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]

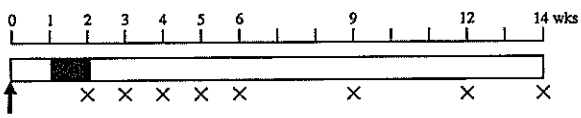

Fig. 1. Experimental protocol. □, basal diet and tap water; ■, 2% DSS; ↑, AOM, 10 mg/kg b.w., i.p.; ×, sacrifice.

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-

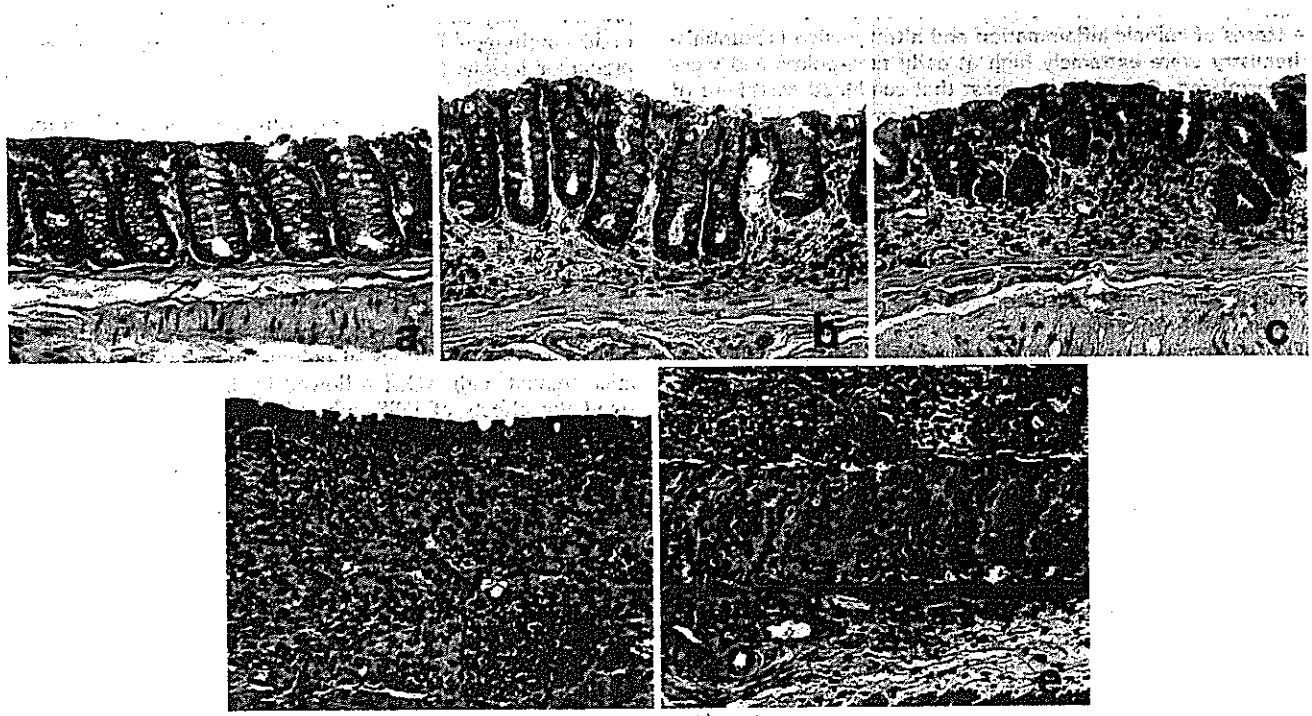

Fig. 2. Histology and various grades of colitis: (a) normal colonic mucosa (grade 0); (b) shortening of the basal one-third of the crypts with slight inflammation and edema in the lamina propria (grade 1); (c) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (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 (UI-I, 100% incidence with a multiplicity of 8.20±1.94; and UI-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 UI-I (0.25±0.43 multiplicity) at week 14. UI-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

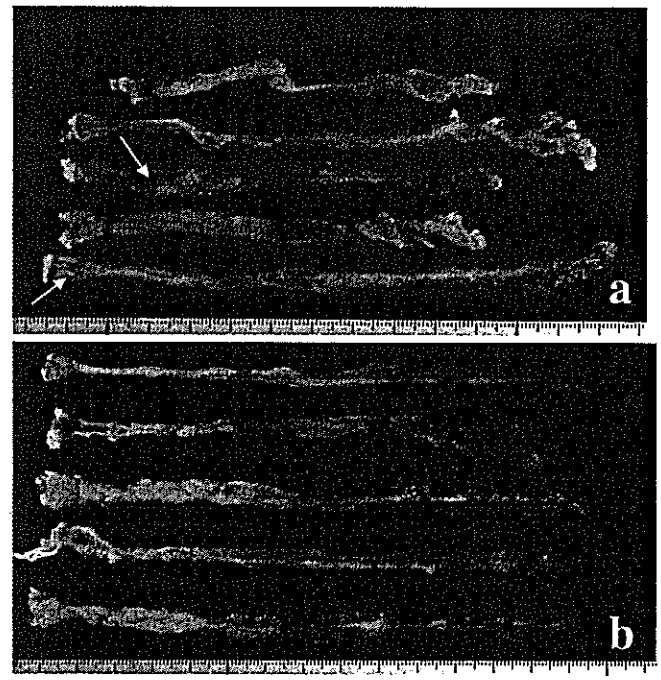

Fig. 4. Macroscopic view of the colon of mice treated with 2% DSS. (a) A small nodular tumor (arrow) is seen in the distal colon of 2 mice at week 3. (b) Nodular, polypoid, or caterpillar-like tumors are found in the distal and/or middle colon of all mice at week 12.

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.

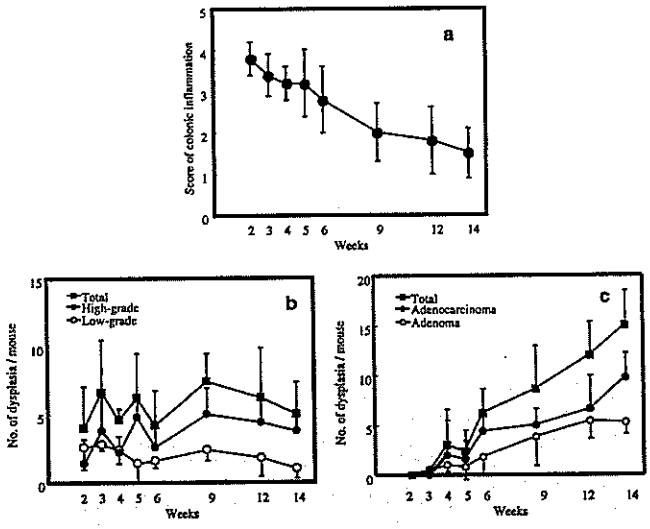

Fig. 5. (a) Colonic inflammation score at each time-point, (b) multiplicity of colonic dysplasia at each time-point, (c) multiplicity of colonic neoplasms at each time-point.

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

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

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.

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).

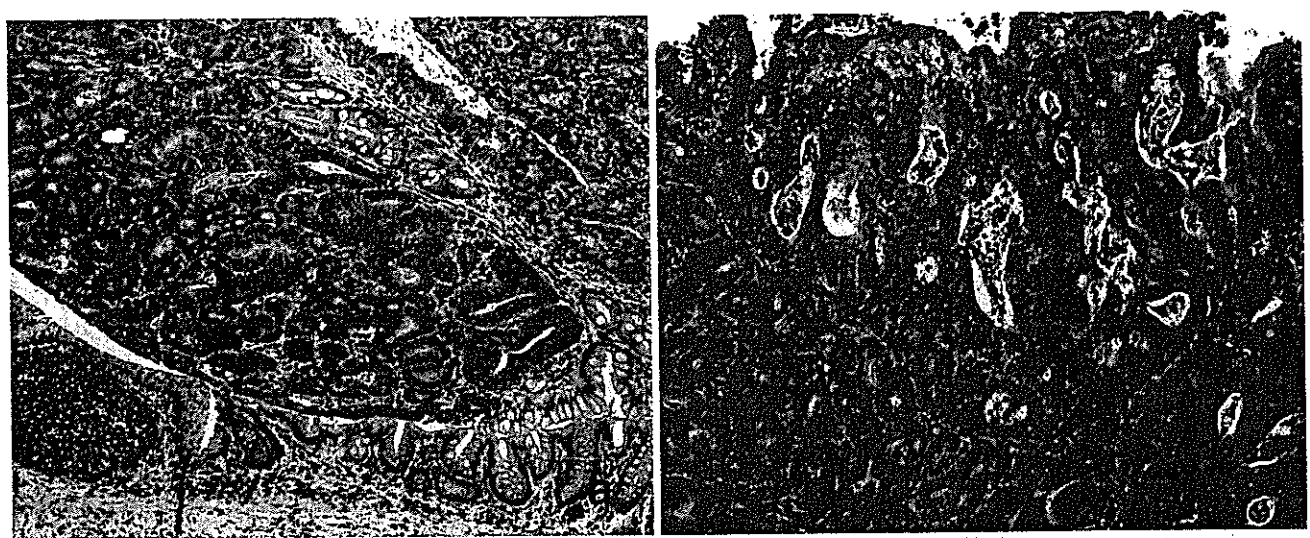

Fig. 6. Histopathology of (a) tubular adenoma and (b) adenocarcinoma. Hematoxylin and eosin stain. Original magnifications, (a) ×10 and (b) ×20.

