Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate

Rikako Suzuki,[1, 2] Hiroyuki Kohno,[2] Shigeyuki Sugie[2] and Takuji Tanaka[2, 3]

[1]Research Fellow of the Japan Society for the Promotion of Science, 6 Ichiban-cho, Chiyoda-ku, Tokyo 102-8471; and [2]Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293

(Received June 24, 2004/Revised July 26, 2004/Accepted August 2, 2004)

Previously, we proposed a novel mouse model for colitis-related colon carcinogenesis using azoxymethane (AOM) and dextran sodium sulfate (DSS) (*Cancer Sci* 2003; 94: 965–73). In the current study, sequential analysis of pathological alterations during carcinogenesis in our model was conducted to establish the influence of inflammation caused by DSS on colon carcinogenesis in this model. Male ICR mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight) and given 2% (w/v) DSS in the drinking water for 7 days, starting 1 week after the AOM injection. They were sequentially sacrificed at weeks 2, 3, 4, 5, 6, 9, 12, and 14 for histopathological and immunohistochemical examinations. Colonic adenomas were found in 2 (40% incidence and 0.40±0.49 multiplicity) of 5 mice at week 3 and colon carcinomas developed in 2 (40% incidence and 2.00±3.52 multiplicity) of 5 mice at week 4. Their incidence gradually increased with time and reached 100% (6.20±2.48 multiplicity) at week 6. At week 14, the multiplicity of adenocarcinoma was 9.75±2.49 (100% incidence). In addition, colonic dysplasia was noted at all time-points. The scores of colonic inflammation and nitrotyrosine immunohistochemistry were extremely high at early time-points and were well correlated. Our results suggest that combined treatment of mice with AOM and DSS generates neoplasms in the colonic mucosa via dysplastic lesions induced by nitrosative stress. (Cancer Sci 2004; 95: 721–727)

In the developed countries, colorectal cancer (CRC) is one of the commonest non-smoking related causes of cancer deaths. Remarkable differences in the incidence worldwide have led to the hypothesis that this variation could be explained largely by environmental influences.[1)]

The occurrence of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is affected by several factors, including race and geography.[2, 3)] Life style, particularly intake of high amount of animal fat, is involved in the occurrence of IBD.[4)] Over recent decades the incidence of IBD has been rising throughout the world, including Japan, as it has been in the last 50 years in Europe and North America.[5)] This increase may be caused by increased intake of animal fat.[6)] CRC is one of the most serious complications of IBD, especially UC and CD. The risk of CRC becomes greater with increasing extent and duration of the disease. A recent meta-analysis has estimated the incidence rate at 7 per 1000 person-years duration and 12 per 1000 persons per year duration in the second and third decades of UC, respectively.[7)] The risk for CRC development in CD patients is also high.[8)] The highest risk of CRC in CD patients was reported to be 26.6 (standardized incidence ratio), although the estimate applied only to patients younger than 21 years of age.[9)] Gillen *et al*.[10)] compared the CRC risk in patients with UC and CD and found an 18-fold increase in the risk of developing CRC in extensive CD and a 19-fold increase in risk in extensive UC when compared with the general population.

The pathogenesis of IBD-related CRC is still unclear, although there have been various attempts to investigate the pathogenesis using animal models. In earlier animal models for investigating the pathogenesis of UC, carageenan was used,[11)] but dextran sodium sulfate (DSS) has been the most widely used chemical to induce colitis.[12)] Also, this DSS animal model is available for examining IBD-related CRC. Intestinal tumors in rats fed 5% DSS in the diet developed between 134 and 215 days.[13)] Colorectal carcinomas developed in rats fed DSS for as long as 6 months.[14)] Recently, Cooper *et al*.[15)] reported a relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis which is similar to that of human UC-associated dysplasia and cancer regarding histopathology. However, these studies basically need a long experimental period or repeated administration of DSS.[14–16)] In addition, our previous study demonstrated that treatment with the non-genotoxic carcinogen DSS enhances the development of putative precursor lesions (aberrant crypt foci) for colonic adenocarcinoma in rats.[17)]

In the first report of our experimental studies of inflammation-related carcinogenesis in mouse colon, we proposed a novel mouse model, using azoxymethane (AOM) and DSS.[18)] In our model, exposure to a single dose of AOM followed by 1-week treatment with 2% DSS could induce a number of colonic epithelial malignancies within 20 weeks. Moreover, the first colonic adenocarcinoma was found as early as 12 weeks.[18)] However, time-course analysis of pathological alterations was not performed. Therefore, in the present study, we investigated the time-course of alterations of colonic morphology in male ICR mice treated with AOM followed by DSS, in order to understand the effects of DSS-induced inflammation on colon carcinogenesis in this model. Since inducible nitric oxide synthase (iNOS) is expressed in inflamed colonic mucosa and is associated with the production of peroxynitrite and nitration of cellular protein in the colon in human IBD[19, 20)] and chemically induced colitis of rodents,[21)] we also immunohistochemically assessed the expression of nitrotyrosine, which is a specific marker of nitrosative stress,[22)] in the colon.

Materials and Methods

Animals, chemicals, and diets. In this study, 5-week-old male

[3]To whom correspondence should be addressed.
E-mail: takutt@kanazawa-med.ac.jp

Abbreviations: AOM, azoxymethane; DSS, dextran sodium sulfate; CRC, colorectal cancer; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; NO, nitric oxide; COX, cyclooxygenase.

