

TABLE II - INCIDENCE AND MULTIPLICITY OF SMALL INTESTINAL POLYPS AT WEEK 5

Genotype	Sex	Incidence (multiplicity) of small intestinal polyps at						Total	
		Proximal region		Middle region		Distal region		2% DSS	Tap water
		2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water		
<i>Apc^{Min/+}</i>	Male	7/7, 100% (9.4±2.4) ¹	9/9, 100% (9.1±2.1)	7/7, 100% (11.6±2.6) ²	9/9, 100% (16.0±3.7)	7/7, 100% (42.9±10.4) ³	9/9, 100% (24.7±5.8)	7/7, 100% (64.3±13.3) ²	9/9, 100% (49.8±9.8)
	Female	7/7, 100% (7.2±2.4)	10/10, 100% (8.5±2.3)	7/7, 100% (8.7±2.3) ⁴	10/10, 100% (14.1±3.0)	7/7, 100% (35.3±4.8) ⁴	10/10, 100% (20.0±5.1)	7/7, 100% (51.2±5.4) ⁵	10/10, 100% (42.6±9.3)
<i>Apc^{+/+}</i>	Male	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)
	Female	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)

¹The number of polyps per mouse (Mean±SD).-²Significantly different from *Apc^{Min/+}* males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).-³Significantly different from *Apc^{Min/+}* males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).-⁴Significantly different from *Apc^{Min/+}* females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).-⁵Significantly different from *Apc^{Min/+}* females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).

TABLE III - SCORES OF INFLAMMATION AND NITROTYROSINE IMMUNOHISTOCHEMISTRY OF COLONIC MUCOSA AT WEEK 5

Genotype	Sex	Score of inflammation (number of mice examined)		Score of nitrotyrosine- immunohistochemistry (number of mice examined)	
		2% DSS	Tap water	2% DSS	Tap water
		<i>Apc^{Min/+}</i>	Male	2.86±0.69 ^{1,2} (7)	0.22±0.44 (9)
Female	2.14±0.69 ³ (7)		0.20±0.42 (10)	2.14±0.69 ³ (7)	0.10±0.32 (10)
<i>Apc^{+/+}</i>	Male	2.33±0.65 ⁴ (12)	0.24±0.44 (17)	2.25±1.06 ⁴ (12)	0.12±0.33 (17)
	Female	2.10±0.74 ⁵ (10)	0.18±0.41 (11)	2.14±0.69 ⁵ (10)	0.09±0.30 (11)

¹Mean ± SD.-²Significantly different from *Apc^{Min/+}* males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).-³Significantly different from *Apc^{Min/+}* females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).-⁴Significantly different from *Apc^{+/+}* males received tap water by one-way ANOVA with Bonferroni correction ($P < 0.01$).-⁵Significantly different from *Apc^{+/+}* females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.001$).

Immunoreactivity of nitrotyrosine was noted in the cryptal cells with or without disruption, infiltrated mononuclear inflammatory cells, and endothelial cells of the small vessels in the colonic mucosa and submucosa in *Apc^{Min/+}* and *Apc^{+/+}* mice that received 2% DSS. Among them, the stainability was strong in the infiltrated mononuclear inflammatory cells. Adenoma cells (Fig. 3j), adenocarcinoma cells (Fig. 3k) and dysplastic cryptal cells (Fig. 3l) also showed moderately positive immunoreactivity of nitrotyrosine in their cytoplasm. The intensity in the colonic lesions in *Apc^{Min/+}* mice given 2% DSS was strong when compared to that observed in *Apc^{+/+}* mice given tap water alone. As summarized in Table III, scores of nitrotyrosine-immunoreactivity in the colonic mucosa of *Apc^{Min/+}* and *Apc^{+/+}* mice of both sexes given 2% DSS were significantly greater than those given tap water alone ($p < 0.001$). The score in the time-course observation indicated that the value decreased after the cessation of 2% DSS (Fig. 2e), as was the value of inflammation (Fig. 2d).

p53 immunoreactivity was observed in the nuclei of neoplastic cells (adenoma and adenocarcinoma cells) with a variety of stainability, which developed in the colon of *Apc^{Min/+}* mice treated with DSS (Fig. 3m,n) but not in those given tap water alone. Also, the nuclei of dysplastic crypts were positive for p53 antibody (Fig. 3o). Surrounding the mucosal ulcer, some nuclei of regenerative hyperplastic crypts in the colon were weakly positive for p53 antibody in the colon of *Apc^{Min/+}* mice treated with DSS (data not shown). No stainability of p53 was observed in the small intestinal polyps (data not shown) in *Apc^{Min/+}* mice treated with or without DSS.

Apc allelic loss in colonic neoplasms

One hundred percent (14 of 14) of adenocarcinomas and 0% (0 of 3) of histologically normal colonic mucosa from male *Apc^{Min/+}* mice that received 2% DSS showed LOH of *Apc*. In male *Apc^{Min/+}*

mice that received tap water alone, 100% (2 of 2) of adenocarcinomas showed LOH of *Apc* and 0% (0 of 3) of histologically normal colonic mucosa was negative for LOH.

Mutation of β -catenin and K-Ras genes

β -Catenin and K-ras mutations were not detected in any of the colonic adenocarcinomas examined.

Discussion

In our study, we investigated the influences of the inflammation induced by 1-week exposure of 2% DSS in the drinking water on intestinal carcinogenesis in *Apc^{Min/+}* mice and found that the treatment resulted in a much higher incidence and multiplicity of large intestinal neoplasms in *Apc^{Min/+}* mice up to 5 weeks. Also, the treatment significantly increased the number of small intestinal polyps (tubular adenomas) at the distal regions. Thus, we developed an *Apc^{Min/+}* mouse model with multiple colonic neoplasms, which develop within 4 weeks after 1-week exposure DSS, in addition to the increase in the number of small intestinal polyps. Regardless of the types of gene and gender, all mice treated with 2% DSS had intestinal mucosal inflammation with various degrees. However DSS treatment did not induce preneoplastic and neoplastic lesions in the large bowel wild-type (*Apc^{+/+}*) mice of either sex. This report describing rapid development of a number of colonic neoplasms in *Apc^{Min/+}* mice within a short-term period (5 weeks) support an earlier work by Cooper *et al.*,¹⁴ who found that treatment with 2 cycles of 4% DSS results in 40% incidence of colon cancer with a multiplicity of 0.67±0.27 in female Min mice at 42 days. Our findings suggest that the development of colonic dysplastic crypts and/or neoplasms in the short-term (up to 5 weeks) needs both the gene (*Apc*) mutation and subsequent inflammatory stimuli, but not either alone under the current exper-

