3H/HeN (−6%). These results suggest that the different susceptibilities of the inbred mouse strains to AOM/DSS-induced colon carcinogenesis might correlate with different sensitivities to AOM or DSS, with only slight contradictions among the substrains.

AOM is widely used as a colonic carcinogen to investigate the pathogenesis and modification of colon carcinogenesis in rodents (11–13). AOM requires metabolic activation to exert its carcinogenic action. Cytochrome P450 (CYP) is known to play a prominent role in the modulation of the xenobiotic metabolism, including chemical carcinogens. CYP 2E1 is one of the important factors for converting AOM to methylazoxymethanol, which can produce DNA adduct formation and also produce the initiation event (40,41). Although we did not investigate the activity of CYP 2E1, it may be possible that the expression and/or content of CYP 2E1 differ among the strains examined. This may be indicated by the findings that the relative liver weight of Balb/c, which had the highest susceptibility of AOM/DSS-induced colon carcinogenesis, was higher than that of other strains of mice in the current study (data not shown).

The influence of nitrosation stress caused by DSS is also an important factor for AOM/DSS-induced mouse colon carcinogenesis, since a powerful tumor-promoting activity of DSS has been observed in this model (8,32,35,42). We found a close association between the score of nitrotyrosine and the occurrence of tumors in the current study. Nitrotyrosine-immunohistochemical scores of each strain of mice in the 'AOM → DSS' and 'DSS alone' groups were much greater than those of the 'AOM alone' and 'untreated' groups. The scores of the 'AOM → DSS' group were relatively higher than those of the 'DSS alone' group in all strains of mice and the order was Balb/c > C57BL/6N > C3H/HeN > DBA/2N in these two groups. Such inflammation could influence tumorigenesis, although the inflammation score did not completely correspond with the frequency of colonic tumors in the current study. Indeed, the score of inflammation in the mice receiving both AOM and DSS was higher than that of the mice administered DSS alone. An investigation of additional factors is needed to precisely elucidate the strain differences in the susceptibility to colon carcinogenesis. Recently Greten *et al.* (43) reported interesting findings, namely that a specific inactivation of the IκB kinase (IKK)/NF-κB pathway can attenuate the formation of inflammation-associated colon tumors in *villin-Cre/Ikkβ*$^{F/\Delta}$ mice. They also suggested that IKKβ might be involved in inflammation-related carcinogenesis.

In conclusion, we herein demonstrated the differences in the genetic susceptibility to AOM/DSS-induced colon tumorigenesis among four inbred strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice and found the Balb/c mice to be the most sensitive. Our findings suggest that the genetic background thus plays an important role in the cancer risk in colitis-related colon tumorigenesis. In addition, strain differences in the susceptibility of colon carcinogenesis induced by AOM and DSS might be influenced by the response to nitrosation stress due to inflammation as determined by the genetic background.

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Predominant T helper type 2-inflammatory responses promote murine colon cancers

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Colon cancer is one of the most serious complications of inflammatory bowel diseases, especially ulcerative colitis (UC). Previous studies have shown that characteristic immunological event during inflammation in UC is the expression of T helper-type 2 (Th2) cell-derived cytokines. In this study, we investigated the influence of a predominant Th2-type cytokine response in colitis on carcinogen-induced colon tumors. Wild type (WT), interferon gamma (IFN-γ) gene deficient (−/−) [Th2 dominant] or interleukin (IL)-4$^{-/-}$ [Th1-dominant] mice of BALB/c background were used in this study. To compare tumor formation, mice were given the carcinogen azoxymethane (AOM) and intrarectal administration of trinitrobenzene sulfonic acid (TNBS), to induce colitis. Thirty-three weeks after initial treatment, the total colon was examined. When IFN-γ$^{-/-}$ mice were treated with AOM and TNBS, significantly higher number of tumors were seen (8.4 ± 1.7) than in WT (3.3 ± 2.9) or IL-4$^{-/-}$ (3.1 ± 3.4) mice, which received identical treatments. A separate set of experiment, using less doses of AOM and TNBS also showed the higher frequency of tumor formation in IFN-γ$^{-/-}$ mice than in IL-4$^{-/-}$ mice. Histologically, the tumors were well- or moderately-differentiated adenocarcinomas. No invasion into the submucosal or serosal layers of the intestine was seen. In immunohistological staining, some tumors in IFN-γ$^{-/-}$ mice showed distinct nuclear expression of β-catenin, in contrast to the strong membrane staining seen in tumors of IL-4$^{-/-}$ mice. In conclusion, colonic inflammation associated with Th2-donimant cytokine responses enhanced the formation of malignant neoplasms.

Key words: colitis; cancer; interferon-γ; interleukin-4; carcinogenesis

Colorectal cancer is one of the most serious complications in inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD).[1] Of note, patients with longstanding, extensive UC have a high cancer risk, ~16.5% at 30 years, after initial diagnosis.[2,3] It has long been noted that cancer arises from regions of chronic inflammation, and the inflammatory cells and cytokines of the immune system found in tumors are more likely to contribute to tumor growth and progression.[4] In animal models, colitis induced by dextran sulfate sodium (DSS) are associated with dysplasia and cancer.[5–7] Recent studies on liver cancer[8] and colon cancer[9] models suggested that transcription factor NF-κB, which is a key player of inflammatory responses, does not affect initiation, but acts in tumor promotion. Thus, inflammation may significantly affect the process of cancer in UC. In fact, cancers in UC have several distinct features from colorectal cancers in non-IBD patients.[10] First, tumors in UC are often multiple, which is to be expected from precancerous dysplastic changes found in UC mucosa. Second, cancer in UC is often flat and infiltrating. Third, there is a higher incidence of high-grade, mucinous carcinomas than seen in non-IBD cancer. At a molecular level, p53 gene mutations or p53 protein overexpression, which is a late event in the development of sporadic colorectal carcinoma, have been commonly reported as early events in the dysplasia–carcinoma sequences in UC-associated carcinomas.[11–14] These results provide evidence that UC-associated cancer may develop along a pathway that is different from that of sporadic colorectal cancer.

Although pathogenesis of IBD is unknown, fluctuating but constant inflammatory responses at the local site is the major pathological finding. Past studies have shown that local immunological events during chronic inflammation in UC and CD are different. The presence of activated Th1 cells in the intestine is the characteristic of CD, with high expression of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α).[15,16] On the other hand, elevated expression of T helper-type 2 (Th2) cell-derived cytokines is often seen in UC.[17,18] By use of various murine models, we and others have shown that such distinct cytokine responses actually involved in the unique pathological changes in CD and UC. For example, we noted distinct pathological differences in the hapten-induced colitis in Th1 *versus* Th2-dominant mice.[19,20] In Th2 dominant mice, fibrosis and diffuse atrophic changes in epithelial cells were seen, while acute ulcers were the major lesions of colitis in Th1 dominant mice. Other groups have reported that administration of the sensitizing agent, oxazolone, induced colitis with diffuse epithelial damage. In this model, a Th2 type cytokine, interleukin (IL)-13 was the major effector cytokine.[21,22] Further, transfer of Th2-dominant T cells to T cell-deficient recipient mice resulted in ileal villus atrophy and goblet cell metaplasia,[23] while transfer of Th1 dominant T cells induced erosive gastritis with enhanced surface epithelial cell apoptosis.[24] These results suggest that a predominance of either Th1- or Th2-type cytokines in inflammatory responses has a major influence on the pathology and tissue remodeling in the chronic inflammation, and eventually affects controlling epithelial cell differentiation, as well as their turnover. Thus, differential upregulation of inflammatory cytokines in UC may directly contribute to malignant progression. However, data on the participation of a predominance of Th1 or Th2 cytokines in mucosal immunity in colonic carcinogenesis is limited.

The possible factors which lead to dysplasia and malignant transformation in UC need to be more thoroughly investigated. In this study, we used models of Th1- or Th2-dominant colitis model together with azoxymethane (AOM)-induced carcinogenesis and sequential and morphological analysis, paying particular attention to the tissue tropism of carcinogenesis. Our findings show that a Th2 cytokine dominant colitis increases the frequency and changes in pathological features of colonic neoplasms.

Abbreviations: AOM, azoxymethane; DSS, dextran sulfate sodium; GI, gastrointestinal; H&E, hematoxylin and eosin; IFN, interferon; IL, interleukin; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

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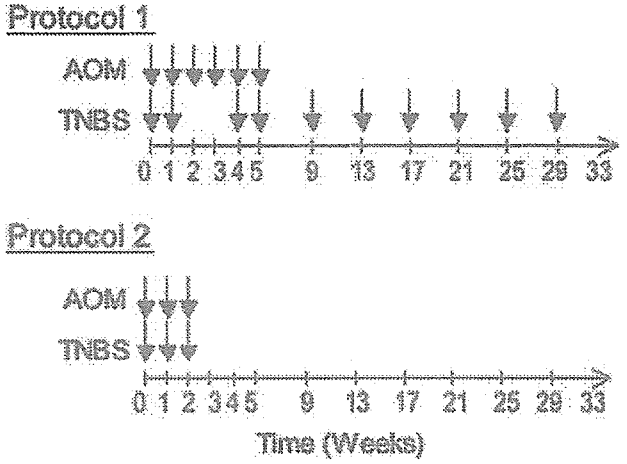

FIGURE 1 – Experimental design used in this study.