Crj:CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo) were used. They were acclimated for 1 week with tap water and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo) *ad libitum*, before the start of the experimentation. Mice were then randomized by body weight into 8 groups. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were housed under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle), and temperature (23±2°C). A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH).

Experimental procedure. All mice received a single intraperitoneal (i.p.) injection of AOM at a dose level of 10 mg/kg body weight. Starting 1 week after the AOM injection, animals were exposed to 2% DSS in the drinking water for 7 days. They were then sacrificed by ether overdose at weeks 2, 3, 4, 5, 6, 9, 12, and 14 (Fig. 1). At autopsy, their large bowel was flushed with saline, and excised. The large bowel (from the ileocecal junction to the anal verge) was measured, cut open longitudinally along the main axis, and then washed with saline. After macroscopic inspection, it was cut, and fixed in 10% buffered formalin for at least 24 h. Paraffin-embedded sections were made by routine procedures.

Histopathological analysis. The histopathological alterations in the colon were examined on hematoxylin and eosin-stained sections. Colitis with or without ulceration was scored on hematoxylin and eosin-stained sections, according to the following morphological criteria described by Cooper *et al.*[23]: grade 0, normal colonic mucosa (Fig. 2a); grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa (Fig. 2b): grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa (Fig. 2c); grade 3, loss of the entire crypts with severe inflammation in the mucosa, but with retention of the surface epithelium (Fig. 2d); and grade 4, loss of the entire crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria, and submucosa (Fig. 2e). High- or low-grade dysplasia of colonic mucosa (Fig. 3) was diagnosed according to the criteria described by Riddell *et al.*[24] and Pascal.[25] Colonic neoplasms were diagnosed according to the description by Ward.[26]

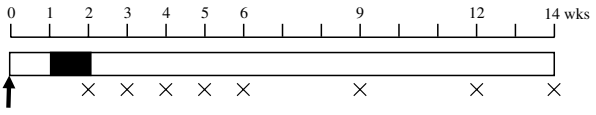

Fig. 1. Experimental protocol. □, basal diet and tap water; ■, 2% DSS; ↑, AOM, 10 mg/kg b.w., i.p.; ×, sacrifice.

Immunohistochemistry of nitrotyrosine. Immunohistochemistry of nitrotyrosine was used to evaluate tyrosine nitration, a marker of nitrosative damage in the colon. Paraffin embedded sections (4 μm) of the distal colon (1 cm from the anus) were deparaffinized, treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity, and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides with a blocking solution (0.1 *M* PBS containing 0.1% Triton X-100 and 2% normal goat serum) for 2 h. Sections were incubated overnight with a primary rabbit polyclonal anti-nitrotyrosine (diluted 1:500, Upstate Biotechnology, Lake Placid, NY) 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). Reaction products were developed by immersing the sections in 3,3′-di-

