Table 2. Incidence of large bowel neoplasms in mice treated with AOM and various doses of DSS.

GROUP no.	TREATMENT (no. of mice examined)	INCIDENCE (MULTIPLICITY) OF LARGE BOWEL NEOPLASMS		
		Total	Adenoma	Adenocarcinoma
1	AOM→2%DSS (4)	100%* (4.00±3.37)	75%** (1.25±1.26)	100%* (2.75±2.22)
2	AOM→1%DSS (5)	100%*** (2.40±2.19)	80%** (1.00±0.71)	60% (1.40±2.07)
3	AOM→0.5%DSS (5)	20% (0.20±0.45)	20% (0.20±0.45)	0%
4	AOM→0.25%DSS (5)	0%	0%	0%
5	AOM→0.1%DSS (4)	0%	0%	0%
6	AOM (5)	0%	0%	0%
7	2%DSS (5)	0%	0%	0%
8	None (5)	0%	0%	0%

Numbers in parentheses are multiplicity (mean±SD) of large bowel tumors. *, **, ***: Significantly different from group 6 by Fisher's exact probability test (*P<0.01, **P<0.05, and ***P<0.005).

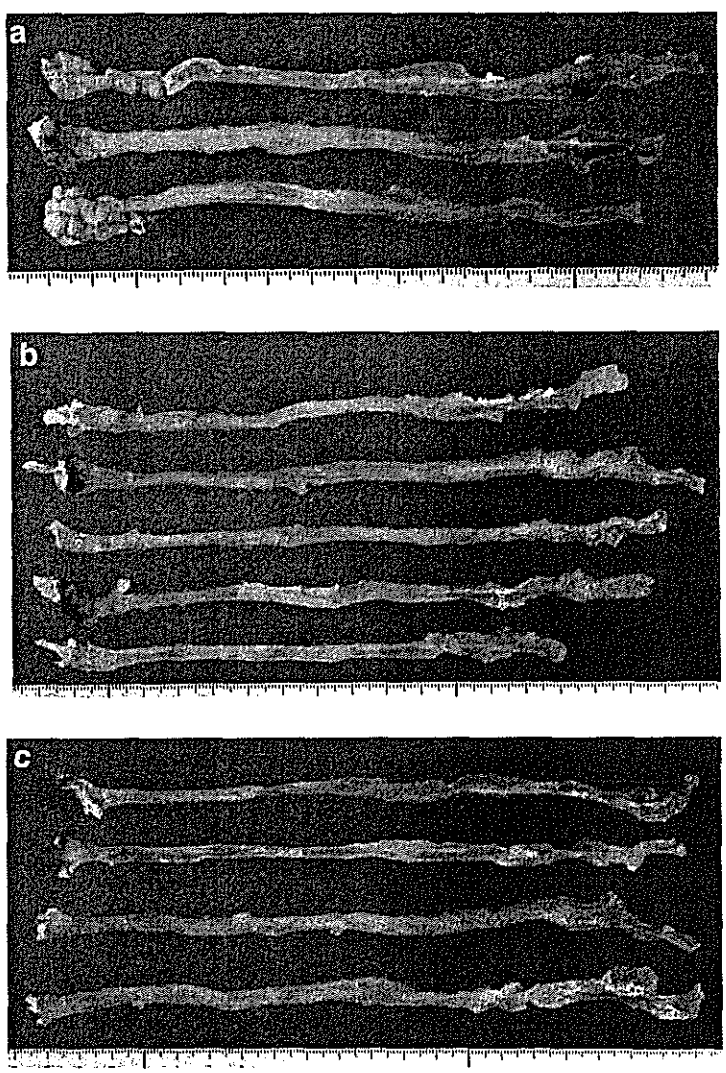

Fig. 2. Macroscopic view of the large bowel. **a.** A number of colonic tumors are seen in mice given AOM→2% DSS. **b.** A number of colonic tumors are seen in mice treated with AOM→1% DSS. **c.** One colonic tumor is present in a mouse received AOM→0.05% DSS. Arrow heads indicate colonic tumors.

distal colon of mice in groups 1 (AOM→2% DSS), 2 (AOM→1% DSS), and 3 (AOM→0.5% DSS) (Fig. 2). They were histologically tubular adenoma (Fig. 3a) or adenocarcinoma (Fig. 3b). As summarized in Table 2, the incidences of total large bowel neoplasms in groups 1 (100%, $P<0.01$) and 2 (100%, $P<0.005$) were significantly greater than group 3 (20%). As for colonic adenoma, the incidences in groups 1 (75%, $P<0.05$) and 2 (80%, $P<0.05$) were statistically higher than group 3 (20%). There was a significant difference ($P<0.01$) in the incidence of colonic adenocarcinoma between group 1 (100%) and group 2 (60%). In mice of group 3, no colonic adenocarcinomas were noted. No colonic neoplasms were found in mice of groups 4 (AOM→0.25% DSS), 5 (AOM→0.1% DSS), 6 (AOM alone), 7 (2% DSS alone), and 8 (untreated control). The multiplicity of colonic neoplasms was increased in proportion to the dose level of DSS, but the difference was not significant among the groups. To determine the dose-response effect of various levels of DSS on the multiplicity of colonic neoplasm, we used simple linear regression. The squared correlation coefficients for the multiplicities of total tumor, adenoma, and adenocarcinoma obtained by the regression were 0.98 ($P<0.01$), 0.94 ($P<0.05$), and 0.98 ($P<0.01$), respectively. These values suggested a dose-response promotion effect of DSS on development of colon tumors.

Effects of various doses of DSS on the occurrence of colonic mucosal inflammation, ulcer, and dysplasia

Colonic mucosal inflammation, ulcer or dysplasia

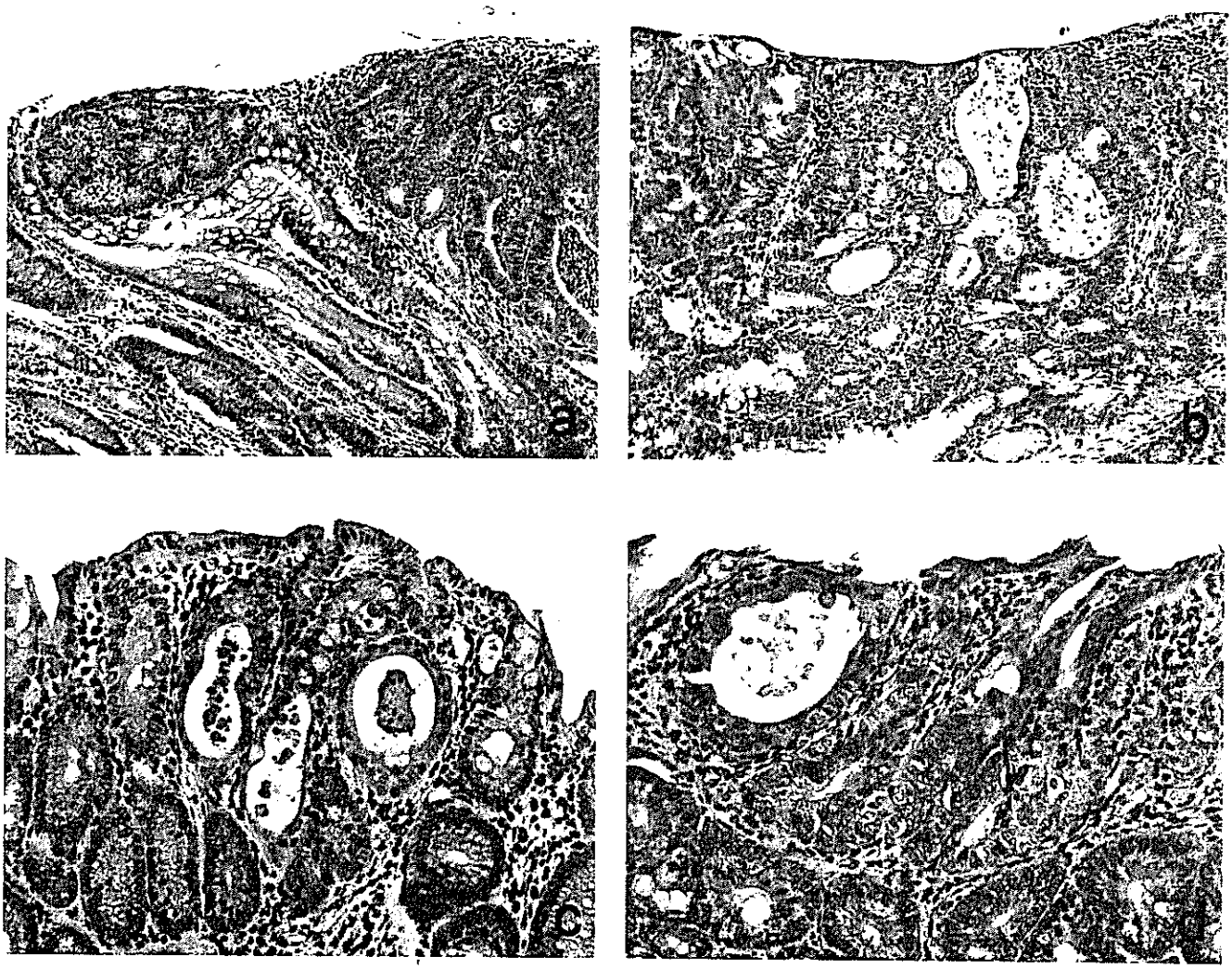

Fig. 3. Histopathology of colonic lesions in mice treated with AOM→2% DSS. **a.** Two tubular adenomas are seen. **b.** A tumor is histologically well differentiated tubular adenocarcinoma. **c.** Low-grade dysplasia with slight nuclear atypia. **d.** High-grade dysplasia with marked nuclear atypia. Hematoxylin and eosin stain, original magnification, a, b, x 10; c, d, x 20

were mainly found in the middle and distal parts of the colon. The severity of inflammation was greater in the distal region than other regions of the colon. Inflammation score of colonic mucosa is illustrated in Fig. 4. The score of group 1 (AOM→2% DSS, 2.25±0.50) was significantly higher than that of group 3 (AOM→0.5% DSS, 1.40±0.55, P<0.05), group 5 (AOM→0.1% DSS, 1.00±0.82, P<0.05), group 6 (AOM alone, 0.40±0.89, P<0.01), group 7 (2% DSS alone, 0.80±0.84, P<0.05), and group 8 (no treatment, 0.20±0.45, P<0.001). Group 2 (AOM→1% DSS, 2.00±0.71, P<0.005), group 3 (P<0.01), and group 4 (AOM→0.25% DSS, 1.20±0.84, P<0.05) were statistically higher than that of group 8. The value of group 2 was also significantly greater than group 6 (P<0.05). The correlation coefficient for the correlation between the inflammation score and dose of DSS was 0.95 (P<0.05).