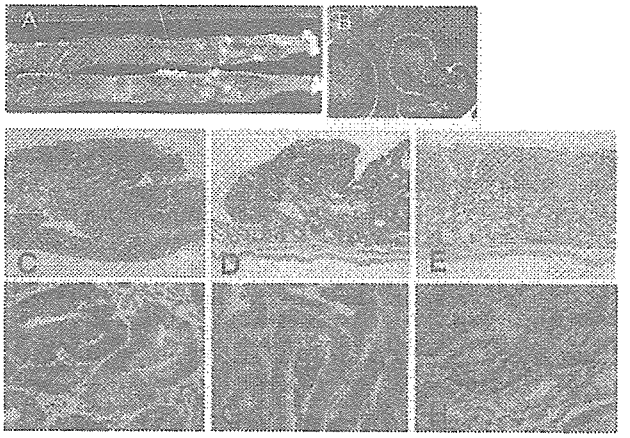

FIGURE 2 – Pathological features of colonic tumors. (*a*) Macroscopic appearance of typical tumors. Colons from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS are shown. (*b*) Zoom stereomicroscopic appearance of tumors in panel A after staining with methylene blue. Polypoid cancer seen in WT (*c*) and IL-$4^{-/-}$ (*d*) mice treated with AOM plus TNBS, stained by H&E. (*e*) Flat-elevated cancer seen in IFN-$\gamma^{-/-}$ mice (H&E staining). Immunostaining for β-catenin of well-differentiated adenocarcinomas from WT (*f*) and IL-$4^{-/-}$ (*g*) mice showing a cell membrane pattern and moderately differentiated adenocarcinoma from IFN-$\gamma^{-/-}$ mice (*f*) showing a nuclear pattern.

Material and methods

Mice

Wild type (WT), IFN-γ gene knockout (IFN-$\gamma^{-/-}$), IL-4 gene disrupted (IL-$4^{-/-}$) mice, all on the BALB/c background were originally obtained from Jackson Laboratory (Bar Harbor, ME). The colony was maintained under pathogen-free conditions in the Immunocompromized Mouse Facility of the Research Institute, International Medical Center of Japan (IMCJ). All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and according to the approval of the local ethics committee in the IMCJ.

Induction of colitis and colon tumors

Treatment of mice was initiated when mice were 8 weeks of age. Trinitrobenzene sulfonic acid (TNBS) colitis was induced, as described previously.[19,20] A 2% solution of TNBS (Research Organics, Cleveland, OH) in PBS:ethanol (1:1 by volume) was administered intrarectally to mice, anesthetized with ketamine (Sankyo Co. Ltd., Tokyo, Japan) and xylazine (Sigma, St. Louis, MO). A dose of TNBS of 36 μg/g of body weight was given. The colon carcinogen AOM was purchased from Sigma, and a dose of 10 μg/g of body weight in saline was injected intraperitoneally. TNBS and AOM were given, as indicated (Fig. 1). In some experimental groups, treatment with either TNBS or AOM was performed. In protocol 1, six doses of AOM or 4 doses of TNBS were given during the first 5 weeks, and then TNBS was given every 4 weeks on 6 occasions, to mimic recurrence of inflammation. In protocol 2, three doses of TNBS and AOM were given together in the first 2 weeks, and colonic tissues were examined after 33 weeks. To examine the spontaneous development of tumors, some cytokine knockout mice were kept untreated, until they were 40 weeks of age.

Macroscopic and histological examination

Colonic tissues were opened longitudinally, fixed in 10% formalin overnight at 4°C, washed in PBS, stained with 2% methylene blue for contrast, and then the numbers of macroscopically visible tumors were assessed. Some specimens were examined with a zoom stereomicroscope. Tumors were removed along with the surrounding normal colonic tissues and embedded in paraffin blocks; then, 4-μm sections were prepared and stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, 3-μm-thick paraffin sections were deparaffinated in xylene, rehydrated and heated at 95°C in 10 mM citrated buffer (pH 6.0) for 10 min. After treatment with 3% H_2O_2, followed by 0.25% goat serum in PBS for blocking, sections were incubated with monoclonal antibodies to p53 (DO-1, 1:200) or β-catenin (1:800, both from BD Transduction Laboratories, CA) overnight at 4°C. Mouse IgG was used as a negative control. Bound antibody was detected using Vectastain ABD-kit (Vector Laboratories, Burlingame, CA) according to vendor's protocol, and deaminobenzidine was used as substrate for peroxidase.

Statistics

The results were compared by the Mann–Whitney test or χ^2 test, using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for the Macintosh computer.

Results

Effects of inflammation and cytokine deficiency on the formation of colon tumors

In our study, polypoid or sessile elevated lesions were macroscopically visible in the middle to distal colon, and these were enumerated (Figs. 2*a* and 2*b*). The results of protocol 1 are summarized in Table I. When WT mice were treated with only AOM in protocol 1, 3.3 tumors/mouse were seen after 33 weeks. In IFN-$\gamma^{-/-}$ mice, the incidence of tumors induced with AOM was not significantly different from that of WT mice. IL-$4^{-/-}$ mice, treated with AOM only, did not develop tumors, except for 1 mouse with a single tumor, although aberrant crypt foci (ACF) were frequently seen by following zoom stereomicroscopy. Induction of TNBS colitis, in addition to AOM treatment, did not change the numbers of tumors in WT mice. In contrast, induction of colitis induced enhanced formation of tumors in both IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice. Of note, the number of tumors in IFN-$\gamma^{-/-}$ mouse reached to 8 tumors/mouse, which was significantly higher when compared with those in IL-$4^{-/-}$ or WT mice. The incidence of tumor bearing mice was 100% in the IFN-$\gamma^{-/-}$ group. There were no significant differences in the size of the tumors, which developed in each experimental group. In protocol 1, most of the deaths occurred mostly in the first 8 weeks. The exceptions were that some IL-$4^{-/-}$ mice treated with TNBS died in later period, coinciding with our previous report that IL-$4^{-/-}$ mice are more susceptible to TNBS colitis.[19] Further, we did not see any tumors in the mice which died before 33 weeks, including IL-$4^{-/-}$ mice.

TABLE I – NUMBER OF TUMORS IN CYTOKINE DEFICIENT MICE (PROTOCOL 1)

Mice	Treatment	No. of mice survived/total	Incidence of tumor	No. of tumors/mouse	Size of tumors (mm^3)	Total number of tumors
WT	AOM	7/12 (58)[1]	5/7 (71)	3.3 ± 2.9	2.2 ± 1.3	23
WT	AOM + TNBS	10/17 (59)	6/10 (60)	2.0 ± 2.5	2.9 ± 1.3	20
WT	TNBS	5/5 (100)	0/5 (0)	0 ± 0		0
IFN-$\gamma^{-/-}$	AOM	7/16 (43)	2/7 (28)	1.4 ± 2.3	2.7 ± 1.9	10
IFN-$\gamma^{-/-}$	AOM + TNBS	7/11 (64)	7/7 (100)[2]	8.3 ± 1.8[3]	2.8 ± 1.8	58
IFN-$\gamma^{-/-}$	TNBS	8/10 (80)	0/8 (0)	0 ± 0		0
IFN-$\gamma^{-/-}$	Untreated	8/8 (100)	0/8 (0)	0 ± 0		0
IL-$4^{-/-}$	AOM	15/17 (88)	1/15 (7)	0.1 ± 0.3[4]	1.6	1
IL-$4^{-/-}$	AOM+TNBS	7/11 (64)	5/7 (71)[5]	3.1 ± 3.4	1.7 ± 1.2	22
IL-$4^{-/-}$	TNBS	2/7 (29)	0/2 (0)	0 ± 0		0
IL-$4^{-/-}$	Untreated	4/4 (100)	0/4 (0)	0 ± 0		0

[1]Values in parentheses indicate percentages.–[2]Difference from IFN-$\gamma^{-/-}$ or IL-$4^{-/-}$ mice treated AOM was statistically significant ($p<0.02$).–[3]Differences from all other experimental groups were statistically significant ($p < 0.02$).–[4]Differences from WT mice with AOM or AOM+TNBS were statistically significant ($p<0.02$).–[5]Difference from IL-$4^{-/-}$ mice treated AOM was statistically significant ($p < 0.02$).

