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

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

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

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

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

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

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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]

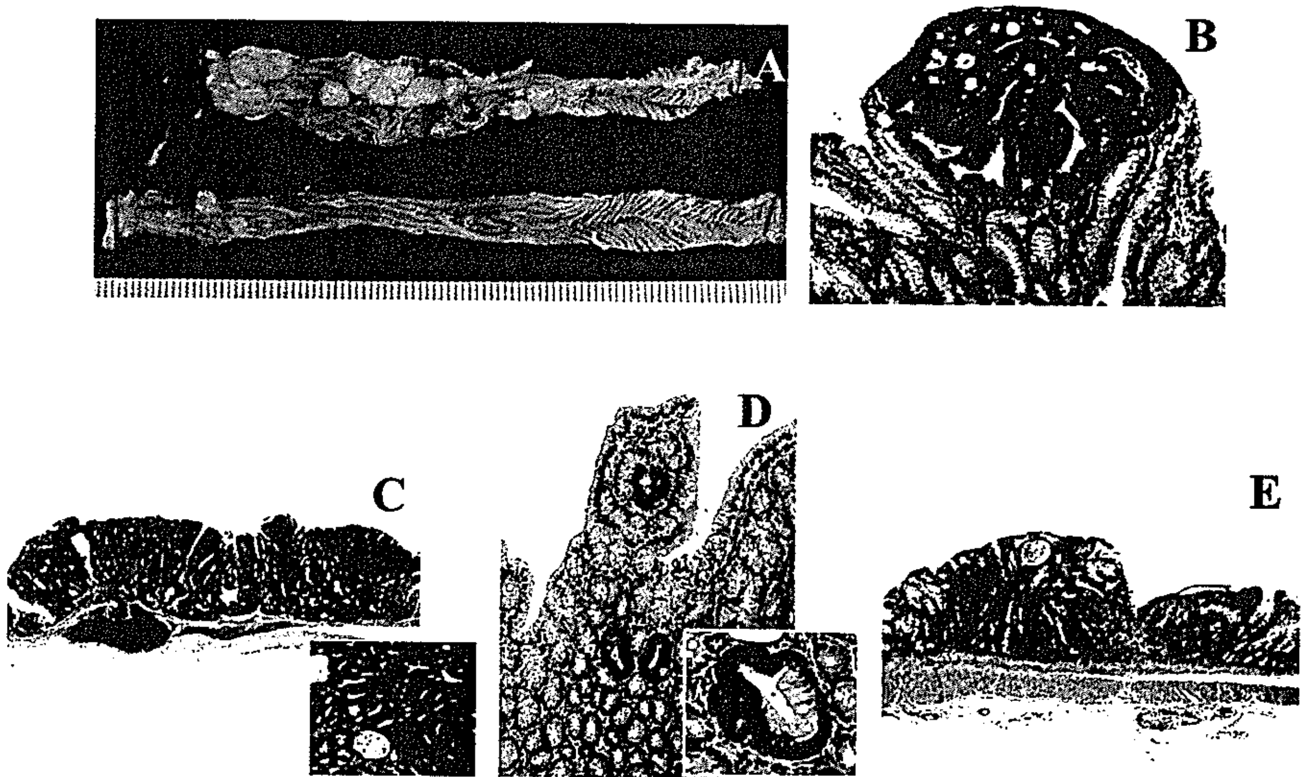

FIGURE 1 – Macroscopic view of the large bowel (*a*) and histopathology of the colonic lesions (*b*–*e*) of male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*) Male $Apc^{Min/+}$ mice given 2% DSS had multiple colonic tumors (upper), while male $Apc^{Min/+}$ mice given tap had a few colonic tumors (lower); (*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	Colonic tumors: incidence (multiplicity)						Dysplastic crypts	
		Total: incidence (multiplicity[1])		AD[2]		ADC[2]			
		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

