

Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane

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Summary. We previously reported a powerful tumor-promoting ability of dextran sodium sulfate (DSS) in a novel mouse model for colitis-related colon carcinogenesis initiated with azoxymethane (AOM). To determine the dose-dependent influence of DSS in our animal model, male ICR mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight), followed by DSS at dose levels of 2, 1, 0.5, 0.25, and 0.1% (w/v) in drinking water for 1 week. All animals were sacrificed at week 14 and histological alterations in their colon and nitrotyrosine immunohistochemistry were examined to evaluate the nitrosative stress. In the mice which received AOM and 2% DSS, the incidences (multiplicity) of colonic tubular adenoma and adenocarcinoma were 75% (1.25 ± 1.26 /mouse) and 100% (2.75 ± 2.22 /mouse), respectively. Mice given AOM and 1% DSS had 80% incidence of adenoma (1.00 ± 0.71 /mouse) and 60% incidence of adenocarcinoma (1.40 ± 2.07 /mouse) in the colon. In a mouse treated with AOM and 0.5% DSS, only one colonic adenoma (20% incidence with 0.20 ± 0.45 multiplicity) developed. Higher frequency of high-grade colonic dysplasia was noted in mice given AOM and 2% or 1% DSS when compared with mice treated with AOM and lower doses of DSS. Also, scoring of inflammation and nitrotyrosine immunoreactivity suggested that severe inflammation and nitrosation stress caused by high-doses (2% and 1%) of DSS contribute its tumor-promoting effects in mouse colon carcinogenesis initiated with a low dose of AOM. Thus, our findings indicate that a tumor-promoting effect of DSS was dose-dependent (1% or more) and the effect might occur under the condition of inflammation and nitrosation stress.

Key words: Dose-dependency, Promotion, DSS, AOM, Mouse colon carcinogenesis

Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (Eaden et al., 2001; van Hogezaand et al., 2002) are relatively common in North America, Europe, and Australia. It is well-known that UC patients have a high risk of colorectal cancer (CRC) (Devroede et al., 1971; Kewenter et al., 1978; Greenstein et al., 1979): patients with UC have a 2.0-8.2 relative risk of CRC compared with the normal population, accounting for about 2% of CRC (Hardy et al., 2000).

We recently developed a novel mouse model for colitis-related colon carcinogenesis (Tanaka et al., 2003). In this model, male ICR mice were initiated with a single dose (10 mg/kg body weight) of azoxymethane (AOM) by intraperitoneal (i.p.) injection, and then followed by one-week exposure to 2% dextran sodium sulfate (DSS) in drinking water, starting one week after the injection of AOM. 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 into the experimental schedule. These findings suggest a powerful tumor-promoting effect of DSS in our model.

The effects of various tumor-promoters on carcinogenesis are known to be dose-dependent (Pereira et al., 1986) and a lower dose appeared to exhibit a threshold (Maekawa et al., 1992). In colon carcinogenesis, tumor-promoting effect of dietary fat depends on the amount of dietary fat (Reddy and Maera, 1984). A non-genotoxic carcinogen DSS is widely used for induction of colitis (Okayasu et al., 1990; Cooper et al., 1993), since administration of DSS through diet or drinking water to rodents could induce colonic inflammation which resembled the symptomatic and histopathological findings in humans UC (Okayasu et al., 1990; Cooper et al., 1993). Also, colonic malignancies develop in chronic inflammation induced by long-term administration of DSS, which is similar to

human cases where colorectal adenocarcinoma occurs via the dysplasia-carcinoma sequence (Yamada et al., 1992; Tamaru et al., 1993; Cooper et al., 2000). Severity of mucosal injury caused by DSS relates to the administration dose and duration of DSS (Kitajima et al., 1999; Egger et al., 2000; Shimizu et al., 2003). These findings suggest that the tumor-promoting effect of DSS in our model (Tanaka et al., 2003) is dose-dependent and may be related to mucosal damage by DSS (Tanaka et al., 2001).