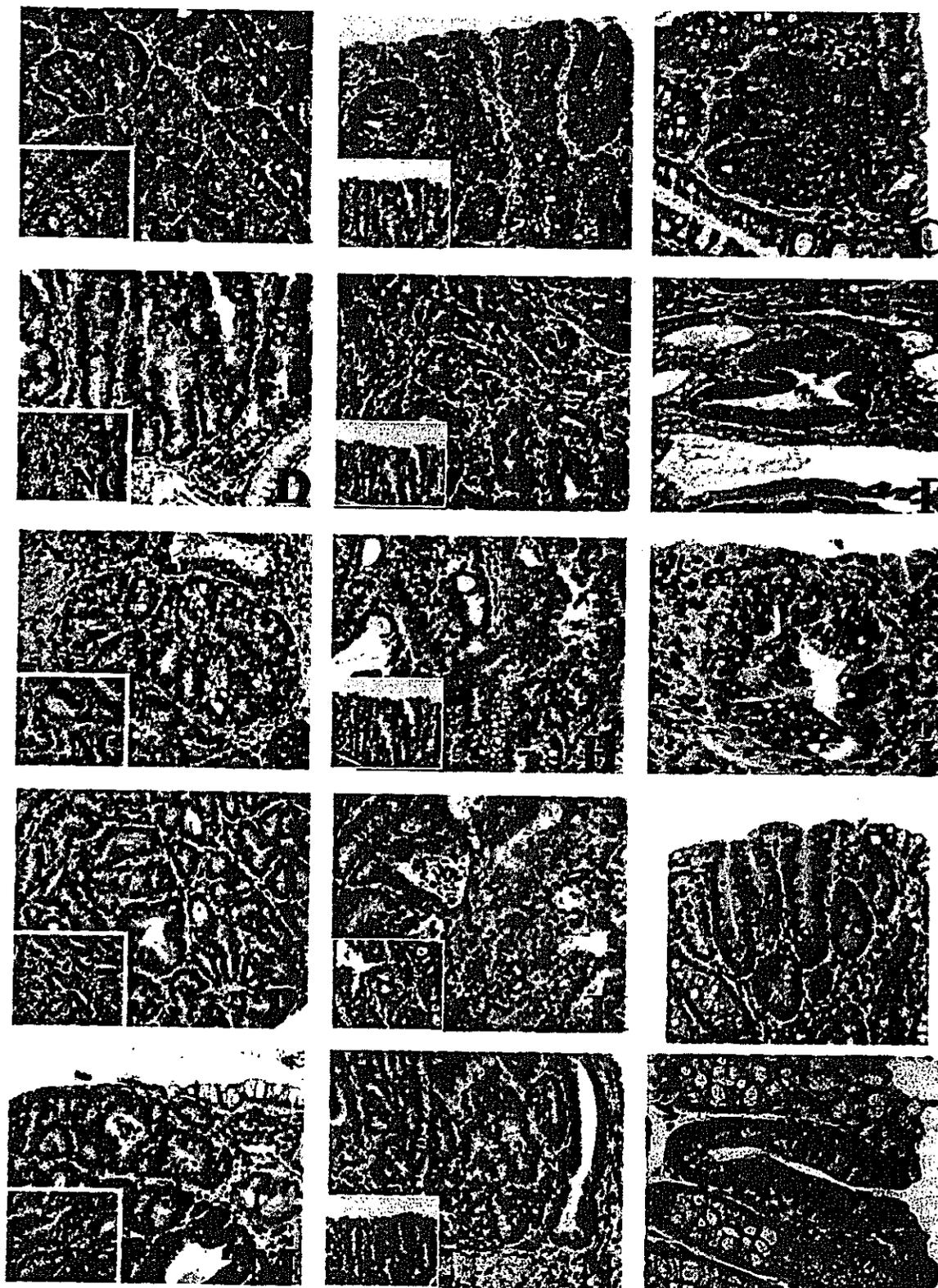


FIGURE 3 – Immunohistochemistry of the colonic lesions developed in male *Apc*^{Min/+} mice treated with 2% DSS. (a)–(c), β-catenin immunohistochemistry; (d)–(f), COX-2 immunohistochemistry; (g)–(i), iNOS immunohistochemistry; (j)–(l), nitrotyrosine immunohistochemistry and (m)–(o), p53 immunohistochemistry. Adenomas (a, d, g, i and m), adenocarcinomas (b, e, h, k and n), and dysplastic crypts (c, f, j, l and o) show positive reaction with a variety of intensity against β-catenin, COX-2, iNOS, nitrotyrosine and p53 antibodies. Inserts of a, d, g, j and m are negative controls (NC) immunostained without antibodies show negative reactions. Inserts of b, e, h, k and n are immunohistochemistry of adenocarcinomas developed in *Apc*^{Min/+} mice given tap water. Original magnification: (a), (b), (d), (e), (g), (h), (j), (k), (l), (m) and (n), ×100; (c), (f), (i) and (o), ×200; inserts, ×200.

imental condition. Our results also support the findings of our previous works,^{8,9,12} suggesting the importance of inflammatory stimuli as a promotion event after the initiation events (genetic alterations) in colon carcinogenesis. There were no differences between males and females in the effects of DSS on large and small intestinal carcinogenesis of *Apc*^{Min/+}, and the histopathology of colonic lesions including neoplasms was similar in both sexes.

As for the development of small intestinal polyps, treatment with DSS significantly increased their number and size, especially at the distal portion of the small intestine. Macrophages engulfing DSS particles were observed in the large intestine and surrounding lymph nodes of mice 1 day after DSS exposure, and then found in the jejunum and ileum 7 days after DSS treatment.⁵¹ In our study, mild mucosal inflammation was observed in the distal portion of the small intestine of mice given 2% DSS. Thus, DSS could also influence the formation of small intestinal polyp in *Apc*^{Min/+} mice. The Min mouse has been regarded as a human FAP model in spite of the fact that the polyps (adenomas) develop in the small intestine. Although the biological pathways in human colon and Min intestine are assumed to be similar, our model described here could be applied for investigation of the genesis, pathophysiology and chemoprevention of human FAP and/or inflammation-related colon tumorigenesis.

In our study, sequential observation on the pathological alteration in the large intestines of female *Apc*^{Min/+} mice after 1-week exposure to 2% DSS revealed that the frequencies of dysplastic crypts and colonic neoplasms (adenoma and adenocarcinoma) gradually increased over time (Fig. 2a,b), indicating that dysplastic crypts⁴³ or adenomatous lesions⁴¹ are precursor lesions for colon carcinoma and DSS treatment could promote their growth. The findings support an earlier report by Cooper *et al.*,¹⁴ but their incidence of colonic cancer was low: 22% in Min mice exposed to 1-cycle of DSS (administration 4% DSS for 4 days and H₂O for 17 days) and 40% in Min mice exposed to 2-cycle of DSS. The discrepancy existing in these 2 studies may be due to the differences in the treatment period and the dose and molecular weight of DSS. In the present study, the incidence of colonic adenocarcinoma was 80% at week 4 and 100% at week 5 (Fig. 2a). When compared to our previous study on the effects of DSS on chemically induced colon carcinogenesis,⁹ where we observed 40% and 100% incidences of colonic epithelial malignancy at week 4 and week 6, respectively, in male ICR mice, it is likely that deletion of the *Apc* gene plays an important role in colitis-associated carcinogenesis, as suggested by Cooper *et al.*¹⁴

