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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 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,

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

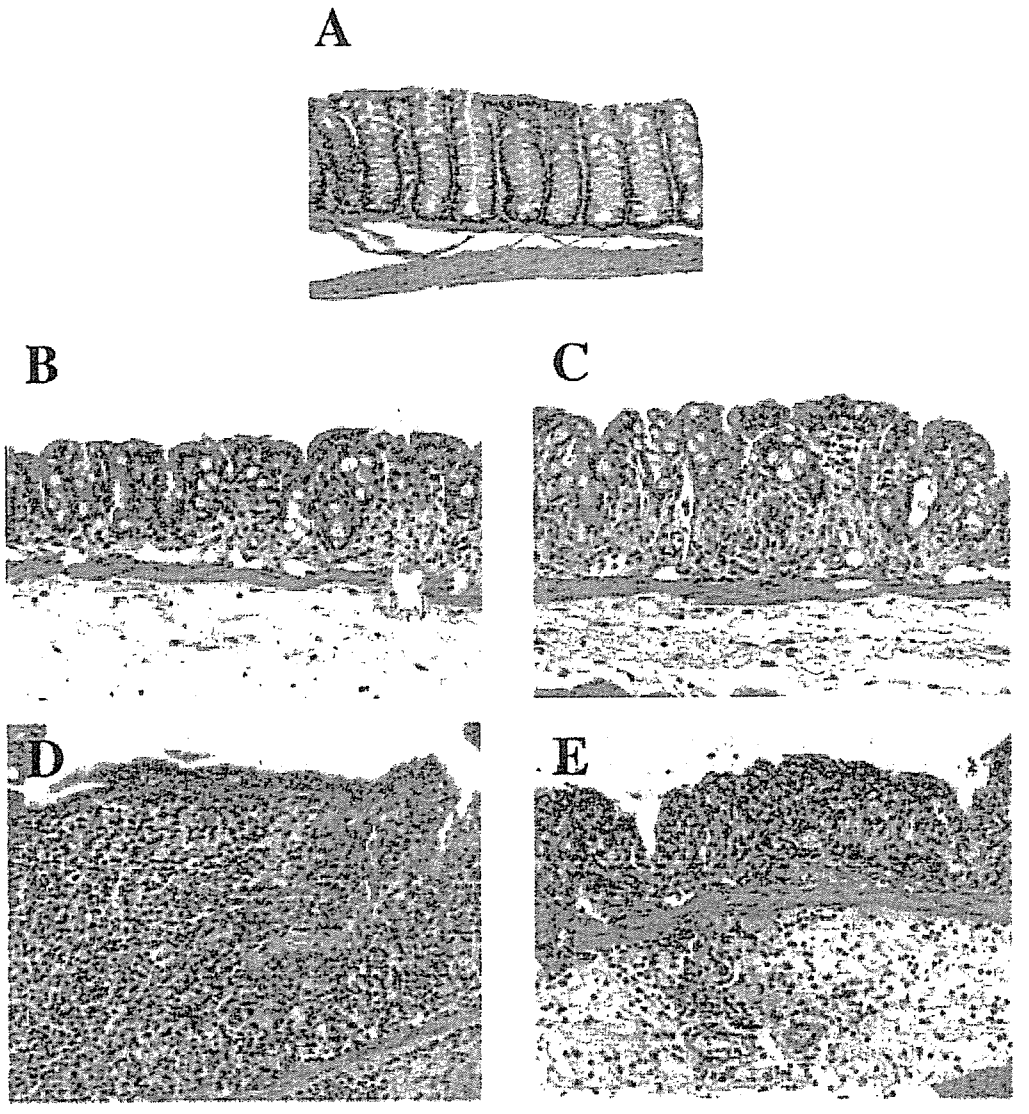

Fig. 1. Various grades of colitis. (**A**) Normal colon mucosa (Grade 0); (**B**) shortening the basal one-third of the crypts with slight inflammation and edema in the lamina propria (Grade 1); (**C**) loss of the basal two-thirds of the crypts with moderate inflammation in the lamina propria (Grade 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×.

(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 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°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

of all crypts and the surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. Intestinal neoplasms were diagnosed according to the criteria described by Pozharisski (34).

Immunohistochemistry

Nitrotyrosine immunohistochemistry was carried out on 4-μm-thick paraffin-embedded sections from the colons in all four strains of mice administered 1% DSS alone as previously described (8,35). The deparaffinized sections were incubated overnight with a primary rabbit polyclonal anti-nitrotyrosine (diluted 1:1500, CHEMICON International, CA) or with a control solution. Control sections included buffer alone or non-specific purified rabbit secondary antibody and avidin–biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The color was developed using 3,3′-diaminobenzidine-4HCl as the chromogen. The stained sections were examined for the localization and intensity of immunoreactivity by microscopy (Olympus AX70, Olympus Optical, Tokyo, Japan). To the degree of nitrotyrosine stainability, the following grading system (Grade 0–4) was applied: Grade 0, no immunoreactivity and no positive cells; Grade 1, weak immunoreactivity and <10% positive cells; Grade 2, mild immunoreactivity and 10–30% positive cells; Grade 3, moderate immunoreactivity and 31–60% positive cells; and Grade 4, strong immunoreactivity and 61–100% positive cells with extensive immunoreactivity (36).

Statistical analysis

Where applicable, the data were analyzed using one-way ANOVA with either Bonferroni correction or Fisher's exact probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), with $P < 0.05$ as the criterion of significance.