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

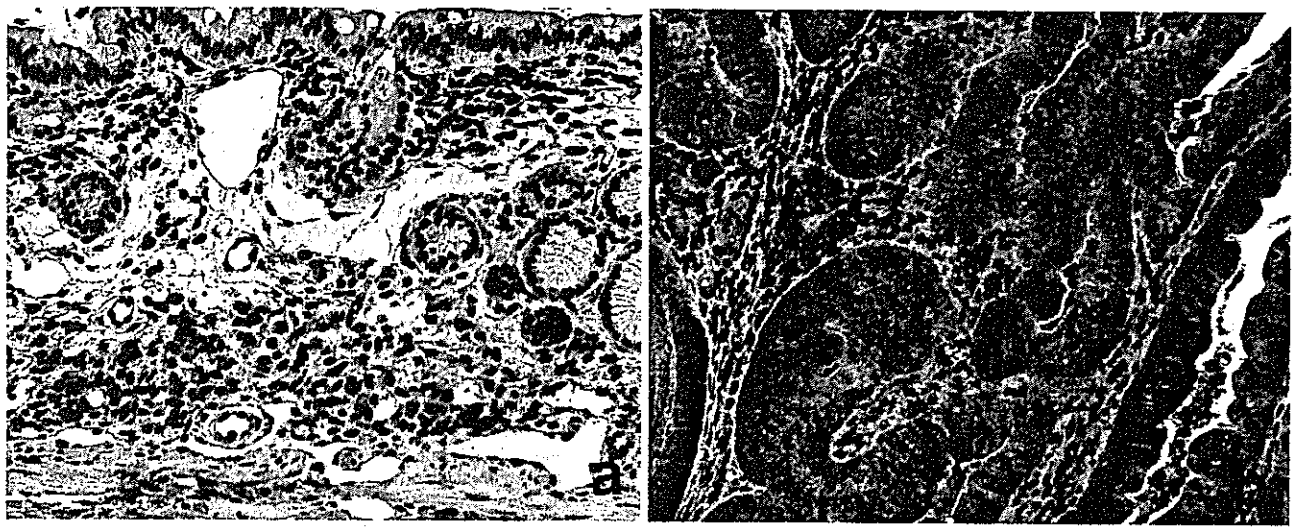

Fig. 7. Immunohistochemistry of nitrotyrösine. (a) Strongly positive immunoreactivity of nitrotyrosine in inflammatory cells (mostly macrophages) infiltrated in the lamina propria and (b) moderate immunoreactivity of nitrotyrosine in adenocarcinoma cells with surrounding macrophages showing strong immunoreactivity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

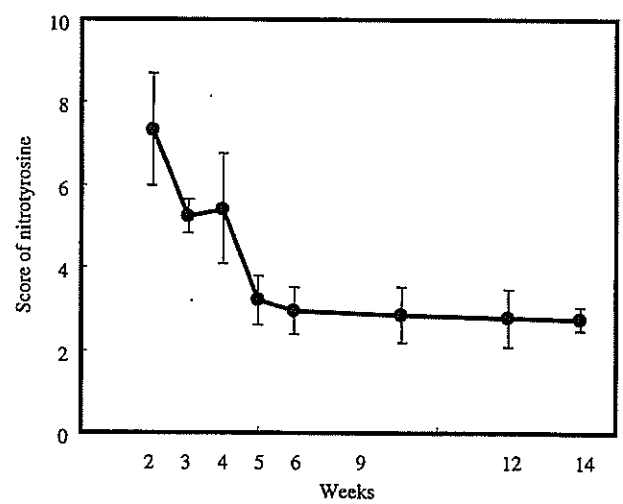

Fig. 8. Score of nitrotyrosine immunoreactivity at each time-point.

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.

BioFactors 21 (2004) 111–114
IOS Press

Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats

Rikako Suzuki[a,b,*], Hiroyuki Kohno[a], Akira Murakami[c], Koichi Koshimizu[c], Hajime Ohigashi[c], Masamichi Yano[d], Harukuni Tokuda[e], Hoyoku Nishino[e] and Takuji Tanaka[a]

[a]*The First Department of Pathology, Kanazawa Medical University, Ishikawa 920-0293, Japan*
[b]*Research Fellow of the Japan Society for the Promotion of Science, Tokyo 102-8471, Japan*
[c]*Division of Applied Life Science, Graduate School of Agriculture, Kyoto University, Kyoto 606-0085, Japan*
[d]*National Institute of Fruit Tree Science, Shizuoka 424-0292, Japan*
[e]*Department of Biochemistry, Kyoto Prefecture University of Medicine, Kyoto 602-0841, Japan*

Abstract. The inhibitory effects of dietary feeding of citrus nobiletin on azoxymethane (AOM)-induced rat colon carcinogenesis using a long-term bioassay were investigated. Five-week old male F344 rats were initiated with two weekly subcutaneous injections of AOM (20 mg/kg bw) to induce colonic tumors. They were also given the diets containing 0.01% or 0.05% nobiletin for 34 weeks, starting one week after the last dosing of AOM. At the end of the study, the incidence of colonic adenocarcinoma were 67% in the AOM alone group, 55% in the AOM→0.01% nobiletin group, 35% ($p < 0.05$) in the AOM→0.05% nobiletin group. Also, nobiletin feeding reduced the cell-proliferation activity, increased the apoptotic index, and decreased the prostaglandin E_2 content in colonic adenocarcinoma and/or colonic mucosa. These findings might suggest that citrus nobiletin has chemopreventive ability against AOM-induced rat colon carcinogenesis.

Keywords: Nobiletin, inhibition, colon carcinogenesis, rats

1. Introduction

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) is a polymethoxy flavonoid extracted from citrus fruits (Fig. 1) [2]. The compound is reported to inhibit proliferation of human cancer cells [4] and exert anti-mutagenic activity [12]. We previously reported the protective effect of dietary nobiletin on the development of aberrant crypt foci (ACF) with high crypt multiplicity in a short-term *in vivo* assay [5]. These findings suggest a possible inhibitory effect of nobiletin on colon carcinogenesis.

In the current study, the possible modifying effect of nobiletin on azoxymethane (AOM)-induced rat colon tumorigenesis was investigated. Also, several biomarkers for cancer chemoprevention studies were assayed for mechanistic investigation.

*Address for correspondence: Dr. Rikako Suzuki, The First Department of Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa, 920-0293, Japan. Tel.: +81 76 286 2211; Fax: +81 76 286 6926; E-mail: rikako@kanazawa-med.ac.jp.

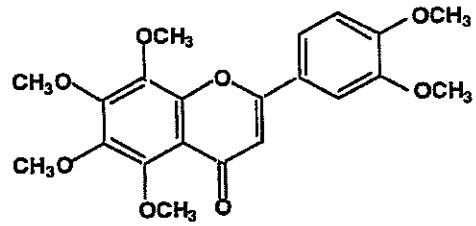

Fig. 1. Chemical structure of nobiletin.

2. Materials and methods

2.1. Experimental protocol

Male F344 rats (5 weeks old, SCL, Inc., Shizuoka, Japan) were divided into 5 experimental groups as shown in Tables. Rats in groups 1–3 were initiated with AOM (20 mg/kg body weight, two weekly s.c. injections, Sigma Chemical Co. (St. Louis, MO, USA). Nobiletin isolated from *Citrus unshiu* (>99% purity) in diet at a dose level of 0.01% (group 2) or 0.05% (group 3) was given to rats for 34 weeks, starting one week after the last injection of AOM. Group 4 was given 0.05% nobiletin-containing diet alone and group 4 served as an untreated control. The experiment was terminated at 36 weeks.

2.2. Assays for biomarkers

Tissue polyamine levels in the mucosal extraction were determined [6]. The scraped colonic mucosa of 4 rats from each group was also used for prostaglandin (PG) E_2 determination using a commercial experimental kit (Cayman, Ann Arbor, MI). The proliferating cell nuclear antigen (PCNA)-labeling and apoptotic indices were immunohistochemically determined in all colonic tumors using a DAKO LSAB 2 kit/HRP (DAKO Japan, Kyoto, Japan) with a mouse monoclonal primary antibody against PCNA (PC10, DAKO Japan) and a rabbit polyclonal primary antibody against ssDNA (DAKO Japan).

2.3. Statistics

The data were analyzed by Student's t-test, Welch's t-test, Fisher's exact probability test or Chi square test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Incidence and multiplicity of the intestinal neoplasms

As shown in Table 1 the incidence and multiplicity of colonic adenocarcinoma were 67% and 1.33 ± 1.28/rat in AOM-alone group. Feeding of 0.01 or 0.05% nobiletin significantly reduced the frequency and multiplicity of colonic malignancy.

Table 1
Incidence of large bowel tumors

Group no.	Treatment (No. of rats)	AD Incidence/Multiplicity	ADC Incidence/Multiplicity
1	AOM (21)	33%/0.38 ± 0.58[a]	67%/1.33 ± 1.28
2	AOM → 0.01% nobiletin (20)	45%/0.65 ± 0.79	55%/0.65 ± 0.65[b]
3	AOM → 0.05% nobiletin (20)	50%/0.50 ± 0.50	35%[c]/0.40 ± 0.58[d]
4	0.05% nobiletin (8)	0/0	0/0
5	None (8)	0/0	0/0

AD=adenoma; ADC=adenocarcinoma.
[a]Mean ± SD.
[b,d]Significantly different from group 1 by Welch's t-test ([b] $p < 0.05$ and [d] $p < 0.01$).
[c]Significantly different from group 1 by Chi square test ($p < 0.05$).

Table 2
Colonic polyamine levels, PGE_2 contents, PCNA-labeling, and apoptotic indices of rats

Group no.	Treatment	Polyamine contents (nmol/mg protein)	PGE_2 content (pg/mg protein)		PCNA-labeling index (%)		Apoptotic index (%)	
			Surrounding mucosa	ADC	AD	ADC	AD	ADC
1	AOM	16.8 ± 1.2[a,b] (5)	97.0 ± 22.2[c] (5)	111.1 ± 35.3 (14)	31 ± 4 (7)	54 ± 6 (14)	4.73 ± 0.81 (7)	4.31 ± 0.53 (14)
2	AOM → 0.01% nobiletin	14.5 ± 0.7[d] (5)	74.2 ± 17.9 (5)	84.6 ± 23.9[e] (11)	29 ± 2 (9)	44 ± 6[f] (11)	4.64 ± 0.21 (9)	4.80 ± 0.83 (11)
3	AOM → 0.05% nobiletin	14.3 ± 1.2[g] (5)	65.8 ± 8.9[g] (5)	77.6 ± 20.2[e] (7)	25 ± 4[d] (10)	44 ± 5[h] (7)	4.40 ± 0.31 (10)	5.08 ± 0.44[i] (7)
4	0.05% nobiletin	13.3 ± 0.4 (5)	58.0 ± 15.9 (5)	–	–	–	–	–
5	None	13.5 ± 1.0 (5)	62.0 ± 15.5 (5)	–	–	–	–	–

AD=adenoma; ADC=adenocarcinoma.
Nos. in parentheses were nos. of samples examined.
[a]Mean ± SD.
[b,c]Significantly different from group 5 by Student's t-test ([b] $p < 0.002$ and [c] $p < 0.05$).
[d–f]Significantly different from group 1 by Student's t-test ([d] $p < 0.01$, [e] $p < 0.05$, [f] $p < 0.001$, [g] $p < 0.02$, [h] $p < 0.002$, and [i] $p < 0.005$).