TABLE II – NUMBERS OF TUMORS IN CYTOKINE DEFICIENT MICE (PROTOCOL 2)

Mice	Treatment	No. of mice survived/total	Incidence of tumor formation	No. of tumors/mouse	Total number of tumors
IFN-$\gamma^{-/-}$	AOM + TNBS	19/24 (79)[1]	10/19[2] (53)	0.8 ± 0.9[2]	15
IL-$4^{-/-}$	AOM + TNBS	12/24 (50)	0/12 (0)	0	0

[1]Values in parentheses indicate percentages.–[2]Differences from IL-$4^{-/-}$ mice were statistically significant ($p < 0.02$).

It indicated that mortality was not associated with cancer, but more with susceptibility to TNBS colitis or toxicity of AOM in each mouse strain.

Next, to conform the difference between IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice treated with both AOM and TNBS, we have chosen protocol 2 (Fig. 1), reducing the dose of AOM and TNBS. A change in the dose of AOM improved the survival of IFN-$\gamma^{-/-}$ mice; however, this dose change did not affect the survival of IL-$4^{-/-}$ mice, probably because of their higher sensitivity to TNBS colitis than IFN-$\gamma^{-/-}$ mice (Table II). In this protocol, death occurred within the first 3 weeks, and no tumor-caused death was observed in both groups. Although the numbers of tumor/mouse decreased in this protocol, we saw similar differences between the IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice groups (Table II). No visible tumors were seen in IL-$4^{-/-}$ mice. In contrast, 10 of 19 IFN-$\gamma^{-/-}$ mice formed more than 1 tumor in the colon.

Microscopic findings

Tumors formed in protocol 1 were subjected to microscopic examination. The majority of these were polypoid lesions (Figs. 2*c* and 2*d*), and some of them were flat, elevated lesions (Fig. 2*e*). Tumors histologically examined were all diagnosed as well-differentiated or moderately differentiated adenocarcinoma. There was also colonic dysplasia involving 1–2 crypts, in mouse groups that had treatment with AOM. In each groups of mice, there were no differences in the severity of cell infiltration into tumors. In IFN-$\gamma^{-/-}$ mice with TNBS colitis, deformity of crypts was the most evident, as previously reported[20]; however, there were no clear relations to tumor location. In WT mice, the ratio of moderately differentiated adenocarcinoma from mice treated with both AOM and TNBS tended to be higher than those treated only with AOM, in which well-differentiated adenocarcinoma was the major type (Fig. 3). The majority of tumors from IFN-$\gamma^{-/-}$ mice were moderately differentiated adenocarcinomas in both AOM or AOM plus TNBS treated groups (Fig. 3). In protocol 2, fourteen out of 15 tumors from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS were also diagnosed as moderately differentiated adenocarcinomas. One tumor was a well-differentiated adenocarcinoma.

Immunohistochemistry

Immunostaining for p53 and β-catenin was performed. Staining with anti-p53 monoclonal antibody was generally weak, and obviously enhanced nuclear staining was seen in only 2 tumors in IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS, 2 from IL-$4^{-/-}$ mice

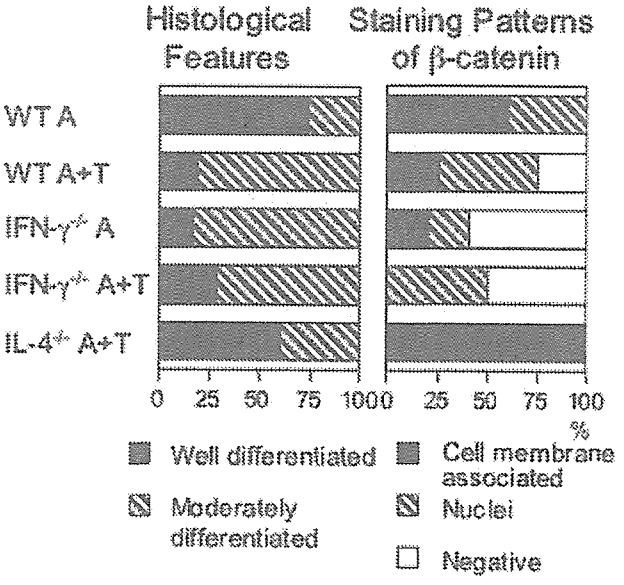

FIGURE 3 – Percentage of histological diagnosis and β-catenin staining pattern of colon tumors. Tumors (6–10) from each mouse group in protocol 1 were evaluated for their histological grading and expression of β-catenin. A, treated with AOM; T, treated with TNBS.

treated with AOM plus TNBS and 2 tumors from WT mice treated with AOM (data not shown). In contrast, a differential staining pattern with β-catenin was seen. Well-differentiated adenocarcinomas in WT and IL-$4^{-/-}$ mice showed typical staining of cell membrane, which was much stronger than that in normal epithelial cells (Figs. 2*f* and 2*g*). In contrast, nuclear staining was frequently observed in tumors from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS (Fig. 2*h*). All tumors from IL-$4^{-/-}$ mice showed a cell membrane pattern, including the one from mice treated with only AOM. There was no tumor with a membrane-staining pattern from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS (Fig. 3). Thus, nuclear and membrane localization of β-catenin was the characteristics of moderately differentiated adenocarcinomas in IFN-$\gamma^{-/-}$ mice and well-differentiated adenocarcinoma in IL-$4^{-/-}$ mice, respectively (Fig. 3).

Discussion

In the present study comparing WT, IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice, several new findings in colitis-related colon cancer were revealed. First, in the absence of IFN-γ, induction of TNBS colitis and AOM strongly enhanced tumor formation in the colon. Importantly, colitis also affected the histological degree of differentiation, and frequently induced nuclear translocation of β-catenin.

Although the precise mechanism of enhancement of tumor formation is not yet clear, one possibility is that a defect of tumor immunosurveillance occurs in IFN-$\gamma^{-/-}$ mice. Previous studies showed that IFN-$\gamma^{-/-}$ mice develop chemical carcinogen methylcholanthrene-induced sarcoma,[25] spontaneous lymphomas and lung adenocarcinomas[26] more frequently than do WT mice. In our studies, there were no significant differences in the frequency of tumor formation between WT and IFN-$\gamma^{-/-}$ mice, when inflammation was not induced. In contrast, in the presence of colitis, the number of tumors in IFN-$\gamma^{-/-}$ mice was markedly increased, while tumor frequency in WT mice was not increased. This result in WT mice is distinct from studies reporting the development of AOM-induced tumors within a short term in WT mice when DSS colitis was induced,[27,28] although there has been no report testing IFN-$\gamma^{-/-}$ mice. In DSS colitis, diffuse loss of epithelium is the primary pathological finding, which might increase the chance of carcinogenesis and promotion during vigorous epithelial cell regeneration. On the other hand, since TNBS colitis is characterized by a focal ulcer and a strong hapten (trinitrophenyl residue)-specific immune response of T and B cells, IFN-γ produced as a part of adoptive immunity in the inflammation seems to be important for protection from the colonic tumor. In other words, IFN-γ production may not be required for tumor surveillance in non-inflamed, steady state colons of BALB/c mice. A significant incidence of lung carcinoma, but no report of colon carcinoma during the life span of more than 600 days in BALB/c IFN-$\gamma^{-/-}$ mice, also supports this notion.[26] The importance of IFN-γ in tumor surveillance produced by T cells as a part of adoptive immunity was also shown in the previous study that RAG$2^{-/-}$ x STAT$1^{-/-}$ double knockout mice showed increased susceptibly to methylcholanthrene-induced carcinogenesis, but did not display a significantly greater tumor incidence, when compared with mice that lacked either RAG2 or STAT1 genes.[25] Thus, it is feasible that the colonic mucosa of UC, which fails to induce prevailing upregulation of IFN-γ but produces Th2-type cytokines, can frequently develop colonic carcinoma. On the other hand, our results also showed that addition of TNBS colitis to AOM increased the incidence of tumor in IL-$4^{-/-}$ mice, although the number was much less than in IFN-$\gamma^{-/-}$ mice. This result suggested that aberrant Th1-dominant inflammatory responses might also increase the tumor risk, although it was not comparable to that in Th2 dominant condition.