In our study, we investigated the immunohistochemical expression of β -catenin, COX-2, iNOS and p53, in the colonic lesions developed in *Apc*^{Min/+} mice that received 2% DSS. The results on immunohistochemistry against these antibodies except for p53 expression in the lesions were similar to those observed in our previous studies, where the lesions were induced by AOM^{8,9}, HCAs¹² or DMH¹¹ followed by DSS in ICR mice, suggesting the similarity of histopathology and immunohistochemistry, and biological nature of the lesions observed in ICR mice given a colonic carcinogen and DSS and *Apc*^{Min/+} mice treated with DSS. Increased immunohistochemical expression of COX-2 and iNOS in the colonic tumors of either *Apc*^{Min/+} mice that received 2% DSS was confirmed by reverse transcription-polymerase chain reaction (data not shown). The findings of nitrotyrosine immunohistochemistry in the current study are also in accordance with those in our previous study⁹ and suggest that oxidative/nitrosative stress strongly promotes the development of colonic neoplasms in *Apc*^{Min/+} mice. iNOS has been shown to be the only isoform involved in stimulating tumor growth, probably through an increase in vascular endothelial growth factor production.⁵² Moreover, NO regulates COX-2 expression.⁵³ Our results on the immu-

nohistochemistry of iNOS and COX-2 indicate that the inflammatory response, the interaction between NO synthase and COX pathways may stand at the center of the pathophysiological basis of inflammation-related colon carcinogenesis in *Apc*^{Min/+} mice treated with DSS, as are the cases of inflammatory diseases,⁵⁴ and chemically induced colon carcinogenesis.⁵³

In the current study, we also screened for mutations of β -catenin and K-ras in colon tumors developed in male *Apc*^{Min/+} mice. In contrast with previous reports,^{12,53,55} we did not detect the mutations of these genes in any of the colonic adenocarcinomas examined. However, our results are not surprising. Suzui *et al.*⁵⁶ reported that adenocarcinomas developed in *Apc*^{Min/+} mice treated with AOM did not have β -catenin gene mutations. In our study, cytoplasmic and/or nuclear accumulation of β -catenin protein was detected in the colonic neoplasms, but β -catenin gene mutations were not present. In the FAP patients, mutations of *APC* are common, but mutations of β -catenin were rare.^{57,58} In addition, β -catenin germline mutations were not found in FAP patients with germline *APC* mutations.⁵⁷ Thus, concerning the β -catenin mutation, the colon tumors developed in the current animal model may imitate the colon carcinogenesis as in the FAP patients, that is, by a second hit in the *APC* gene such as loss of *Apc*⁺ allele or somatic mutations in the *Apc* gene. Immunohistochemical staining with an antibody for the C-terminal of *Apc* showed the loss of wild-type *Apc* in colonic tumors in *Apc*^{Min/+} mice (data not shown). As for the mutation of K-ras, no mutations were found in the colonic adenocarcinomas examined in the current study. Our results on K-ras mutations are in accordance with IBD-related colon carcinogenesis⁵⁹ and suggest that activation of the K-ras gene is not essential for the development and growth of colonic neoplasms in our model.

p53 gene mutation occurs in the late stage of human colon carcinogenesis.^{33,59} In our study, p53 immunohistochemistry revealed positive reaction in the nuclei of neoplastic cells in *Apc*^{Min/+} mice treated with DSS, although we did not examine its mutation in our study. The accumulation of p53 shown in our study is interesting and may be important for colon cancer development in *Apc*-deficient mice, since an increased p53 mutation load in the inflamed colon tissue from UC patients being a high-risk for colon cancer,⁶⁰ and a potential mechanism link between NO and p53 in UC and sporadic colon cancer⁶¹ were reported. In addition, COX-2, iNOS and p53 are suggested to be fundamental "play-makers" of the angiogenesis processes.⁶²

Taken together, our results suggest that a novel *Apc*^{Min/+} mouse model with DSS may provide new insight into the genesis and chemoprevention of colon cancer development in FAP patients. In our model, a single allele *Apc* gene followed by appropriate promotional stimuli is sufficient for the development and growth of colonic neoplasms in *Apc*^{Min/+} mice, and COX-2, iNOS, p53, oxidative/nitrosative stress and interactions of these may play important roles in colon carcinogenesis in *Apc*^{Min/+} mice given DSS. Our model can be applied for investigating the pathogenesis in carcinogenesis of IBD, since the Wnt/ β -catenin signaling pathway may be involved in carcinogenesis of UC.^{59,63} Our ongoing microarray analysis will provide new information of the mechanism(s) for the effects of DSS on large and small intestinal tumorigenesis in *Apc*^{Min/+} mice.

Acknowledgements

Dr. R. Suzuki is a Research Fellow of the Japan Society for the Promotion of Science, 6 Ichiban-cho, Chiyoda-ku, Tokyo 102-8471, Japan.

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Cancer Letters 238 (2006) 69–75

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Lack of enhancing effects of degraded λ -carrageenan on the development of β -catenin-accumulated crypts in male DBA/2J mice initiated with azoxymethane

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Received 10 February 2005; received in revised form 28 May 2005; accepted 13 June 2005

Abstract

Effect of degraded λ -carrageenan, which induces colitis in rodents, on the development of β -catenin-accumulated crypts (BCAC) being putative precancer lesions of colon cancer was investigated in male DBA/2J mice initiated with azoxymethane (AOM). In a preliminary experiment, male DBA/2J mice among seven different strains (A/J, BALB/c, C3H/HeN, C57BL/6J, CBA/N, DBA/1J, and DBA/2J) of male mice were most sensitive to degraded λ -carrageenan. Therefore, male DBA/2J mice were intraperitoneally injected AOM (10 mg/kg body weight), and then 2% degraded λ -carrageenan in drinking water for one or two weeks, starting one week after dosing of AOM. Thereafter animals were no further treated up to week 26. At week 26, the frequency of BCAC in the colonic mucosa was 12.50 ± 2.46 in the AOM alone group, 11.30 ± 3.50 in the AOM/degraded λ -carrageenan (for one week) group, and 11.60 ± 2.27 in the AOM/degraded λ -carrageenan (for two weeks) group. The findings suggest that degraded λ -carrageenan treatment for one or two weeks did not affect the occurrence of BCAC. Our results may indicate no enhancing or promoting effects of degraded λ -carrageenan on colon carcinogenesis in mice initiated with AOM. © 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Azoxymethane; Carrageenan; β -catenin-accumulated crypts (BCAC); Mice

1. Introduction

Carrageenan (CARR) is a sulfated polysaccharide extracted from a variety of red seaweeds and

consisting mainly of varying amounts of the ammonium, calcium, magnesium, potassium or sodium salts of sulfate esters of galactose and 3,6-anhydrogalactose copolymers [1]. The principle copolymers are designated κ -, λ - and ι - and differ both in structure and in their ability to form gels upon the addition of potassium ions to dilute solutions of carrageenan [2]. Native CARR is used in foods,

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cosmetics, pharmaceuticals and other products in which their ability to stabilize mixtures, emulsify ingredients and thicken or gel solutions are utilized. Degraded CARR (dCARR) has been produced from extracts of *Eucheuma spinosum*, the principal component of which is ι -CARR, by treatment with dilute hydrochloride. It has an average molecular weight of 20,000–40,000. dCARR has long been used as an antipeptic agent in Europe [3]. Oral administration of dCARR induces mucosal ulceration of the large intestine of mice [4], guinea pigs [5], rabbits [6], rats [7], and rhesus monkeys [8]. Furthermore, Fabian et al. [9] reported colorectal squamous cell metaplasia and colon adenomatous polyps in rats by prolonged oral administration of dCARR.