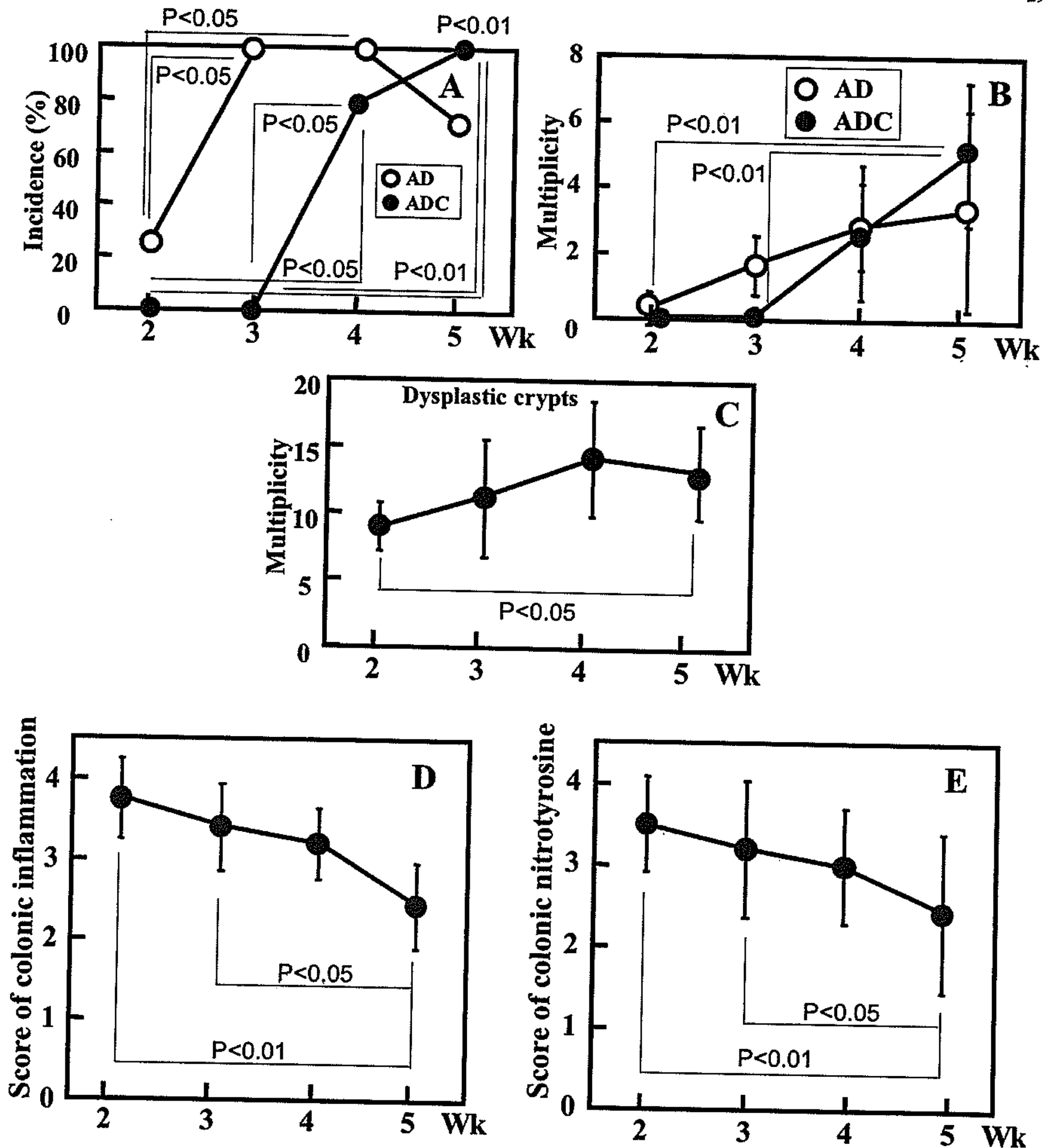

FIGURE 2 – Time-course observation of colonic lesions in female $Apc^{Min/+}$ mice treated with 2% DSS. The incidence (*a*) and multiplicity (*b*) of colonic neoplasms and the multiplicity (*c*) of colonic dysplastic crypt were gradually increased with time. Scores of colonic inflammation (*d*) and nitrotyrosine-positivity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