3.2. *Assays for biomarkers*

The data on assays of several biomarkers are summarized in Table 2. Polyamine content and PGE_2 level of colonic mucosa without tumors in group 1 were significantly greater than that of group 5. Polyamine contents in groups 2 and 3 were significantly smaller than group 1. PGE_2 levels in colonic mucosa without tumors of groups 3 was significantly smaller than group 1. In colonic adenocarcinomas of groups 2 and 3 were significantly reduced the level of PGE_2 compared with group 1. The PCNA-positive indices of adenocarcinomas developed in rats of groups 2 and 3 were significantly smaller than in group 1. Apoptotic indices of adenocarcinomas developed in rats of groups 3 was significantly larger than in group 1.

4. Discussion

The results described here indicate that dietary feeding of nobiletin effectively suppresses AOM-induced large bowel carcinogenesis in rats.

Nobiletin was reported to inhibit increased cell-proliferation activity [4]. In this study, nobiletin feeding caused reduction in expression of cell proliferation biomarkers such as PCNA-labeling index in colonic tumors and polyamine level in non-lesional colonic mucosa. In addition, dietary nobiletin increased apoptotic index in the colonic adenocarcinoma, as found in an *in vitro* study [13]. Thus, it is likely that the inhibition of AOM-induced colonic adenocarcinoma formation for animals consuming nobiletin is due in part to the alteration of cell proliferating activity in the colonic mucosa and neoplasms.

In this study, administration of nobiletin reduced biosynthesis of PGE_2 in colonic adenocarcinomas and in their surrounding mucosa. Eicosanoids including PGE_2, the metabolites of arachidonic acid (AA) through the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, have a variety of biological activities. AA products synthesized via these pathways could modulate colon carcinogenesis [1] and some inhibitors of the AA cascade possess chemopreventive activity in colon carcinogenesis [3,11]. Although, we did not investigate expression of COX and LOX in colonic mucosa in the current study, nobiletin is reported to suppress the COX-2 expression in RAW 264.7 cells treated with lipopolysaccharide and IFN-γ, suggesting that nobiletin may affect both pathways of AA.

In conclusion, the results of this study suggest that dietary nobiletin has a beneficial effect on chemically-induced rat colon carcinogenesis. Our findings and recent studies on possible anti-metastatic ability of nobiletin [8,10] may suggest need for further investigations of biological functions and its mechanisms of nobiletin for fighting cancer development.

Acknowledgement

This study was supported in part by the Grant-in-Aid (13–15) for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

References

[1] A. Bennett, A. Civier, C.N. Hensby, P.B. Melhuish and I.F. Stamford, *Gut* **28** (1987), 315–318.
[2] J. Chen, A.M. Montanari and W.W. Widmer, *J. Agric. Food Chem.* **45** (1997), 364–368.
[3] M. Cuendet and J.M. Pezzuto, *Drug Metabol. Drug Interact.* **17** (2000), 109–157.
[4] C. Kandaswami, E. Perkins, D.S. Soloniuk, G. Drzewiecki and E.J. Middleton, *Cancer Lett.* **56** (1991), 147–152.
[5] H. Kohno, S. Yoshitani, Y. Tsukio, A. Murakami, K. Koshimizu, M. Yano, H. Tokuda, H. Nishino, H. Ohigashi and T. Tanaka, *Life Sci.* **69** (2001), 901–913.
[6] T. Koide, S. Sakai, Y. Kawada, A. Hara and H. Sawada, *Acta Urol. Jpn.* **36** (1990), 1103–1108.
[7] N.K. Mak, Y.L. Wong-Leung, S.C. Chan, J. Wen, K.N. Leung and M.C. Fung, *Life Sci.* **58** (1996), 1269–1976.
[8] A. Minagawa, Y. Otani, T. Kubota, N. Wada, T. Furukawa, K. Kumai, K. Kameyama, Y. Okada, M. Fujii, M. Yano, T. Sato, A. Ito and M. Kitajima, *Jpn. J. Cancer Res.* **92** (2001), 1322–1328.
[9] A. Murakami, Y. Nakamura, T. Tanaka, H. Tokuda, H. Nishino, S. Kuwahara, Y. Takahashi, K. Ogawa, M. Yano, T. Koshiba, K. Koshimizu, K. Torikai and H. Ohigashi, *Cancer Res.* **60** (2000), 5059–5066.
[10] T. Sato, L. Koike, Y. Miyata, M. Hirata, Y. Mimaki, Y. Sashida, M. Yano and A. Ito, *Cancer Res.* **62** (2002), 1025–1029.
[11] V.E. Steele, C.A. Holmes, E.T. Hawk, L. Kopelovich, R.A. Lubet, J.A. Crowell, C.C. Sigman and G.J. Kelloff, *Cancer Epidemiol. Biomarkers Prev.* **8** (1999), 467–483.
[12] M.W. Wall, M.C. Wani, G. Manukumar, P. Abraham, H. Taylor, T.J. Hughes, J. Warner and R. McGivney, *J. Nat. Prod.* **51** (1998), 1084–1091.
[13] Q. Zheng, Y. Hirose, N. Yoshimi, A. Murakami, K. Koshimizu, H. Ohigashi, K. Sakata, Y. Matsumoto, S.Y. and H. Mori, *J. Cancer Res. Clin. Oncol.* **128** (2002), 539–546.

Carcinogenesis vol.26 no.1 pp.229–238, 2005
doi:10.1093/carcin/bgh292

Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and dextran sodium sulfate in male ICR mice possess β-*catenin* gene mutations and increases immunoreactivity for β-catenin, cyclooxygenase-2 and inducible nitric oxide synthase

Takuji Tanaka[1,4], Rikako Suzuki[1,2], Hiroyuki Kohno[1], Shigeyuki Sugie[1], Mami Takahashi[3] and Keiji Wakabayashi[3]

[1]The Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan, [2]Resarch Fellow of the Japan Science for the Promotion of Science, FS Building, 8 Ichibancho, Chiyoda-, Tokyo 102-8472, Japan and [3]Cancer Prevention Basic Research Project, National Cancer Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

[4]To whom correspondence should be addressed
Email: takutt@kanazawa-med.ac.jp

Heterocyclic amines are known to be important environmental carcinogens in several organs including the colon. The aim of this study was to induce colonic epithelial malignancies within a short-term period and analyze the expression of cycooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and β-catenin, and mutations of β-*catenin* gene in induced tumors. Male Crj: CD-1 mice were given a single i.g. administration (200 mg/kg body wt) of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) or 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) followed by 2% dextran sodium sulfate (DSS) in the drinking water for a week. The expression of β-catenin, COX-2 and iNOS was immunohistochemically assessed in colonic epithelial lesions and the β-*catenin* gene mutations in colonic adenocarcinomas induced were analyzed by the single strand conformation polymorphism method, restriction enzyme fragment length polymorphism and direct sequencing. At week 16, a high incidence of colonic neoplasms with dysplastic lesions developed in mice that received PhIP and DSS, but only a few developed in those given MeIQx and DSS. Immunohistochemically, the adenocarcinomas induced were all positive for three proteins. All seven adenocarcinomas induced by PhIP and DSS have mutations. The findings suggest that DSS exerts powerful tumor-promoting effects on PhIP-initiated colon carcinogenesis in mice and this mouse model is useful for investigating environment-related colon carcinogenesis within a short-term period.

Introduction

Dietary factors intensively influence the occurrence of certain types of epithelial malignancies, such as colorectal and breast cancers (1). In Japan, the incidence of colorectal malignancy has been increasing with westernization of the dietary habits of Japanese people (2,3). Therefore, dietary factors involving the occurrence of this malignancy should be investigated extensively in order to control the disease.

In 1976, Sugimura's scientific group found new mutagenic chemicals, classified as heterocyclic amines (HCAs), from cooked meat and fish and heating amino acids and proteins (1,4,5). Thereafter, extensive work with rodents revealed the carcinogenicity of 10 HCAs, including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (6). The target organs for their tumorigenicity are liver, urinary bladder, intestine, blood vessels, skin, mammary gland, oral cavity, prostate, hematopoietic system, etc. (1,4–8). In 1991, Gerhardsson de Verdier *et al.* (9) reported that regular consumers of well-done fried meat led to colorectal and pancreatic malignancies. This was supported by the findings of the involvement of well-done cooked meat consumption and exposure of HCAs, specifically PhIP and MeIQx, in the occurrence of human colorectal and breast cancers (10,11). Thus, the intake of well-done meats containing HCAs is suspected to be associated with an increased risk of certain types of cancer in humans (5,7,12). Among various types of HCAs, PhIP and MeIQx are most abundant in cooked food. Thus, in view of environmental (especially dietary) factors and carcinogenesis, investigations (using genetic analysis and animal models) of the involvement of HCAs, particularly PhIP and MeIQx, in human colon carcinogenesis and genetic analysis are important (4).