In the current study, we investigated the influence of various doses of DSS on our AOM/DSS-induced mouse colon carcinogenesis model to determine the lowest dose of DSS, which can exert its tumor-promoting ability, for utilizing the model for detecting the modifying effects of xenobiotics on colon carcinogenesis. Also, the immunohistochemistry of nitrotyrosine, which is a marker of the formation of peroxynitrite and its interaction with protein tyrosines (Singer et al., 1996), in the colon was performed to determine the possible involvement of inflammation damage by inducible nitric oxide synthase (iNOS), which is induced in inflamed colonic mucosa and is associated with the production of peroxynitrite and nitration of cellular protein in the colon of both human IBD (Singer et al., 1996; Kimura et al., 1998) and chemically-induced colitis of rodents (Zingarelli et al., 1999), in our model.

Materials and methods

Animals, chemicals and diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo), 5 weeks old, were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. Mice were housed in plastic cages (4 or 5 mice/cage) under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle), and temperature (23±2 °C). Drinking water and a pelleted basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) were available *ad libitum*. They were quarantined for the first 7 days after arriving, and then randomized by body weight into experimental and control groups. 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 prepared every day by dissolving in distilled water at a concentration of 2, 1, 0.5, 0.25, and 0.1% (w/v).

Experimental procedure

Male ICR mice were divided into 8 groups, as shown in Fig. 1. Mice of groups 1 through 5 were given a single i.p. injection of AOM at a dose of 10 mg/kg body weight. Starting 1 week after the injection, animals of groups 1 through 5 received 2, 1, 0.5, 0.25, and 0.1% DSS in the drinking water for 7 days, respectively, and

then were given no further treatment for 12 weeks. Group 6 was given a single i.p. injection of AOM (10 mg/kg body weight) alone. Mice of group 7 received 2% DSS the same as groups 1 through 5. Group 8 was an untreated control. All animals were sacrificed at the end of the study (week 14) by ether overdose. Their large bowel was flushed with saline and excised. Their length (from the ileocecal junction to the anal verge) was measured, cut open longitudinally along the main axis, and then washed with saline. The entire colon was macroscopically inspected, cut, fixed in 10% buffered formalin for at least 24 h, and embedded paraffin for histopathological and immunohistochemical examinations.

Histopathological analysis

Histopathology (mucosal inflammation with or without ulceration, dysplasia, and neoplasms) in the entire colon was analyzed on hematoxylin and eosin-stained sections. Colitis was graded according to the following morphological criteria (Cooper et al., 1993): showing normal appearance (grade 0); shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa (grade 1); loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa (grade 2); loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium (grade 3); and presence of mucosal ulcer with severe inflammation (neutrophils, lymphocytes, macrophages, and plasma cells infiltration) in the mucosa, submucosa, muscularis propria, and/or subserosa (grade 4). The scoring was made on the entire colon with or without proliferative lesions and expressed as mean average score / mouse. Colonic mucosa dysplasia (low- and high-grade) and colonic neoplasms were diagnosed according to the earlier reports (Ward, 1974; Riddell et al., 1983; Pascal,

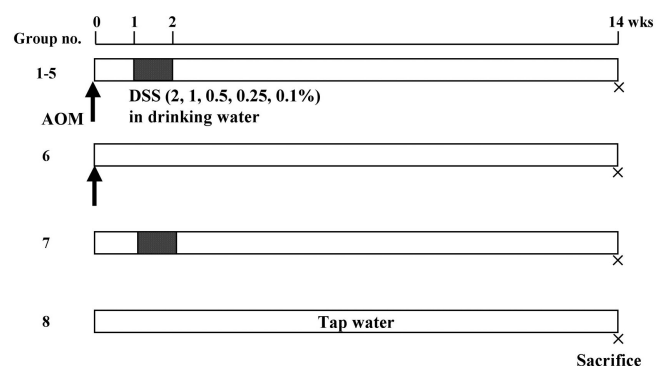


Fig. 1. Experimental protocol. Treatments are as follows: Group 1, AOM (10 mg/kg body weight)→2%DSS; Group 2, AOM→1%DSS; Group 3, AOM→0.5%DSS; Group 4, AOM→0.25%DSS; Group 5, AOM→0.1%DSS; Group 6, AOM alone; Group 7, 2%DSS alone; and Group 8, untreated.