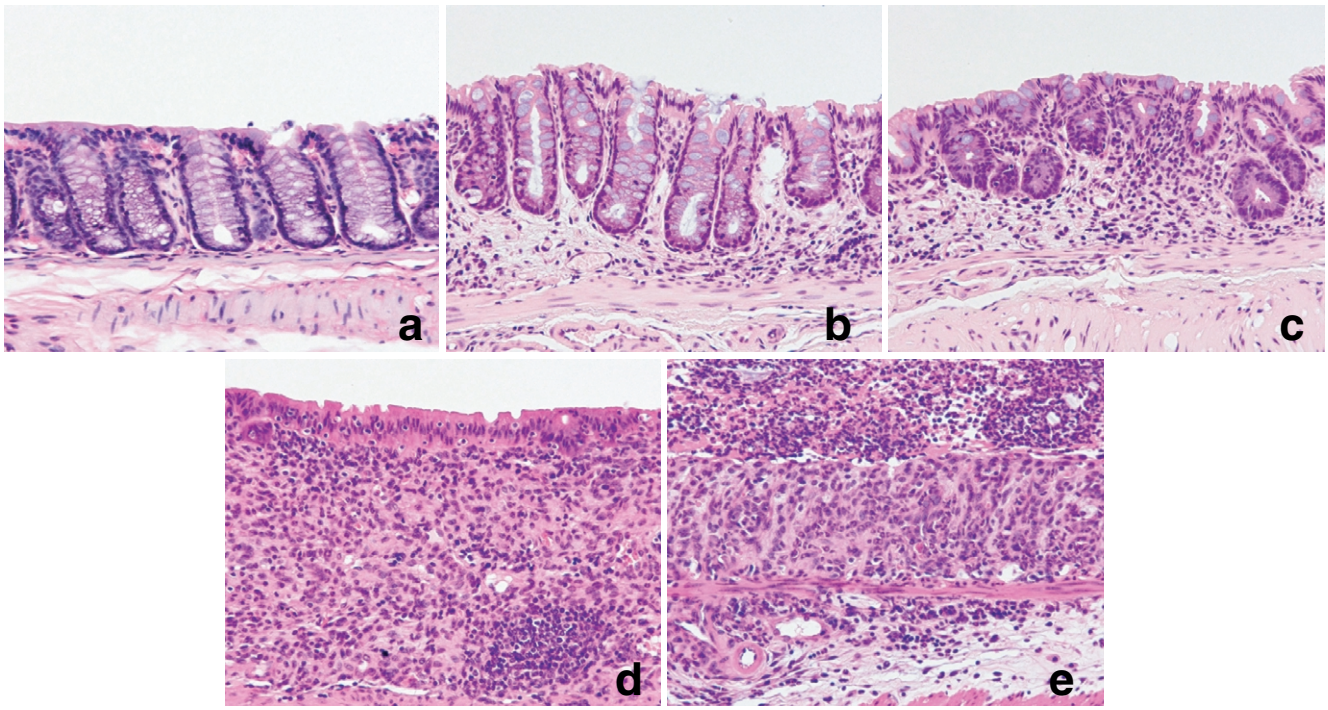

Fig. 2. Histology and various grades of colitis: (a) normal colonic mucosa (grade 0); (b) shortening of the basal one-third of the crypts with slight inflammation and edema in the lamina propria (grade 1); (c) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (grade 2); (d) loss of the entire crypts with severe inflammation in the lamina propria, but with retention of the surface epithelium (grade 3); and (e) loss of the entire crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria, and submucosa. The exudates containing cell debris, inflammatory cells, fibrin, and mucus covers the damaged mucosa (grade 4). Hematoxylin and eosin stain. Original magnifications, ×20.

aminobenzidine 4HCl solution (Sigma Chemical Co.) containing 0.1% hydrogen peroxide. To quantitate the degree of nitrotyrosine stainability, a grading system (grades 0–4) was used: grade 0, no immunoreactivity; grades 1–3, increasing degrees of intermediate immunoreactivity; and grade 4, extensive immunoreactivity.[21]

Statistical analysis. All measurements were compared by the use of Student's *t* test or Welch's *t* test for paired samples.

Results

General observations. A few mice had bloody stools at week 2, but no such clinical symptoms were observed thereafter. At week 8, anal prolapse due to tumor development in the distal colon was noted in a few mice. Mean body and liver weights and mean lengths of large bowel at different time-points are shown in Table 1. No remarkable changes of mean body and liver weights were observed after week 4 or 5. No significant changes in the lengths of large bowel were noted after week 4. At week 14, the mean body weight, liver weight, and length of colon of untreated mice was 41.0±3.4 g, 2.78±0.26 g, and 13.1±0.9 cm, respectively (data was not shown in Table 1). The mean liver weight (2.27±0.37) of mice given 2% DSS at week 14 was significantly lower than that of untreated mice ($P<0.05$).

Colitis and colonic dysplasia. Colitis in the current study was present with or without colonic dysplasia in the middle or distal colon (Fig. 4a). As shown in Fig. 5a, the inflammation score was the highest (3.80±0.45) at week 2, and then gradually decreased during the study. The scores at weeks 2, 3 (3.40±0.55), 4 (3.20±0.45), 5 (3.20±0.84), and 6 (2.80±0.84) were much greater than that at week 14 (1.50±0.58). Mucosal ulceration was found at week 2, soon after the cessation of DSS exposure (Ul-I, 100% incidence with a multiplicity of 8.20±1.94; and Ul-II, 80% incidence with a multiplicity of 1.80±2.14). After this time-point, the incidence of mucosal ulceration was decreased with time, accompanied with regeneration, and there was a 25% incidence of Ul-I (0.25±0.43 multiplicity) at week 14. Ul-II ulcer was not found in the colon after week 6. Colonic dysplasia was also present at week 2 (Table 2). During the ex-

Table 1. Body and liver weights and lengths of colon

Wks	No. of mice examined	Body wt (g)	Liver wt (g)	Length of large bowel (cm)
2	5	23.8±2.2	1.41±0.13	6.7±0.4
3	5	28.6±2.2	2.10±0.80	8.7±1.2
4	5	36.4±3.0	2.50±0.40	10.7±1.1
5	5	39.8±2.2	3.10±0.70	11.2±0.6
6	5	39.6±1.9	2.78±0.40	11.2±0.6
9	5	40.3±1.9	2.65±0.30	11.6±0.6
12	5	40.1±1.6	2.98±0.10	11.1±0.4
14	4	39.7±1.0	2.27±0.37	11.8±1.0