Relationship between inflammation and cancer development is noted for a long time [10]. Examples are hepatocellular carcinoma/chronic hepatitis with hepatitis C and B viruses infection [11,12], gastric cancer/*H. pylori* infection [13], human papilloma virus infection/uterine cervical cancer [14], and colon cancer/inflammatory bowel disease including ulcerative colitis and Crohn's disease [15]. Recently we have developed a new mouse model for colitis-related colon carcinogenesis [16], and have found a powerful tumor-promoting effect of dextran sodium sulfate (DSS, molecular weight: 40,000) on colon carcinogenesis. In our studies, mice were given a genotoxic colonic carcinogen azoxymethane (AOM) [16], 1,2-dimethylhydrazine (DMH) [17] or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine [18], and followed by one-week treatment of a colitis-induced agent DSS (molecular weight 40,000) and the treatments resulted in a high incidence of colonic tumors. This model can be used to determine the tumor-initiation [17,18], tumor-promotion [19,20], and tumor-inhibition activities of xenobiotics [21], when the experimental protocol is slightly modified.

In an experimental animal model of colitis or ulcerative colitis, CARR as well as DSS is widely used [22]. Colonic inflammation produced by oral administration of 1.5% solution of degraded λ -carrageenan (d- λ CARR) is similar to that found in human inflammatory bowel disease [23]. Although carcinogenicity [24,25] and tumor-promoting ability [26] of dCARR in the colon were previously reported, such effects become a mark of an alteration [27,28]. However, in the colonic mucosa with colitis induced

by d λ CARR, IgG- and IgM-containing cells were increased in number, as observed in human ulcerative colitis [29]. Development of a focal but high-grade dysplasia with mild inflammation in the colon was reported when 1% d- λ CARR in drinking water was given to rabbits [30]. Suzuki et al. reported that d- λ CARR could inhibit gap junctional intercellular communication in rat liver epithelial cells. Their findings may suggest tumor-promoting effects of d- λ CARR, since most tumor-promoting compounds are known to inhibit gap junctional intercellular communication [31].

In the current study, we conducted an experiment to determine whether degraded d- λ CARR with about 30,000 molecular weight exerts tumor-promoting effects on colon tumorigenesis in mice initiated with AOM [16]. In this experiment we utilized β -catenin-accumulated crypts (BCAC) as an endpoint biomarker for colon tumorigenesis, since BCAC are reliable biomarkers for detecting the modifying effects of xenobiotics on colon carcinogenesis in mice and rats [32–35].

2. Materials and methods

2.1. Animals, chemicals, and diet

Seven strains (A/J, BALB/c, C3H/HeN, C57BL/6J, CBA/N, DBA/1J, and DBA/2J mice) of mice used in this study were obtained from Japan SLC, Inc., Shizuoka, Japan. AOM was purchased from Sigma Chemical Co., St Louis, MO, USA and d- λ CARR (30,000 molecular weight, Cat no.030-14251) from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. All mice used for the experiment were maintained in the well-controlled room with a high-efficiency particulate air (HEPA) filter, a 12 h lighting (7:00–19:00), 25 ± 2 °C room temperature, and $55 \pm 15\%$ humidity. Mice (5 mice/cage) were housed in polycarbonate cages measuring $W225 \times D338 \times H140$ mm (Japan CLEA, Inc., Tokyo, Japan) with the floor covered with a sheet of roll paper (Japan SLC). MF (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as a basal diet throughout the study. Groundwater that was chlorine-treated and subjected to ultraviolet disinfection was used as drinking water in a bottle, except when d- λ CARR was

administered. In handling the mice for the experiment, we fully complied with the 'Guidelines Concerning Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

2.2. Experimental procedure

Before the study, a preliminary experiment was conducted: seven different strains of mice were treated with 2% d- λ CARR to determine their sensitivity to the chemicals, since there are no reports on species difference of d- λ CARR-induced colitis in mice. We selected 2% as a dose of d- λ CARR in this study, since the dose is the highest to dissolve in tap water. Ten mice of each strain of mice were given 2% d- λ CARR in drinking water for one week whether this treatment could induce colitis in these strains of mice, and then they were sacrificed one week after d- λ CARR exposure for examining the presence of colitis. Histopathology of their colon revealed that inflammation was found in the distal colon of DBA/1J and DBA/2J mice: the degree (grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa) of colitis in DBA/2J mice was greater than that (grade 1, shortening and loss of the basal one-third of the actual crypts with mild

inflammation and edema in the mucosa) in DBA/1J mice, based on the criteria (grades 0–4) described by Cooper et al. [36]. However, colitis was not observed in the colon of other strains.

Based on these results, we used DBA/2J mice to determine modifying effect of d- λ CARR on the occurrence of BCAC in the colon (Fig. 1). Thirty-five DBA/2J mice (Japan SLC Inc.) mice were divided into four groups after one-week acclimatization. Group 1 (10 mice) was given a single intraperitoneal injection of 10 mg/kg body weight AOM. Groups 2 (ten mice) and 3 (ten mice) were treated with AOM, and followed by one-week and two-week exposure of 2% d- λ CARR in drinking water, respectively. They received no further treatment thereafter and sacrificed at week 26. Group 4 (five mice) served as untreated control.

2.3. Histopathology and immunohistochemistry

All animals were sacrificed at the end of the study (week 26) and their large bowels were flushed with saline, and excised. The length of large bowels (from the ileocecal junction to the anal verge) was measured. They were cut open longitudinally along the main axis, and then washed saline. The large bowels were macroscopically inspected, cut, fixed in 10% buffered formalin for at least 24 h, and routinely processed for making paraffin-embedded sections for

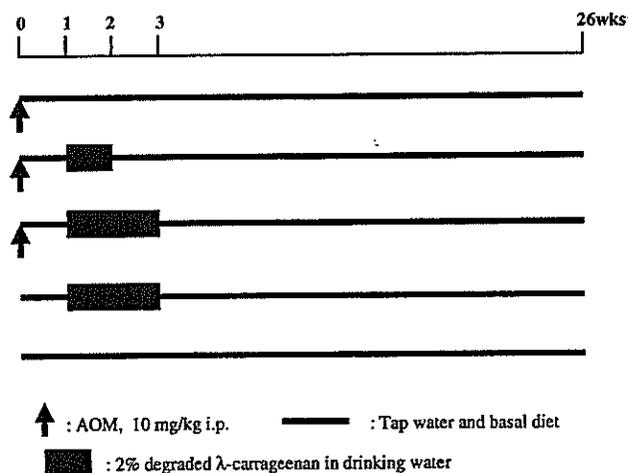


Fig. 1. Experimental protocol.

histopathology. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections. For histopathological examination, immunohistochemistry of BCAC, and enumeration of BCAC, large bowel was divided into the four segments: 0–2 cm (distal), 2–4 cm (distal–middle), 4–6 cm (proximal–middle), and 6–9 cm (proximal) each from the anal verge—whereby they were embedded in such a way that they could be thinly sliced off along the horizontal surface of the large intestinal mucosa. Then, six serial sections from each segment were subjected to H&E. (one section) and β -catenin immunohistochemical staining (five sections) [35].

Immunohistochemistry for β -catenin was performed on 4- μ m-thick paraffin-embedded sections from all segments of the colons, using the labeled streptavidin-biotin method (LSAB KIT; DAKO, Glostrup, Denmark) or TRITC-conjugated secondary antibody (DAKO) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65 °C, deparaffinized in xylene, and rehydrated through graded alcohols at room temperature. A 0.05 M Tris-HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4 °C with a primary antibody against β -catenin protein (diluted 1:1000, Transduction Laboratories, Lexington, KY, USA). Horseradish peroxidase activity was visualized by treatment with H₂O₂ and diaminobenzidine for

5 min. Negative control sections were immunostained without the primary antibody. Immunoreactivities were regarded as positive if the apparent stainings were detected in cytoplasm and/or nuclei for determining BCAC.