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1994). To determine the multiplicity of the colonic mucosal ulcer and dysplasia, the colon was cut into three equal parts from the anus, and then each part was cut in half longitudinally. Each tissue fixed in 10% buffered formalin was totally submitted as multiple transverse sections for histological processing. This averaged two pieces/tissue and 12 pieces / total colon. The colon lesions were counted on all slides stained with hematoxylin and eosin, the sum was divided by the number of slides, and expressed as mean \pm SD.

Nitrotyrosine immunohistochemistry

Immunohistochemistry was used to evaluate tyrosine nitration, a marker of nitrosative damage in the colon. Paraffin-embedded sections (4 μ m) of the colon were deparaffinized, treated with 0.3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides with a blocking solution (0.1M PBS containing 0.1% triton X-100 and 2% normal goat serum) for 2 hours. Sections were incubated overnight with a primary rabbit polyclonal anti-nitrotyrosine (diluted 1:500, Upstate Biotechnology, Lake Placid, New York, USA) or with control solution. Control sections included buffer alone or non-specific purified rabbit secondary antibody and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Color was developed using 3-3'-diaminobenzidine-4HCl as the chromogen. To quantitate the degree of nitrotyrosine stainability, the grading system (grade 0-4) was used according to the following criteria (Zingarelli et al., 1999): grade 0, no immunoreactivity; grades 1-3, increasing degrees of intermediate immunoreactivity; and grade 4, extensive immunoreactivity. The nitrotyrosine immunohistochemistry was scored on the serial immunostained sections that were made for counting colonic mucosal ulcer and dysplasia.

Statistical analysis

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

Results

General observation

The intake of DSS or tap water did not significantly differ among the groups (data not shown). A few mice receiving AOM and 2% or 1% DSS in the drinking water had bloody stools after the DSS administration. However, no such symptoms were observed in the groups which received AOM and other doses of DSS. Mean body, liver, and relative liver weights (g/100 g body weight) are listed in Table 1. The mean body weight of group 1 (AOM \rightarrow 2% DSS), group 2 (AOM \rightarrow 1% DSS), and group 3 (AOM \rightarrow 0.5% DSS) were significantly lower than that of groups 6 (AOM alone, $P<0.001$ vs. group 1, and $P<0.05$ vs. groups 2 and 3) and 8 (no treatment, $P<0.05$ vs. groups 1 and 2, and $P<0.01$ vs. groups 3). Also, the mean body weight of group 1 was lower ($P<0.05$) than that of group 7 (2% DSS alone). Although the mean liver weight of group 1 was significantly smaller than that of group 6 ($P<0.005$) and group 8 ($P<0.05$), relative liver weights had no statistical differences among the groups. Mean lengths of large bowel of all groups at the end of the study are also given in Table 1. Significant differences were observed between group 1 and group 6 ($P<0.05$) or group 8 ($P<0.01$).