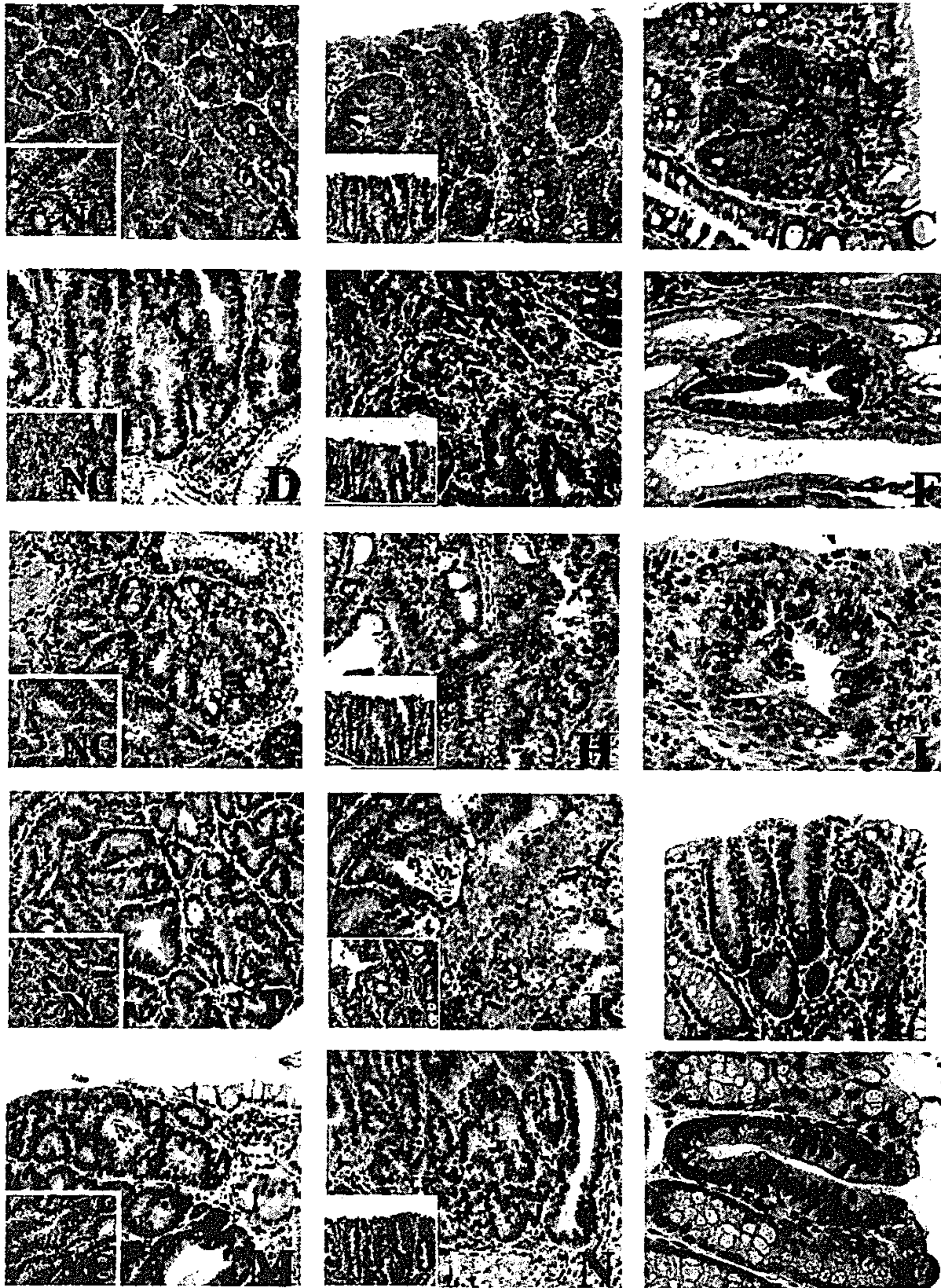

FIGURE 3 – Immunohistochemistry of the colonic lesions developed in male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*)–(*c*), β-catenin immunohistochemistry; (*d*)–(*f*), COX-2 immunohistochemistry; (*g*)–(*i*), iNOS immunohistochemistry; (*j*)–(*l*), nitrotyrosine immunohistochemistry and (*m*)–(*o*),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

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Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice