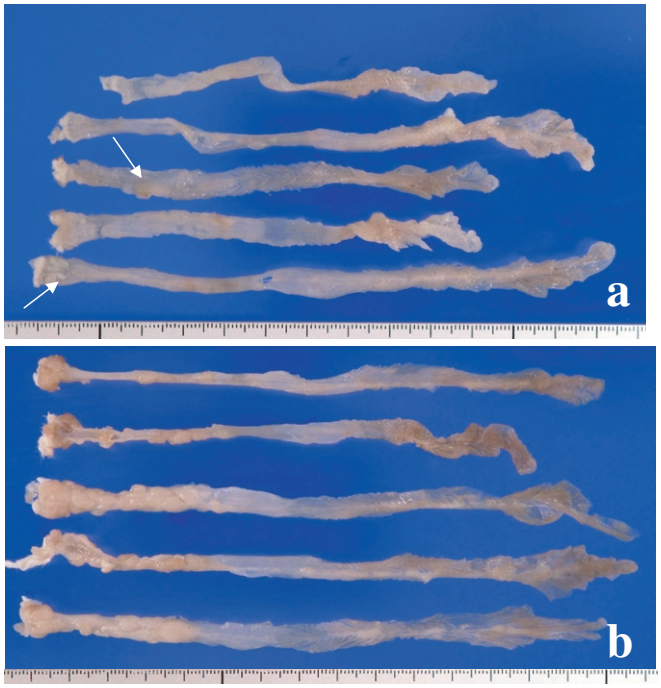

Fig. 4. Macroscopic view of the colon of mice treated with 2% DSS. (a) A small nodular tumor (arrow) is seen in the distal colon of 2 mice at week 3. (b) Nodular, polypoid, or caterpillar-like tumors are found in the distal and/or middle colon of all mice at week 12.

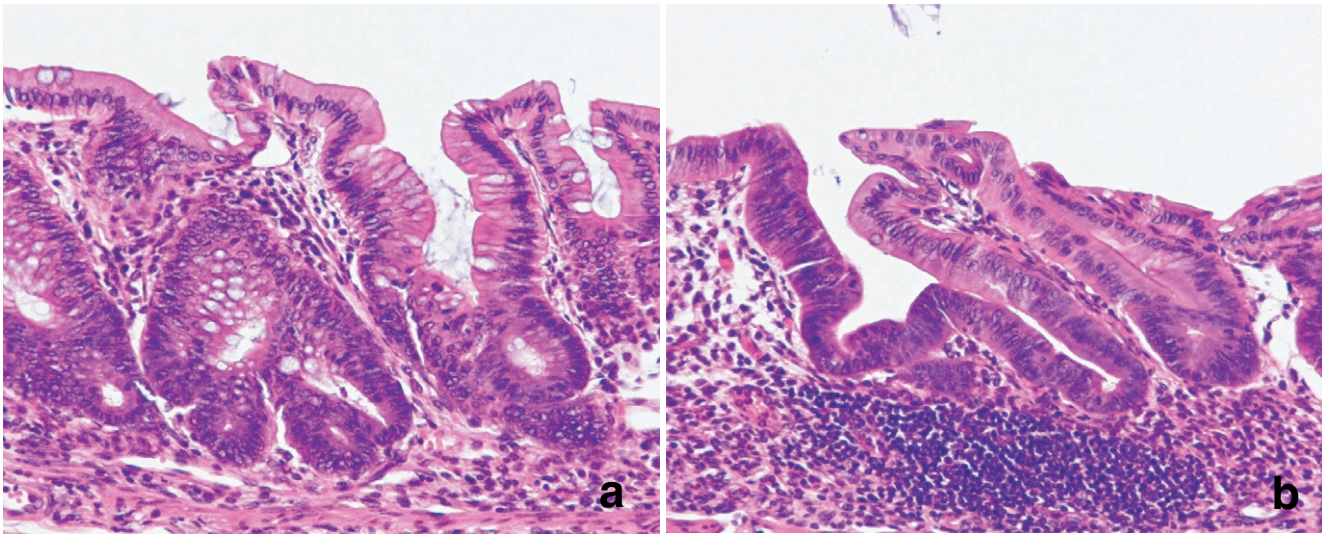

Fig. 3. Histopathology of colonic dysplasia. (a) Low-grade dysplasia, mild dysplastic changes with decreased goblet cells extend to the surface epithelium; and (b) high-grade dysplasia, a greater degree of cytologic atypia is evident. The nuclei are enlarged and have irregular contours. There is also a loss of normal polarity. Hematoxylin and eosin stain. Original magnifications, ×20.

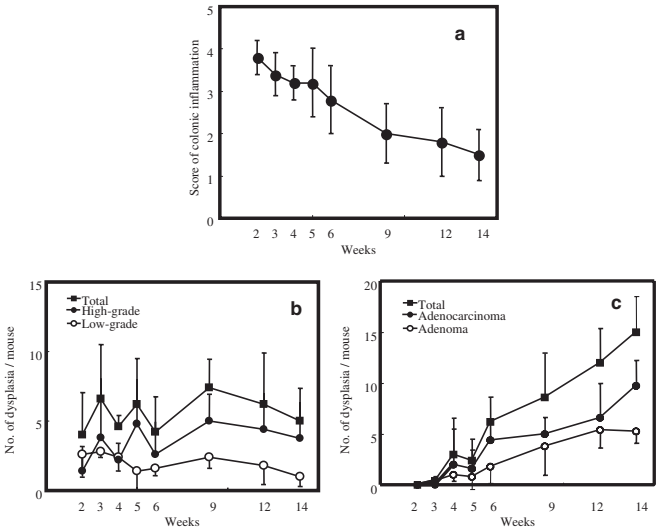

Fig. 5. (a) Colonic inflammation score at each time-point, (b) multiplicity of colonic dysplasia at each time-point, (c) multiplicity of colonic neoplasms at each time-point.

periment, the incidence of high-grade dysplasia gradually increased with time: 40% at week 2 and 100% at weeks 4–14. The multiplicity of high-grade dysplasia was 1.40±1.74/mouse at week 2 and thereafter the value slightly increased (Fig. 5b). However, the multiplicity of low-grade dysplasia decreased with time.