Effects of various doses of DSS on the development of large bowel neoplasms

Macroscopically, flat, nodular, polypoid or caterpillar-like tumors were present in the middle and

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

GROUP no.	TREATMENT (No of mice examined)	BODY WEIGHT (g)	LIVER WEIGHT (g)	RELATIVE LIVER WEIGHT (g/100g body weight)	LENGTH OF LARGE BOWEL (cm)
1	AOM \rightarrow 2%DSS (4)	37.4 \pm 5.1 ^{a,b,c,d}	2.27 \pm 0.43 ^{d,e}	6.02 \pm 0.40	11.8 \pm 1.1 ^{f,g}
2	AOM \rightarrow 1%DSS (5)	42.9 \pm 2.7 ^{d,f}	2.95 \pm 0.16	6.91 \pm 0.68	13.7 \pm 1.1
3	AOM \rightarrow 0.5%DSS (5)	42.5 \pm 2.5 ^{f,g}	2.90 \pm 0.23	6.83 \pm 0.70	13.7 \pm 0.7
4	AOM \rightarrow 0.25%DSS (5)	45.4 \pm 3.6	3.02 \pm 0.25	6.67 \pm 0.64	13.2 \pm 1.0
5	AOM \rightarrow 0.1%DSS (4)	48.9 \pm 7.0	3.14 \pm 0.79	6.37 \pm 0.93	13.2 \pm 1.3
6	AOM (5)	49.6 \pm 4.6	3.14 \pm 0.17	6.38 \pm 0.55	14.1 \pm 1.3
7	2%DSS (5)	45.1 \pm 3.4	2.99 \pm 0.63	6.60 \pm 1.07	13.2 \pm 0.8
8	None (5)	47.3 \pm 1.5	2.96 \pm 0.15	6.27 \pm 0.36	13.8 \pm 0.5

^a: mean \pm SD; ^b: significantly different from group 6 by Student's *t*-test ($P<0.001$); ^c: significantly different from group 7 by Student's *t*-test ($P<0.05$); ^d: significantly different from group 8 by Welch's *t*-test ($P<0.05$); ^e: significantly different from group 6 by Welch's *t*-test ($P<0.005$); ^f: significantly different from group 6 by Student's *t*-test ($P<0.05$); ^g: significantly different from group 8 by Student's *t*-test ($P<0.01$).

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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).

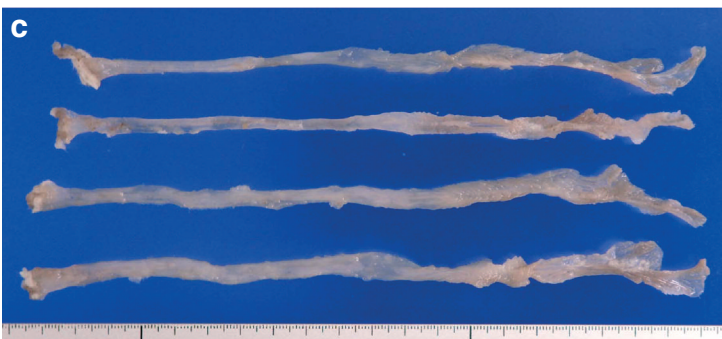


Fig. 2. Macroscopic view of the large bowel. **a.** A number of colonic tumors are seen in mice given AOM→2% DSS. **b.** A number of colonic tumors are seen in mice treated with AOM→1% DSS. **c.** One colonic tumor is present in a mouse received AOM→0.05% DSS. Arrow heads indicate colonic tumors.

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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