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We have recently developed a mouse model for colitis-related colon carcinogenesis by a combined treatment with azoxymethane (AOM) and dextran sodium sulfate (DSS) in male ICR mice. However, strain differences in the sensitivity to AOM/DSS-induced colon carcinogenesis in mice have yet to be elucidated. The aim of this study was to determine the presence of any genetically determined differences in sensitivity to our model of colon carcinogenesis in four inbred strains of mice. Male Balb/c, C3H/HeN, C57BL/6N and DBA/2N mice were given a single intraperitoneal injection of AOM (10 mg/kg body wt), followed by 1% DSS (w/v) in drinking water for 4 days, and thereafter they received no further treatment for up to 16 weeks. At the end of the study (Week 18), all mice were killed and a histopathological analysis of their colon was performed. The incidence of colonic adenocarcinoma was 100% with a multiplicity (no. of tumors/mouse) of 7.7 ± 4.3 in the Balb/c mice and 50% with a multiplicity of 1.0 ± 1.2 in the C57BL/6N mice. On the other hand, only a few colonic adenomas, but no adenocarcinomas, developed in the C3H/HeN mice (29% incidence with a multiplicity of 0.7 ± 1.5) and the DBA/2N mice (20% incidence with a multiplicity of 0.2 ± 0.4). The inflammation and immunohistochemical nitrotyrosine-positivity scores of the mice treated with AOM and DSS in the decreasing order were as follows: C3H/HeN > Balb/c > DBA/2N > C57BL/6N and Balb/c > C57BL/6N > C3H/HeN > DBA/2N, respectively. Our results thus indicated the presence of strain differences in the susceptibility to AOM/DSS-induced colonic tumorigenesis. These differences may have been directly influenced by the response to nitrosation stress due to the inflammation caused by DSS.

Introduction

Colorectal cancer (CRC) is one of the most common malignant neoplasms in both sexes (1). In Western countries, this malignancy is one of the most leading causes of cancer deaths (1). In patients with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease, the risk of CRC development is higher than in the general population (2–5). In sporadic and IBD-related CRC, the expression of inducible nitric oxide synthase and cyclooxygenase-2, both of which are associated with inflammation, has been reported to be elevated (6,7). As a result, inflammation is suggested to play an important role in IBD-related CRC (2).

In our recent series of studies on inflammation-related colon carcinogenesis, we developed a novel model of colitis-related colon carcinogenesis using ICR mice. In this animal model, ICR mice received a single dose of a different colonic carcinogen, consisting of either azoxymethane (AOM) (8), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (9) or 1, 2-dimethylhydrazine (10), followed by a 1-week exposure to 2% dextran sodium sulfate (DSS) in their drinking water, which thus resulted in a high incidence of colonic epithelial malignancy within 20 weeks (8–10). We have previously proposed that the colonic inflammation and nitrosative stress caused by DSS exposure contributes to the development of cryptal dysplasia and neoplasms in the colon (8–10).

AOM is a colonic genotoxic carcinogen that is extensively used for the investigation of large bowel carcinogenesis in rodents (11–13). A synthetic sulfate polysaccharide, DSS, is a non-genotoxic colonic carcinogen that is widely used to produce colitis in rodents, which shares most features with human UC (14–18). It is well known that different strains of mice have different sensitivities to xenobiotic including AOM and DSS (19–28). For example, the Balb/CJ strain is known to be susceptible to AOM (26), whereas, the C3H (29), C57BL/6J (26) and DBA/2 (25) strains are less sensitive to AOM. Regarding the sensitivity to DSS in several mouse strains, Balb/c, C3H/HeJ, and C57BL/6J mice are relatively susceptible to DSS, while DBA/2J mice have been reported to be virtually resistant (27,28). It may therefore be possible that the differences in the genetic background of the mice differently affect the colon carcinogenesis induced by AOM and DSS.

The current study was conducted to determine the different sensitivities to AOM/DSS-induced colon carcinogenesis in four different inbred mouse strains, namely Balb/c, C3H/HeN, C57BL/6N and DBA/2N, by evaluating the incidence and multiplicity of colonic tumors. In addition, an immunohistochemical analysis of nitrotyrosine, a marker of both formation of peroxynitrite (30) and perhaps the inflammation-associated carcinogenesis (31), was done to evaluate whether nitrosative stress is involved in the strain difference sensitivity to AOM/DSS-induced colon tumorigenesis.

Materials and methods

Animals, chemicals and diets

For the study 5-week-old male mice of Balb/c, C3H/HeN, C57BL/6N and DBA/2N strains were obtained from Charles River Japan, (Tokyo, Japan). AOM was purchased from the Sigma-Aldrich (St Louis, MO). DSS with a molecular weight of 36 000–50 000 was purchased from ICN Biochemicals,

Abbreviations: AOM, azoxymethane; CRC, colorectal cancer; CYP, Cytochrome P450; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; IKK, IκB kinase; LPS, lipopolysaccharide; UC, ulcerative colitis.