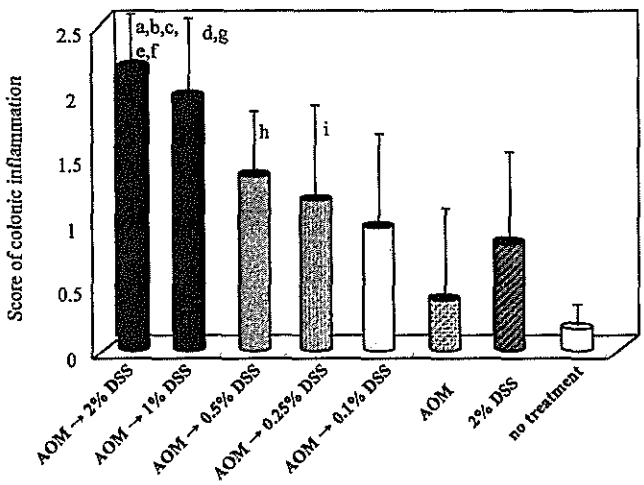

Fig. 4. Inflammation score. Statistical analysis using Student's t-test or Welch's t-test indicates significant difference: a (P<0.05), vs. the AOM→0.5% DSS group; b (P<0.05), vs. the AOM→0.1% DSS group; c (P<0.01) and d (P<0.05), vs. the AOM alone group; e (P<0.05), vs. the 2% DSS alone group; and f (P<0.001), g (P<0.005), h (P<0.01), and i (P<0.05), vs. the "no treatment group".

The frequencies of colonic mucosal ulceration and dysplasia are shown in Table 3. DSS administration increased dose-dependently the incidence and frequency of mucosal ulceration of the colon. However, the correlation coefficient was 0.59 (P>0.1). This might be caused by the high value of group 2 (6.80±1.92). All mice belonging to groups 1 through 5, which were initiated with AOM and followed by various doses of DSS exposure, developed mucosal dysplasia with low- (Fig. 3c) and/or high-grade (Fig. 3d), and one mouse in group 6 had low-grade dysplasia (Table 3). The multiplicities of total and high-grade dysplasia in groups 1 and 2 were much greater than those of groups 3 through 5. The incidences of the total dysplasia and low-grade dysplasia were 100% in groups 1-5 (P<0.05 vs. group 6). Their multiplicities significantly increased when the dose of DSS increased (P<0.05, P<0.01, P<0.005 or P<0.001). The incidences of high-grade

Table 3. Incidence of large bowel ulceration and dysplasia in mice treated with AOM and various doses of DSS.

GROUP	TREATMENT	INCIDENCE OF MUCOSAL ULCER	INCIDENCE (MULTIPLICITY) OF COLONIC MUCOSA		
no.	(no of mice examined)	(multiplicity)	Total	Low-grade	High-grade
1	AOM→2%DSS (4)	100%* (4.00±0.82[a,b,c])	100%** (12.50±4.65[d,e,f,g])	100%** (4.00±1.83[g])	100%* (8.50±2.89[e,h,i])
2	AOM→1%DSS (5)	100%* (6.80±1.92[c,d,j])	100%** (9.00±3.94[d,e,f,k])	100%** (3.80±2.17[g])	100%* (5.20±2.86[e,f])
3	AOM→0.5%DSS (5)	100%* (3.60±1.95[f])	100%** (3.80±3.11)	100%** (2.00±1.22[g])	80%** (1.80±1.92)
4	AOM→0.25%DSS (5)	80%** (1.40±1.14)	100%** (2.80±1.30[k])	100%** (2.00±0.71[l])	60% (0.80±0.84)
5	AOM→0.1%DSS (4)	50% (0.50±0.58)	100%** (2.50±0.58[m])	100%** (1.75±0.50[m])	50% (0.75±0.96)
6	AOM (5)	0%	20% (0.20±0.45)	20% (0.20±0.45)	0%
7	2%DSS (5)	0%	0%	0%	0%
8	None (5)	0%	0%	0%	0%

Numbers in parentheses are multiplicity (mean±SD) of large bowel tumors. *,**: significantly different from group 6 by Fisher's exact probability test (*P<0.01 and **P<0.05); [a]: significantly different from group 2 by Student's t-test (P<0.05); [b]: significantly different from group 4 by Student's t-test (P<0.01); [c]: significantly different from group 5 by Student's t-test (P<0.001); [d]: significantly different from group 3 by Student's t-test (P<0.05); [e]: significantly different from group 4 by Welch's t-test (P<0.05); [f]: significantly different from group 5 by Welch's t-test (P<0.05); [g]: significantly different from group 6 by Welch's t-test (P<0.05); [h]: significantly different from group 3 by Student's t-test (P<0.005); [i]: significantly different from group 5 by Student's t-test (P<0.005); [j]: significantly different from group 4 by Student's t-test (P<0.001); [k]: significantly different from group 6 by Welch's t-test (P<0.01); [l]: significantly different from group 6 by Student's t-test (P<0.005); [m]: significantly different from group 6 by Student's t-test (P<0.001).

dysplasia in groups 1 ($P<0.01$), 2 ($P<0.01$), and 3 ($P<0.05$) were significantly higher than group 6. The multiplicities of the lesion increased with increasing the concentration of DSS. The correlation coefficients for the multiplicities of total dysplasia, low-grade dysplasia, and high-grade dysplasia were 0.98 ($P<0.01$), 0.91 ($P<0.05$), and 0.99 ($P<0.01$), respectively. These values indicated that DSS exposure has a dose-dependent promoting effect on the development of colonic dysplasia.

Immunohistochemistry nitrotyrosine

Nitrotyrosine immunoreactivity was mainly observed in mononuclear cells infiltrated in the colonic mucosa with the lesions (Fig. 5). The stainability was very weak in the cryptal cells and neoplastic cells if present (Fig. 5). The score of nitrotyrosine immunohistochemistry is shown in Fig. 6. The scores of groups 1 (AOM→2% DSS, 3.08±0.31) and 2 (AOM→1% DSS, 2.54±0.40) were significantly higher than that of group 3 (AOM→0.5% DSS, 0.96±0.15, $P<0.001$), group 4 (AOM→0.25% DSS, 0.60±0.22, $P<0.001$), group 5 (AOM→0.1% DSS, 0.53±0.22, $P<0.001$), group 6 (AOM alone, 0.2±0.45, $P<0.001$) and group 8 (no treatment, 0.28±0.15, $P<0.001$). Also a significant difference ($P<0.005$) was found between groups 1 and 7 (2% DSS alone, 0.60±0.89). Immunohistochemical nitrotyrosine score of group 3 was significantly greater than that of groups 4 ($P<0.05$), 5 ($P<0.01$), 6 ($P<0.05$), and 8 ($P<0.001$). A significant difference was also noted between groups 4 and 8 ($P<0.05$). The dose dependent effect of DSS on the scores of nitrotyrosine immunohistochemistry was demonstrated by the calculated correlation coefficient (0.95, $P<0.05$).

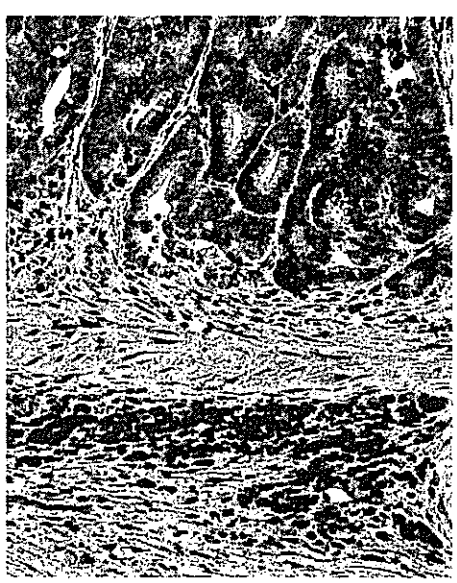

Fig. 5. Nitrotyrosine immunohistochemistry of the colon of a mouse from group 1 (AOM→2% DSS). The positive reaction is noted inflammatory cells under the dysplastic lesion. Original magnification, x 20

Discussion

We recently reported that exposure to DSS in the drinking water at a dose of 2% for a week after a single i.p. injection of AOM (10 mg/kg body weight) could produce a number of colonic neoplasms with ß-catenin gene mutation in male ICR mice within a short-term period (20 weeks) (Tanaka et al., 2003), suggesting a powerful tumor-promoting activity of DSS. Subsequent time-course observation confirmed the tumor-promoting effect of DSS on AOM-initiated mouse colon carcinogenesis and suggested involvement of inflammation and nitrosative stress (Suzuki et al., 2004). The current study conducted to determine the lowest dose of DSS with tumor-promoting ability in our model revealed that a number of colonic neoplasms (adenoma and adenocarcinoma) developed in mice treated with 1% or 2% DSS after AOM administration and the incidence and multiplicity of the groups given these dose levels of DSS were almost similar. In addition, only a few colonic neoplasms developed in mice give 0.5% DSS after AOM exposure. These results clearly indicate that treatment with 1% or more of DSS could exert its tumor-promoting ability after the initiation with a low dose of AOM in male ICR mice. Our recent work using female ICR mice also indicates that 1% is the lowest dose of DSS, which can promote AOM-induced colon carcinogenesis (manuscript in preparation).

Since colonic adenocarcinoma developed as early as 12 weeks in our previous work with an experimental period of 20 weeks (Tanaka et al., 2003), we shortened the experimental period (14 weeks) in the present study

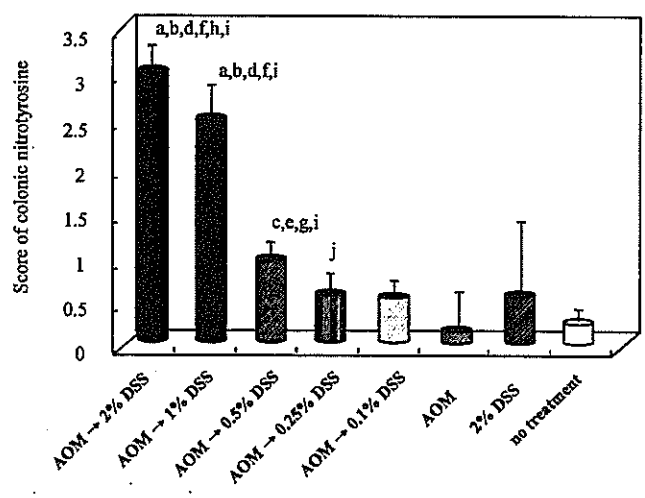

Fig. 6. Score for nitrotyrosine immunohistochemistry. Statistical analysis using Student's t-test or Welch's t-test indicates significant difference: a ($P<0.001$), vs. the AOM→0.5% DSS group; b ($P<0.001$) and c ($P<0.05$), vs. the AOM→0.25% DSS group; d ($P<0.001$) and e ($P<0.01$), vs. the AOM→0.1% DSS group; f ($P<0.001$) and g ($P<0.05$), vs. the AOM alone group; h ($P<0.005$), vs. the 2% DSS alone group; and i ($P<0.001$) and j ($P<0.05$), vs. the "no treatment group".

to investigate the dose-dependent effects of DSS on the occurrence of cryptal dysplasia, which is considered precursor lesions in colitis-related colon carcinogenesis in both humans (Riddell et al., 1983) and rodents (Cooper et al., 2000). As a result, the incidence of mucosal dysplasia was 100% in all groups treated with 2%, 1%, 0.5%, 0.25% or 0.1% DSS after AOM exposure (groups 1 through 5). However, the multiplicities of the lesion gradually increased with the dose of DSS. The values of high-grade dysplasia in groups 1 (AOM→2% DSS) and 2 (AOM→1% DSS) were over 2-fold of groups through 3-5. These findings suggest a dose-dependent tumor-promoting effect of DSS in AOM-initiated mouse colon carcinogenesis and the presence of the threshold. Although there are many studies showing that DSS exposure could induce colonic dysplasia and neoplasms, a long experimental period, high concentration of DSS and/or cycle treatment with DSS require induction of the lesions (Cooper et al., 2000; Kullmann et al., 2001; Takesue et al., 2001; Okayasu et al., 2002). Recently, Cooper et al. reported an interesting mouse model of colitis with dysplasia (Cooper et al., 2000), where mice were exposed to four cycles of 5% DSS in the drinking water (one cycle: 7 days DSS followed by 14 days of H_2O) and the incidence of dysplasia was 13.9% at week 12. On the other hand, in our mouse model cryptal dysplasia occurred in all mice given even 0.1% DSS. The results may be different by the use of a different strain of mice and the presence of initiation treatment.