Since there is an interaction between Th1 and Th2 cytokines that they suppress the effect of each other, the presence of an excess amount of Th2 cytokines in IFN-$\gamma^{-/-}$ would have a significant effect. We have previously analyzed the cytokine production by colonic $CD4^+$ cells in WT, IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice in naïve colon, as well as in acute (day 1) and chronic (day 10) phase of TNBS colitis. Colonic $CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice produced very large amounts of IL-4 and IL-5. In contrast, secretion of IFN-γ by colonic $CD4^+$ T cells from IL-$4^{-/-}$ mice was higher than in colonic $CD4^+$ T cells from WT mice.[19,20] In protocol 1 of current study, the number of tumors in IL-$4^{-/-}$ mice was much lower than those of WT or IFN-$\gamma^{-/-}$ mice. In protocol 2, none of the IL-$4^{-/-}$ mice developed tumors, in contrast to the 53% incidence in IFN-$\gamma^{-/-}$ mice. These results suggest that IL-4 may have a direct effect on promoting tumor formation. In fact, many malignant tumors express the IL-4 receptor, which is able to bind to both IL-4 and IL-13 and also the high affinity decoy receptor of IL-13, IL-13 receptor α2.[29] However, the function of IL-4 and IL-13 in tumor cells, especially in colonic cancer is not yet clear, and the output effect via these receptors still remains to be investigated. Early studies showed that IL-4 and IL-13 had antitumor activity in mice by growth inhibition[30] through IL-4 receptor.[31] However, subversion of host antitumor defenses has also been demonstrated for IL-13. Recent studies using tumor cell lines demonstrated that STAT6, IL-4 and IL-13 were capable of inhibiting tumor rejection.[32–34] Thus, Th2 type cytokines appear to antagonize tumor immunosurveillance.

In our study, IL-$4^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice showed distinct expression patterns for β-catenin, cell membrane and nuclear staining in IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice, respectively, while WT mice had tumors of both patterns. This again suggests that Th2-cytokine predominance directly influences the mutation of DNA in epithelial cells. Indeed, exogenous IL-4 and IL-13 decreased the levels of membrane staining of β-catenin in keratinocytes, without changes of total protein levels of β-catenin, while IFN-γ enhanced membrane staining.[35] In our model, gene mutation induced by AOM might facilitate nuclear translocation of β-catenin, which had localized in cytoplasm, but not in cell membrane by the action of IL-4 and IL-13, secreted as inflammatory responses of IFN-$\gamma^{-/-}$ mice. Thus, we propose that enhancement of tumor formation in IFN-$\gamma^{-/-}$ mice is due both to a lack of immunosurveillance by IFN-γ and promotion of carcinogenesis by excess Th2 type cytokines. In this context, epithelial cells in the process of tissue repair in the inflamed colon are susceptible to the absence of IFN-γ and the presence of excess amount of IL-4 and IL-13.

In summary, our results show that a predominance of Th2 type cytokines in the inflamed colon, which mimics mucosal immunity in UC, promotes aberrant β-catenin expression and tumor formation. This model will be useful to further clarify the mechanism of colitic-cancer and for our search for targets or new agents for prevention of colon cancer.

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Effect of Running Training on DMH-Induced Aberrant Crypt Foci in Rat Colon

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ABSTRACT

FUKU, N., M. OCHIAI, S. TERADA, E. FUJIMOTO, H. NAKAGAMA, and I. TABATA. Effect of Running Training on DMH-Induced Aberrant Crypt Foci in Rat Colon. *Med. Sci. Sports Exerc.*,Vol. 39, No. 1, pp. 70–74, 2007. **Purpose:** We examined the effects of treadmill-running training on the induction of aberrant crypt foci (ACF), which is the first step of colon cancer induction, in the colonic mucosa of rats injected with 1,2-dimethylhydrazine (DMH). **Methods:** Four-week-old F344 rats (N = 38) were randomly assigned to training (19 rats) and control (19 rats) groups. After a week, all rats were given DMH (20 $mg \cdot kg^{-1}$ body weight) once a week for 2 wk. Running training was started at age 7 wk (speed: 10 $m \cdot min^{-1}$, 0% grade, 120 $min \cdot d^{-1}$, 5 $d \cdot wk^{-1}$). After 4 wk of training, the rats were sacrificed and the colon was removed, opened, and counted for ACF with 0.2% methylene blue staining. **Results:** Running training resulted in lower body- ($P < 0.01$) and adipose fat weight ($P < 0.05$). The numbers of ACF and total AC were significantly lower in the running training group than in the control group ($P < 0.05$). The occurrences of one, three, and five aberrant crypts per focus were also significantly lower in the running training group than in the control group ($P < 0.05$). The ratios of total AC/ACF did not significantly differ between the running training and control groups. **Conclusions:** The results of the present investigation suggest that low-intensity running training inhibits the DMH-induced initiation of colon ACF development. **Key Words:** EXERCISE, ACF, 1,2-DIMETHYLHYDRAZINE, COLON CANCER, PRIMARY PREVENTION, PHYSICAL ACTIVITY

Cancer of the large intestine is classified into colon and rectal cancers. The incidence of colon cancer is increasing at a faster rate than that of rectal cancer in recent years in advanced countries, including Japan. Colon cancers develop after the multistep accumulation of genetic and epigenetic induction of oncogenes in both humans and experimental animals (9,14,19).

The proposed multisteps of colon carcinogenesis may start when aberrant crypt foci (ACF) appear in the colon (37). ACF were defined as lesions composed of enlarged crypts, slightly elevated above the surrounding mucosa and more densely stained with methylene blue than normal crypts (3). ACF are considered to be putative preneoplastic colon lesions that may be early indicators of colon carcinogenesis (4,10,18).

Epidemiological evidence has suggested that physical activity has a protective effect on colon cancer incidence (11,13,30). However, few experimental studies have been conducted to elucidate the mechanisms of exercise-related effects on colon cancer. For example, a few earlier animal studies found that both voluntary (1,26) and treadmill (36) running training reduced tumor incidence after the administration of 1,2-dimethylhydrazine (DMH) or azoxymethane. Furthermore, no studies have examined the effects of physical exercise training on colon cancer as they might be related to the multistep nature of colon carcinogenesis, although Demarzo et al. (7) recently reported that a single session of exhaustive exercise increased the number of ACF DMH-induced rat colons. Up to now, there is no study demonstrating that exercise training affects the number of ACF, which is a putative initial step of colon carcinogenesis of rats. Therefore, we investigated the effects of running exercise training on the number of DMH-induced ACF, because previous studies suggested that physical training of this type has a protective effect on colon tumor incidence in rats.

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MATERIALS AND METHODS

Materials

All chemicals, including 1,2-dimethylhydrazine (DMH), a carcinogenic chemical of the colon, was purchased from SIGMA (St. Louis, MO).

Exercise Protocols

Animal care. All experimentation was conducted in accordance with policy statement of the American College of Sports Medicine on research with experimental animals and was approved by the animal care and use committee of National Institute of Health and Nutrition. Four-week-old Fischer 344 male rats were purchased from CLEA Japan, Tokyo. The animals were housed in rooms lighted from 7 a.m. to 7 p.m. and were maintained on an *ad libitum* diet of standard chow and water. The room temperature was maintained at 20–22°C.

Experimental design. After 1 wk of acclimatization to the housing environment (5 wk of age), the rats were randomly assigned to one of two groups: the treadmill-running training group (N = 19) or the control group (N = 19). All rats were given a subcutaneous injection of DMH at a dose level of 20 $mg \cdot kg^{-1}$ body weight, once a week for 2 wk. The DMH was dissolved in 0.1 mM EDTA (pH 6.5) immediately before the administration.

One week after the last injection of DMH (i.e., at age 7 wk), running training was started. The training rats ran for 120 $min \cdot d^{-1}$ (two 60-min bouts separated by 10 min of rest) on a flat motorized treadmill (Natsume, Tokyo, Japan). On the first day, the rats were accustomed to running at a speed of 10 $m \cdot min^{-1}$ by gradually increasing the treadmill speed to the fixed speed. The running speed was maintained for the following 4 wk of training. The intensity of this training was considered to be low because this exercise could be continued for more than 6 h without exhaustion, as reported elsewhere (34).

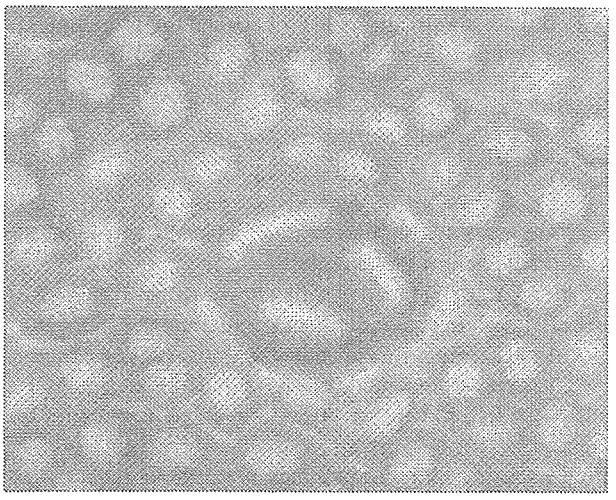

FIGURE 1—1,2-dimethylhydrazine–induced ACF in methylene blue–stained colonic mucosa. In particular, ACF is indicated by the three aberrant crypts per focus that have large crypts, altered luminal openings, and thickened epithelia. This micrograph shows an ACF that consists of three AC. ACF, aberrant crypt foci; AC, aberrant crypts.