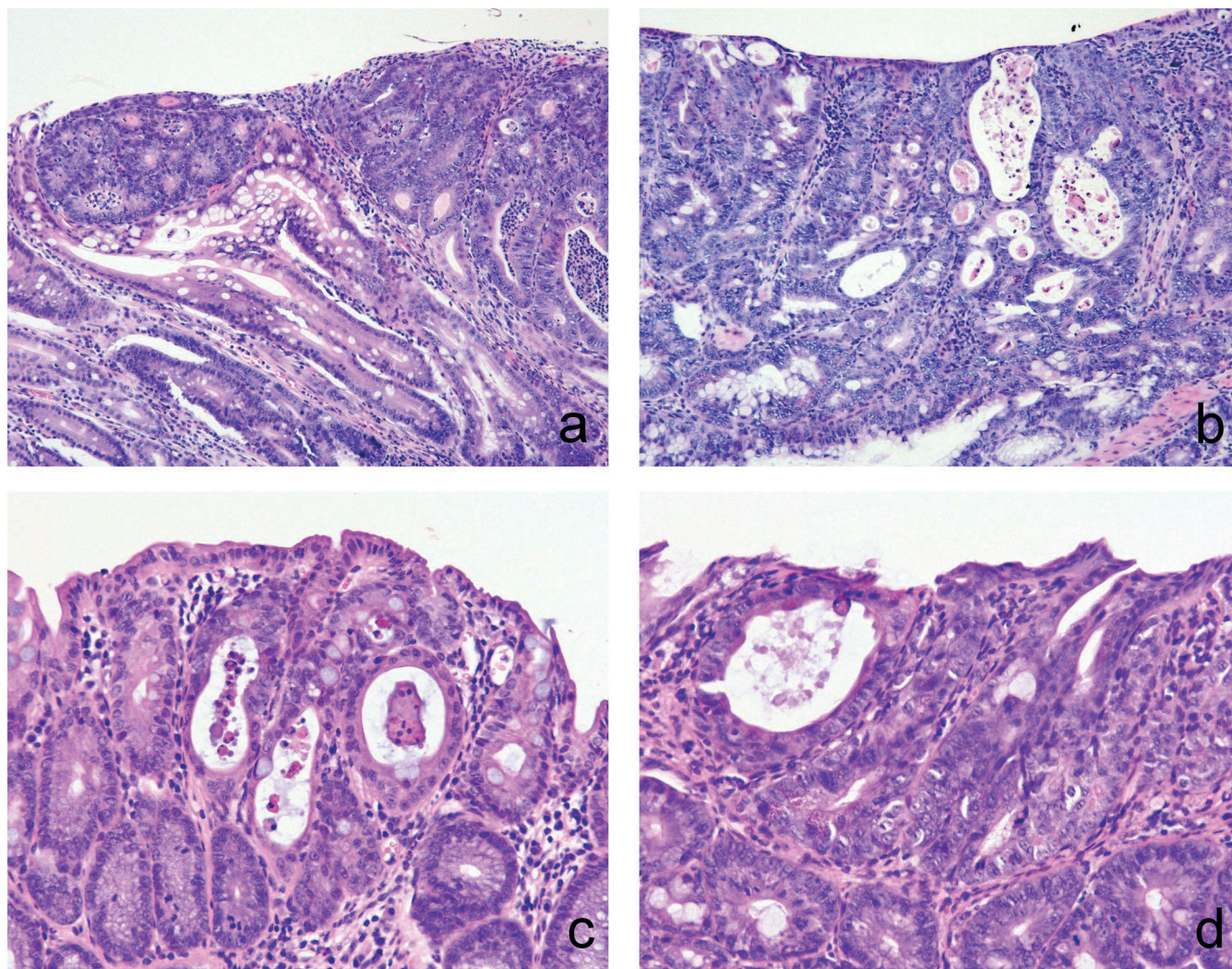


Fig. 3. Histopathology of colonic lesions in mice treated with AOM→2% DSS. **a.** Two tubular adenomas are seen. **b.** A tumor is histologically well differentiated tubular adenocarcinoma. **c.** Low-grade dysplasia with slight nuclear atypia. **d.** High-grade dysplasia with marked nuclear atypia.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

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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,