In the current study, multiplicity of mucosal ulcer in mice given AOM and DSS was increased with increasing the dose of DSS except for those treated with AOM→1% DSS (Table 3). Treatment with 2% DSS alone did not cause mucosal ulcer, but colonic inflammation was noted even at week 14 and the severity was greater than AOM alone treatment (Fig. 4), as found in nitrotyrosine-immunohistochemistry (Fig. 6). In other groups, the score for nitrotyrosine immunoreactivity, a good biomarker for "nitrating species" (Halliwell, 1997), also paralleled with inflammation score. Both the scores in the groups exposed to more than 0.25% DSS were significantly higher than that of an untreated group. Thus, to induce a number of large bowel neoplasms, more than 1% DSS after AOM exposure was needed. These results also suggest that administration of 0.25% DSS can induce inflammation in the colon of mice, but this dose did not exert tumor-promoting effect in this experiment. Mice that received 2% or 1% DSS after AOM initiation produced a number of colonic adenocarcinoma. Scores of inflammation and nitrotyrosine immunoreactivity in mice treated with AOM→2% or 1% DSS were much greater than those that received AOM and other doses of DSS. In patients with UC, reactive oxygen and nitrogen species are over-produced (Rachmilewitz et al., 1993; Grisham, 1994; Lundberg et al., 1994; Buffinton and Doe, 1995; Oudkerk Pool et al., 1995; Lih-Brody et al., 1996; Singer et al., 1996; Kimura et al., 1997) and oxidative and nitrosative stress also may contribute to the increased CRC risk in these individuals (Babbs, 1992). Interestingly, mice with a number of colonic tumors had high scores of inflammation and nitrotyrosine in the current study. These findings indicate that inflammatory damage by production of nitric oxide (NO) is important to form the colonic adenocarcinoma in this model. Although we did not examine the expression of iNOS in the current study, our previous study demonstrated over-expression of iNOS in adenocarcinoma in this animal model (Tanaka et al., 2003). Thus, colonic damage from NO was partly caused through the iNOS pathway in our model.

An experimental model using pretreatment of AOM and administration of DSS was reported using CBA/J mice (Mitamura et al., 2002). In their study, male and female CBA/J mice aged 14 weeks were given i.p. injection with AOM (8 mg/kg body weight), and followed by 3% DSS (MW 61,600) exposure in drinking water for 7 days followed by tap water for the subsequent 14 days, and sacrificed at 19 weeks of age. Thirty-three colonic dysplastic lesions were found in mice, but there was no information about the colonic tumors. We need further experiments to know that combined treatment with AOM and DSS is able to induce a high incidence of colonic dysplasia as well as epithelial malignancy in other mouse strains with different susceptibilities for AOM and/or DSS. Such studies are underway in our laboratory.

In conclusion, our results demonstrate that 1% or more of DSS is sufficient to exert its powerful tumor-promoting effects in the colon of male ICR mice initiated with a low-dose of AOM within a short-term period (14 weeks).

Acknowledgements. We are indebted to Ms. S. Yamamoto for her excellent technical assistance and staff of the Research Animal facility of Kanazawa Medical University for taking good care of the animals. This study was supported in part by a Grant-in-Aid for the Third-Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, labour and welfare, Japan; by a Grant-in-Aid (nos. 15592007 and 15•2052) from the Ministry of Education, Culture, Sports, Science and Technology, Japan: and by grants (H2004-6 and C2004-4) from Kanazawa Medical University.

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Accepted December 17, 2004

β-*Catenin* mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate

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(Received October 29, 2004/Revised Novermber 24, 2004/Accepted November 26, 2004/Online publication 17 February, 2005)

In a previous study, we developed a novel mouse model for colitis-related carcinogenesis, utilizing a single dose of azoxymethane (AOM) followed by dextran sodium sulfate (DSS) in drinking water. In the present study, we investigated whether colonic neoplasms can be developed in mice initiated with a single injection of another genotoxic colonic carcinogen 1,2-dimethylhydrazine (DMH), instead of AOM and followed by exposure of DSS in drinking water. Male crj: CD-1 (ICR) mice were given a single intraperitoneal administration (10, 20 or 40 mg/kg body weight) of DMH and 1-week oral exposure (2% in drinking water) of a non-genotoxic carcinogen, DSS. All animals were killed at week 20, histological alterations and immunohistochemical expression of β-catenin, cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) were examined in induced colonic epithelial lesions (colonic dysplasias and neoplasms). Also, the β-*catenin* gene mutations in paraffin-embedded colonic adenocarcinomas were analyzed by the single strand conformation polymorphism method, restriction enzyme fragment length polymorphism and direct sequencing. The incidences of colonic neoplasms with dysplastic lesions developed were 100% with 2.29 ± 0.95 multiplicity, and 100% with 10.38 ± 4.00 multiplicity in mice given DMH at doses of 10 mg/kg or 20 mg/kg and 2%DSS, respectively. Although approximately half of the mice given DMH at a dose of 40 mg/kg bodyweight were dead after 2–3 days after the injection, mice who received DMH 40 mg/kg and 2%DSS had 100% incidence of colonic neoplasms with 9.75 ± 6.29 multiplicity. Immunohistochemical investigation revealed that adnocarcinomas, induced by DMH at all doses and 2%DSS, showed positive reactivities against β-catenin, COX-2 and iNOS. In DMH/DSS-induced adenocarcinomas, 10 of 11 (90.9%) adenocacrcinomas had β-*catenin* gene mutations. Half of the mutations were detected at codon 37 or 41, encoding serine and threonine that are direct targets for phosphorylation by glycogen synthase kinase-3β. The present results suggests that, as in the previously reported model (AOM/DSS) our experimental protocol, DMH initiation followed by DSS, may provide a novel and useful mouse model for investigating inflammation-related colon carcinogenesis and for identifying xenobiotics with modifying effects. (*Cancer Sci* 2005; 96: 69–76)

Colorectal cancer (CRC) is one of the most common non-smoking related cancers. The risk for CRC is associated with extent and duration of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease.[1,2] The etiopathogenesis of IBD remains uncertain, although it is generally assumed that chronic inflammation is the primary driving force.[3] To understand the pathogenesis of IBD and IBD-related CRC, several animal models were reported. The chemically induced and genetic models of colonic inflammation do not completely mimic the disease situation found in UC patients,[4] although they are more readily available, reproducible and conductive to therapeutic and mechanistic studies. Most used is an animal model with dextran sodium sulfate (DSS) administration through the diet, or drinking fluid. A non-genotoxic carcinogen, DSS[5] induces colonic inflammation in rodents with clinical and histopathological similarity to human UC.[6] However, the colitis model using DSS needs a long period or cycle administration of DSS to induce colitis and colitis-related CRC, and the incidence and/or multiplicity of induced tumors are relatively low.[7] Recently, we developed a novel mouse model for inflammation-related colon carcinogenesis utilizing a single and low dose of azoxymethane (AOM), a metabolite of 1,2-dimethylhydrazine (DMH), followed by a strong tumor-promoter DSS in drinking water.[8] This combined treatment with AOM and DSS resulted in a high incidence and greater multiplicity of colonic neoplasms within 20 weeks. Moreover, the first colonic malignancy was observed as early as 12 weeks of the experimental schedule. This model can be used for detecting the chemicals with weak colonic carcinogenicity in mice within a short-term period and for analyzing gene mutations in induced colonic neoplasms. The colon carcinogen DMH has been widely used to study chemically-induced colon cancer in rodents. Regardless of the mode of administration, DMH specifically induces colorectal tumors.[9] DMH-induced colon tumors in rodents are very close to human colon cancer with regard to morphology, pattern of growth and clinical manifestations.[10] Colorectal adenocarcinomas, induced by DMH in mice, often invade into the submucosa and muscular layer, but those induced by AOM and methylazoxymethanol did not show such biological and histological natures.[10,11] However, the major weakness of the model, using DMH, is that multiple injections of DMH and long-term experimental period are required to induce colon tumors in laboratory animals. β-Catenin, acting as a structural protein at cell–cell adherens junctions and as a transcriptional activator mediating Wnt signal transduction,[12] participates in a large cytoplasmic protein complex, which contains the tumor suppressor gene product of adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β) and axin/conductin.[13] Frequent mutation of the β-*catenin* gene was found in chemically induced colonic neoplasms in rodents.[14,15] For example, β-*catenin* mutations were frequently observed in AOM-induced colon tumors in rats and mice.[15,16] In rats, 32% of colonic adenocarcinomas, induced by DMH, possessed β-*catenin* gene mutations.[17] Mutation of the *APC* gene is known to repress the degradation and result in accumulation of β-catenin.[18] About 80% of colorectal neoplasms harbor mutations in the *APC* gene and half of the reminder have β-*catenin* mutation.[19–21] In the colonic neoplasms (adenomas and adenocarcinomas), β-catenin was universally localized to the cytoplasm and/or nucleus.[22] In addition, altered expression of

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β-catenin was reported in inflammation-related colonic cancer in rodents[8,23] and humans.[24] These findings suggest that the mutation of β-*catenin* gene plays an important role in the development of colon carcinogenesis in rodents as well as in humans. In the current study, we tried to induce colonic neoplasms in mice with a single administration of DMH at three dose levels followed by a 1-week exposure of DSS in drinking water. In addition, we analyzed mutations of the β-*catenin* gene in induced colonic adenocarcinomas and compared with those found in colonic malignancies induced by AOM and DSS.[8]

Materials and Methods

Animals, chemicals and diets. Male Crj: CD-1 (ICR) mice (Charles River Japan Inc., Tokyo, Japan) aged 5 weeks were used. They were maintained at the Animal Facility of Kanazawa Medical University according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (four or five mice/cage) with free access to drinking water and a pelleted basal diet (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12:12 h light : dark cycle) and temperature (23 ± 2°C). After 7-days of quarantine, they were randomized by body weight into experimental and control groups. DMH was purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). DSS with a molecular weight of 36 000–50 000 was obtained from ICN Biochemicals, Inc. (Aurora, OH, USA [Cat no. 160110]).