TABLE 1. The effect of treadmill-running training on body weight, muscle weight of the heart and soleus, adipose tissue weight of the peritoneum and epididymides, and citrate synthase activity of soleus muscle in rats.

	Control Group (N)	Training Group (N)
BW (g)	216.7 ± 3.5 (19)	199.1 ± 4.0 (19)$^{\#\#}$
Heart weight ($mg \cdot g^{-1}$ BW)	2.89 ± 0.04 (11)	3.00 ± 0.04 (10)$^{\#}$
Soleus weight ($mg \cdot g^{-1}$ BW)	0.33 ± 0.01 (19)	0.37 ± 0.01 (19)$^{\#\#}$
Peritoneal adipose tissue weight ($mg \cdot g^{-1}$ BW)	15.0 ± 0.7 (19)	10.2 ± 0.6 (19)$^{\#\#\#}$
Epididymides adipose tissue weight ($mg \cdot g^{-1}$ BW)	15.1 ± 0.7 (19)	10.2 ± 0.6 (19)$^{\#}$
Brown adipose tissue weight ($mg \cdot g^{-1}$ BW)	1.50 ± 0.07 (11)	1.28 ± 0.10 (10)
Citrate synthase activity in soleus muscle ($\mu mol \cdot g^{-1}$ wet tissue$\cdot min^{-1}$)	35.9 ± 1.0 (19)	40.3 ± 1.2 (19)$^{\#\#}$

Values are means ± SE. BW, body weight. $^{\#}$,$^{\#\#}$,$^{\#\#\#}$ indicate significant differences from the control group at levels of $P < 0.05$, 0.01, and 0.001, respectively, by t-test.

Two or three days after the last bout of exercise, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 $mg \cdot kg^{-1}$ body weight), and the heart and soleus muscles were excised, weighed, quickly clamp-frozen in liquid nitrogen, and stored at −80°C until analysis. Then, the colons were dissected and gently flushed with 10% neutralized formalin to remove residual bowel contents, cut open longitudinally, fixed flat between filter papers, and submerged in 10% neutralized formalin overnight at 4°C (23). Peritoneal fat, epididymides fat, and brown adipose tissue (BAT) were excised and weighed.

Detection of ACF. Fixed colons were stained with 0.2% methylene blue, as described by Bird (3). The number of ACF and total number of aberrant crypts (AC) comprising ACF were counted for each colon. The ratio of total AC/ACF was calculated to assess ACF multiplicity. As shown in Figure 1, ACF were identified as lesions composed of enlarged crypts, with an increased pericryptal area, a slightly elevated appearance above the surrounding mucosa with an oval or slitlike orifice, and a higher staining intensity with 0.2% methylene blue than normal crypts (3).

Citrate synthase activity in skeletal muscle. Ten percent homogenates were made from the muscle in 175 mM KCl, 10 mM glutathione, and 2 mM EDTA, pH 7.4. The homogenate was frozen and thawed four times and mixed thoroughly before enzymatic measurements. As a maker of oxidative enzyme, citrate synthase (CS) activity in the soleus muscle was measured using Srere's method (31).

Statistical Analysis

All data are shown as the mean ± SE. Quantitative clinical data were compared between run-trained rats and controls by use of the unpaired t-test. ACF-related data were also compared by use of the Mann–Whitney rank sum test because the numbers of ACF were not normally distributed. These data were analyzed by use of SigmaStat for Windows (SPSS Inc., Chicago, IL). Differences were considered significant when the P value was less than 0.05.

RESULTS

Physical characteristics and citrate synthase activity. The body weight of the training rats was

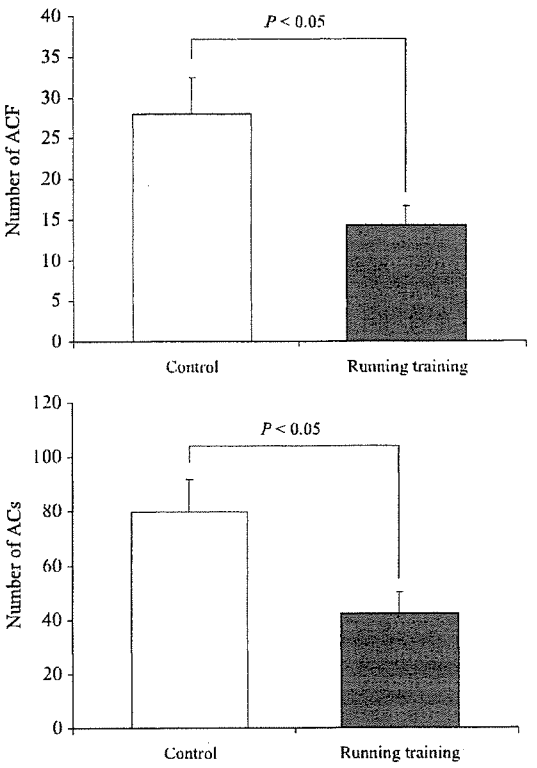

FIGURE 2—Effect of running training on the number of 1,2-dimethylhydrazine-induced ACF (*upper*) and AC (*lower*) in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

significantly less than that of the control rats ($P < 0.01$) (Table 1). The weight of heart and soleus, expressed relative to body weight, was significantly heavier in the training group compared with the control group ($P < 0.05$ and $P < 0.001$, respectively), whereas the relative adipose tissue weight of the peritoneum and epididymides was significantly lighter in the training group compared with the control group ($P < 0.001$ and $P < 0.05$, respectively).

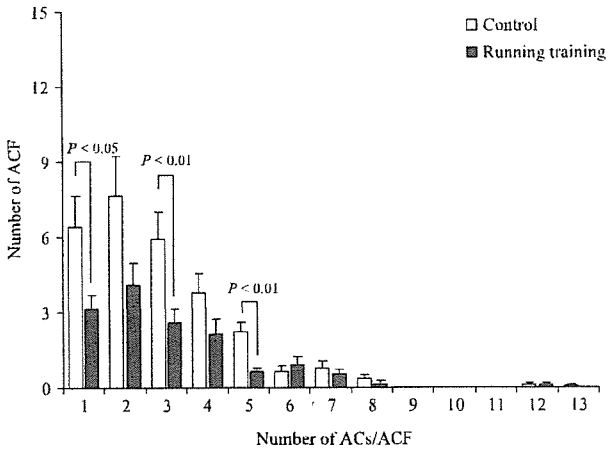

FIGURE 3—Effect of running training on the number of 1,2-dimethylhydrazine-induced AC per focus in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

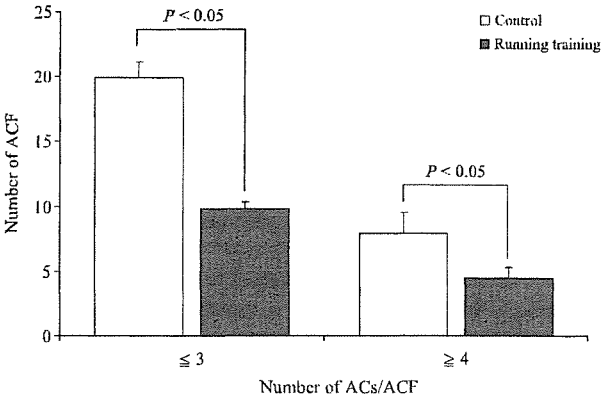

FIGURE 4—Effect of running training on the number of 1,2-dimethylhydrazine-induced small ACF (aberrant crypts per focus ≤ 3) and large ACF (aberrant crypts per focus ≥ 4) in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

The relative BAT weight of the training rats tended to be lower than that of the control group ($P = 0.07$). CS activity in the soleus muscle of the training group was significantly higher than in the control group ($P < 0.05$).

Induction of ACF and AC. As shown in Figure 2 upper panel, the number of ACF of training-group rats was significantly less than that observed in the control group ($P < 0.05$). The number of total AC was also significantly less in the training group than in the control group ($P < 0.05$; Fig. 2 lower panel). As shown in Figure 3, the occurrences of one, three, and five aberrant crypts per focus were significantly smaller in the training group than in the control group ($P < 0.05$). Furthermore, running training decreased the number of not only small ACF, which consists of less than or equal to three AC ($P < 0.05$), but also large ACF (≥ 4 AC) ($P < 0.05$), as compared with the control group (Fig. 4). However, the ratio of total AC/ACF, which indicates the average size of induced ACF, did not significantly differ between the training and control groups (2.9 ± 0.2 vs 2.9 ± 0.7, $P > 0.10$).

DISCUSSION

The main finding of the present study was that short-term, low-intensity running training reduced DMH-induced ACF production in the rat colon.