In animal experiments, dietary feeding with PhIP induces aberrant crypt foci (ACF), which are putative precursor lesions for large bowel adenocarcinomas (13), in both rats (14) and mice (15). Dietary exposure to PhIP for 52 weeks, as other HCAs, such as 2-amino-6-methyldipyrido[1,2-*a*:3′, 2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-a:3′,2′-*d*] imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), produces large intestinal carcinomas in rats (16), but not in mice (6). Ochiai *et al.* (17) reported recently that administration of 300 p.p.m. PhIP in a high-fat diet after 40 weeks followed by continuous feeding with a high-fat diet, mice developed small intestinal neoplasms. Although colonic adenocarcinomas developed in C57BL/6N and BBN female mice fed the diet containing 300 p.p.m. MeIQ for 92 weeks (18), there were no reports on the colonic carcinogenicity of MeIQx in rats and mice (6). However, MeIQx in a diet could induce ACF in both rats (19) and mice (20). These findings suggest a weak cancer initiating capability of both food-borne carcinogens in the large bowel of rats and mice, and therefore a long-term administration of PhIP and MeIQx is required for the induction of intestinal adenocarcinomas in rodents. In addition, the incidence and multiplicity of ACF and colonic malignancies except malignant lymphoma (17) induced by these two HCAs was relatively low. Therefore, we needed a novel and efficient animal model for

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; APC, adenomatous polyposis coli; COX, cyclooxygenase; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; HCA, heterocyclic amine; iNOS, inducible nitric oxide synthase; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

determining the possible involvement and mode of action of these two HCAs in human colon carcinogenesis.

β-Catenin, acting as a structural protein at cell–cell adherent junctions and as a transcriptional activator mediating Wnt signal transduction (21), participates in a large cytoplasmic protein complex, which contains the serine/threonine protein kinase glycogen synthase kinase-3β (GSK-3β), the tumor suppressor gene product of adenomatous polyposis coli (APC), and axin/conductin (22). Frequent mutation of the β-*catenin* gene was found in chemically induced colonic neoplasms in rodents (23–25). For example, β-catenin mutations were observed frequently in azoxymethane (AOM)-induced colon tumors in rats and mice (25,26). Also, colon adenocarcinomas induced by IQ or PhIP in rats have mutations in the β-*catenin* gene (23). Mutation of the *APC* gene is known to repress the degradation and result in the accumulation of the β-catenin (27). About 80% of colorectal neoplasms harbor mutations in the *APC* gene and half of the reminder have β-*catenin* gene mutations (28–30). In the colonic adenomas and adenocarcinomas, β-catenin was universally localized to the cytoplasm and/or nucleus (31). These findings suggest that the mutation of the β-*catenin* gene plays an important role in the development of colon carcinogenesis in rodents as well as in humans.

We recently have developed a novel mouse model for inflammation-related colon carcinogenesis utilizing a single and low dose of AOM, a specific colonic carcinogen in rodents, followed by a strong tumor-promoter dextran sodium sulfate (DSS) in drinking water (32). 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. Using this model with a slight modification, in the current study, we tried to induce colonic neoplasms in mice gavaged with a single dose of PhIP or MeIQx followed by a 1-week exposure of DSS in the drinking water. In addition, we analyzed mutations of the β-*catenin* gene in induced colonic adenocarcinomas and compared them with those found in colonic malignancies induced by AOM and DSS (32). Immunohistochemical expression of β-catenin, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were also evaluated in colonic neoplasms.

Materials and methods

Animals, chemicals and diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) aged 5 weeks were used. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (4 or 5 mice/cage) with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C). After 7 days quarantine, they were randomized by body weight into experimental and control groups. PhIP and MeIQx were purchased from Wako Pure Chemical (Osaka, Japan). DSS (molecular weight 40 000) was obtained from ICN Biochemicals (Aurora, OH).

Experimental procedure

A total of 38 male ICR mice were divided into six experimental and control groups. Groups 1 (nine mice) and 2 (10 mice) were given a single i.g. intubation of PhIP (200 mg/kg body wt) and MeIQx (200 mg/kg body wt), respectively. Starting 1 week after the intubation, animals in groups 1 and 2 were given 2% (w/v) DSS in the drinking water for 7 days, and then followed without any further treatment for 14 weeks. Groups 3 (five mice) and 4 (five mice) were given PhIP and MeIQx alone, respectively. Group 5 (five mice) was given 2% DSS alone. Group 6 (five mice) was untreated. All animals were killed at week 16 by ether overdose. The large bowels were flushed with saline, excised, the length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed with saline. Macroscopic inspection of the large bowels was carefully carried out and they were cut and fixed in 10% buffered formalin for at least 24 h. Histological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. Some tumors were frozen at −80°C. On H&E-stained sections, histological alterations, such as mucosal ulceration, dysplasia and colonic neoplasms, were examined. Colonic neoplasms were diagnosed according to the description by Ward (33). Histopathological examination was also carried out on other organs.

Immunohistochemistry

Using the protocol of our previous study (32), immunohistochemistry for β-catenin, COX-2 and iNOS was performed on 3-μm-thick paraffin-embedded sections from the colons of mice in groups 1 and 2, 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 re-hydrated 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% BSA and incubated overnight at 4°C with primary antibodies, such as anti-β-catenin mouse monoclonal antibody (diluted 1:1000, Transduction Laboratories, Lexington, KY), 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 IgG blocking reagent (Vector Laboratories, Burlingame, CA) 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, 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, Tokyo, Japan) and recorded.

DNA extraction

For analysis of β-catenin mutations, seven colonic adenocarcinomas (three paraffin-embedded and four frozen materials) from PhIP/DSS-treated mice and one paraffin-embedded adenocarcinoma from a mouse treated with MeIQx/DSS were used. Also, 14 adenocarcinomas embedded in paraffin from our previous experiment (32) were used for analysis of β-catenin mutations for comparison. DNA was extracted from frozen tissue using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) or from paraffin-embedded sections using DEXPAT™ (TaKaRa Shuzo, Shiga, Japan). Tumor tissues were scraped off from paraffin sections using needles to avoid contamination with their surrounding tissue.

PCR-single strand conformation polymorphism (SSCP) analysis

DNA from colonic adenocarcinomas was PCR-amplified with primers (5′-primer, GCTGACCTGATGGAGTTGGA; 3′-primer, GCTACTTGCTCTTGCGTGAA), which were designed to amplify exon 3 of the β-*catenin* gene containing the consensus sequence for GSK-3β phosphorylation (25). The length of the PCR product with these primers is 227 bp. The primers were synthesized with a 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) and purified with an OPC cartridge (Applied Biosystems). PCR for non-radioisotopic SSCP was performed in 50 μl of reaction mixture consisting of 0.5 μM of each primer, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 200 μM each dNTP, 2.5 U AmpliTaq Gold™ (Perkin Elmer) and 0.5–5 μl of template DNA. The mixture was heated at 94°C for 9 min and subjected to 40 or 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 3 min) using a Perkin Elmer-Cetus thermal cycler. The PCR products were purified and concentrated to 20 μl using Microcon 100 (Amicon, Beverley, MA). Ten volumes of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol were added to 0.5 μl of purified PCR products, heated to 90°C for 3 min and applied to 10% polyacrylamide gels containing 5% glycerol. Electrophoresis was carried out at 300 V for 2 h 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 2-D Silver Staining Solution II (Daiichi Chemical DNA, Tokyo, Japan).

Restriction fragment length polymorphism (RFLP) 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 *Hin*fI 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

With 1 μl of the purified PCR products and 5'DyeAmidite-667-labeled 5' or 3' PCR primers (synthesized by Pharmacia Biotech, Tokyo, Japan), cycle sequencing reactions were carried out using a Thermo Sequenase™ fluorescent labeled primer cycle sequencing kit (Amersham) and the sequences were determined with an ALF express™ DNA sequencer (Pharmacia Biotech).

Statistical analysis

All measurements were compared by Student's *t*-test, Welch's *t*-test, χ^2 test or Fisher's exact probability test for paired samples.

Results

General observation

During the study, bloody stools were found during and soon after DSS exposure (days 12–21) in a few mice that received 2% DSS in the drinking water and their body weight gains were slightly decreased (data not shown). However, thereafter no such clinical symptoms were observed. The body and liver weights, and the lengths of the large bowel of mice in all groups were measured at the end of the study (week 16) and are listed in Table I. The mean body weight of group 1 (PhIP→2% DSS) was significantly larger than that of group 3 (PhIP alone, $P < 0.02$) or 6 (untreated, $P < 0.05$). The mean length of the large bowel of mice in group 1 was significantly lower than that of mice in group 3 ($P < 0.01$). The mean length of the large bowel in the mice of group 2 (MeIQx→2% DSS) was significantly smaller than that of mice in group 4 (MeIQx alone, $P < 0.005$). These shortenings were caused by DSS-induced inflammation in the colonic mucosa. Those values in groups 3 ($P < 0.02$) and 4 ($P < 0.05$) were significantly greater than that of group 6.

Pathological findings

Macroscopically, nodular, polypoid or flat-type colonic tumors were observed in the middle and distal colon of all mice in groups 1 (Figure 1a) and 2 (Figure 1b). Their histopathology was well or moderately differentiated tubular adenocarcinoma (Figures 2a, 3a, 4 and 5a) or tubular adenoma (Figures 2b and 3b). A few tumors were diagnosed as adenocarcinoma in adenoma (Figure 4). There were no tumors in any organs other than the large bowel in these two groups. As shown in Table II, the incidence of adenocarcinoma and tubular adenoma in group 1 was 56% with a multiplicity of 0.78 ± 0.97 and 33% with 0.44 ± 0.73 multiplicity, respectively. Two mice in group 2 had colonic neoplasms: two small tubular adenomas (20% incidence with 0.20 ± 0.42 multiplicity) and one well-differentiated tubular adenocarcinoma (10% incidence with 0.10 ± 0.32 multiplicity) in the distal colon (Table II). In the mice of groups 3–6, no neoplasms developed in any organs including the large bowel. Besides colonic neoplasms, all mice of groups 1 and 9 (90%) of 10 mice of group 2 had colonic dysplasia (Figures 2c and 3c). Their multiplicities were 3.11 ± 1.45 in group 1 and 2.30 ± 1.62 in group 2. As indicated in Table III, colonic dysplasia also developed in the mice of groups 3 (40% incidence with a multiplicity of 0.60 ± 0.80) and 4 (40% incidence with a multiplicity of 0.60 ± 0.80). There were no dysplastic lesions in the mice of groups 5 and 6. In addition, colonic mucosal ulceration was found in the distal colon of mice in groups 1, 2 and 5 (Table III).