Experimental procedure. A total of 43 male ICR mice were divided into seven experimental and control groups. DMH was dissolved in 0.9% saline and the pH adjusted to 6.5 using 0.25 M NaOH. Groups 1 (seven mice), 2 (eight mice) and 3 (eight mice) were given a single intraperitoneal (i.p.) injection of DMH at a dose of 10, 20 or 40 mg/kg body weight, respectively. Starting 1 week after the injection, animals in groups 1–3 were given 2% (w/v) DSS in drinking water for 7 days, and then followed without any further treatment for 18 weeks. Groups 4 (five mice) and 5 (five mice) were given DMH 20 and 40 mg/kg body weight alone, respectively. Group 6 (five mice) was given 2% DSS alone. Group 7 (five mice) was an untreated control. All animals were killed at week 20 by ether overdose. At the termination of the study, all organs, including small and large intestines, in the mice were carefully inspected for macroscopic pathological lesions. The large bowels were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis, and then washed with saline. Macroscopic inspection on the large bowels was carefully carried out and they were cut and fixed in 10% buffered formalin for at least 24 h. Formalin-fixed colonic tissues were routinely processed for histological examination. Histological diagnosis was performed on hematoxylin–eosin (HE) stained section. Eleven colonic tumors, histologically diagnosed as adenocarcinoma, were stored in a deep-freezer at −80°C for analyzing β-*catenin* mutation. On HE-stained sections, histological alterations, such as mucosal ulceration, dysplasia and colonic neoplasms, were examined. Colitis with or without ulceration was scored on HE-stained sections, according to the following morphological criteria described by Cooper *et al.*:[25] grade 0, normal colonic mucosa; grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa: grade 2, loss of the basal two-thirds 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, musculaaris propria and/or subserosa. High- or low-grade of dysplasia of colonic mucosa was diagnosed according to the criteria described by Riddell *et al.*[26] and Pascal.[27] Colonic neoplasms were diagnosed according to the description by Ward.[28] Histopathological examination was also carried out in other organs.

Immunohistochemistry. As in our previous study,[8] immunohistochemistry for β-catenin, cyclooxygenase (COX)-2 and nitric oxide synthase (iNOS), was performed on 3-μm-thick paraffin-embedded sections from colons of mice in all groups, utilizing the labeled streptavidin–biotin method using a LSAB Kit (DAKO, Glostrup, Denmark) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated through graded ethanols at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. Sections were treated for 40 min at room temperature with 2% bovine serum albumin, and incubated overnight at 4°C with primary antibodies, such as anti-β-catenin mouse monoclonal antibody (diluted 1:1000; Transduction Laboratories, Lexington, KY, USA), anti-COX-2 mouse monoclonal antibody (diluted 1:200; Transduction Laboratories), and anti-iNOS mouse monoclonal antibody (cat. no. N32020-150; diluted 1:250, Transduction Laboratories). To reduce the non-specific staining of mouse tissue by the mouse antibodies, a mouse on mouse immunoglobulin G blocking reagent (Vector Laboratories Inc., Burlingame, CA, USA) was applied for 1 h. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck Ltd, Tokyo, Japan). For each case, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted. Intensity and localization of immunoreactivities against all primary antibodies used were examined on all sections using a microscope (Olympus BX41, Olympus Optical Co., Ltd, Tokyo, Japan) and recorded.

DNA extraction. For analysis of β-*catenin* mutations, 11 colonic adenocarcinomas developed in DMH (10 or 20 mg/kg body weight)/DSS-treated mice were used. DNA was extracted from frozen tissue using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA).

Polymerase chain reaction-single strand conformation polymorphism analysis. DNA from colonic adenocarcinomas was polymerase chain reaction (PCR)-amplified with primers (5′-primer, GCTG-ACCTGATGGAGTTGGA; 3′-primer, GCTACTTGCTCTT-GCGTGAA), which were designed to amplify exon 3 of the β-*catenin* gene containing the consensus sequence for GSK-3β phosphorylation.[15] The length of the PCR product with these primers is 227 bp. The primers were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). PCR for non-radioisotopic single strand conformation polymorphism (SSCP) was performed in 50 μL of reaction mixture consisting of 0.5 μM of each primer, 1 × PCR buffer (Takara Bio, Otsu, Japan), 250 μM each dNTP, 2.5 U TaKaRa Ex Taq (Takara Bio) and 1 μL of template DNA. The mixture was heated at 94°C for 1 min and subjected to 30 cycles of denaturation (94°C, 0.5 min), annealing (55°C, 0.5 min) and extension (72°C, 1 min) using a using a TaKaRa PCR Thermal Cycler Dice (Takara Bio). The amplified PCR product was analyzed for its mobility-shifted bands using a GenePhor (Amersham Biosciences Corp., NJ, USA) with a GeneGel Clean (Amersham Biosciences Corp.) according to the manufacturer's protocol. Electrophoresis was carried out at 90 V for 25 min and then 500 V for 50 min at 20°C, and the gels were soaked in 10% trichloroacetic acid and in 50% methanol for 10 min each. DNA bands were detected by silver staining using 2D Silver Staining Solution II (Daiichi Chemical DNA Co., Tokyo, Japan).

Restriction fragment length polymorphism assay for PCR products of β-*catenin*. To detect β-catenin mutations at codons 32, 33 and 34, PCR products were treated with a restriction enzyme HinfI

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(Wako Pure Chemical Industries, Tokyo, Japan) and electrophoresed on 5% agarose gels. Recognition sequences of *Hin*fI are GANTC. The PCR product of 227 bp is digested by *Hin*fI to 82, 7 and 138 bp in the case of the wild-type, to 89 and 138 bp with mutations at the first or second bases of codons 32 or 33, and to 82 and 145 bp with mutations at the second or third bases of codons 34 or 35.

Direct DNA sequencing. The PCR products were purified and concentrated to 20 μL using Microcon 100 (Amicon Inc., Beverley, MA, USA). With 2 μL of the purified PCR products and 5′ or 3′ PCR primers, cycle sequencing reactions were carried out using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and purified by isopropanol precipitation. The sequences were determined with an ABI PRISM 310 Genetic Analyzer (PerkinElmer, Wellesley, MA, USA).

Statistical analysis. All measurements were compared by Student's *t*-test, Welch's *t*-test, chi-squared test or Fisher's exact probability test for multiple group comparisons.

Results

General observations. Approximately half of the mice injected 40 mg/kg body weight of DMH (four mice of group 3, and two mice of group 5) died of hepatotoxicity of DMH 2–3 days after the injection. This was confirmed by histological examination of liver. Also bloody stool was found during and soon after of DSS exposure (days 12–21) in a few mice who received 2% DSS in drinking water, and their body weight gains were slightly decreased (data not shown). Thereafter, however, no such clinical symptoms were observed. The body and liver weights, and lengths of large bowel of mice in all groups at the end of the study (week 20) are listed in Table 1. There were no significant differences among the groups in these measurements.

Pathological findings. Macroscopically, nodular, polypoid or flat-type colonic tumors were observed in the middle and distal colon of all mice in groups 1–3 (Fig. 1), but not in the small intestine. Their histopathology was well- or moderately-differentiated tubular adenocarcinoma (Fig. 2a) or tubular adenoma (Fig. 2b). Histologically, there were no tumors in any organs other than the large bowel in these groups. The incidences and multiplicities of large bowel adenoma, adenocarcinoma and total tumors (adenoma + adenocarcinoma) are summarized in Table 2. The incidences of total tumors and adenocarcinoma in mice of given DMH/DSS (groups 1–3) were 100%. The multiplicities of total tumors, adenoma and adenocarcinoma in groups 2 and 3 were significantly higher than those of group 1 ($P \leq 0.001$, $P \leq 0.01$ or $P \leq 0.05$, respectively). In mice of groups 4–7, no neoplasms developed in any organs including large bowel. Besides colonic neoplasms, all mice in groups 1–3 had colonic dysplasia (Fig. 2c). Their multiplicities were 4.71 ± 2.29, 7.13 ± 1.27 and 8.25 ± 3.10 in groups 1, 2 and 3, respecively (Table 3). The multiplicities of total dysplasia and high-grade dysplasia in groups 2 and 3 were significantly greater than those of group 1 ($P \leq 0.02$, $P \leq 0.01$ or $P \leq 0.05$, respectively). There were no such dysplatic lesions in mice of groups 4–7. In addition, colonic mucosal ulceration (grade 1) was found in the distal colon of mice in groups 1, 2, 3 and 5 (Table 3).

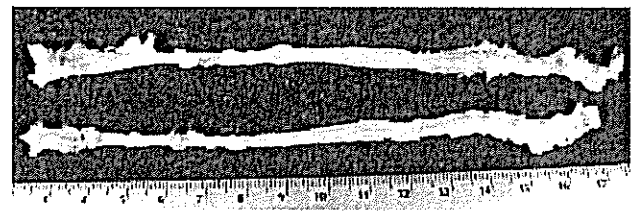

Fig. 1. Representative macroscopic view of the colonc from group 2 (1,2-dimethylhydrazine [DMH] 20 mg/kg body weight →2% dextran sodium sulfate [2%DSS]). Note the numerous polypoid tumors in the colon.

Table 1. Body weights, liver weights, and lengths of large bowel in each group

Group no.	Treatment (no. mice examined)	Body weight (g)	Liver weight (g)	Length of large bowel (cm)
1	DMH 10 mg/kg→2%DSS (7)	42.6 ± 2.7[†]	2.47 ± 0.29	13.8 ± 1.3
2	DMH 20 mg/kg→2%DSS (8)	43.3 ± 3.0	2.63 ± 0.35	13.5 ± 1.5
3	DMH 40 mg/kg→2%DSS (4)	44.3 ± 3.9	2.64 ± 0.21	13.9 ± 1.2
4	DMH 20 mg/kg (5)	45.9 ± 3.6	2.81 ± 0.41	14.5 ± 1.1
5	DMH 40 mg/kg (3)	43.2 ± 1.9	2.86 ± 0.46	14.9 ± 0.1
6	2%DSS (5)	42.1 ± 5.5	2.82 ± 0.31	13.9 ± 1.0
7	None (5)	44.6 ± 3.2	2.32 ± 0.45	15.0 ± 0.9

[†]Mean ± standard deviation. DMH, 1,2-Dimethylhydrazine; DSS, dextran sodium sulfate.