Colon carcinogenesis is well known to be a multistep process involving multiple genetic alterations (15,37,38). In humans, mutation of adenomatous polyposis coli (APC) gene is regarded as the initial event in ACF, followed by additional mutation of *K-ras* gene in adenomas; further mutation of the *p53* gene is the progressive event in carcinomas (14,33). In rodents, β-catenin mutations are frequently observed in colon tumors and in dysplastic ACF induced by azoxymethane (32). APC and/or K-ras mutations are also occasionally observed in rodents, as they are in humans (32), and ACF has been considered a very initial lesion in a

multistep colorectal tumorigenesis model (14). After ACF were first described in animals, similar lesions were found in humans (28). Because previous studies have suggested that low-intensity, long-term treadmill-running training prevented the incidence or development of colon tumors in a rat model injected subcutaneously with azoxymethane (36), it is reasonable to speculate that exercise training may exert its effect on one or more steps in colon carcinogenesis. To date, however, it remains unknown at which step of the carcinogenic process (e.g., ACF, adenomas (early, intermediate, and late), or carcinomas) physical exercise training exerts its preventive effect in rodents injected with carcinogens. The present results suggest that training in rats suppressed the first step, the initiation of ACF development in the rat colon. This is the first observation regarding the effect of physical exercise on ACF, the development of which is considered the first step in colon carcinogenesis. Furthermore, Colbert et al. (5), using the APC^{Min} mouse model, reported a trend toward fewer polyps in the colon in treadmill-exercised males compared with nonexercised mice. From the present investigation, it is obvious that the earlier phase of colon carcinogenesis is inhibited by exercise training. Therefore, it is of great importance when considering efficient chemopreventive effects on cancer development. Several hypotheses have been suggested to explain the preventive effects of exercise/physical activity on colon carcinogenesis—for example, shortened gastrointestinal transit time as a result of exercise (6); energy balance (16); reduced levels of blood insulin, which might be a growth factor for colon cancer cells (12); enhanced immune activity–related NK cells (20); enhanced free-radical scavenger system (8); changed prostaglandin levels (17); and decreased obesity (25). However, mechanisms related to the exercise-induced decrease in AC and/or ACF are not known at all. Therefore, only a few hypotheses can be raised. First, as Lasko and Bird et al. (16) have reported that caloric restriction–induced weight loss suppressed the increase in the number of ACF after the injection of azoxymethane in rats, it may be possible that energy balance (29), including energy expenditure and reduced food intake (24) or reduced nonexercise activity level (35) by exercise training, may exert a suppressive effect similar to that of caloric restriction, inhibiting the initiation or proliferation of ACF on the colonic mucosa. In fact, the results of the present study indicate that the body weight and/or adipose tissue weight of the peritoneum and epididymides were significantly lower in the running group than in the control group. Therefore, it is plausible that body- or fat weight loss yielded by physical exercise may reduce the initiation of ACF. Another plausible mechanism is that exercise might exhibit its preventive effects on mutation induction in the APC, *K-ras*, and/or *p53* genes through the induction of some detoxification enzymes for oxidative stresses. A third possibility is the commitment of moderate levels of physical exercise on the improvement of lipid metabolism. High fat levels in serum and low expression levels of lipid metabolism–related genes such as lipoprotein lipase in the liver and colon are now considered to have some significant impact on the development of intestinal tumors in the APC^{Min} mouse model (21,22). Further studies are expected to investigate the molecular mechanisms underlying exercise-induced effects on AC/ACF formation.

Recently, Demarzo and Garcia (7) reported that a single bout of exhaustive swimming exercise with a 2% weight tied to the tail was related to an elevated number of colonic ACF in untrained rats treated with DMH, when compared with a control group. Because this report included no description of the exercise protocol, such as a period of acclimatization that is usually given before acute bouts of exercise (27), it is not known how much "stress" was imposed on the exercised rats in the study. Therefore, we could not discuss the different effects of ACF production between the two studies in terms of exercise training–induced stress. On the other hand, the intensity of the swimming exercise with a 2% weight tied to the tail might be higher than that of the running training adopted in the present investigation. Thus, the exercise intensity may be a key factor determining the number of ACF after exercise. In fact, unaccustomed exhaustive and/or high-intensity exercise increases systemic free-radical generation in experimental animals (2). On the other hand, in the present study, we showed that low-intensity physical exercise for 2 h may decrease the development of colonic ACF in experimental animals. Furthermore, stress related to exercise at times during which the rats normally sleep may influence the ACF number in the colon. Therefore, voluntary exercise during the night cycle may be a better alternative exercise protocol than the "forced daytime" treadmill running adopted in the present investigation. However, because the number of ACF in the trained rats in the present study was actually lower than in the nonexercise control group, the overall effects of treadmill running on ACF production are favorable. Therefore, the benefits of the exercise training adopted in the present study are thought to outweigh the disadvantages of exercise-related stress. A future study using voluntary exercise should be conducted to clarify this issue.

The number of AC with a specific number of ACF (1,3,5) in the trained rats was lower than in the control group in the present investigation. However, running training did not affect the overall mean AC/ACF ratios of the rat colon induced by DMH. Thus, it is suggested that the physical exercise training adopted in the present investigation may not be effective for preventing the proliferation of ACF in rat colonic mucosa.

In conclusion, the results of the present investigation demonstrated that low-intensity running training inhibits the initiation of ACF in the rat colon induced by DMH. Furthermore, the present investigation suggests that increasing physical activity might be effective for primary prevention of colon cancer incidence, not only for rats but also for humans, by affecting the first step of cancer induction. However, the clinical implications and pathophysiological mechanisms of these findings warrant further investigation.

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Prostaglandin E receptor subtype EP_1 deficiency inhibits colon cancer development

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Prostaglandin E_2 exerts its biological activity through binding to its membrane receptors, E-prostanoid (EP) $receptors_{1-4}$. Our previous finding that lack of EP_1 receptor inhibits the early stages of colon carcinogenesis led us to investigate whether EP_1 receptor deficiency reduces colon cancer development induced by azoxymethane (AOM) using EP_1 receptor knockout mice. At 6 weeks of age 33 homozygous EP_1-deficient ($EP_1^{-/-}$) mice and 28 wild-type ($EP_1^{+/+}$) mice were given i.p. AOM (10 mg/kg body wt) once a week for 6 weeks. At 56 weeks of age all animals were killed and intestinal tumors were examined. The results clearly indicated that lack of EP_1 receptor significantly reduced colon cancer incidence (27 versus 57%, $P < 0.05$) and multiplicity (0.30 versus 0.76, $P < 0.05$) as well as tumor volume (12.2 versus 75.6 mm^3, $P < 0.05$). In $EP_1^{-/-}$ mice, silver stained nucleolar organization region protein count as cell proliferation marker was significantly reduced (1.35 versus 2.17, $P < 0.001$) and apoptosis was significantly increased (0.685 versus 0.077, $P < 0.001$) in colon tumors induced by AOM compared with those in $EP_1^{+/+}$ mice. We confirmed that EP_1 receptor mRNA was overexpressed in colon cancers of $EP_1^{+/+}$ mice using reverse transcription–polymerase chain reaction. These results provide strong evidence that the EP_1 receptor is of major importance for colon cancer development and it could be a new target for a mechanism-based chemoprevention strategy against colon cancer development.

Introduction

Colon cancer development appears to be closely linked with alterations in the arachidonic acid cascade and non-steroidal anti-inflammatory drugs reduce the risk of colon cancer development in human (1) and the incidence of carcinogen-induced colon cancers in rodents (2) through inhibition of cyclooxygenase (COX) activity. There are two isozymes of COX, referred to as COX-1 and COX-2. COX-2, the inducible form, is known to be overexpressed in colon cancers in rodents (3) and humans (4,5), whereas COX-1 is constitutively expressed and may contribute to various physiological functions. Celecoxib, a selective COX-2 inhibitor, has shown strong chemopreventive effects against colon cancer development in animal models (6,7) and may significantly reduce the numbers of colorectal polyps in familial adenomatous polyposis patients (8). There is accumulating evidence that COX-2 plays a pivotal role in colon carcinogenesis from genetic and pharmacological studies (9,10). Recently, however, there was a report using knockout mice that COX-1 contributed to colon carcinogenesis as well as COX-2 (11). These results suggest that not only COX-2 but also COX-1 contribute to colon carcinogenesis. Several reports have documented increased levels of prostaglandin E_2 (PGE_2), one of the major prostanoids, in colon cancer tissues compared with surrounding normal appearing mucosa of rodents and humans (12,13). Taken together, both COX enzymes and the levels of their product PGE_2 may be important factors and may play roles in colon carcinogenesis. In addition, recent studies provide evidence that PGE_2 administration enhances AOM-induced colon carcinogenesis in male F344 rats (14). Thus, we hypothesized that decreased signaling via the PGE_2 pathway may be associated with inhibition of colon cancer development. PGE_2 exerts its biological activity through binding to the membrane receptors E-prostanoid (EP) $receptors_{1-4}$. Therefore, it is likely that lack of specific receptors may contribute to the inhibitory effects on colon carcinogenesis. Recent development of mice lacking the genes encoding these receptors (15–17) has allowed us to investigate which types of receptors are involved in colon carcinogenesis. In our previous studies, EP_1 or EP_4 receptor deficiency and specific antagonists against these receptors significantly reduced the number of azoxymethane (AOM)-induced aberrant crypt foci, which are thought to be preneoplastic lesions in the colon (18,19). The antagonists also inhibited intestinal polyp formation in *Min* mice, suggesting that receptors EP_1 and EP_4 play important roles in the early stages of colon carcinogenesis. However, the question remains as to whether EP_1 receptor deficiency really has an impact on colon cancer development. Therefore, we designed the present study to analyze the effects of EP_1 receptor deficiency on colon cancer development induced by AOM using knockout mice.