Table I. Body weight and length of large bowel at the end of the experiment

Group no.	Treatment (no. of mice examined)	Body weight (g)	Length of large bowel (cm)
1	PhIP → 2% DSS (9)	46.5 ± 4.0[a–c]	13.2 ± 0.9[d]
2	MeIQx → 2% DSS (10)	46.6 ± 4.0	13.2 ± 0.5[e]
3	PhIP (5)	40.7 ± 2.8	14.7 ± 0.7[f]
4	MeIQx (5)	43.9 ± 2.1	14.9 ± 1.1[g]
5	2% DSS (4)	40.1 ± 3.8	13.5 ± 0.5
6	None (5)	41.1 ± 3.4	13.1 ± 0.9

[a]Mean ± SD.
[b]Significantly different from group 3 ($P < 0.02$).
[c]Significantly different from group 6 ($P < 0.05$).
[d]Significantly different from group 3 ($P < 0.01$).
[e]Significantly different from group 4 ($P < 0.005$).
[f]Significantly different from group 6 ($P < 0.02$).
[g]Significantly different from group 6 ($P < 0.05$).

Immunohistochemical findings

The immunoreactivities against β-catenin, COX-2 and iNOS were found in all colonic lesions, including neoplasms (four adenomas and seven adenocarcinomas in group 1; and two adenomas and one adenocarcinoma in group 2) and dysplastic lesions (28 in group 1; and 23 in group 2). The immunoreactivity showed dark brown reaction products with a slight variation in the intensity and distribution (Figures 5b–d and 6b–d). Strong β-catenin expression was seen in the nucleus and cytoplasm of adenocarcinoma cells (Figures 5b and 6b). 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. Non-lesional cryptal cells showed weak positivity of β-catenin in their cell membrane. In addition, positive reaction against the β-catenin antibody was found in the vascular endothelium, infiltrated inflammatory cells and ganglion cells in myenteric (Auerbach's) plexus. Strong COX-2 immunoreactivity was also found in adenocarcinoma (Figures 5c and 6c) and adenoma cells in their cytoplasm. Dysplastic cells showed weak positivity for COX-2, when compared with neoplastic cells. Non-lesional cryptal cells at lower part of crypts were weakly positive for COX-2, while a strongly positive reaction of COX-2 was seen in the endothelium of small blood vessels and inflammatory cells infiltrated in the lamina propria. Smooth muscle cells and fibroblasts in the wall of the large bowel showed a weak reaction of COX-2. iNOS-immunohistochemistry showed strong immunoreactivity in the cytoplasm of adenocarcinoma (Figures 5d and 6d) and adenoma cells: the intensity was greater in carcinoma cells when compared with adenoma cells. Also, dysplastic cells were positive for iNOS in their cytoplasm, but the intensity was weaker than adenoma cells. The faint positive 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 and 5.

Mutation in β-catenin gene

In the current experiment, mutations of exon 3 of the β-*catenin* gene were investigated by PCR–SSCP and RFLP analyses

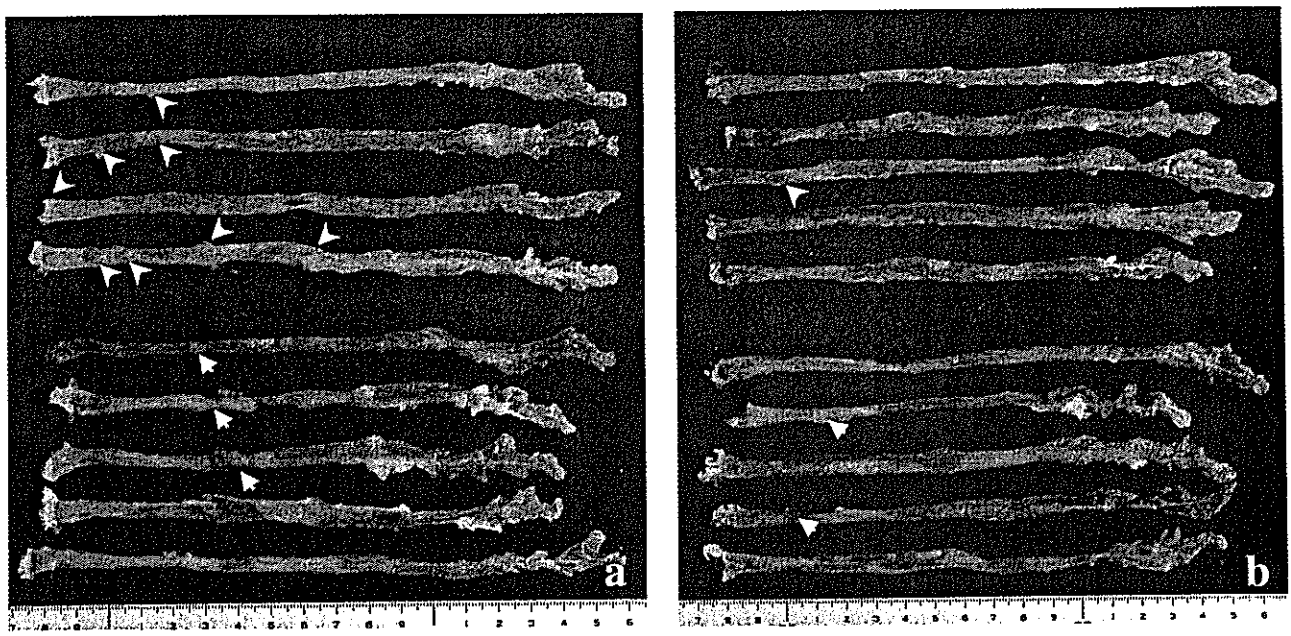

Fig. 1. Macroscopic view of large bowels. (a) Mice treated with PhIP/DSS (group 1) and (b) those given MeIQx/DSS (group 2). Arrows indicate colonic tumors.

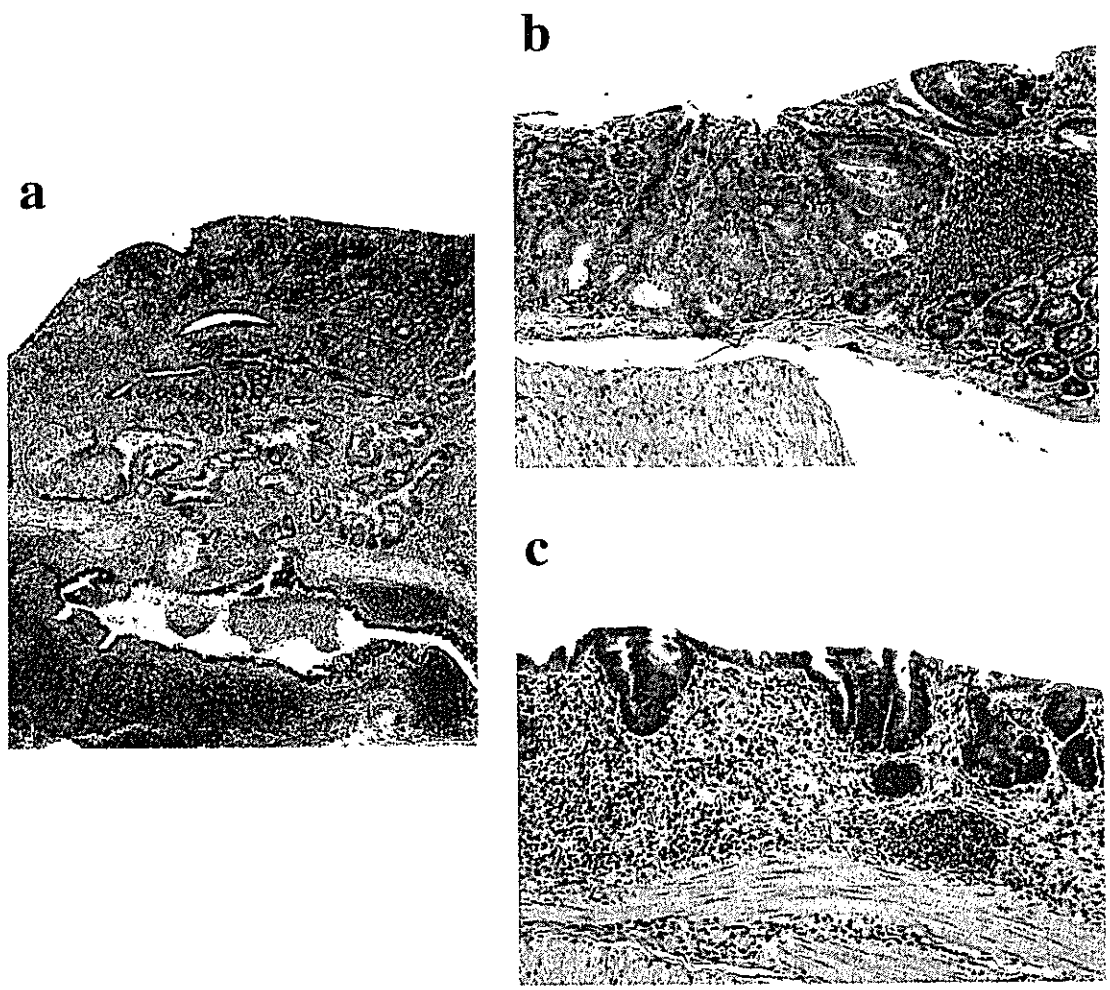

Fig. 2. Histopathology of colonic lesions developed in mice treated with PhIP/DSS. (a) A tubular adenocarcinoma invaded into the submucosa; (b) a tubular adenoma; and (c) dysplasia with mucosal ulceration. H&E stain, original magnification, (a) ×20, (b) ×50, (c) ×50.