Table 2. Incidence of large bowel neoplasms in mice treated with 1,2-dimethylhydrazine and dextran sodium sulfate

Group no.	Treatment (no. mice examined)	No. mice with large bowel neoplasms		
		Total (%) (multiplicity)	Adenoma (%) (multiplicity)	Adenocarcinoma (%) (multiplicity)
1	DMH 10 mg/kg→2%DSS (7)	100 (2.29 ± 0.95)	57.1 (1.00 ± 1.15)	100 (1.29 ± 0.50)
2	DMH 20 mg/kg→2%DSS (8)	100 (10.38 ± 4.00)*	87.5 (4.63 ± 3.29)**	100 (5.75 ± 1.83)*
3	DMH 40 mg/kg→2%DSS (4)	100 (9.75 ± 6.29)**	100 (5.25 ± 4.65)***	100 (4.50 ± 1.73)*
4	DMH 20 mg/kg (5)	0	0	0
5	DMH 40 mg/kg (3)	0	0	0
6	2%DSS (5)	0	0	0
7	None (5)	0	0	0

Significantly different from group 1 by Student's *t*-test (*$P < 0.001$, **$P < 0.01$, and ***$P < 0.05$). DMH, 1,2-Dimethylhydrazine; DSS, dextran sodium sulfate. Numbers in parentheses are multiplicity (mean ± standard deviation) of large bowel tumors.

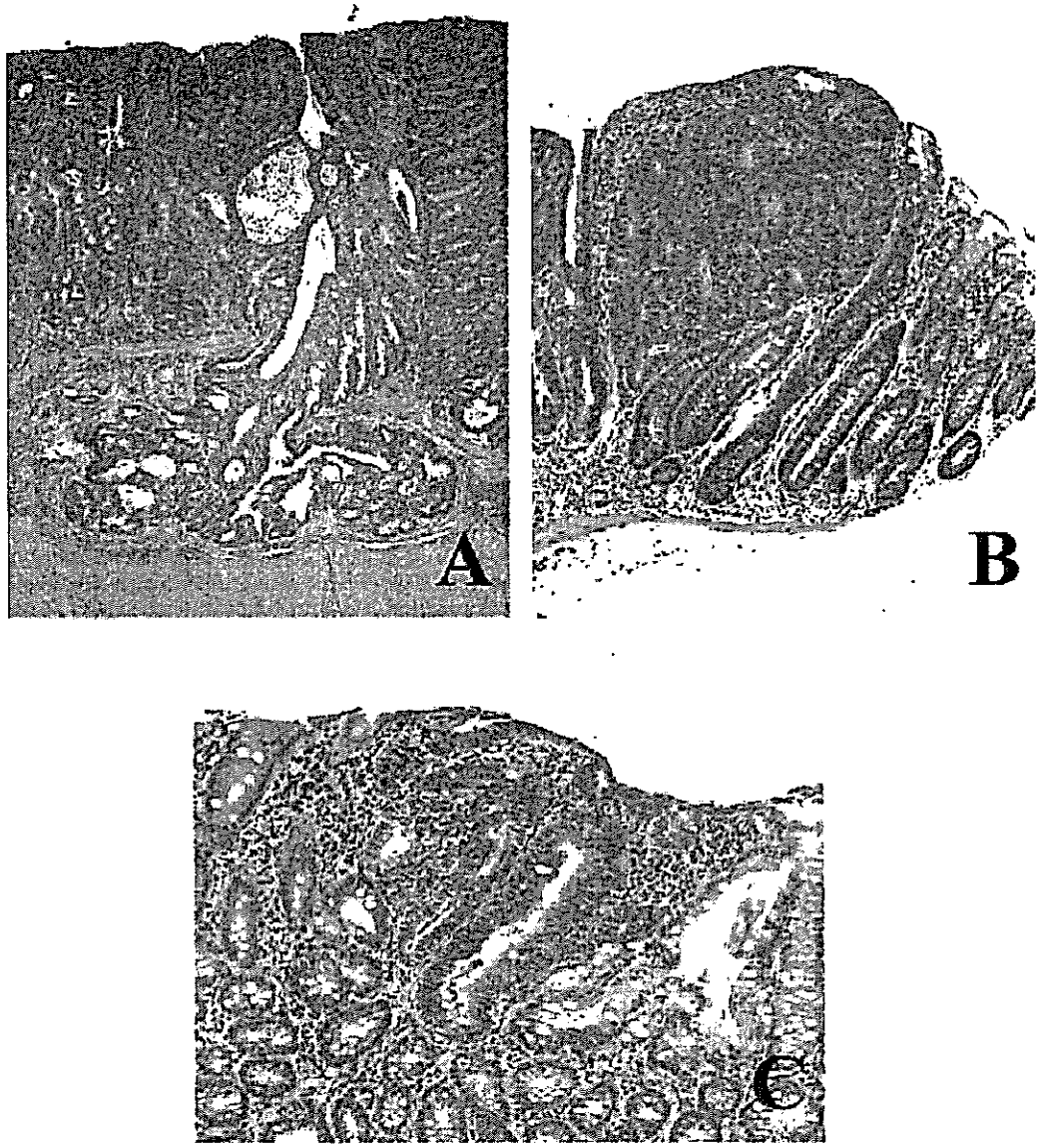

Fig. 2. Histopathology of colonic neoplasms developed in mice. (a) adenocarcinoma (b) adenoma, and (c) dysplasia. Hematoxylin–eosion stain. Original magnification (a) ×4, (b) ×10, and (c) ×20.

Immunohistochemical findings. The immunoreactivities against β-catenin, COX-2 and iNOS were noted in all colonic lesions, including neoplasms and dysplastic lesions. The immunoreactivity showed dark brown reaction products with slight variation in the intensity and distribution. Strong β-catenin expression was seen in the nucleus and cytoplasm of adenocarcinoma cells (Fig. 3a). Although the intensity was relatively weaker than carcinoma cells, adenoma cells showed positivity for β-catenin in their cytoplasm and cell membrane. β-Catenin immunoreactivity was also found in the cell membrane and cytoplasm of dysplastic cells, but intensity was weaker than adenoma cells. Non-lesional cryptal cells showed weak positivity of β-catenin in their cell membrane. In addition, positive reaction against β-catenin antibody was found in the cytoplasm of vascular endothelium, infiltrated inflammatory cells, and ganglion cells in myenteric (Auerbach's) plexus. Strong COX-2 immunoreactivity was found in adenocarcinoma cytoplasm (Fig. 3b). Adenoma cells also were found in their cytoplasm, and the intensity was weaker than adenocarcinoma cells. Dysplastic cells showed weak positivity for COX-2 when compared to neoplastic cells. Non-lesional cryptal cells at lower part of crypts were weakly positive for COX-2, and the stainability was lower than dysplastic crypts. Strongly positive reaction of COX-2 was also seen in the endothelium of small blood vessels, and inflammatory cells infiltrated in the lamina propria. Smooth muscle cells and fibroblasts in the wall of the large bowel showed weak reaction of COX-2. iNOS-immunohistochemistry showed strong immunoreactivity in the cytoplasm of adenocarcinoma (Fig. 3c) and adenoma cells; the intensity was greater in carcinoma cells when compared to adenoma cells. Also, dysplastic cells were positive for iNOS in their cytoplasm, but the intensity was weaker than adenoma cells. The faint positive

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Table 3. Incidence of large bowel ulceration and dysplasia in mice treated with 1,2-dimethylhydrazine and dextran sodium sulfate

Group no.	Treatment (no. mice examined)	Incidence of mucosal ulcer (%) (multiplicity)	Incidence of colonic dysplasia (multiplicity)		
			Total	Low-grade	High-grade
1	DMH 10 mg/kg→2%DSS (7)	100 (2.43 ± 1.40)	100 (4.71 ± 2.29)	100 (3.00 ± 1.29)	100% (1.71 ± 1.11)
2	DMH 20 mg/kg→2%DSS (8)	100 (1.86 ± 0.4)	100 (7.13 ± 1.25)*	100 (2.88 ± 1.25)	100% (4.25 ± 1.58)**
3	DMH 40 mg/kg→2%DSS (4)	100 (2.50 ± 1.29)	100 (8.25 ± 3.10)	100 (4.00 ± 1.41)	100% (4.25 ± 2.22)***
4	DMH 20 mg/kg (5)	0 (0)	0 (0)	0 (0)	0 (0)
5	DMH 40 mg/kg (3)	0 (0)	0 (0)	0 (0)	0 (0)
6	2%DSS (5)	40 (0.51 ± 0.32)	0 (0)	0 (0)	0 (0)
7	None (5)	0 (0)	0 (0)	0 (0)	0 (0)

Significantly different from group 1 by Student's *t*-test (*$P < 0.02$, **$P < 0.01$, and ***$P < 0.05$). DMH, 1,2-Dimethylhydrazine; DSS, dextran sodium sulfate. Numbers in parentheses are multiplicity (mean ± standard deviation) of large bowel tumors.

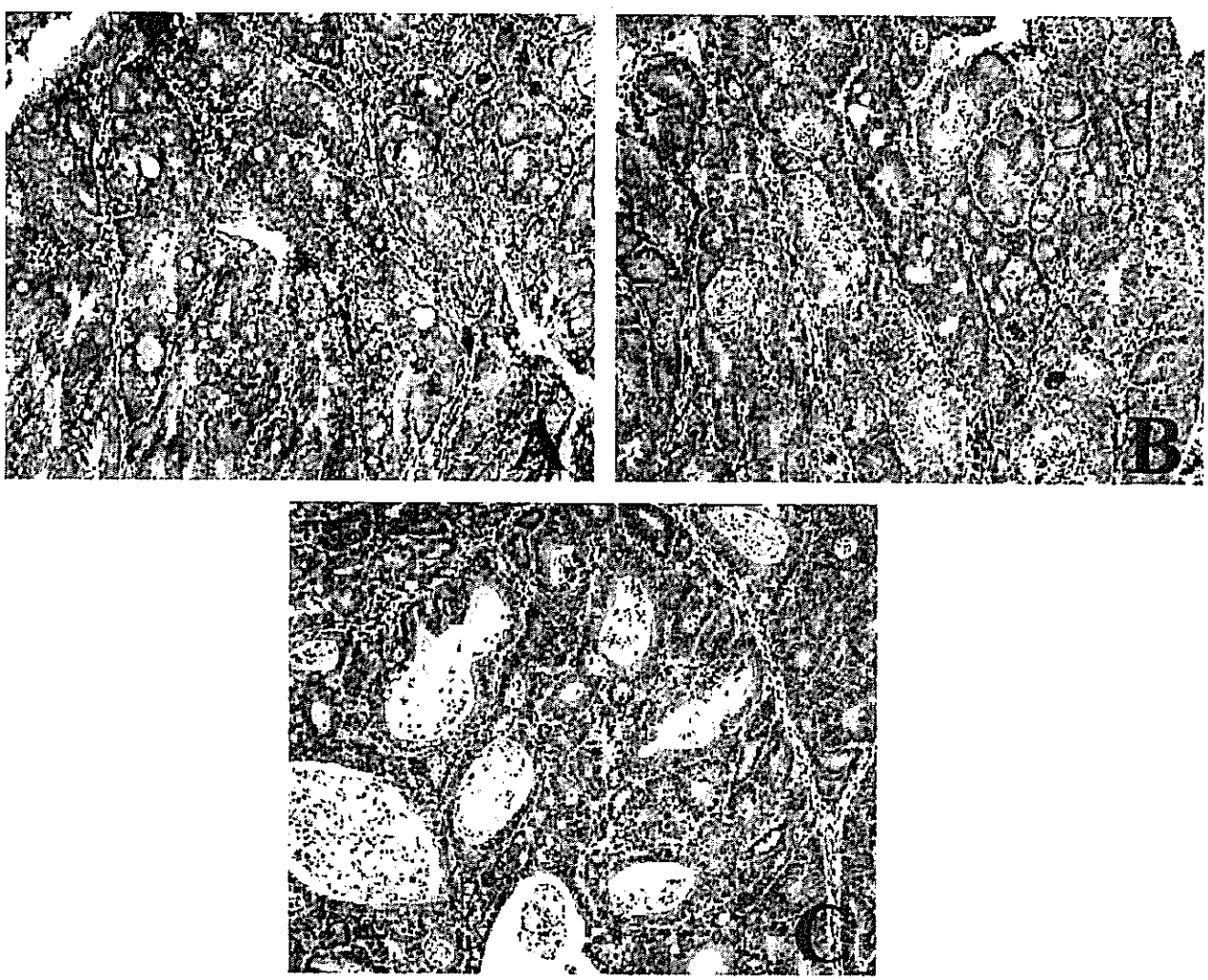

Fig. 3. Immunohistochemistry of (a) β-catenin, (b) cyclooxygenase (COX-2), and (c) nitric oxide synthase (iNOS), and immunofluorescent staining of β-catenin in colonic adenocarcinoma in mice. Original magnification (a–c) ×10.