3. Results

No clinical signs of toxicity due to the treatments were noted in mice. Also, there were no changes of weight gains of mice in any group during the study (data not shown). At sacrifice shortening of the large bowels, which is a typical finding caused by colitis-induced compounds, of mice treated with d- λ CARR was not observed (Table 1). Although macroscopic and microscopic examinations did not reveal the development of colonic neoplasms, a large number of BCAC that were detected on H&E-stained (Fig. 2a) and β -catenin immunohistochemical stained (Fig. 2b)-sections were present in the large intestinal mucosa of mice in the groups given AOM. The frequencies of BCAC in all groups are summarized in Table 1. The mean numbers of BCAC per colon did not significantly differ among the groups. The distribution of BCAC in the colon is also listed in Table 1. The highest frequency of BCAC developed in the distal part of the colon in all groups given AOM, and the order was found in the distal > distal–middle > proximal–middle > proximal. The differences in the number of BCAC and the distribution of BCAC were not statistically significant among the groups.

Table 1
Effects of degraded λ -carrageenan (d- λ CARR) on the length of large bowel and occurrence of β -catenin-accumulated crypts (BCAC) in male DBA/2J mice

Treatment	No. of mice examined	Length of large bowel (cm)	No. of BCAC \pm /segment)				
			Total	Proximal	Proximal–middle	Distal–middle	Distal
AOM alone	10	14.00 \pm 1.25 ^a	12.50 \pm 2.46	0.40 \pm 0.52	2.80 \pm 1.32	3.30 \pm 1.34	6.00 \pm 1.41
AOM \rightarrow 2% d- λ CARR \pm one week)	10	14.07 \pm 1.29	11.30 \pm 3.50	0.50 \pm 0.71	2.40 \pm 1.17	3.20 \pm 1.40	5.20 \pm 1.75
AOM \rightarrow 2% d- λ CARR \pm two week)	10	14.05 \pm 0.52	11.60 \pm 2.27	0.30 \pm 0.48	2.70 \pm 0.98	3.20 \pm 1.14	5.40 \pm 1.65
None	5	13.40 \pm 0.82	0	0	0	0	0

^a Mean \pm SD.

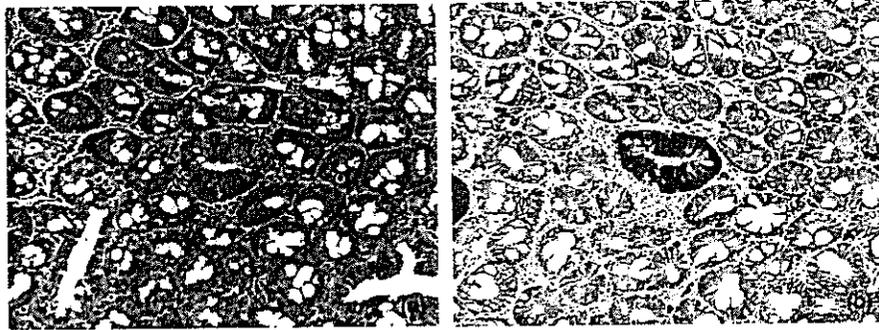


Fig. 2. Histopathology (a) and immunohistochemistry (b) of BCAC developed in the colon of a male DBA/2J mouse treated with AOM. (a) H & E stain and (b) β -catenin immunohistochemistry, Original magnification, (a) and (b), $\times 100$.

4. Discussion

BCAC develop when β -catenin protein accumulates in large volume in the cytoplasm and/or the nuclei as a result of suppressed phosphorylation of β -catenin protein due to an abnormality in the Wnt signaling pathway [32,33,35]. In addition to aberrant crypt foci (ACF) [37–40], BCAC are considered as reliable precancerous lesions for colonic adenocarcinoma [35, 41,42]. The lesions are now used as one of the biomarkers for determining the modifying effects of xenobiotics on colon carcinogenesis in rodents [34], since in spite of ACF the lesions pose the biological nature closed to that of colonic neoplasms [35,41,42].

CARR is widely used in commercial food preparation to improve the texture of processed foods. CARR is classified into three groups, κ -, ι -, and λ -CARR, by its chemical structure. While κ -CARR and ι -CARR produce gels according to their 3,6-anhydro ring in the sugar, λ -CARR does not form gels in water, but interacts strongly with proteins. λ -CARR is added in various milk products (up to 2% of concentration by weight chocolate milk), and in meat products (up to 0.5% in ham) as an emulsifier, stabilizer, or thickener in the food industry. A wide variety of biological effects of CARR, including those on carcinogenesis were reported in rodents, but most were those of intact (undegraded) CARR. Although a review to doubt safety of CARR was published recently [27], but the safety is emphasized in another review article [28]. CARR ingested in the gel form is stable to the conditions of passage through the digestive tract. Because of its large molecular weight, CARR

remains within the lumen of the digestive tract and is not absorbed [43]. Therefore, low molecular weight of CARR, such as dCARR, should be used for estimating its biological effects including those on carcinogenesis.

As for the effects of λ CARR on tumorigenesis, λ CARR was suspected to have a role in dietary mechanism of mammary carcinogenesis by the findings that λ -CARR affects cell death of human myoepithelial cells in an in vitro study [44]. Also, λ -CARR inhibited gap-junctional intracellular communication in rat liver epithelial cells in an in vitro assay system [45]. These reports may suggest the involvement of λ -CARR in carcinogenesis, especially the latter suspected its tumor-promoting effects. However, our results described here may suggest lack of tumor-promotion effects of d- λ CARR on colon carcinogenesis. In the current study, the duration of d- λ CARR exposure was limited for evaluating the tumor-promoting ability of xenobiotics. However, in our mouse colon carcinogenesis model [16], the short-period of administration of test compounds after the initiation is enough to enhance colon tumor development, if they are colitis-inducing agents. d- λ CARR in drinking water at a dose level of 1% could induce colitis as well as cryptal dysplasia in rabbits [30]. Our preliminary experiment also confirmed that one-week exposure of 2% d- λ CARR in drinking water is able to induce colitis in DBA/1J and DBA/2J mice. Therefore, the administration period of d- λ CARR in the current study could be accepted for assessing its modifying effects on colon tumorigenesis under the present experimental condition.

Our findings described are in line with those reported by Hagiwara et al. [46], who found no

tumor-promoting activity of λ -CARR (Viscarin S) in rat colon carcinogenesis at doses up to 5% in diet for 32 weeks after the initiation with DMH, although their experimental condition differed from our own. While they used the incidence and multiplicity of colonic tumors as the end-point for their experiment, we employed BCAC, that is a reliable precursor lesion for colonic epithelial malignancies [41,42], as the end-point biomarker.

In conclusion, the present study demonstrated that one-week or two-week administration of 2% d- λ CARR in drinking water after the initiation with AOM did not affect the development of BCAC in male DBA/2J mice, suggesting lack of modifying effect of d- λ CARR on colon carcinogenesis.

Acknowledgements

We wish to thank Kyoko Takahashi, Tomoko Kajita and Misato Yasuda for their technical assistance, and Yoshihisa Wakita and Shinya Tanahashi for care of the animals. 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.