Incidence and multiplicity of large bowel neoplasms. Macroscopically, flat, nodular, polypoid, or caterpillar-like colonic tumors (Fig. 4b) were found in the middle and distal colon of mice. Table 3 summarizes the incidence of large bowel neoplasms at each time-point. Fig. 5c illustrates the multiplicity of colonic neoplasms at each time-point. At week 2, no colonic neoplasms developed. At week 3, colonic tubular adenoma (Fig. 6a) developed in 2 of 5 mice (40% incidence with a multiplicity of 0.40±0.49). As for colonic adenocarcinoma (Fig. 6b), the incidence and multiplicity were 40% and 2.00±3.52 at week 4, and 60% and 1.60±1.85 at week 5. Thereafter, the incidence of colonic adenocarcinoma was 100% and the multiplicity was increased. As shown in Table 4, all adenocarcinomas were located in colonic mucosa, at weeks 4 and 5, but the number of adenocarcinomas infiltrating into submucosa and muscularis propria was increased after week 6.

Table 2. Incidence of dysplasia in the colon

Wks	No. of mice examined	Incidence of dysplasia (%)		
		Total	Low-grade	High-grade
2	5	80	80	40
3	5	100	100	80
4	5	100	100	100
5	5	100	80	100
6	5	100	100	100
9	5	100	100	100
12	5	100	80	100
14	4	100	75	100

Table 3. Incidence of colonic neoplasms

Wks	No. of mice examined	Incidence of colonic neoplasms (%)		
		Total	Adenoma	Adenocarcinoma
2	5	0	0	0
3	5	40	40	0
4	5	80	80	40
5	5	80	40	60
6	5	100	80	100
9	5	100	100	100
12	5	100	100	100
14	4	100	100	100

Nitrotyrosine immunohistochemistry. Immunoreactivity of nitrotyrosine was noted in the cryptal cells with or without disruption, infiltrated mononuclear inflammatory cells, and endothelial cells of the small vessels in the mucosa and submucosa. Stainability was strong in the infiltrated mononuclear inflammatory cells (Fig. 7, a and b). Adenocarcinoma cells also showed weakly or moderately positive immunoreactivity of nitrotyrosine (Fig. 7b). As shown in Fig. 8, scoring of the immunoreactivity revealed that the highest score of nitrotyrosine was at week 2 (7.32±1.35) and the scores at weeks 2, 3 (5.22±0.40), and 4 (5.40±1.32) were much higher than that at week 14 (2.75±0.25).

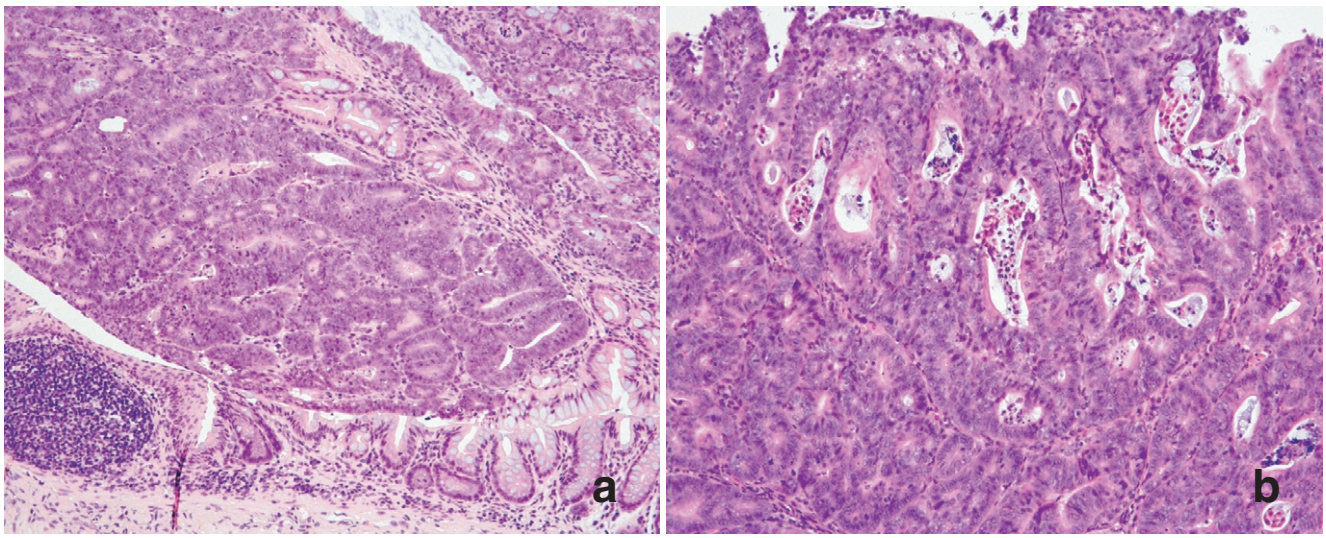

Fig. 6. Histopathology of (a) tubular adenoma and (b) adenocarcinoma. Hematoxylin and eosin stain. Original magnifications, (a) ×10 and (b) ×20.