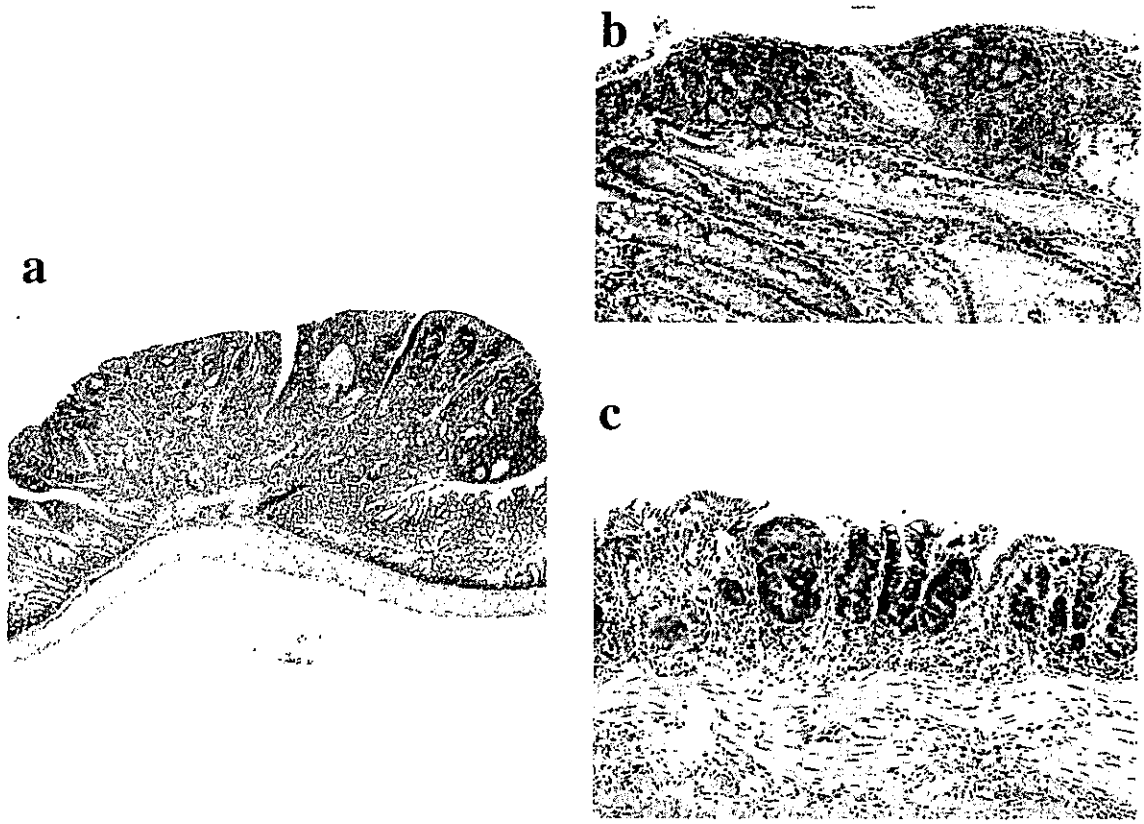

Fig. 3. Histopathology of colonic lesions developed in mice treated with MeIQx/DSS. (a) A polypoid tubular adenocarcinoma; (b) tubular adenomas; and (c) dysplasia with mucosal ulceration. H&E stain, original magnification, (a) ×10, (b) ×50, (c) ×50.

Fig. 4. Histopathology of an adenocarcinoma in adenoma developed in the large bowel of a mouse treated with PhIP/DSS. H&E stain, original magnification, ×50.

(Figures 7 and 8). Among the PhIP/DSS-treated mice, β-*catenin* genes of all of the seven colonic adenocarcinomas (100%) have mutations restricted to codons 32 and 34 (Table IV). In codon 32, the mutations found were GAT (Asp) to AAT (Asn) (PhIP/DSS-2, 3, 4, 5), to CAT (His) (PhIP/DSS-1) and TAT (Tyr) (PhIP/DSS-6) and in codon 34 GGA (Gly) to GTA (Val) (PhIP/DSS-7). However, mutation in the β-*catenin* gene was not detected in the MeIQx/DSS-induced colonic adenocarcinoma (Figures 7 and 8; Table IV), although only one sample was investigated. In these analyses, positive controls with mutations in codons 32 and 34 showed band shifts (Figures 7 and 8).

With respect to AOM/DSS-induced mouse colonic adenocarcinomas (32), β-*catenin* mutations in codons 32 through to 34 were detected in 11 (79%) out of 14 (Figures 7 and 8; Table V). These mutations included GAT (Asp) to AAT (Asn) (AOM/DSS-6, 11, 13), to GGT (Gly) (AOM/DSS-3) at codon 32, TCT (Ser) to TTT (Phe) at codon 33 (AOM/DSS-4, 8, 10), and GGA (Gly) to GAA (Glu) (AOM/DSS-7, 9, 12, 14) at codon 34 (Table V; Figure 8). Except for a mutation at the second base of codon 32 (AOM/DSS-3), all were G:C to A:T transitions (Figure 9).

Discussion

The results of the present work indicate that a single i.g. intubation of PhIP (200 mg/kg body wt) followed by 1-week exposure of 2% DSS in drinking water could produce colonic adenocarcinomas with 56% incidence and 0.78 ± 0.97 multiplicity in male ICR mice within 16 weeks. Also, a single gavage with MeIQx (200 mg/kg body wt) followed by 2% DSS in the drinking water induced colonic epithelial malignancy, although their incidence (10%) and multiplicity (0.10 ± 0.32) was low. Colonic adenocarcinomas induced by this treatment schedule were immunohistochemically positive for β-catenin, COX-2 and iNOS. Moreover, all examined adenocarcinomas in mice treated with PhIP/DSS and MeIQx/DSS had β-catenin mutations.

Table II. Incidence and multiplicity of colonic neoplasms induced by PhIP or MeIQx followed by DSS

Group no.	Treatment (no. of mice examined)	Total		Adenoma		Adenocarcinoma	
		Incidence	Multiplicity	Incidence	Multiplicity	Incidence	Multiplicity
1	PhIP→2% DSS (9)	78%	1.22 ± 1.20[a]	33%	0.44 ± 0.73	56%	0.78 ± 0.97
2	MeIQx→2% DSS (10)	30%	0.30 ± 0.48	20%	0.20 ± 0.42	10%	0.10 ± 0.32
3	PhIP (5)	0	0	0	0	0	0
4	MeIQx (5)	0	0	0	0	0	0
5	2% DSS (4)	0	0	0	0	0	0
6	None (5)	0	0	0	0	0	0

[a]Mean ± SD.

Table III. Incidence and multiplicity of dysplastic lesions in the large bowel

Group no.	Treatment (no. of mice examined)	Dysplastic lesions		Mucosal ulceration	
		Incidence	Multiplicity	Incidence	Multiplicity
1	PhIP→2% DSS (9)	100%	3.11 ± 1.45	100%	1.78 ± 0.92
2	MeIQx→2% DSS (10)	90%	2.30 ± 1.62	50%	0.80 ± 1.17
3	PhIP (5)	40%	0.60 ± 0.80	0%	0
4	MeIQx (5)	40%	0.60 ± 0.80	0%	0
5	2% DSS (4)	0%	0	100%	1.58 ± 0.87
6	None (5)	0%	0	0%	0

Several researchers have made great efforts to establish an efficient experimental animal model for colon carcinogenesis induced by PhIP or MeIQx (34,35). Nakagama's group developed efficient animal models for PhIP-induced colon carcinogenesis, where rats were given cycle treatments with dietary PhIP (300 or 400 p.p.m.) and a high-fat diet or PhIP in the diet followed by a high-fat diet (36,37). Tsukamoto *et al.* (35) also reported that i.g. administration of PhIP (three times a week for 7 weeks at a dose of 100 mg/kg body wt) could efficiently induce large intestinal tumors within 50 weeks in male F344 rats. Indeed, ACF and colonic neoplasms could develop in rats using such protocols, but their incidence and multiplicity were low and the experimental period was long. As for intestinal carcinogenicity of PhIP in mice, dietary PhIP (300 p.p.m.) in a high-fat diet for 40 weeks and followed by a high-fat diet without PhIP for 30 or 45 weeks induced small intestinal and cecal tumors (adenomas and adenocarcinomas) (17). There were no reports on MeIQx-induced colonic epithelial malignancies, although dietary feeding with MeIQx could produce ACF in mice (20). In the current study we could develop large bowel neoplasms within 16 weeks by an experimental protocol of a single and low-dose i.g. administration of PhIP or MeIQx (200 mg/kg body wt) followed by 1-week exposure of 2% DSS in the drinking water. The findings also indicated a powerful tumor-promoting ability of DSS, as found in our previous experiment using AOM as a carcinogen (32). Thus, our model can be applied to investigate colonic carcinogenesis induced by environmental carcinogens (HCAs, etc.) and/or modulators (promoters and chemopreventors) (32,34) and to genetic analysis of the susceptibility to colon tumorigenesis (38).

There have been no studies on the mutation of β-*catenin* in mouse colonic tumors induced by HCAs, although β-*catenin* mutations at codons 32 and 34 are the most common in PhIP-induced colonic neoplasms in rats (35–37). Our results on β-catenin mutations are in accordance with these findings (35–37). To our knowledge, the mutation of β-catenin, GAT→CAT or TAT, at codon 32 was not reported. To identify whether these mutations came from DSS or were characteristics of the mice, more detailed analysis should be carried out. All mutations are involved in guanine, consistent with a report that PhIP preferentially forms DNA adducts at guanines (39). In the present study, we could not find a β-*catenin* gene mutation in a mouse colonic adenocarcinoma produced by MeIQx/DSS, but we will analyze the mutation in our on-going experiments where more colonic tumors will develop in mice treated with MeIQx and DSS. In AOM/DSS-induced colon tumors (32), mutations of the β-*catenin* gene were present in codons 32 through to 34. The location was slightly different from a report documenting that β-catenin mutations in mouse colon tumors induced by AOM were found in codons 33, 37 and 41, encoding serine and threonine that are direct targets for phosphorylation by GSK-3β (26). Codon 34 also encodes neighboring serine and threonine residues (26). Mutations of codons 33 and 34 were detected in both the present investigation and another report (26). However, mutation of codon 32 was not found in the other report (26). Therefore, it may be speculated that mutations of codons 33 and 34 might be caused by AOM exposure and that of codon 32 by DSS administration, as Koesters *et al.* (40) reported that the different mutational spectra observed in *Ctnnb1* directly relates to the particular carcinogenic treatment. We did not identify the mutations of codons 37 and 41 in the present study. This might be explained by the different experimental protocol between the present study and the previous report on β-catenin mutations in mouse colon cancer, where mice were treated with AOM at a dose of 10 mg/kg body wt, once a week for 6 weeks and the