The inhibitory mechanism was investigated by determining cell proliferation and apoptosis in colon tumors. An assessment of EP receptor expression in colonic normal mucosa and cancers of mice with or without EP_1 receptors using the reverse transcription–polymerase chain reaction (RT-PCR) method was also examined. The mouse *EP1* receptor gene, consisting of three exons, is predominantly expressed in the kidney and in the hypothalamus of the brain. Mouse *PKN*, a newly discovered gene encoding a protein kinase related to the protein

Abbreviations: AgNOR, silver stained nucleolar organizer region protein; AOM, azoxymethane; COX, cyclooxygenase; EP, E-prostanoid; NORs, nucleolar organizer regions; PGE_2, prostaglandin E_2; PKN, protein kinase N; RT-PCR, reverse transcription–polymerase chain reaction.

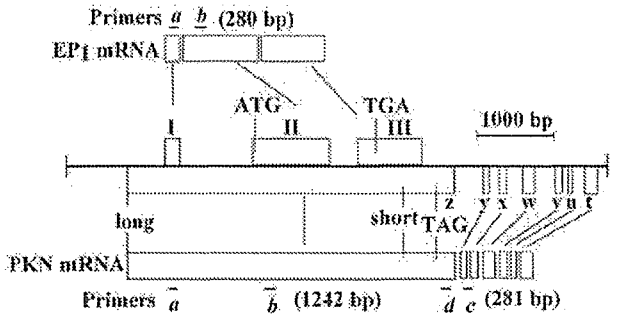

Fig. 1. Gene organization at the mouse *EP$_1$/PKN* gene locus and the transcripts produced. The center line represents the 7.2 kb of sequenced genomic DNA. Open boxes represent exons. The *EP$_1$* gene exons are numbered I–III and are shown above the line. Under the line are the seven last exons of the *PKN* gene. Solid bars illustrate mRNAs produced at this locus. *a*, *b*, *c* and *d* indicate primer positions within the locus.

kinase C family (20), overlaps the whole *EP$_1$* gene in a tail-to-tail manner (21), indicating that the whole *EP$_1$* gene is contained within the 3′-untranslated region of a long protein kinase N (*PKN*) gene transcript (Figure 1). Our *EP$_1$* knockout mice were made by inserting the neomycin-resistance gene into the FspI site of exon 2, which is located immediately before the sequence encoding the first transmembrane domain (15). Therefore, this EP_1 receptor knockout strategy may influence a long *PKN* gene transcript. In this report, to confirm that the *PKN* gene is not a player in AOM-induced colon carcinogenesis, expression of the *PKN* gene in mice with or without EP_1 receptor was also investigated using the RT-PCR method.

Materials and methods

Animals and chemicals

The mouse gene encoding the EP_1 receptor was disrupted by gene knockout methods using homologous recombination, as reported previously (15). The chimeric mice generated were back-crossed with C57BL/6Cr mice and the resulting wild-type and homozygous mutant males of the F_2 progeny were used at 6 weeks. The genotypes of the knockout mice were confirmed by PCR according to the method described previously (15). The animals were housed in plastic cages at 24 ± 2°C and 55% humidity with a 12 h light/dark cycle and maintained on powder diet (AIN-76A; Dyets Inc., Bethlehem, PA). AOM was purchased from Sigma Chemical Co. (St Louis, MO). The study was performed with the approval of the Institutional Animal Care and Use Committee.

Experimental procedure

At 6 weeks of age, 33 homozygous EP_1-deficient ($EP_1^{-/-}$)and 28 wild-type ($EP_1^{+/+}$) mice were given i.p. injections of AOM (10 mg/kg body wt) once a week for 6 weeks. All mice were provided with food and tap water *ad libitum*, weighed weekly and killed with ether at 56 weeks of age. Complete autopsies were performed and, after laparotomy, the entire stomach and intestines were resected and opened longitudinally and the contents were flushed with normal saline. Using a dissection microscope, large intestinal tumors were noted grossly for their location, number and size. The length (L), width (W) and depth (D) of each tumor were measured with calipers and tumor volume was calculated using the formula $V = L \cdot W \cdot D \cdot \pi/6$ (6). Colon tumors and normal tissues were fixed in 10% buffered formalin and embedded in paraffin blocks for histological evaluation. Diagnosis of intestinal tumors using hematoxylin and eosin stained sections was performed according to the classification of Pozharisski (22). Half of the colon tumors sized >~20 mm^3 and normal mucosa from each group were snap frozen with liquid nitrogen for analysis of RT-PCR.

Silver stained nucleolar organizer region protein (AgNOR) count and apoptotic index

Two serial sections (3 μm in thickness) of colon tumors were used for AgNOR staining and *in situ* end-labeling of fragmented DNA using Apo-BrdU-IHC™ (Chemicon International Inc., Temecula, CA). AgNOR staining was carried out according to the method described previously (23). For determination of AgNOR number in cell nuclei, AgNOR was counted on silver stained sections using a microscope at a magnification of ×400. The other sections were stained with Apo-BrdU-IHC according to the manufacturer's instructions. For analysis of apoptosis, only well-defined and darkly stained cells were counted using a microscope. All tumor cells were counted for AgNOR number and apoptotic index in a section of colon tumor. Five tumors in each group provided data for AgNOR count and apoptotic index. The percentage of labeled cells (apoptotic index) was determined by calculating (labeled cell number:total cell number) × 100.

EP receptor and PKN mRNA expression

Using the RT-PCR method, *EP* receptor and *PKN* mRNA expression in colon tumors and normal mucosa was investigated. Total RNA was isolated from colon tumors and normal mucosa using Isogen (Nippon Gene Co. Inc., Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription with random 9mers was used to generate cDNAs from 0.8 μg total RNA extract using reverse transcriptase (AMV Reverse Transcriptase XL) and a Takara RNA LA PCR kit (Takara Biomedical Co. Inc., Tokyo, Japan). The *EP$_1$* receptor (24) and *PKN* (20) primers were made for PCR amplification of the resulting cDNA (Figure 1): forward primer (*a* in Figure 1) for exon I, 5′-CCTGCATCCTGAGCAGCACT-3′ (nt 33–52); reverse primer (*b* in Figure 1) for the middle of exon II, 5′-TGGCGACGAACAACAGGAAG-3′ (nt 293–312); these being considered as reverse and forward primers for *PKN* (nt 4104–4123 and 2882–2901), respectively. The expected PCR products were 280 bp for the *EP$_1$* receptor and 1242 bp for *PKN*. The *EP$_1$* gene is located in the 3′-untranslated region of the *PKN* gene. To investigate the effects of the *EP$_1$* receptor knockout strategy on *PKN* gene expression, we used primers in the *PKN* encoding region as follows: forward primer (*c* in Figure 1), 5′-GAGAAGGCTACTGCGGAGGA-3′ (nt 562–581); reverse primer (*d* in Figure 1); 5′-CGGCCACAAAGTCGAAATC-3′ (nt 824–842). The expected PCR product was 281 bp. The following primers for the other EP receptors and β-actin were used for PCR amplification of the resulting cDNA: *EP$_2$* (402 bp) forward primer, 5′-AGGACTTCGATGGCAGAGGAGAC-3′ (nt 903–925); reverse primer 5′-CAGCCCCTTACACTTCTCCAATG-3′ (nt 1282–1304) (25); *EP$_3$* (438 bp) forward primer, 5′-CCGGGCACGTGGT-GCTTCAT-3′ (nt 657–676); reverse primer, 5′-TAGCAGCAGATAAACC-CAGG-3′ (nt 1075–1094) (26); *EP$_4$* forward primer, 5′-GCCATAGAGAA-GATCAAGTGCCT-3′ (nt 1150–1172); reverse primer, 5′-CCCACTAACCT-CATCCACCAA-3′ (nt 1480–1500) (27); β-actin (203 bp) forward primer, 5′-TCCTCCCTGGAGAAGAGCTA-3′ (nt 763–782); reverse primer, 5′-CCAGACAGCACTGTGTTGGC-3′ (nt 946–965). PCR conditions were 94°C for 120 s and then 30 cycles of 94°C for 30 s, 62–66°C for 60 s and 72°C for 60 s. β-Actin was used as the internal control for normalization of sample amounts. Agarose gels (1.5%) were stained with ethidium bromide. All assays were performed in triplicate.