Table 4. Invasiveness of colonic adenocarcinomas

Wks	Total no. of colonic adenocarcinoma (no. of mice with adenocarcinoma)	Depth of invasion			
		Mucosa	Submucosa	Muscularis propria	Serosa
2	0 (0/5)	0	0	0	0
3	0 (0/5)	0	0	0	0
4	10 (2/5)	10 (100%)	0	0	0
5	8 (3/5)	8 (100%)	0	0	0
6	22 (5/5)	21 (95%)	1 (5%)	0	0
9	25 (5/5)	23 (92%)	2 (8%)	0	0
12	33 (5/5)	27 (82%)	5 (15%)	1 (3%)	0
14	39 (4/4)	32 (82%)	6 (15%)	1 (3%)	0

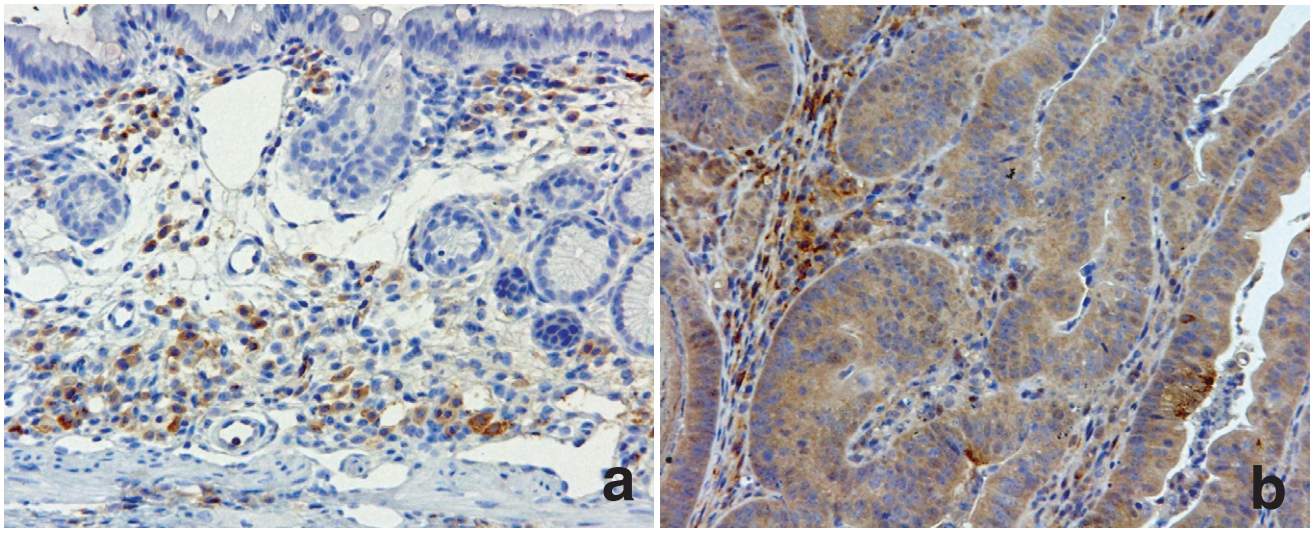

Fig. 7. Immunohistochemistry of nitrotyrosine. (a) Strongly positive immunoreactivity of nitrotyrosine in inflammatory cells (mostly macrophages) infiltrated in the lamina propria and (b) moderate immunoreactivity of nitrotyrosine in adenocarcinoma cells with surrounding macrophages showing strong immunoreactivity of nitrotyrosine. Original magnifications, ×20.

Discussion

In the current study, treatment with a single dose of genotoxic colonic carcinogen AOM (10 mg/kg body weight, i.p. injection) followed by 1-week oral exposure to a non-genotoxic carcinogen DSS (2% in drinking water) could induce colonic adenoma within 3 weeks after the start of the experiment. Colonic adenocarcinoma also developed as early as 4 weeks. These findings support our earlier work[18] demonstrating a powerful tumor-promoting activity of DSS, which has colitis-inducing ability in the mouse colon.

In the current study, DSS induced colitis in the middle and distal parts of the colon, as reported by others.[23, 27] Besides these inflammatory changes, low- or high-grade dysplasia was present at all time-points and colonic neoplasms were observed even at week 3 or 4 in the current study. Severe colitis with ulceration soon after the end of 2% DSS exposure in this study might be caused by a direct cytotoxic effect of DSS.[28] Kitajima *et al*. demonstrated the presence of macrophages phagocyting DSS in the middle and distal colon of mice the day after the oral administration of 5% DSS,[29] suggesting that DSS could be absorbed in the colonic mucosa and act as a tumor-promoter in the colon initiated with a low dose of AOM.