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β -Catenin-accumulated crypts in the colonic mucosa of juvenile $Apc^{Min/+}$ mice

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Received 2 May 2005; received in revised form 25 July 2005; accepted 28 July 2005

Abstract

Although $Apc^{Min/+}$ mice are widely used for an animal model of human familial adenomatous polyposis (FAP), a majority of intestinal polyps locate in the small intestine. We recently reported that numerous β -catenin-accumulated crypts (BCAC), which are reliable precursor lesions for colonic adenocarcinoma, develop in the large bowel of aged $Apc^{Min/+}$ mice. In this study, we determined the presence and location of BCAC in the large intestine of juvenile $Apc^{Min/+}$ mice (3 and 5 weeks of age). Surprisingly, BCAC were noted in the colon of even $Apc^{Min/+}$ mice of both ages, and mainly located in the distal and middle segments of the colon. Also, a few microadenomas were detected in $Apc^{Min/+}$ mice of 5-week old. Our results may indicate need of further investigation of the colorectal mucosa of $Apc^{Min/+}$ mice for examining colorectal carcinogenesis using $Apc^{Min/+}$ mice.

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Keywords: β -Catenin-accumulated crypts; C57BL/6J- $Apc^{Min/+}$; Juvenile mice

1. Introduction

Aberrant crypt foci (ACF) and BCAC are widely used as markers for evaluating colorectal carcinogenic risk in rodents [1,2] and humans [3]. ACF proposed by Bird [1,2], are morphologically distinguished from their surrounding crypts on colonic mucosa stained

with methylene blue [1]. While they are considered as putative precursor lesions for colonic adenocarcinoma and frequently used for preclinical cancer chemoprevention studies [4,5], we have recently proposed that β -catenin-accumulated crypts (BCAC) rather than ACF are reliable precancerous lesions for colonic adenocarcinoma [6–8].

Mutant mouse lineage being predisposed to $Apc^{Min/+}$ is regarded as one of the models for colorectal tumorigenesis [9]. Originally, this lineage was established from an ethylnitrosourea-treated

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C57BL/6J male mouse, and its phenotype is a fully penetrant autosomal dominant trait. The dominant mutation is known to be located in *Apc*, the mouse homologue of the human *APC* gene, resulting in truncation of the gene product at amino acid 850 [10]. Although homozygous *Apc*^{Min/Min} mice die as embryos [11], *Apc*^{Min/+} mice develop multiple intestinal tumors in the small intestine. However, unlike in human familial adenomatous polyposis (FAP), colonic neoplasms are rarely detected in the large intestine. We previously documented that numerous BCAC, which could be useful for detecting the modifying effects of xenobiotics in colon carcinogenesis in mice and rats [6,12], develop in the colorectal mucosa of adult *Apc*^{Min/+} mice aged over 20 weeks [13]. The presence of spontaneous ACF [14] or dysplastic ACF (ACF_{Min}) [15] are also known in *Apc*^{Min/+} mice, but their frequency is low.

Since certain hit(s) on the BCAC may lead to tumor development in *Apc*^{Min/+} mice [16], we, in the present study, turned attention to the presence of BCAC in the large bowel of juvenile *Apc*^{Min/+} mice and determined where BCAC develop.

2. Materials and methods

2.1. Animals

The mice were bred at our laboratory, from inbred mice originally purchased from The Jackson Laboratory (Bar Harbor, ME). The *Apc*^{Min/+} pedigree was maintained by mating *Apc*^{+/+} females with *Apc*^{Min/+} males, and procedures to secure inbreeding were followed. The *Apc*^{Min/+} mice were identified by allele-specific PCR on DNA isolated from tail. All mice used for the experiment were maintained in the well-controlled room with a high-efficiency particulate air (HEPA) filter, a 12 h lighting (7:00–19:00), 25 ± 2 °C room temperature, and 55 ± 15% humidity. Mice (5 mice/cage) were housed in polycarbonate cages measuring W225 × D338 × H140 mm (Japan CLEA, Inc., Tokyo, Japan) with the floor covered with a sheet of roll paper (Japan SLC). Water and diet were given ad libitum. The mice were given a basal diet, MF (Oriental Yeast Co., Ltd, Tokyo, Japan), during gestation and until 5 weeks of age. We fully complied with the 'Guidelines Concerning

Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

2.2. Experimental procedure

A total of 54 mice were used in this study: *Apc*^{Min/+} mice of 3 weeks of age (7 females and 8 males) or 5 weeks of age (7 females and 6 males) and *Apc*^{+/+} mice of 3 weeks of age (8 females and 6 males) or 5 weeks of age (5 females and 7 males). The *Apc*^{Min/+} mice were autopsied at 3-week and 5-week of age respectively for measurement of the large intestines as well as detection of ACF and BCAC in the large intestinal mucosa. At sacrifice the large bowels were removed, flushed with saline, fixed flat in 10% buffered formalin for 24 h at room temperature, and then processed for histopathological evaluation by routine procedures [17]. To identify aberrant classical ACF [1], mucosal surface of the colons were stained with methylene blue. In brief, fixed colons were placed in 0.5% solution of methylene blue in distilled water for 30 s. They were then placed mucosal side up on a microscope slide and ACF were counted under a light microscope at a magnification of ×40.

2.3. Tissue preparation

To identify intramucosal lesions ACF and BCAC, colon was divided into three (distal, middle, and proximal) segments and embedded in paraffin. A total of 162 segments were examined by using an en face preparation and 3–5-µm thick serial sections were made [6,7]. For each case, two serial sections were used to analyze the intramucosal lesions.

2.4. Histopathology and immunohistochemistry

Two serial sections were subjected to hematoxylin and eosin (H and E) staining for histopathology and β-catenin immunohistochemistry for enumeration of BCAC [12]. Immunohistochemistry for β-catenin was performed on 4-µm-thick paraffin-embedded sections from all segments of the colons, using the labeled streptavidin-biotin method (LSAB KIT; DAKO, Glostrup, Denmark) with microwave

accentuation. The paraffin-embedded sections were heated for 30 min at 65 °C, deparaffinized in xylene, and rehydrated through graded alcohols at room temperature. A 0.05 M Tris–HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4 °C with a primary antibody against β -catenin protein (diluted 1:1000, Transduction Laboratories, Lexington, KY, USA). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. Negative control sections were immunostained without the primary antibody. Immunoreactivities were regarded as positive if the apparent stainings were detected in cytoplasm and/or nuclei for determining BCAC.

2.5. Statistical analysis

Data on the frequency of BCAC were analyzed by two-way ANOVA, Bonferroni post hoc tests, and a $P < 0.05$ was considered significant.