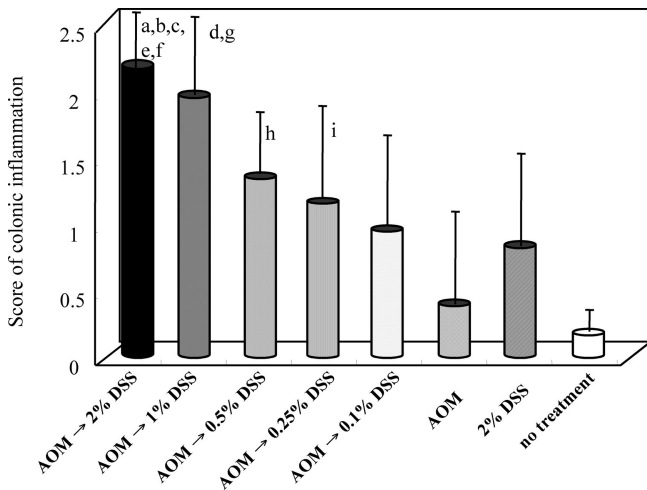


Fig. 4. Inflammation score. Statistical analysis using Student's t-test or Welch's t-test indicates significant difference: a ($P<0.05$), vs. the AOM→0.5% DSS group; b ($P<0.05$), vs. the AOM→0.1% DSS group; c ($P<0.01$) and d ($P<0.05$), vs. the AOM alone group; e ($P<0.05$), vs. the 2% DSS alone group;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

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 ($P<0.05$).

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 no.	TREATMENT (no of mice examined)	INCIDENCE OF MUCOSAL ULCER (multiplicity)	INCIDENCE (MULTIPLICITY) OF COLONIC MUCOSA		
			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,i})	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 ($P<0.001$).

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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, $P<0.05$).

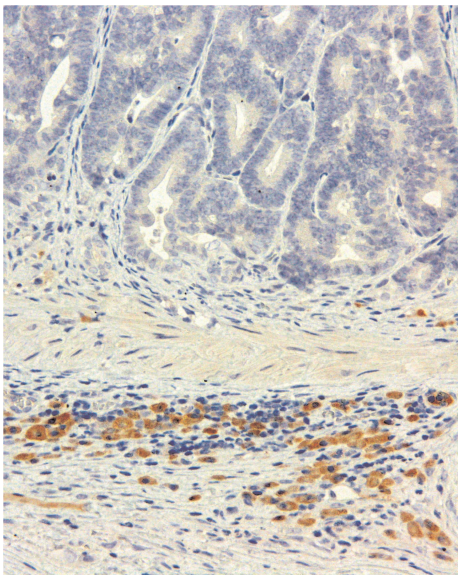


Fig. 5. Nitrotyrosine immunohistochemistry of the colon of a mouse from group 1 (AOM→2% DSS). The positive reaction is noted in inflammatory cells under the dysplastic lesion. Original magnification, x 20

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 β -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 given 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

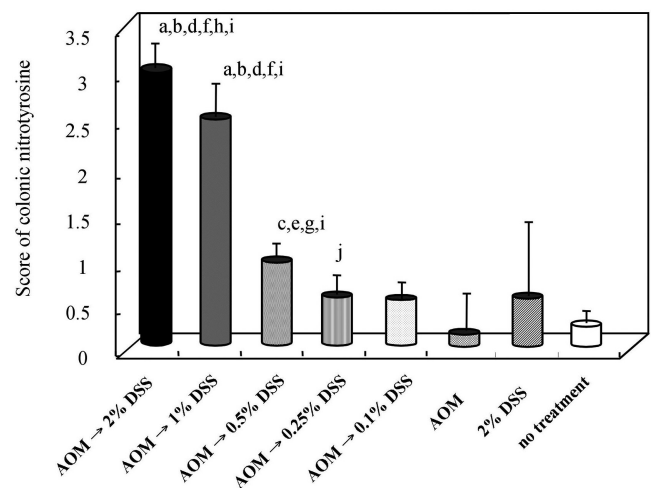


Fig. 6. Score for nitrotyrosine immunohistochemistry. Statistical analysis using Student's t-test or Welch's t-test indicates significant difference: a ($P<0.001$), vs. the AOM→0.5% DSS group; b ($P<0.001$) and c ($P<0.05$), vs. the AOM→0.25% DSS group; d ($P<0.001$) and e ($P<0.01$), vs. the AOM→0.1% 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 H₂O) 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