Inflammatory damage in UC is associated with increased production of nitric oxide (NO) through the iNOS pathway.[30] In accordance with this report, a good biomarker for "nitrating species," nitrotyrosine,[31] showed a high immunohistochemical reaction score for 2 weeks (from week 2 to week 4), beginning soon after the cessation of DSS exposure, in this study. Also, changes of inflammation score with or without ulceration paralleled the nitrotyrosine-immunohistochemical score during the study. High scores of these parameters were noted at the earlier time-points, and then both scores decreased with time: the scores for nitrotyrosine positivity and inflammation reached a

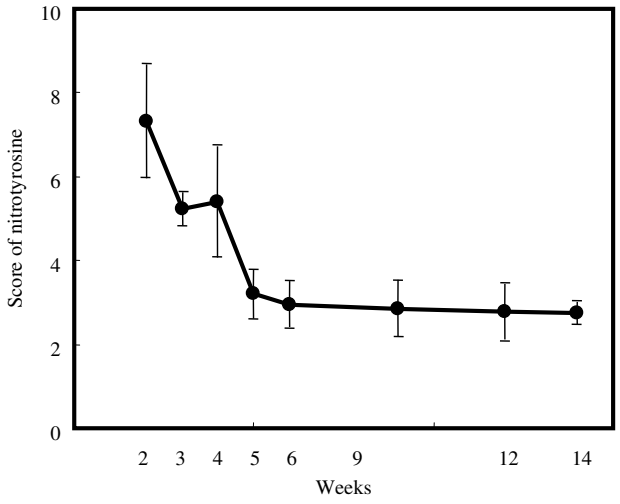

Fig. 8. Score of nitrotyrosine immunoreactivity at each time-point.

plateau after week 5 and week 9, respectively. iNOS is reported to be over-expressed in colonic tumors of humans.[32] In our previous investigation[18] with this model, immunohistochemical expression of iNOS and cyclooxygenase (COX)-2 was found in colonic adenocarcinomas. In this study, colonic adenocarcinoma was noted as early as at 4 weeks, when nitrotyrosine immunoreactivity was still high. This supports our previous report showing a powerful tumor-promoting effect of DSS in this model.[18] Although we did not investigate the immunohistochemical expression of COX-2 or iNOS in this study, we observed the positive reaction of both enzymes in colonic neoplasms as well as their surrounding inflammatory cells in this model.[18] We consider that these enzymes are involved in colitis-related colon carcinogenesis.[18] In fact, increased expression of iNOS and COX-2 was reported in colonic epithelial malignancy and pre-malignancy induced by chemical carcinogen,[33, 34] and also in human colonic neoplasms.[32, 35] Moreover, increases in the amounts of their reaction products, NO and prostaglandin E_2, might contribute to the development of colonic neoplasms, since specific inhibitors of both enzymes can inhibit colon carcinogenesis in this model (manuscript in preparation).

Oxidative stress accompanied with inflammation contributes to neoplastic transformation.[36] IBD is considered one of the major "oxyradical overload" diseases, whereby chronic inflammation results in a cancer-prone phenotype.[37] Oxidative stress with its associated cellular damage is thought to play a key role in the pathogenesis of the colitis itself,[38] as well as in rat colon carcinogenesis.[39] Indeed, measures of oxidative stress, including 8-oxoguanine and 4-hydroxy-2-nonenal-modified proteins, were increased in colonic mucosa of IBD patients[40–42] and inflamed colonic tissue of rodents treated with DSS.[43, 44] An ongoing study in our laboratory will clarify the contribution of oxidative stress to colon carcinogenesis in our model.

In conclusion, the results in the current study demonstrated that a single dose of AOM followed by 2% DSS produced colonic adenocarcinoma within 4 weeks and resulted in 100% incidence at week 6. Our findings might suggest that genotoxic damage caused by AOM and subsequent severe inflammation induced by DSS result in a high incidence of colonic epithelial malignancy.

We are indebted to Ms. S. Yamamoto for her excellent technical assistance and staff of the Research Animal Facility of Kanazawa Medical University for care of the animals. This study was supported in part by a Grant-in-Aid for the 3rd 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.