Table 4. Expression of β-catenin, nitric oxide synthase and cyclooxygenase in 1,2-dimethylhydrazine/dextran sodium sulfate-induced mouse colon lesions

Protein	Normal mucosa	Dysplasia	Adenoma	Adenocarcinoma
β-Catenin	±–+	+–++	++	+++
	(M[1])	(M, C[2])	(M, C)	(C, N[3])
COX-2	––±	+	++	+++
	(C)	(C)	(C)	(C)
iNOS	––±	+	++	+++
	(C)	(C)	(C)	(C)

[1]Cell membrane; [2]cytoplasm, [3]nucleus. –, No staining; ±, faint and partial staining; +, weak staing; ++, moderate staining, +++, strong staining. COX-2, cyclooxygenase; iNOS, nitric oxide synthase.

Table 5. Mutations in exon 3 of the β-*catenin* gene in 1,2-dimethylhydrazine/dextran sodium sulfate-induced mouse colonic adenocarcinomas

Sample	β-catenin status		Amino acid substitution
DMH 10/DSS-1	Codon 37	TCT→CCT	Ser→Pro
DMH 10/DSS-2	Codon 32	GAT→AAT	Asp→Asn
DMH 10/DSS-3	Codon 37	TCT→CCT	Ser→Pro
DMH 20/DSS-4	Wild type		–
DMH 20/DSS-5	Codon 34	GGA→GAA	Gly→Glu
DMH 20/DSS-6	Codon 34	GGA→GAA	Gly→Glu
DMH 20/DSS-7	Codon 41	ACC→ATC	Thr→Ile
DMH 20/DSS-8	Codon 34	GGA→GAA	Gly→Glu
	Codon 41	ACC→ATC	Thr→Ile
DMH 20/DSS-9	Codon 34	GGA→GAA	Gly→Glu
DMH 20/DSS-10	Codon 41	ACC→ATC	Thr→Ile
DMH 20/DSS-11	Codon 34	GGA→GAA	Gly→Glu

DMH, 1,2-Dimethylhydrazine; DSS, dextran sodium sulfate.

reaction was found in the cytoplasm of non-lesional 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 areas of mucosal ulceration in groups 1, 2, 3 and 6. The results of immunoreactivities against β-catenin, COX-2, and iNOS are summarized in Table 4.

Mutation in β-*catenin* gene. In this study, we analyzed the status of the β-*catenin* gene in the histological sections of DMII/DSS-induced colon adenocarcinomas. We detected β-*catenin* gene mutations in 10 out of 11 colonic adenocarcinomas induced by DMH (10 or 20 mg/kg bodyweight) and 2% DSS (Figs 4,5). All mutations detected in colon adenocarcinomas converged at codons 32, 34, 37 and 41, all being functionally important codons for β-catenin degradation: five were located at the second base of codon 34, three at the second base of codon 41, two at the first base of codon 37 and one at the first base of codon 32. Except for mutation (A : T to G : C) at the first base of codon 37, all were G : C to A : T transitions (Table 5).

Discussion

In the current study, a single i.p. injection of DMH (10, 20 or 40 mg/kg body weight) followed by a 1-week exposure of 2% DSS in drinking water, could produced colonic adenocarcinomas with 100% incidence in male ICR mice within 20 weeks. All of the DMH/DSS-induced colonic adenocarcinomas were immunohistochemically positive for β-catenin, COX-2 and iNOS. Moreover, 10 (91%) out of 11 colonic adenocarcinomas had β-catenin mutations. However, no colonic neoplasms were found in mice treated with DMH alone or DSS alone. These findings indicated a powerful tumor-promoting ability of DSS on DMII-initiated colon carcinogenesis in male ICR mice, as found in our previous experiment using AOM as a carcinogen.[8] In the current study, dosing of 40 mg/kg body weight of DMH was lethal in almost half of the mice. This was caused by hepatotoxicity (necrosis and bleeding in the liver) of DMH at the dose. Therefore, appropriate dose of DMH was considered to be 10 or 20 mg/kg body weight in this model. β-Catenin is a multifunctional molecule involved in the cadherin-mediated cell–cell adhesion and Wnt-APC signal transduction.[29] Regulation of membrane, cytoplasmic and nuclear pools of β-catenin is crucial for modulating its adhesion and signaling functions.[30] Normally, β-catenin is localized in cell–cell junctions with very low levels of β-catenin in the cytoplasm and nucleus. Accumulation of β-catenin in the cytoplasm or nucleus as a consequence of mutant *APC*, β-*catenin* or *Axin*, is associated with colon carcinogenesis.[31] β-Catenin accumulation moves

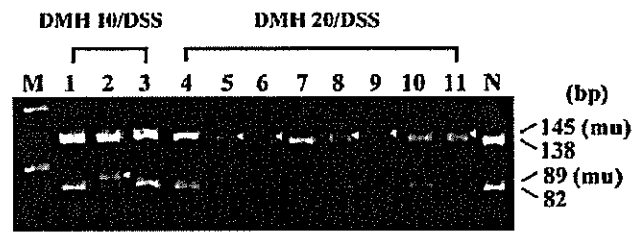

Fig. 5 Restriction fragment length polymorphism (RFLP) analysis of the β-*catenin* gene in mouse colon adenocarcinomas. Lanes 1–11: 1,2-dimethylhydrazine/dextran sodium sulfate (DMH/DSS)-induced mouse colon adenocarcinomas samples. Lanes 1–3: DMH (10 mg/kg bodyweight)/DSS-induced mouse colon adenocarcinoma samples. Lanes 4–11: DMH (20 mg/kg body weight)/DSS-induced mouse colon adenocarcinoma samples. Lane M: DNA size markers. Lane N: Negative control mouse colon mucosa sample. Arrowheads indicate tumor-specific bands.

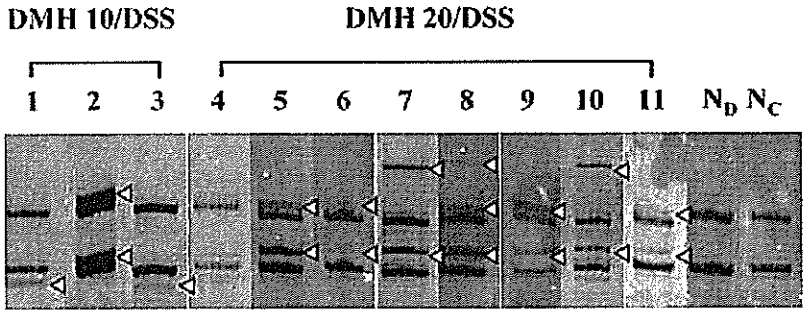

Fig. 4. Polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis of the β-*catenin* gene in mouse colon adenocarcinomas. 1,2-Dimethylhydrazine/dextran sodium sulfate (DMH/DSS)-induced mouse colon adenocarcinomas (lanes 1–11). Lanes 1–3: DMH (10 mg/kg body weight)/DSS-induced mouse colon adenocarcinoma samples. Lanes 4–11: DMH (20 mg/kg body weight)/DSS-induced mouse colon adenocarcinoma samples. Lane N_D: DMH (20 mg/kg body weight)-induced mouse colon mucosa sample. Lane N_C: negative control mouse colon mucosa sample. Arrowheads indicate tumor-specific bands.

© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00020.x

from the cytoplasm to the nucleus when the β-*catenin* or *APC* genes are mutated or the Wnt signaling pathway is activated.[32] Immunohistochemically aberrant expression of β-catenin was reported in colonic neoplasms and dysplasia in a DSS-induced mouse colitis model,[23] and human colitis-related dysplasia and neoplasms.[33,34] The observed frequent mutations in the GSK-3β phosphorylation consensus motif of the β-*catenin* gene appear to be associated with alteration of the cellular localization and functional site of the protein, as shown by immnohistochemical staining in the present study. Our results are comparable to those in a recent report describing altered distribution of β-catenin in UC-related CRC.[35] Although, the β-*catenin* gene is frequently mutated at codons 33, 41 and 45 of the GSK-3β phosphorylation motif in human colon cancers without APC mutations,[36] the mutations of the gene in chemically induced rat colon tumors is found at codons 32, 33 and 34.[15,17] In the present study, we detected β-*catenin* gene mutation of mouse colon adenocarcinomas, induced by DMH/DSS, at codon 32, 34, 37 and 41. The location was slightly different from a report documenting that β-*catenin* gene mutations of mouse colon tumors, induced by AOM, were present at codons 33, 34, 37 and 41, but not at codon 32.[16] Recently, we detected β-*catenin* gene mutations of mouse colon adenocarcinomas, induced by AOM/DSS, at codons 32, 33 and 34.[37] However, in the current protocol, half of the mutations caused by DMH/DSS treatment were at codon 37 and 41, which are important serine and threonine sites for GSK-3β phosphorylation. Koesters *et al.*[17] reported that the different mutational spectra, observed in *Ctnnb1*, directly relates to the particular carcinogenic treatment. They demonstrated that the β-*catenin* mutations at codons 37 and 41 possess higher oncogenic potential.[17] Therefore, it may be speculated that DSS exposure caused a shift in the mutation sites induced by DMH alone treatment. Since mutation of β-*catenin* is reported to be an early event of colorectal carcinogenesis,[38] molecular analysis at early stage of colon carcinogenesis should be carried out in this model. Although the mutations of the *APC* and β-*catenin* are rare in UC-related CRC, as compared with sporadic CRC, nuclear β-catenin expression is related to UC-related CRC development.[39] Since the data on β-*catenin* mutation in UC-related CRC are limited, more studies are required to determine the role of the β-*catenin* mutation in UC-related CRC. Also, expression of *c-myc*, *cyclinD1* and *c-jun*, which are targets of the β-catenin/APC pathway,[17,40,41] may also influence colon carcinogenesis in the present model. Such analysis is underway in our laboratory. Nitric oxide (NO) and prostaglandin, as the main inflammatory mediators, take part in the pathogenesis of IBD, with enhanced expression of iNOS and COX-2 in the morbid colonic mucosa.[42,43] Moreover, expression of the iNOS and COX-2 is increased in human colorectal tumors with inflammation,[44] and in carcinogen-induced colon tumors in rodents.[45] There exist various proposed pathways for NO-induced regulation of COX-2 expression and for modulation of cancer development.[46] They include cross-talk or interactions between endogenous NO and COX-2, and between β-catenin/APC pathway and COX-2.[47] β-Catenin/APC is reported to play a critical role in NO induction of COX-2 in colon epithelial cells.[48] Furthermore, Howe *et al.*[49] demonstrated that β-catenin/Tcf-4 complex transactivates the expression of PEA3, a transcription factor of Ets family, and stimulates COX-2 expression. More recently, it has been reported that NO increases PEA3 expression through β-catenin/APC pathway and directly augments the COX-2 promoter activity of the PEA3/p300 in YAMC cells.[50] In the present study, all colonic neoplasms were immunohistochemically positive for iNOS and COX-2, which was in accordance with the previous reports.[8] Furthermore, strong β-catenin expression was found in the nucleus and cytoplasm of adenocarcinoma cells. It may be possible that the increased expression of iNOS may be related to the altered localization of β-catenin, and/or increased expression of COX-2 in the colonic tumor formation and/or progression. Our recent work suggest involvement of oxidative/nitrosative stress in tumor-promoting effect of DSS on AOM-induced colon carcinogenesis in mice.[51]