3. Results

The classical ACF proposed by Bird [1] were not observed in any mice examined, but BCAC (Fig. 1), mostly less than 50 μ m in diameter, were identified in the colon of $Apc^{Min/+}$ mice of both sexes. There were no BCAC in $Apc^{+/+}$ mice of both sexes. As shown in Table 1, the mean numbers of BCAC in 5 weeks old $Apc^{Min/+}$ of both sexes were significantly greater than those in 3 weeks old $Apc^{Min/+}$ mice of both sexes ($P < 0.001$). As for the distribution of BCAC, the lesions mainly located in the distal and middle segments with a few in the proximal part: 3 weeks of age: distal vs. proximal parts, $P < 0.01$ for females and males, middle vs. proximal parts, $P < 0.01$ for females and $P < 0.001$ for males; and 5 weeks of age: distal vs. proximal parts, $P < 0.001$ for females and $P < 0.001$ for males, middle vs. proximal parts, $P < 0.01$ for females and $P < 0.001$ for males). The mean numbers of BCAC at the distal and middle parts in 5 weeks old $Apc^{Min/+}$ of both sexes were also significantly greater than those in 3 weeks old $Apc^{Min/+}$ mice of both sexes (the distal part:

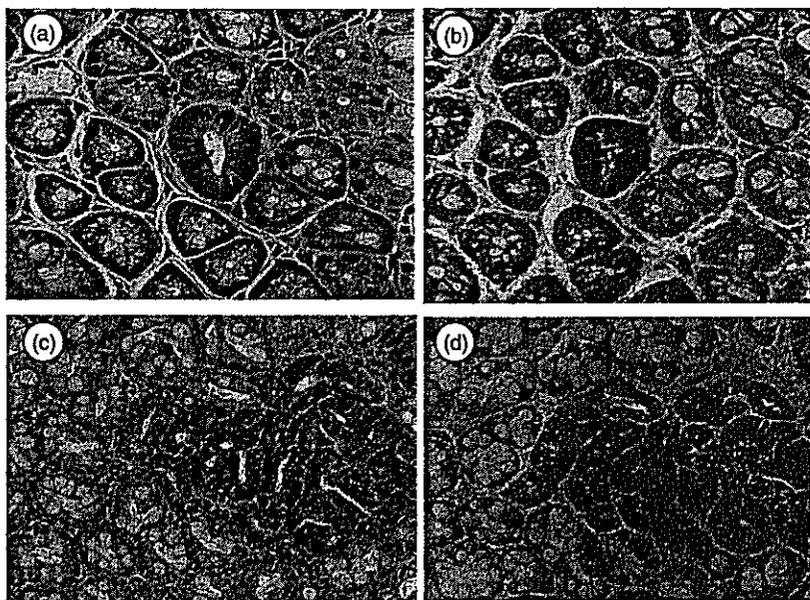


Fig. 1. A BCAC (a, b) found in the middle segment of the colon of a $Apc^{Min/+}$ female mouse aged 5 weeks and a microadenoma (c, d) observed in the middle segment of the colon of a $Apc^{Min/+}$ male mouse aged 5 weeks. A BCAC is consisted of one crypt (a) and positive for β -catenin immunohistochemistry (b). A microadenoma compresses surrounding crypts (c) and shows positive reactivity for β -catenin in the cytoplasm (d). (a, c) H and E stain and (b, d) β -catenin immunohistochemistry. Original magnification, (a–d) $\times 100$.

Table 1
Numbers of BCAC in C57BL/6J-*Apc^{Min/+}* and *APC^{+/+}* mice at 3 and 5 weeks of age

Weeks of age	Genotype	Sex	No. of mice examined	No. of BCAC per segment			
				Total	Distal	Middle	Proximal
3	<i>Apc^{Min/+}</i>	Female	7	3.43±1.13*	1.71±0.76	1.57±0.79	0.14±0.38
	<i>Apc^{+/+}</i>	Female	8	0	0	0	0
	<i>Apc^{Min/+}</i>	Male	8	4.50±1.85	1.88±0.99	2.50±1.31	0.13±0.35
	<i>Apc^{+/+}</i>	Male	6	0	0	0	0
5	<i>Apc^{Min/+}</i>	Female	7	9.86±2.34	4.71±0.76	4.71±1.98	0.43±0.53
	<i>Apc^{+/+}</i>	Female	5	0	0	0	0
	<i>Apc^{Min/+}</i>	Male	6	9.67±2.66	4.83±1.17	4.50±2.17	0.33±0.52
	<i>Apc^{+/+}</i>	Male	7	0	0	0	0

*Mean SD.

Statistical analysis was done by two-way ANOVA, Bonferroni post hoc tests.

$P < 0.001$ for males and females; and the middle part: $P < 0.01$ for females and $P < 0.05$ for males). As illustrated in Fig. 1, most BCAC consisted of one or two crypts at both ages. A single microadenoma developed in a male and a female *Apc^{+/+}* mice of 5 weeks old.

4. Discussion

Apc^{Min/+} mice are heterozygous for a nonsense mutation in the *Apc* gene [13,15]. They develop spontaneously *Apc^{Min/+}* mice similarly to the FAP syndrome in humans [13,15]. However, the distributing pattern of intestinal tumors in *Apc^{Min/+}* mice is different from that in human FAP. Most adenomatous polyps in the FAP patients arise in the colon and, if left untreated, lead to colonic cancers [18]. In contrast, the highest frequency of tumors (adenomas) in *Apc^{Min/+}* mice is seen in the small intestine, whereas small number of tumors develop in the colon [19]. Our results described here showed that there are BCAC that are hardly identified in the whole mount preparations in the colonic mucosa of the *Apc^{Min/+}* mice.

Dysplastic ACF (ACF_{Min}) were reported to be present in the colon of *Apc^{Min/+}* mice at 7 weeks of age and were positive for β -catenin [15]. Their number was small, but increased when *Apc^{Min/+}* mice were treated with a colonic carcinogen azoxymethane (AOM) [15]. We considered that BCAC described here are similar lesions as dysplastic ACF (ACF_{Min}), because of similar size (less than 50 μ m), their increase in number with aging and carcinogen treatment [15] like in the case of BCAC [13]. However, the frequency of spontaneous dysplastic ACF (ACF_{Min}) in the *Apc^{Min/+}* mice at 7 weeks of age [15] was much lower than that found our mice at 3 and 5 weeks of ages. In contrast to classical ACF, ACF_{Min} are not elevated lesions above the surrounding mucosa, their detection depends on methylene blue staining and transillumination [20]. Also, the tissue preparation (cross section vs. whole mount) may contribute to the difference in the numbers of spontaneous dysplastic ACF (ACF_{Min}) [20] and BCAC in the current study. However, our findings that there were no classic ACF in the colon of juvenile *Apc^{Min/+}* mice are in agreement with the reported absence or very rare development of ACF in the colon of *Apc⁴⁷¹⁶* knockout mice and Min mice [20–23].

Surprisingly we observed a microadenomas in the *Apc^{Min/+}* mice of either sex even at 5 weeks of age. In our previous work with aged *Apc^{Min/+}* mice (20–23 weeks of age), the frequency of microadenomas were $17.85 \pm 9.86/\text{cm}^2$ with a diameter of $176.04 \pm 410.84 \mu\text{m}$ in the colon [13]. Thus, our previous [13] and present findings may suggest that BCAC with a few microadenomas are already present in the colon of juvenile *Apc^{Min/+}* mice and increase in their number and growth with aging. This may be likely in the case of human FAP. However, we [13] and others [15] did not observe colonic adenocarcinomas in untreated *Apc^{Min/+}* mice, although multiple exposure of AOM produced a number of this malignancy [24]. As reported microadenomatous crypts have lost of the remaining allele of *Apc* in old *Apc^{Min/+}* mice, suggesting that loss of *Apc* function in such crypts. However, our recent work [16] has demonstrated that exposure of a colitis-inducing agent dextran sodium sulfate results in numerous colonic adenocarcinomas in *Apc^{Min/+}* mice of either sex. Additional promotion (inflammation [25]) stimuli are enough to produce colonic epithelial malignancy in the colon of *Apc^{Min/+}* mice. The findings may indicate the importance of inflammatory stimuli in the progression of BCAC through microadenomas to colonic malignancy in *Apc^{Min/+}* mice, although detailed mechanisms that what kinds of factors involve in a powerful promotion effect of this agent should be clarified.