Statistical analysis

Body weights, tumor incidence, multiplicity, volume and AgNOR count, as well as apoptotic index, were compared between animals with and without EP_1 receptors. Tumor incidence, expressed as the percentage of tumor-bearing animals, was analyzed using Armitage's χ^2 method. Tumor multiplicity, expressed as the mean number of tumors per animal, tumor volume and body weights were analyzed by unpaired Welch's or Student's t-test. AgNOR count, expressed mean number of AgNORs per nucleus and apoptotic index, expressed as percentage of cells with positive staining of Apo-BrdU-IH in tumors, were analyzed by unpaired Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

Body weight changes for $EP_1^{+/+}$ and $EP_1^{-/-}$ animals during the experiment are shown in Figure 2, values for the latter being significantly greater at 35 and 50 weeks after the first dosing of AOM (38.3 versus 42.0 g, $P < 0.05$ and 39.6 versus 44.6 g, $P < 0.01$, respectively). One $EP_1^{+/+}$ mouse at the age of 30 weeks was found to have three colon tumors diagnosed as adenocarcinomas. Therefore, mice alive on that day were counted as effective animals. At 56 weeks of age all survivors were killed and complete autopsies were performed. $EP_1^{-/-}$ mice without AOM treatment had no tumors in their intestines and lived >1 year as previously described (15). No tumors

Table I. Effects of EP_1 receptor deficiency on AOM-induced colon carcinogenesis

Animals	Incidence (% mice with colon tumors)			Multiplicity (tumors/mouse)			Tumor volume (mm^3)
	Total[a]	Adenomas	Adenocarcinomas	Total[a]	Adenomas	Adenocarcinomas	
$EP_1^{+/+}$	16/28 (57%)	0/28 (0%)	16/28 (57%)	0.79 ± 0.82[b]	0	0.79 ± 0.82[b]	75.6 ± 153[b]
$EP_1^{-/-}$	8/26 (31%)	1/26 (4%)	7/26 (27%)[c]	0.35 ± 0.55[d]	0.04 ± 0.19	0.31 ± 0.54[d]	12.2 ± 11.7[d]

[a]Includes adenomas and adenocarcinomas.
[b]Values represent means ± SD.
[c]Significantly different from $EP_1^{+/+}$ mice by χ^2 test ($P < 0.05$).
[d]Significantly different from $EP_1^{+/+}$ mice by Welch's t-test ($P < 0.05$).

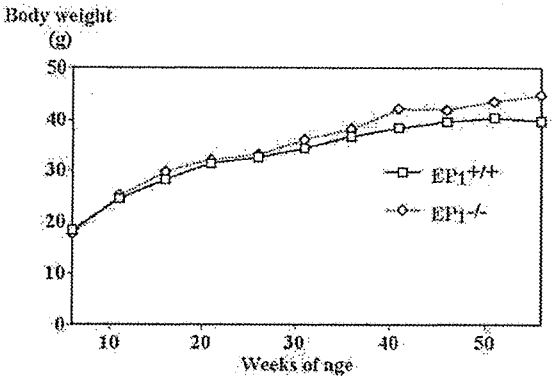

Fig. 2. Body weight changes of $EP_1^{+/+}$ and $EP_1^{-/-}$ mice during the study.

other than colon tumors were observed in AOM-treated mice both with and without EP_1 receptor.

Histopathological examination revealed colon tumors induced by AOM to be adenomas or adenocarcinomas. Table I summarizes the data for effects of EP_1 receptor deficiency on colon tumor development. Colon tumors developed in 8 out of 26 $EP_1^{-/-}$ mice (31%), including 1 mouse with an adenoma and 7 mice with adenocarcinomas. In the $EP_1^{+/+}$ case, 16 out of 28 mice (57%) had adenocarcinomas. In $EP_1^{-/-}$ mice, 0.35 tumors/mouse were found, whereas the value was 0.79 tumors/mouse in $EP_1^{+/+}$ mice ($P < 0.05$). In addition, colon tumor volume was significantly decreased in $EP_1^{-/-}$ mice compared with that in $EP_1^{+/+}$ mice (12.2 versus 75.6 mm^3, $P < 0.05$). Thus, EP_1 receptor deficiency significantly reduced colon cancer development as an end-point. These results strengthen our hypothesis that the EP_1 receptor is involved in colon carcinogenesis and lack of the EP_1 receptor significantly reduces colon cancer incidence and volume.

To elucidate the inhibitory mechanisms, we analyzed cell proliferation and apoptosis in colon tumors of mice with or without the EP_1 receptor. The results are summarized in Table II. Nucleolar organizer regions (NORs) are loops of DNA which contain rRNA genes. They are transcribed by RNA polymerase I and are of vital importance for the ultimate synthesis of proteins (28). AgNORs are acidic proteins associated with the NORs which are selectively stained by a silver colloid technique. A series of studies indicates that the quantity of AgNOR protein is related to the rapidity of cell proliferation and there is evidence of a relationship between AgNOR counts and the prognosis of malignant tumors (29). Mean number of AgNORs per nucleus of tumors in $EP_1^{+/+}$ mice is significantly greater than that of $EP_1^{-/-}$ mice (2.17 versus 1.35, $P < 0.001$). In addition to cell proliferation, we analyzed apoptosis

Table II. Effects of EP_1 receptor deficiency on cell proliferation and apoptosis in colon tumors induced by AOM

Animals	AgNORs count/nucleus	Apoptotic index (%)
$EP_1^{+/+}$	2.17 ± 0.22[a]	0.077 ± 0.067[a]
$EP_1^{-/-}$	1.35 ± 0.11[b]	0.685 ± 0.061[b]

[a]Mean ± SD.
[b]Significantly different from $EP_1^{+/+}$ mice by Student's t-test ($P < 0.001$).

of tumors induced by AOM in both mice. Apoptotic cell count (apoptotic index) in cancers of $EP_1^{-/-}$ mice treated with AOM was significantly higher than that in $EP_1^{+/+}$ mice (0.685 versus 0.077, $P < 0.001$). The mechanisms by which activation of the EP_1 receptor regulates cell proliferation and apoptosis in colon tumors are not known. However, the fact that colon cancers induced by AOM in $EP_1^{-/-}$ mice showed lower cell proliferation rates and higher apoptotic indices than those in $EP_1^{+/+}$ mice suggests that lack of the EP_1 receptor may down-regulate cell proliferation and up-regulate apoptosis, resulting in lower tumor incidence and volume. Further studies to investigate how the EP_1 receptor regulates cell proliferation and apoptosis signaling in colon tumors are required.

To investigate which EP receptors are involved in colon cancer tissues, we analyzed expression of *EP* receptor genes using the RT-PCR method. We first examined the *EP_1* receptor mRNA levels in normal mucosa and cancer tissues in the mice. Representative results on EP receptor expression in normal mucosa and cancer tissues of the colon from $EP_1^{+/+}$ and $EP_1^{-/-}$ mice are shown in Figure 3. Using primers *a* and *b* (Figure 1) we found that the EP_1 receptor was evident in colon cancer, whereas it was not detectable in normal mucosa of $EP_1^{+/+}$ mice (Figure 3, upper panel). As expected, *EP_1* receptor mRNA was not detected either in normal colon mucosa or cancer tissues of $EP_1^{-/-}$ mice. The whole mouse *EP_1* receptor gene is contained within the 3′-untranslated region of a long *PKN* gene transcript. Primers *a* and *b* (Figure 1) were designed to distinguish *EP_1* receptor from *PKN* by product length. Although *PKN* was found to be constitutively expressed in both normal mucosa and cancer of the colon in $EP_1^{+/+}$ mice, interestingly, *PKN* appeared not to be expressed in $EP_1^{-/-}$ mice. These results suggest that the *EP_1* gene knockout strategy may influence the stability of the long form of the *PKN* gene. To clarify the contribution of *PKN* gene instability in *EP_1* knockout mice during colon carcinogenesis, we examined whether the PKN encoding region was influenced in these mice. Using primers *c* and *d* (Figure 1) we found no differences in mRNA levels of the PKN encoding region between normal mucosa and cancer of the colon in both $EP_1^{+/+}$ and $EP_1^{-/-}$ mice (Figure 3, second panel). In addition, expression of