In conclusion, the results in the current study indicate that a single dose of DMH followed by DSS resulted in a high incidence of colonic epithelial malignancies with β-*catenin* mutations within 20 weeks. Furthermore, half of the β-*catenin* mutations, detected in adenocarcinomas, were at codon 37 and 41, and strong β-catenin expression was seen in their nucleus and cytoplasm. Also, our findings suggest the importance of inflammation caused by DSS exposure in mouse colon carcinogenesis. The experimental protocol described here could be applied to investigate detailed molecular mechanism of inflammation-related CRC, as is the AOM/DSS model.[8]

Acknowledgments

This research was supported in part by the Grant-in-Aid (13–15) for Cancer Research from the Ministry of Health, Labour and Welfare of Japan; the Grant-in-Aid for the 3rd Term for a Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan; the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the grant (C2004-4) for the Collaborative Research from Kanazawa Medical University; and the grant (H2004-6) for the Project Research from the High-Technology Center of Kanazawa Medical University.

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© Japanese Cancer Association doi: 10.1111/j.1349-7006.2005.00020.x

BMC Cancer

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Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands

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Published: 16 May 2005

BMC Cancer 2005, 5:46 doi:10.1186/1471-2407-5-46

Received: 15 January 2005
Accepted: 16 May 2005

This article is available from: http://www.biomedcentral.com/1471-2407/5/46

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Abstract

Background: It is generally assumed that inflammatory bowel disease (IBD)-related carcinogenesis occurs as a result of chronic inflammation. We previously developed a novel colitis-related mouse colon carcinogenesis model initiated with azoxymethane (AOM) and followed by dextran sodium sulfate (DSS). In the present study we investigated whether a cyclooxygenase (COX)-2 inhibitor nimesulide and ligands for peroxisome proliferator-activated receptors (PPARs), troglitazone (a PPARγ ligand) and bezafibrate (a PPARα ligand) inhibit colitis-related colon carcinogenesis using our model to evaluate the efficacy of these drugs in prevention of IBD-related colon carcinogenesis.

Methods: Female CD-1 (ICR) mice were given a single intraperitoneal administration of AOM (10 mg/kg body weight) and followed by one-week oral exposure of 2% (w/v) DSS in drinking water, and then maintained on the basal diets mixed with or without nimesulide (0.04%, w/w), troglitazone (0.05%, w/w), and bezafibrate (0.05%, w/w) for 14 weeks. The inhibitory effects of dietary administration of these compounds were determined by histopathological and immunohistochemical analyses.

Results: Feeding with nimesulide and troglitazone significantly inhibited both the incidence and multiplicity of colonic adenocarcinoma induced by AOM/DSS in mice. Bezafibrate feeding significantly reduced the incidence of colonic adenocarcinoma, but did not significantly lower the multiplicity. Feeding with nimesulide and troglitazone decreased the proliferating cell nuclear antigen (PCNA)-labeling index and expression of β-catenin, COX-2, inducible nitric oxide synthase (iNOS) and nitrotyrosine. The treatments increased the apoptosis index in the colonic adenocarcinoma. Feeding with bezafibrate also affected these parameters except for β-catenin expression in the colonic malignancy.

Conclusion: Dietary administration of nimesulide, troglitazone and bezafibrate effectively suppressed the development of colonic epithelial malignancy induced by AOM/DSS in female ICR mice. The results suggest that COX-2 inhibitor and PPAR ligands could serve as an effective agent against colitis-related colon cancer development.

Background

Colorectal cancer (CRC) is one of the leading causes of death in the world. This malignancy is also one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease [1]. Long-term UC patients have an increased risk of developing CRC compared with the general population [2]. The precise mechanisms of the IBD-related carcinogenesis process are largely unclear, although it is generally assumed that IBD-related carcinogenesis occurs as a result of chronic inflammation [3].

Several agents, such as folic acid, short chain fatty acid (butyrate), ursodeoxycholic acid, and 5-aminosalicylic acid, have been suggested to be useful for prevention of CRC in UC [4]. Epidemiological studies have shown that prolonged use of aspirin is associated with a reduced risk of CRC [5]. Consistent with these data, several non-steroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase (COX)-2 inhibitors, suppressed the development of chemically-induced colon carcinomas in rats [6] and intestinal polyps in *Min* mice with a nonsense mutation of the *Apc* gene [7]. In addition, clinical trials have demonstrated that a NSAID sulindac causes regression of adenomas in patients with familial adenomatous polyposis [8]. Nimesulide (4-nitro-2-phenoxymethanesulfonanilide), a selective inhibitor of COX-2, belonging to the sulfonamide class [9], is less ulcerogenic than other NSAIDs [10], and suppresses the formation of aberrant crypt foci (ACF), being putative precancerous lesions of the colon cancer, induced by a colon carcinogen, azoxymethane (AOM) in rats [11]. Moreover, this COX-2 inhibitor could effectively reduce the development of intestinal polyps in *Min* mice [12].

Peroxisome proliferator-activated receptors (PPARs?) are ligand-activated transcription factors, belonging to the nuclear hormone receptor superfamily [13]. Three PPAR isotypes, PPARα, PPARδ(β), and PPARγ, have been identified. PPARγ is highly expressed in fat tissue, and play important roles in adipocyte differentiation and lipid storage [14]. PPARγ is also expressed in a number of epithelial neoplasms, such as cancers in colon, breast, and prostate [13]. PPARγ ligands, including thiazolidinediones (troglitazone and rosiglitazone) and tyrosine analogue (GW7845), can induce apoptosis and adipogenic differentiation, and inhibit tumor growth both *in vitro* and *in vivo* studies [15-17]. We previously reported that pioglitazone, bezafibrate or troglitazone in diet are able to suppress ACF formation induced by dextran sodium sulfate (DSS)/AOM in the rat colon [18]. Osawa *et al.* [19] confirmed our findings by demonstrating that ligands for PPARγ (troglitazone, rosiglitazone, and pioglitazone) suppress the occurrence of colonic tumors in mice initiated with AOM. Niho *et al.* [20] also demonstrated that ligands for PPARα (bezafibrate) and PPARγ (pioglitazone) suppress intestinal polyp formation in *Apc*-deficient mice. Moreover, PPARγ could suppress β-catenin levels and colon carcinogenesis during the early steps of tumor formation [21]. On the other hand, high doses of troglitazone and rosiglitazone can promote polyp formation in the *Min* mouse colon [22,23].

For understanding the pathogenesis of IBD and IBD-related CRC, several animal models have been established. Most used is a mouse model with DSS [24]. Modifying effects of several xenobiotics on IBD-related colon carcinogenesis were reported [25] in animal models of IBD. However, the colitis model using DSS with or without carcinogen needs to a long period and repeated administration of DSS to induce colitis and colitis-related CRC. Recently, an endogenous anti-inflammatory PPARγ pathway was suggested in the intestine, which was found in PPARγ-deficient mice [26,27]. To search novel and effective chemopreventive agents against IBD-related CRC, we recently have developed a novel colitis-related CRC mouse model, in which large bowel adenocarcinomas occur within 20 weeks and their histology and biological characteristics are resemble to those found in human cased [28]. As a part of our search for a safer chemopreventive agent for colitis-related colon cancer, in the present study we examined the chemopreventive ability of nimesulide, troglitazone, and bezafibrate using our mouse colon carcinogenesis model for colitis-related colon carcinogenesis [28].

Methods

Animals, chemicals and diets

Female Crj: CD-1 (ICR) (Charles River Japan Inc., Tokyo, Japan) aged 5 weeks were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. The mice were quarantined for the first 7 days then randomized by body weight into experimental and control groups. All animals were housed in plastic cages (five or six mice/cage) with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 hour light/dark cycle) and temperature (23 ± 2°C). A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). DSS for induction of colitis was dissolved in distilled water at a concentration of 2% (w/v). Nimesulide was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Troglitazone and bezafibrate were kindly supplied by Sankyo Co. (Tokyo, Japan) and Kissei Pharmaceutical Co. (Matsumoto, Japan), respectively. Experimental diet containing nimesulide (0.04%, w/w), troglitazone (0.05%, w/w)