1. Potter JD. Risk factors for colon neoplasia—epidemiology and biology. *Eur J Cancer* 1995; **31**:1033–8.
2. Grossman A, Fireman Z, Lilos P, Novis B, Rozen P, Gilat T. Epidemiology of ulcerative colitis in the Jewish population of central Israel 1970–1980. *Hepatogastroenterology* 1989; **36**: 193–7.
3. Sonnenberg A, McCarty DJ, Jacobsen SJ. Geographic variation of inflammatory bowel disease within the United States. *Gastroenterology* 1991; **100**: 143–9.
4. Reif S, Klein I, Lubin F, Farbstein M, Hallak A, Gilat T. Pre-illness dietary factors in inflammatory bowel disease. *Gut* 1997; **40**: 754–60.
5. Rampton DS, Phil D. New treatments for inflammatory bowel disease. *World J Gastroenterol* 1998; **4**: 369–76.
6. Fujimoto K. IBD and feeding behavior. *Pathol Clin Med* 2002; **20**: 1226–30 (in Japanese).
7. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526–35.
8. van Hogezand RA, Eichhorn RF, Choudry A, Veenendaal RA, Lamers CB. Malignancies in inflammatory bowel disease: fact or fiction? *Scand J Gastroenterol Suppl* 2002; **236**: 48–53.
9. Weedon DD, Shorter RG, Ilstrup DM, Huizenga KA, Taylor WF. Crohn's disease and cancer. *N Engl J Med* 1973; **289**: 1099–103.
10. Gillen CD, Walmsley RS, Prior P, Adndrews HA, Allan RN. Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. *Gut* 1994; **35**: 1590–2.
11. Marcus R, Watt J. Colonic ulceration in young rats fed degraded carrageenan. *Lancet* 1971; **ii**: 765–6.
12. Seril DN, Liao J, Yang GY, Yang CS. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. *Carcinogenesis* 2003; **24**: 353–62.
13. Hirono I, Kuhara K, Hosaka S, Tomizawa S, Golberg L. Induction of intestinal tumors in rats by dextran sulfate sodium. *J Natl Cancer Inst* 1981; **66**: 579–83.
14. Tamaru T, Kobayashi H, Kishimoto S, Kajiyama G, Shimamoto F, Brown WR. Histochemical study of colonic cancer in experimental colitis of rats. *Dig Dis Sci* 1993; **38**: 529–37.
15. Cooper HS, Murthy S, Kido K, Yoshitake H, Flanigan A. Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology, β-catenin and p53 expression and the role of inflammation. *Carcinogenesis* 2000; **21**: 757–68.
16. Yamada M, Ohkusa T, Okayasu I. Occurrence of dysplasia and adenocarcinoma after experimental chronic ulcerative colitis in hamsters induced by dextran sulphate sodium. *Gut* 1992; **33**: 1521–7.
17. Tanaka T, Kohno H, Yoshitani S, Takashima S, Okumura A, Murakami A, Hosokawa M. Ligands for peroxisome proliferator-activated receptors α and γ inhibit chemically induced colitis and formation of aberrant crypt foci in rats. *Cancer Res* 2001; **61**: 2424–8.
18. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* 2003; **94**: 965–73.
19. Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, Stenson WF. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* 1996; **111**: 871–85.
20. Kimura H, Hokari R, Miura S, Shigematsu T, Hirokawa M, Akiba Y, Kurose I, Higuchi H, Fujimori H, Tsuzuki Y, Serizawa H, Ishii H. Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. *Gut* 1998; **42**: 180–7.
21. Zingarelli B, Szabo C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. *Gut* 1999; **45**: 199–209.
22. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 1996; **9**: 836–44.
23. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238–49.
24. Riddell RH, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983; **14**: 931–68.
25. Pascal RR. Dysplasia and early carcinoma in inflammatory bowel disease and colorectal adenomas. *Hum Pathol* 1994; **25**: 1160–71.
26. Ward JM. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab Invest* 1974; **30**: 505–13.
27. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990; **98**: 694–702.
28. Ni J, Chen SF, Hollander D. Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. *Gut* 1996; **39**: 234–41.
29. Kitajima S, Takuma S, Morimoto M. Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. *J Vet Med Sci* 1999; **61**: 67–70.
30. Kankuri E, Hamalainen M, Hukkanen M, Salmenpera P, Kivilaakso E, Vapaatalo H, Moilanen E. Suppression of pro-inflammatory cytokine release by selective inhibition of inducible nitric oxide synthase in mucosal explants from patients with ulcerative colitis. *Scand J Gastroenterol* 2003; **38**: 186–92.
31. Halliwell B. What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation *in vivo*? *FEBS Lett* 1997; **411**: 157–60.
32. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, Klein S, Shields PG, Billiar TR, Harris CC. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. *Cancer Res* 1998; **58**: 334–41.
33. Takahashi M, Mutoh M, Kawamori T, Sugimura T, Wakabayashi K. Altered expression of β-catenin, inducible nitric oxide synthase and cyclooxygenase-

2 in azoxymethane-induced rat colon carcinogenesis. *Carcinogenesis* 2000; **21**: 1319–27.
34. Yamada Y, Yoshimi N, Hirose Y, Matsunaga K, Katayama M, Sakata K, Shimizu M, Kuno T, Mori H. Sequential analysis of morphological and biological properties of β-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res* 2001; **61**: 1874–8.
35. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183–8.
36. Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol* 2004; **44**: 239–67.
37. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; **3**: 276–85.
38. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G7–17.
39. Tanaka T, Kawabata K, Kakumoto M, Hara A, Murakami A, Kuki W, Takahashi Y, Yonei H, Maeda M, Ota T, Odashima S, Yamane T, Koshimizu K, Ohigashi H. Citrus auraptene exerts dose-dependent chemopreventive activity in rat large bowel tumorigenesis: the inhibition correlates with suppression of cell proliferation and lipid peroxidation and with induction of phase II drug-metabolizing enzymes. *Cancer Res* 1998; **58**: 2550–6.
40. Lih-Brody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, Weissman GS, Katz S, Floyd RA, McKinley MJ, Fisher SE, Mullin GE. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 1996; **41**: 2078–86.
41. Bartsch H, Nair J. Potential role of lipid peroxidation derived DNA damage in human colon carcinogenesis: studies on exocyclic base adducts as stable oxidative stress markers. *Cancer Detect Prev* 2002; **26**: 308–12.
42. D'Inca R, Cardin R, Benazzato L, Angriman I, Martines D, Sturniolo GC. Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia. *Inflamm Bowel Dis* 2004; **10**: 23–7.
43. Tardieu D, Jaeg JP, Cadet J, Embvani E, Corpet DE, Petit C. Dextran sulfate enhances the level of an oxidative DNA damage biomarker, 8-oxo-7,8-dihydro-2′-deoxyguanosine, in rat colonic mucosa. *Cancer Lett* 1998; **134**: 1–5.
44. Araki Y, Andoh A, Fujiyama Y. The free radical scavenger edaravone suppresses experimental dextran sulfate sodium-induced colitis in rats. *Int J Mol Med* 2003; **12**: 125–9.