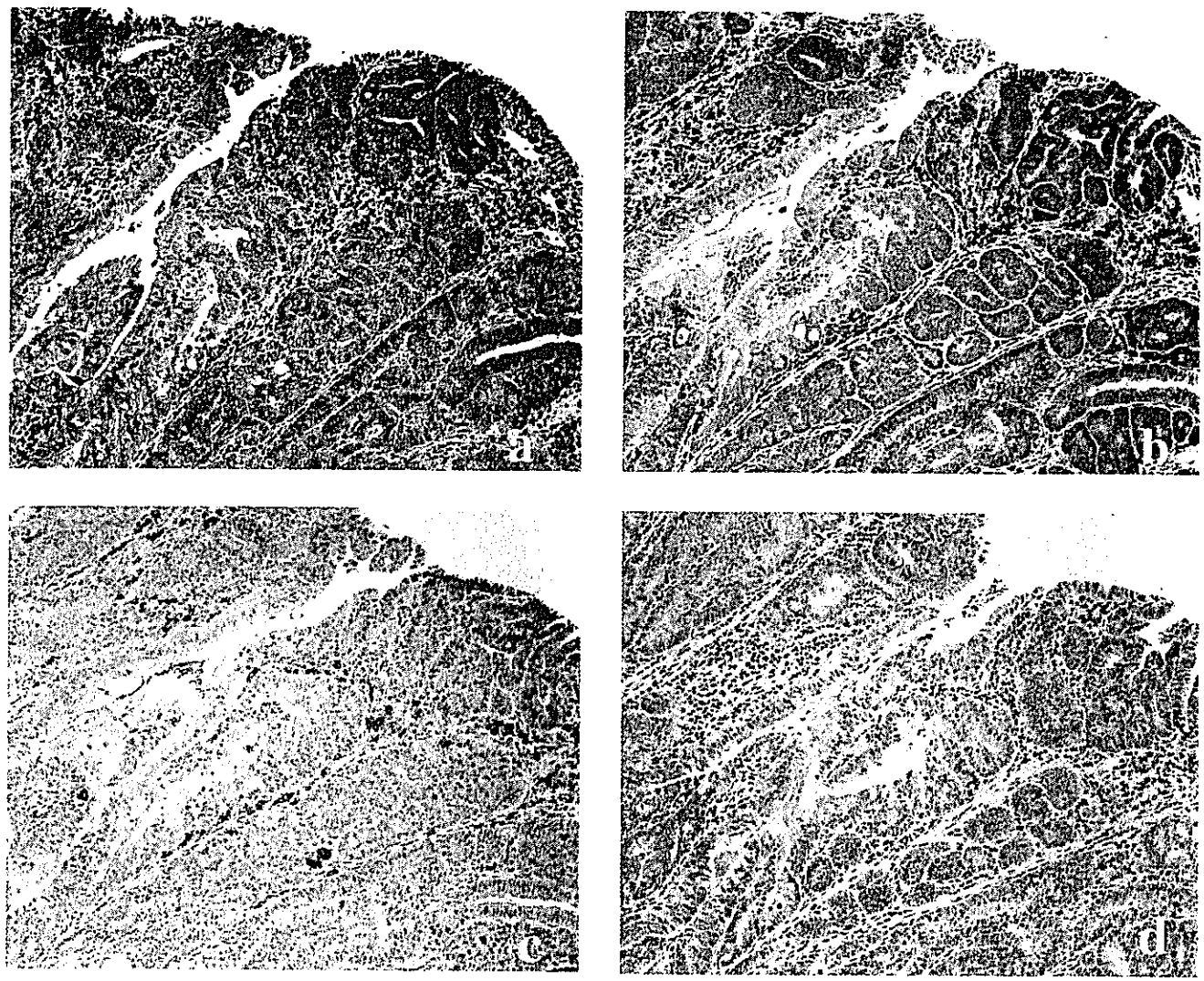

Fig. 5. Histopathology and immunohistochemistry of β-catenin, COX-2, and iNOS in a colonic adenocarcinoma developed in a mouse given PhIP/DSS. (a) H&E staining; (b) β-catenin immunohistochemistry; (c) COX-2 immunohistochemistry; and (d) iNOS immunohistochemistry. Original magnification, (a–d) ×50.

experiment was terminated at week 30 (26). The second G of the CTGGA sequence was commonly mutated to A in codons 32 and 34 of the rat β-*catenin* gene and this site is considered to be a mutational hot-spot with AOM (25,41). In this study, 10 of 11 mutations being G:C→A:T transitions at codons 32, 33 and 34, the mutation of the second G of the CTGGA might be particularly important in colon carcinogenesis. We reported recently the increased expression of COX-2 and iNOS in mouse colon adenocarcinoma in an AOM/DSS mouse colon carcinogenesis model (32). The reaction products of iNOS and COX-2, nitric oxide and prostaglandin E_2 respectively, could contribute to colon tumorigenesis. Also, there is evidence of an involvement of the Wnt–APC–β-catenin/Tcf pathway in COX-2 expression (42–44). Although the relationship between its pathway and iNOS expression is still unclear, β-catenin was accumulated in the cytoplasm and nucleus in the colonic tumors induced by AOM/DSS (32) and those developed in this study. Therefore, mutation of β-catenin may play important roles in mouse and rat colon carcinogenesis. Furthermore, mutation of β-catenin is an early event of colorectal carcinogenesis (35,41); analysis of this at an early stage of colon carcinogenesis should be done in this model. Since *c-myc* and *cyclinD1* were identified as targets of the β-catenin/APC pathway (45,46), this gene expression might also influence colon carcinogenesis in the present model.

In the present study, all colonic neoplasms induced by PhIP or MeIQx followed by DSS were immunohistochemically positive for iNOS and COX-2. The iNOS and COX-2 were reported to be over-expressed in colon tumors or ACF that develop in rats after the administration of the colon-specific carcinogen, AOM (41,47,48). iNOS may regulate COX-2 production of pro-inflammatory prostaglandins, which are known to play a key role in colon tumor development (49). The results in the current study indicate a powerful tumor-promoting ability of DSS, since the doses of PhIP and MeIQx used were too small to induce colonic neoplasms. It is probable that peroxynitrite acts as an oxidant for the heme of COX-2 and activates the enzyme (50,51). Our recent study using an AOM/DSS model (52) has suggested the possibility. Also, nitric oxide

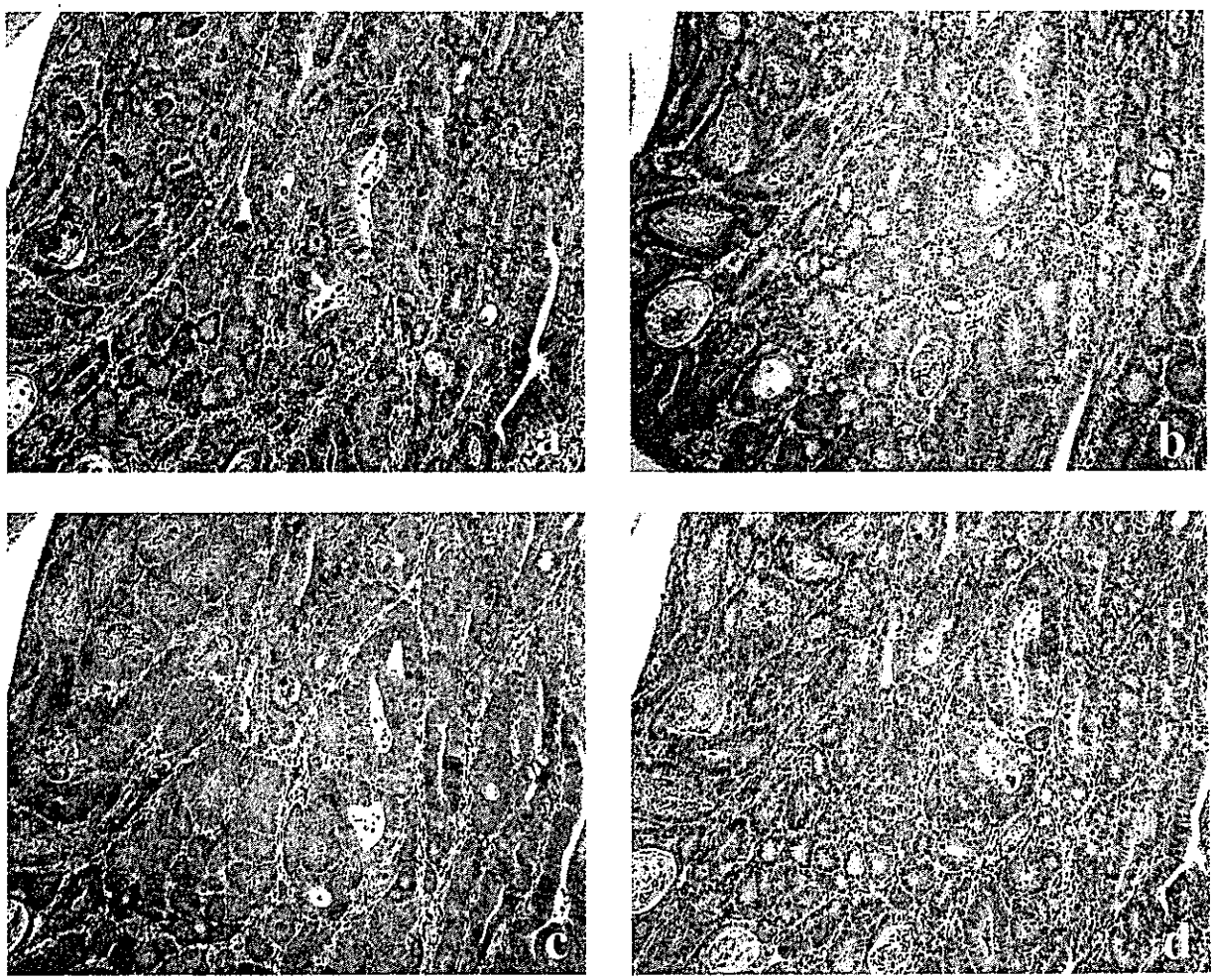

Fig. 6. Histopathology and immunohistochemistry of β-catenin, COX-2, and iNOS in a colonic adenocarcinoma developed in a mouse treated with MeIQx/DSS. (a) H&E staining; (b) β-catenin immunohistochemistry; (c) COX-2 immunohistochemistry; and (d) iNOS immunohistochemistry. Original magnification, (a–d) ×50.