In conclusion, our findings may offer a valuable clue to detection of precancerous colonic lesions and provide a new insight that experimentation using *Apc^{Min/+}* mice can be applied for investigation of colon carcinogenesis as well as small intestinal tumorigenesis.

Acknowledgements

We wish to thank Kyoko Takahashi, Tomoko Kajita and Misato Yasuda for their technical assistance, and Yoshihisa Wakita and Shinya Tanahashi for care of the animals. 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.

15-2052 and 15592007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the grants (H2005-6 and C2005-3) from Kanazawa Medical University.

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

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

Introduction

Colorectal cancer (CRC) is one of the most common malignant neoplasms in both sexes (1). In Western countries, this malignancy is one of the most leading causes of cancer deaths (1). In patients with inflammatory bowel disease (IBD), including

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

ulcerative colitis (UC) and Crohn's disease, the risk of CRC development is higher than in the general population (2–5). In sporadic and IBD-related CRC, the expression of inducible nitric oxide synthase and cyclooxygenase-2, both of which are associated with inflammation, has been reported to be elevated (6,7). As a result, inflammation is suggested to play an important role in IBD-related CRC (2).

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

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

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

Materials and methods

Animals, chemicals and diets

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

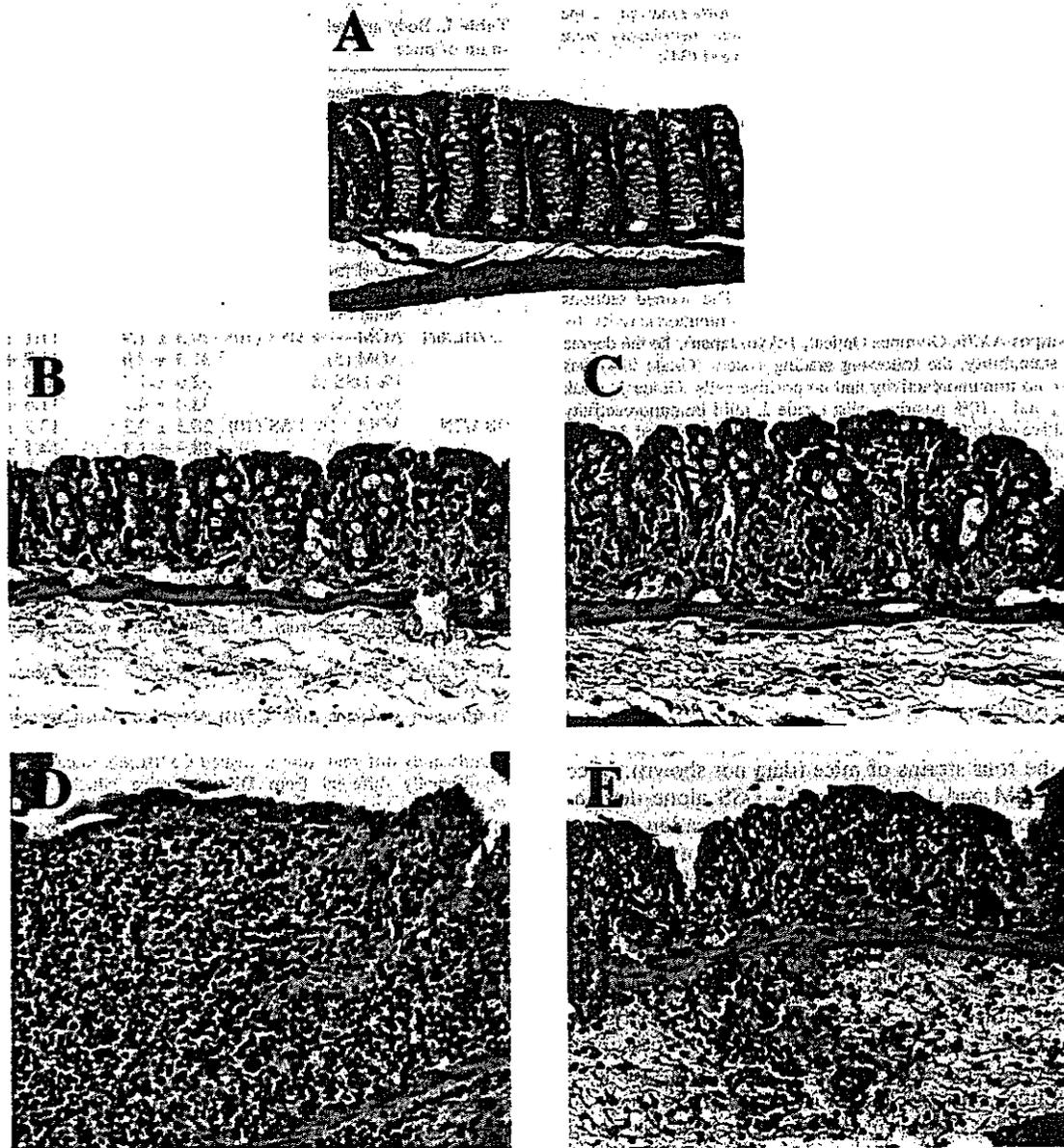


Fig. 1. Various grades of colitis. (A) Normal colon mucosa (Grade 0); (B) shortening the basal one-third of the crypts with slight inflammation and edema in the lamina propria (Grade 1); (C) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (Grade 2); (D) loss of all the crypts with severe inflammation in the lamina propria, but with the surface epithelium still remaining (Grade 3); and (E) a loss of all the crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. An exudate containing cell debris, inflammatory cells, fibrin and mucus covers the damaged mucosa (Grade 4). Hematoxylin and eosin stain. Original magnification, (A-E), 20 \times .

(Cat. No. 160110, Aurora, OH). CRF-1 (Oriental Yeast, Tokyo, Japan) was used as the basal diet throughout the study.

Experimental procedure

After they were brought, the mice were acclimated for 1 week with tap water and a pelleted basal diet, CRF-1, *ad libitum*. The experimental groups in each strain of mice included the AOM and DSS group, the AOM alone group, the DSS alone group and the untreated control group. The experimental protocol in the current study was slightly modified from our original protocol (8). We chose 1% as the dose level of DSS since this dose has been shown to exert sufficient tumor-promoting effects (32). In addition, the duration (4 days) of DSS exposure in drinking water was shortened based on our preliminary investigation, in which 4 days of exposure to DSS was found to enhance AOM-initiated colon carcinogenesis in ICR mice of either sex. All mice were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were maintained under controlled conditions of humidity (50 \pm 10%), light (12/12 h light/dark cycle) and temperature (23 \pm 2 $^{\circ}$ C).

Histopathological analysis

At the end of the experiment (Week 18), all the mice were killed by an ether overdose. At autopsy, their large bowel was flushed with saline and excised. After measuring the length of the large bowel (from the ileocecal junction to the anal verge), it was cut open longitudinally along the main axis and washed with saline. The large bowel was then carefully inspected for the presence of pathological lesions and fixed in 10% buffered formalin for at least 24 h. Paraffin-embedded sections of the large bowel were then made by routine procedures. Any histopathological alterations in the colon were examined on hematoxylin and eosin-stained sections. Colitis was recorded and scored according to the following morphological criteria described by Cooper *et al.* (33): Grade 0 (Figure 1A), normal colonic mucosa; Grade 1 (Figure 1B), shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa; Grade 2 (Figure 1C), loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3 (Figure 1D), loss of all crypts with severe inflammation in the mucosa, but with the surface epithelium still remaining; and Grade 4 (Figure 1E), loss