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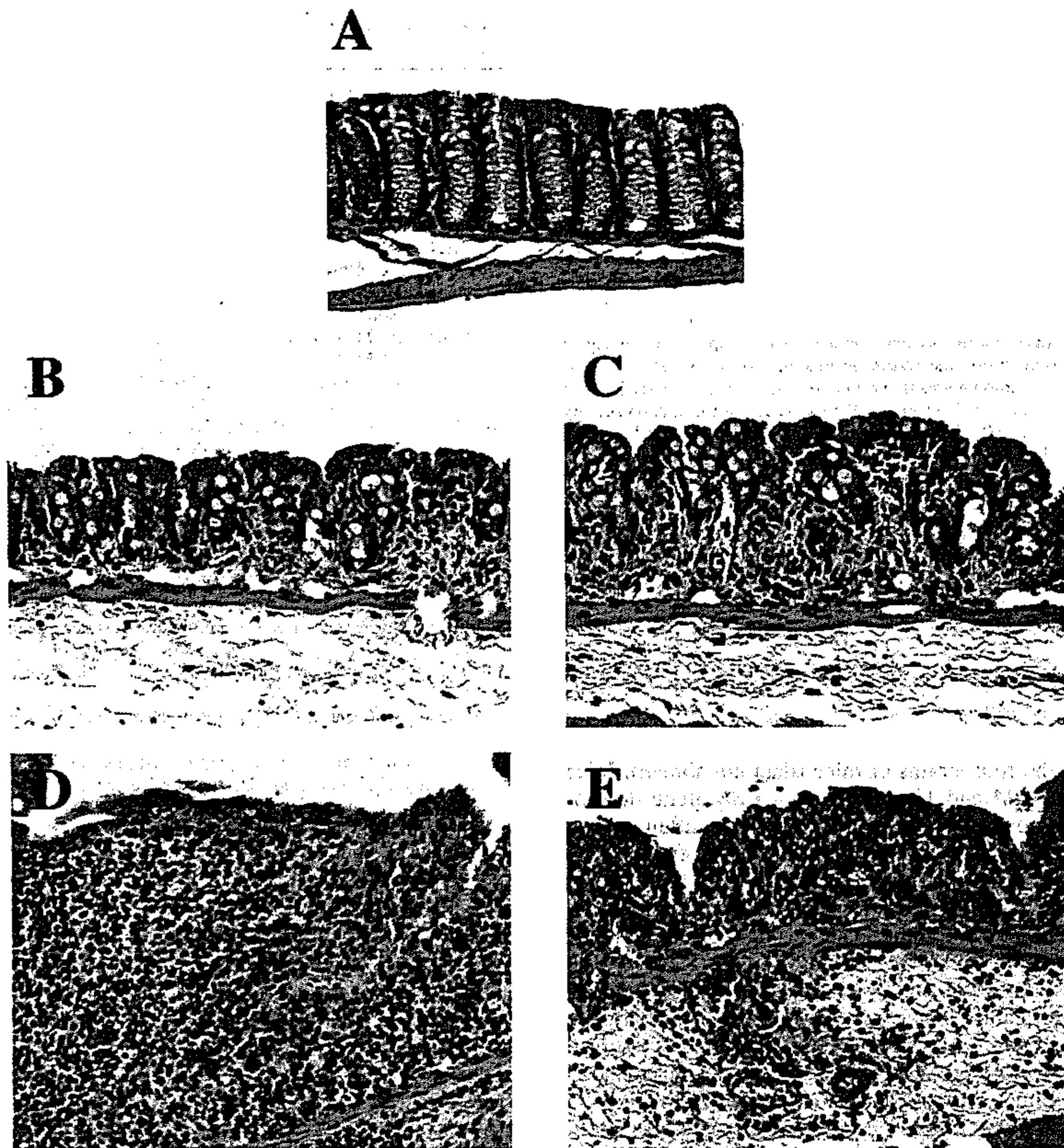

Fig. 1. Various grades of colitis. (A) Normal colon mucosa (Grade 0); (B) shortening the basal one-third of the crypts with slight inflammation and edema in the lamina propria (Grade 1); (C) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (Grade 2); (D) loss 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

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)	$25.1 \pm 3.8^{a,b,c,d}$	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 ($P<0.01$).

of the strains of mice given AOM alone, 1% DSS alone or tap water had any colonic tumors.

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