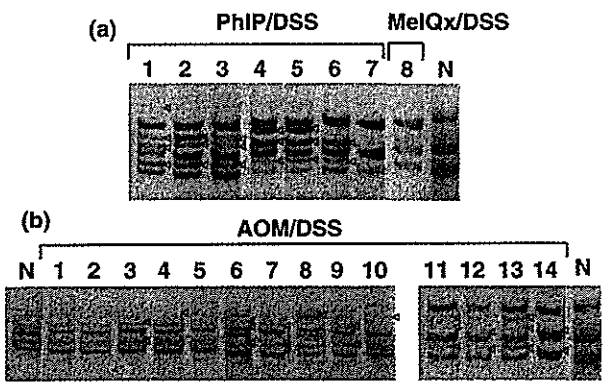

Fig. 7. PCR–SSCP analysis of the β-*catenin* gene in mouse colon adenocarcinomas. (a) PhIP/DSS-induced mouse colon adenocarcinomas (lanes 1–7); and MeIQx/DSS-induced mouse colon adenocarcinomas (lane 8). (b) AOM/DSS-induced mouse colon adenocarcinomas (lanes 1–14). Lanes N, normal colon mucosa samples. Tumor-specific bands are indicated by arrow heads.

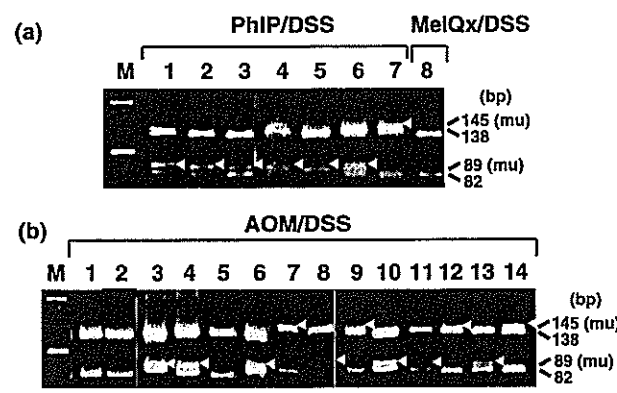

Fig. 8. RFLP analysis of the β-*catenin* gene in mouse colon adenocarcinomas. (a) PhIP/DSS (lanes 1–7) and MeIQx/DSS (lane 8) induced mouse colonic adenocarcinomas. (b) AOM/DSS-induced mouse colon adenocarcinomas (lanes 1–14). Lane M, DNA size markers (X174/*Hae*III digest). Tumor-specific bands are indicated by arrow heads.

and its metabolites may affect tumor formation and/or progression. As found in ulcerative colitis-related colon carcinogenesis in humans, iNOS expression and nitrotyrosine accumulation in inflamed colonic mucosa caused by DSS treatment may be involved in the colonic tumor development in the current study (32,53).

In conclusion, the results in the current study indicate that a single i.g. administration of a low dose of the

Table IV. Mutations in exon 3 of the β-*catenin* gene in PhIP/DSS or MeIQx/DSS-induced mouse colonic adenocarcinomas

Sample		β-Catenin status	Amino acid substitution
PhIP/DSS-1 (paraffin-embedded)	Codon 32	GAT→CAT	Asp→His
PhIP/DSS-2 (paraffin-embedded)	Codon 32	GAT→AAT	Asp→Asn
PhIP/DSS-3 (paraffin-embedded)	Codon 32	GAT→AAT	Asp→Asn
PhIP/DSS-4 (frozen tissue)	Codon 32	GAT→AAT	Asp→Asn
PhIP/DSS-5 (frozen tissue)	Codon 32	GAT→AAT	Asp→Asn
PhIP/DSS-6 (frozen tissue)	Codon 32	GAT→TAT	Asp→Tyr
PhIP/DSS-7 (frozen tissue)	Codon 34	GGA→GTA	Gly→Val
MeIQx/DSS-1 (paraffin-embedded)		Wild-type	

Table V. Mutations in exon 3 of the β-*catenin* gene in AOM/DSS-induced mouse colonic adenocarcinomas

Sample		β-Catenin status	Amino acid substitution
AOM/DSS-1 (paraffin-embedded)		Wild-type	-
AOM/DSS-2 (paraffin-embedded)		Wild-type	-
AOM/DSS-3 (paraffin-embedded)	Codon 32	GAT→GGT	Asp→Gly
AOM/DSS-4 (paraffin-embedded)	Codon 33	TCT→TTT	Ser→Phe
AOM/DSS-5 (paraffin-embedded)		Wild-type	
AOM/DSS-6 (paraffin-embedded)	Codon 32	GAT→AAT	Asp→Asn
AOM/DSS-7 (paraffin-embedded)	Codon 34	GGA→GAA	Gly→Glu
AOM/DSS-8 (paraffin-embedded)	Codon 33	TCT→TTT	Ser→Phe
AOM/DSS-9 (paraffin-embedded)	Codon 34	GGA→GAA	Gly→Glu
AOM/DSS-10 (paraffin-embedded)	Codon 33	TCT→TTT	Ser→Phe
AOM/DSS-11 (paraffin-embedded)	Codon 32	GAT→AAT	Asp→Asn
AOM/DSS-12 (paraffin-embedded)	Codon 34	GGA→GAA	Gly→Glu
AOM/DSS-13 (paraffin-embedded)	Codon 32	GAT→AAT	Asp→Asn
AOM/DSS-14 (paraffin-embedded)	Codon 34	GGA→GAA	Gly→Glu

genotoxic food-derived carcinogens, especially PhIP, followed by DSS resulted in a high incidence of colonic epithelial malignancies with β-catenin mutations within 16 weeks. Also, our findings suggest the importance of inflammation caused by DSS exposure in mouse colon carcinogenesis under this experimental condition. The experimental protocol described here could be applied to investigate colonic carcinogenesis induced by environmental carcinogens and/or modulators and to genetic analysis of the susceptibility to colon tumorigenesis. Such collaborating studies with other laboratories are on-going in order to fight against colon cancer development.

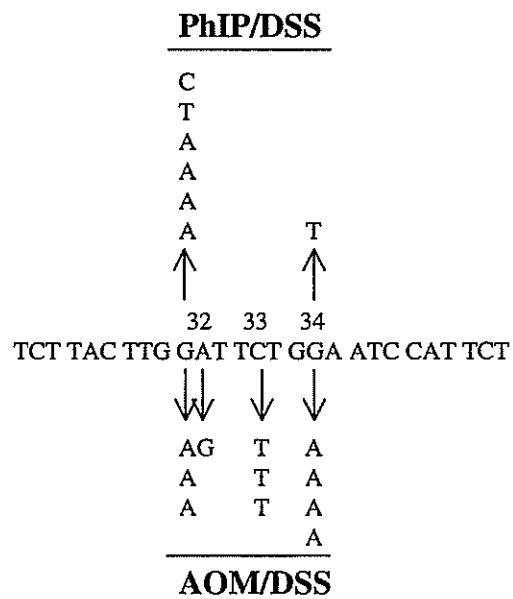

Fig. 9. Sequence of β-*catenin* exon 3 with the codon numbers and β-*catenin* mutations found in PhIP/DSS-, MeIQx/DSS- and AOM/DSS-induced mouse colonic tumors.

Acknowledgements

We express our thanks to the staff of the Research Animal Facility. We also thank Mrs Sotoe Yamamoto for her secretarial assistance. This study was supported in part by the Grant-in-Aid 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 (nos 152052 and 15592007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the grants (H2004-6 and C2004-4) from Kanazawa Medical University.

References

1. Sugimura,T. (2000) Nutrition and dietary carcinogens. *Carcinogenesis*, **21**, 387–395.
2. Kotake,K., Honjo,S., Sugihara,K., Kato,T., Kodaira,S., Takahashi,T., Yasutomi,M., Muto,T. and Koyama,Y. (2003) Changes in colorectal cancer during a 20-year period: an extended report from the multi-institutional registry of large bowel cancer, Japan. *Dis. Colon Rectum*, **46** (10 suppl.), 532–543.
3. Tamakoshi,K., Tokudome,S., Kuriki,K., Takekuma,K. and Toyoshima,H. (2001) Epidemiology and primary prevention of colorectal cancer (in Japanese). *Jpn. J. Cancer Chemother.*, **28**, 146–150.
4. Nagao,M. (1999) A new approach to risk estimation of food-borne carcinogens–heterocyclic amines-based on molecular information. *Mutat. Res.*, **431**, 3–12.
5. Sugimura,T. (1997) Overview of carcinogenic heterocyclic amines. *Mutat. Res.*, **376**, 211–219.
6. Ohgaki,H. (2000) 7.1 Rodents. In Nagao,M. and Sugimura,T. (eds), *Food Borne Carcinogens: Heterocyclic Amines*. John Wiley & Sons, Ltd, New York, NY, pp. 198–228.
7. Weisburger,J.H. (2002) Comments on the history and importance of aromatic and heterocyclic amines in public health. *Mutat. Res.*, **506/507**, 9–20.
8. Wakabayashi,K., Nagao,M., Esumi,H. and Sugimura,T. (1992) Food-derived mutagens and carcinogens. *Cancer Res.*, **52** (suppl.), 2092s–2098s.
9. Gerhardsson de Verdier,M., Hagman,U., Peters,R.K., Steineck,G. and Overvik,E. (1991) Meat, cooking methods and colorectal cancer: a case-referent study in Stockholm. *Int. J. Cancer*, **49**, 520–525.
10. Sinha,R. and Rothman,N. (1997) Exposure assessment of heterocyclic amines (HCAs) in epidemiologic studies. *Mutat. Res.*, **376**, 195–202.
11. Sinha,R. and Norat,T. (2002) *Meat Cooking and Cancer Risk, Volume 156*. IARC Scientific Publications, IARC, Lyon, pp. 181–186.