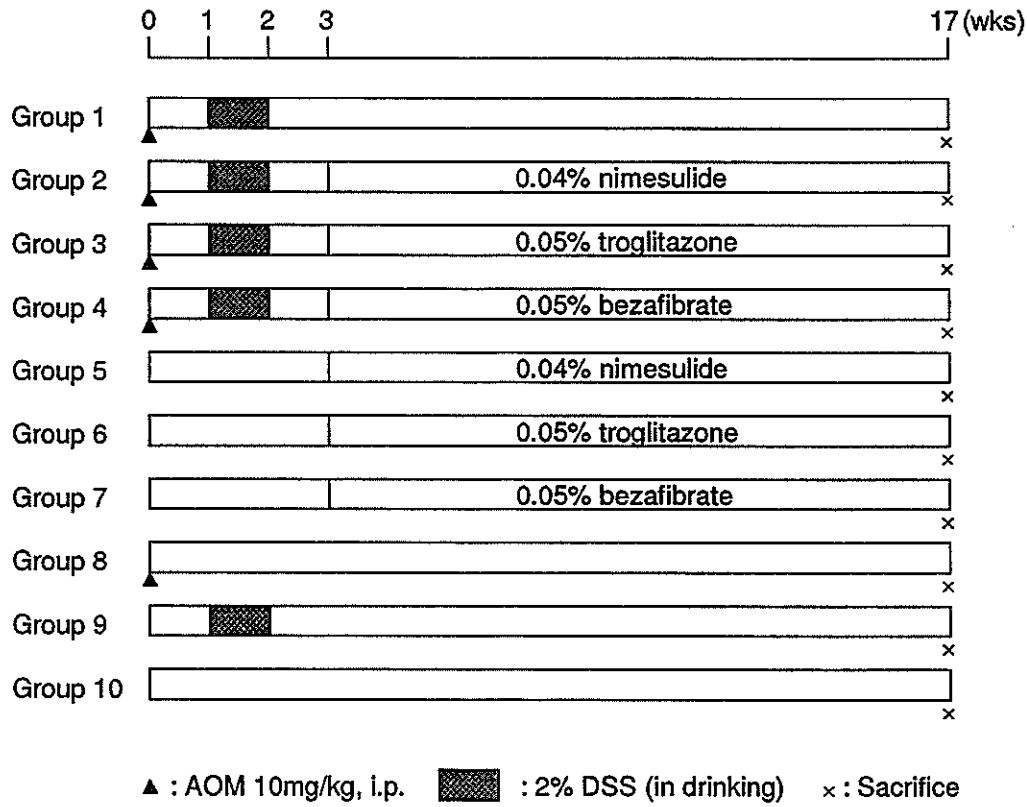

Figure 1
Experimental protocol. Arrows, AOM 10 mg/kg body weight, i.p. injection; densely cross-hatched bars, 2% dextran sodium sulfate (DSS) in drinking water; open bars, basal diet and tap water; crosses, death.

or bezafibrate (0.05%, w/w) was prepared every week by mixing the respective compound in powdered basal diet CRF-1. The dose levels were determined on the basis of previous studies [18,29].

Experimental procedure

A total of 71 female ICR mice were divided into 7 experimental and control groups (Figure 1). Mice in groups 1 through 4 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting one week after the AOM injection, animals in group 1 were administered to 2%DSS in drinking water for 7 days, and then followed without any further treatment for 15 weeks. Mice in groups 2, 3, and 4 were fed the diets containing 0.04% nimesulide, 0.05% troglitazone, and 0.05% bezafibrate, respectively, for 14 weeks, starting 1 week after the stop of DSS administration. Animals in groups 5, 6, and 7 were respectively given the diets containing 0.04% nimesulide, 0.05% troglitazone, and 0.05% bezafibrate alone for 14 weeks. Group 8 was given a single dose of AOM. Group 9 was given 2% DSS for 7 days. Group 10 consisted of untreated mice. All animals were sacrificed at the end of the study (Week 17) by ether overdose. Their large bowels were flushed with saline, excised, measured their length (from ileocecal junction to the anal verge), cut open longitudinally along the main axis, and then washed with

saline. The large bowels were macroscopically inspected, cut, and fixed in 10% buffered formalin for at least 24 hours. Histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H & E) staining. Colonic mucosal dysplasia (mild- and severe-graded) was diagnosed according to the criteria described by Ridell *et al.* [30] and Pascal [31]. Colonic neoplasms were diagnosed according to the description by Ward [32].

Immunohistochemistry

Immunohistochemistry for the proliferating cell nuclear antigen (PCNA), apoptotic nuclei, β-catenin, COX-2, inducible nitric oxide synthase (iNOS), and nitrotyrosine was performed on 4-μm-thick paraffin-embedded sections from colons of mice in each group by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated through graded ethanol at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed according to the method described by Watanabe *et al.* [33]. Apoptotic index was also evaluated by immunohistochemistry for single stranded DNA (ssDNA) [33]. Sections were treated for 40 min at room temperature with 2% BSA and incubated overnight at 4°C with primary antibodies. Primary antibodies included anti-PCNA mouse monoclonal antibody (diluted 1:50; PC10, DAKO Japan), anti-ssDNA rabbit polyclonal antibody (diluted 1:300, DAKO Japan), anti-β-catenin mouse monoclonal antibody (diluted 1:1000, Transduction Laboratories, Lexington, KY, USA), anti-COX-2 rabbit polyclonal antibody (diluted 1:50, IBL Co., Ltd., Gunma, Japan), anti-iNOS rabbit polyclonal antibody (diluted 1:1000, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and anti-nitrotyrosine rabbit polyclonal antibody (diluted 1:500, Upstate Biotechnology, Lake Placid, NY, USA). To reduce the non-specific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 1 h. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck Ltd., Tokyo, Japan). For each case, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted.

Intensity and localization of immunoreactivities against all primary antibodies used were examined on all sections using a microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). The PCNA and apoptotic indices were determined by counting the number of positive cells among at least 200 cells in the lesion, and were indicated as percentages. Each slide for β-catenin, COX-2, iNOS, and nitrotyrosine immunohistochemistry was evaluated for intensity of immunoreactivity on a 0 to 4+ scale. The overall intensity of the staining reaction was scored with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and < 10% of positive cells, 2+ mild immunoreactivity and 10–30% of positive cells, 3+ moderate immunoreactivity and 31–60% of positive cells, and 4+ strong immunoreactivity and 61–100% of positive cells.

Statistical analysis

All measurements were compared by Student's *t*-test, Welch's *t*-test or Fisher's exact probability test for paired samples.

Table 1: Body, liver, relative liver weights, and length of colon.

Group no.	Treatment	No. of mice	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Length of colon (cm)
1	AOM/DSS	10	36.6 ± 5.6 [a]	2.0 ± 0.5	5.5 ± 0.7	11.5 ± 2.1
2	AOM/DSS/0.04% Nimesulide	10	44.8 ± 5.6 [b]	2.3 ± 0.3	5.3 ± 0.7	12.1 ± 1.3
3	AOM/DSS/0.05% Troglitazone	10	43.6 ± 6.3 [c]	2.5 ± 0.5 [d]	5.7 ± 1.0	11.4 ± 1.1
4	AOM/DSS/0.05% Bezafibrate	10	42.4 ± 6.4 [d]	2.8 ± 0.7*	6.6 ± 1.5	12.1 ± 1.4
5	0. 04% Nimesulide	5	39.9 ± 7.3	2.0 ± 0.3	5.0 ± 0.9	12.5 ± 0.7
6	0. 05% Troglitazone	5	39.8 ± 4.0	2.1 ± 0.3	5.2 ± 0.7	11.9 ± 0.9
7	0. 05% Bezafibrate	5	46.8 ± 9.6	2.7 ± 0.5	5.8 ± 0.9	12.6 ± 1.4
8	AOM	5	42.8 ± 2.6	1.9 ± 0.1	4.5 ± 0.4	12.3 ± 1.2
9	DSS	6	34.4 ± 2.8[f]	1.8 ± 0.2	5.2 ± 0.7	12.3 ± 0.6
10	None	5	43.2 ± 6.5	2.0 ± 0.2	4.8 ± 0.8	12.0 ± 0.6

[a] Mean ± SD.

[b-*] Significantly different from group 1 by Student's *t*-test ([b] $P < 0.005$, [c] $P < 0.02$, [d] $P < 0.05$, and * $P < 0.01$).

[f] Significantly different from group 1 by Welch's *t*-test ([f] $P < 0.05$).

Results

General observation

Bloody stool was noted in a few mice received 2% DSS and their body weight gains were slightly decreased during the period of the treatment. However, thereafter no such clinical symptoms were observed. After Week 12, anal prolapsus due to the tumor development in the distal colon was found in a few mice treated with AOM and 2% DSS (group 1). The body weights and lengths of large bowel of mice in all groups at the end of the study are shown in Table 1. The mean body weights of groups 2 (AOM/DSS/0.04% nimesulide, $P < 0.005$), 3 (AOM/DSS/0.04% troglitazone, $P < 0.02$), and 4 (AOM/DSS/0.05% bezafibrate, $P < 0.05$), were significantly higher than that of group 1 (AOM/DSS). The mean body weight of group 9 (DSS alone, $P < 0.05$) was significantly lower than that of group 10 (untreated). The mean liver weights of mice in groups 3 ($P < 0.05$) and 4 ($P < 0.01$) were significantly greater than that of group 1. However, there were no pathological alterations suggesting toxicity of test compounds in the liver, kidneys, lung, and heart of mice (data not shown).

Table 2: Incidence and multiplicity of colonic neoplasia.

Group no.	Treatment	No. of mice	Incidence (no. of mice with neoplasms)			Multiplicity (no. of tumors/mice, means ± SD)		
			Total	Adenoma	Adeno-carcinoma	Total	Adenoma	Adeno-carcinoma
1	AOM/DSS	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	5.2 ± 3.0	2.1 ± 1.8	3.0 ± 1.8
2	AOM/DSS/ 0.04% Nimesulide	10	8/10 (80%)	6/10 (60%)[a]	4/10 (40%)[b]	1.8 ± 1.7[b]	1.2 ± 1.3	0.6 ± 1.0[c]
3	AOM/DSS/ 0.05% Troglitazone	10	9/10 (90%)	9/10 (90%)	4/10 (40%)[b]	2.5 ± 1.8[a]	1.6 ± 1.1	1.2 ± 2.5[a]
4	AOM/DSS/ 0.05% Bezafibrate	10	8/10 (80%)	7/10 (70%)	6/10 (60%)[a]	2.6 ± 2.5[a]	1.1 ± 1.0[a]	1.8 ± 2.6
5	0.04% Nimesulide	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0
6	0.05% Troglitazone	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0
7	0.05% Bezafibrate	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0
8	AOM	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0
9	DSS	6	0/6 (0%)	0/6 (0%)	0/5 (0%)	0	0	0
10	None	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0	0	0

[a,b,c] Significantly different from group 1 by Fisher's exact probability test or Student's *t*-test ([a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.005$).

Table 3: Incidence of multiplicity colonic mucosal ulcer and dysplasia.

Group no.	Treatment	Incidence (%)				Multiplicity (no. of lesions / mouse, means ± SD)			
		Mucosal ulcer	Total dysplasia	Dysplasia with:		Mucosal ulcer	Total dysplasia	Dysplasia with:	
				Mild atypia	Severe atypia			Mild atypia	Severe atypia
1	AOM/DSS	40%	90%	80%	50%	0.5 ± 0.7	3.2 ± 1.5	1.4 ± 1.0	1.1 ± 1.3
2	AOM/DSS/0.04% Nimesulide	10%	90%	80%	50%	0.1 ± 0.3	2.2 ± 2.3	1.2 ± 0.9	0.6 ± 0.7
3	AOM/DSS/0.05% Troglitazone	20%	90%	50%	30%	0.3 ± 0.7	2.1 ± 2.2	0.7 ± 0.8	0.8 ± 1.6
4	AOM/DSS/0.05% Bezafibrate	30%	80%	60%	20%	0.4 ± 0.7	1.9 ± 1.8	0.9 ± 1.0	0.4 ± 0.8