Results

General observation

The intake of DSS-containing tap water did not significantly differ among the four strains of mice (data not shown). Mice that received AOM and 1% DSS or 1% DSS alone demonstrated bloody stools either during DSS administration or soon after the cessation of DSS exposure. The degree of this symptom varied among the strains: Balb/c and C3H/HeN mice showed severe symptoms while C57BL/6N and DBA/2N mice showed mild symptoms. The mean body weight and colon length of the mice are summarized in Table I. The mean body weight of the Balb/c mice, which received AOM/DSS, was significantly lower than that of the C3H/HeN mice ($P < 0.01$) and C57BL/6N mice ($P < 0.01$), which were given AOM and DSS. A significant difference on the mean body weight was found between the AOM/DSS group and the untreated group ($P < 0.001$) in Balb/c mice. As listed in Table I, the mean lengths of the colon in the Balb/c mice ($P < 0.001$) and C3H/HeN mice ($P < 0.001$) that were treated with AOM/DSS were statistically longer than in the C57BL/6N mice. A significant difference ($P < 0.001$) was also observed between the C57BL/6N and DBA/2N mice that were exposed to AOM/DSS. The C57BL/6N mice given AOM alone has a significantly shorter colon than the Balb/c ($P < 0.01$) and DBA/2N mice ($P < 0.01$) treated with AOM alone. As for the untreated group, the colon length of the C57BL/6N mice was significantly shorter than that of the Balb/c ($P < 0.01$) and DBA/2N mice ($P < 0.01$).

Incidence and multiplicity of large bowel neoplasms

Macroscopically, colonic neoplasms developed with a different incidence and multiplicity for each strain of mice that received AOM and 1% DSS. Flat, nodular, polypoid or caterpillar-like tumors were mainly located in the middle and/or distal colon if any tumors existed (Figure 2). Histopathologically, they were tubular adenoma (Figure 3A) or adenocarcinoma (Figure 3B). Dysplastic lesions were also observed in the colonic mucosa surrounding the tumors. None of the strains of mice given AOM alone, 1% DSS alone or tap water had any colonic tumors.

Table I. Body and relative liver weights and lengths of colon in each strain of mice

Strain	Treatment (no. of mice examined)	Body weight (g)	Length of colon (cm)
Balb/c	AOM→1% DSS (10)	25.1 ± 3.8[a,b,c,d]	12.7 ± 1.0[e]
	AOM (4)	30.9 ± 0.8	14.0 ± 1.0[f]
	1% DSS (5)	34.1 ± 2.0	13.0 ± 0.6
	None (5)	32.4 ± 1.1	13.7 ± 0.5[g]
C3H/HeN	AOM→1% DSS (7)	30.2 ± 0.6	12.7 ± 1.3[e]
	AOM (5)	32.6 ± 2.2	12.5 ± 0.6
	1% DSS (5)	32.2 ± 1.2	13.1 ± 1.1
	None (3)	31.8 ± 1.1	11.9 ± 0.6
C57BL/6N	AOM→1% DSS (10)	29.3 ± 1.9	11.1 ± 0.6[h]
	AOM (5)	31.3 ± 2.0	11.7 ± 0.5[i]
	1% DSS (5)	32.0 ± 1.7	12.8 ± 0.9
	None (5)	33.0 ± 4.7	11.6 ± 1.0[j]
DBA/2N	AOM→1% DSS (10)	28.3 ± 2.3	13.2 ± 1.0
	AOM (5)	28.9 ± 1.3	14.1 ± 0.9
	1% DSS (5)	30.5 ± 0.6	14.0 ± 0.8
	None (5)	30.7 ± 1.4	13.6 ± 1.7

[a]Mean ± SD.
[b]Significantly different from untreated Balb/c mice ($P < 0.001$).
[c]Significantly different from C3H/HeN mice which received AOM/DSS ($P < 0.01$).
[d]Significantly different from C57BL/6N mice which received AOM/DSS ($P < 0.01$).
[e]Significantly different from C57BL/6N mice which received AOM/DSS ($P < 0.001$).
[f]Significantly different from C57BL/6N mice which received AOM alone ($P < 0.01$).
[g]Significantly different from untreated C57BL/6N mice ($P < 0.01$).
[h]Significantly different from DBA/2N mice which received AOM/DSS ($P < 0.001$).
[i]Significantly different from DBA/2N mice which received AOM alone ($P < 0.01$).
[j]Significantly different from untreated DBA/2N mice ($P < 0.01$).

The incidence (percent of mice with tumors) of colonic neoplasms is summarized in Figure 4A. The incidence of colonic neoplasms in the Balb/c mice (100%) was significantly higher than in the C3H/HeN mice (29%, $P = 0.0034$) and the DBA/2N mice (20%, $P = 0.0004$). A statistically significant difference ($P = 0.0115$) was also noted between the C57BL/6N (80%) and the DBA/2N mice. The order of the incidence of colonic adenoma was Balb/c mice (90%) > C57BL/6N mice (70%) > C3H/HeN mice (29%) > DBA/2N mice (20%). The incidence of adenoma in Balb/c mice was statistically greater than in C3H/HeN mice ($P = 0.0175$) and DBA/2N mice ($P = 0.0027$), and the difference between C57BL/6N mice and DBA/2N mice was statistically significant ($P = 0.0349$). The incidence of colonic adenocarcinoma was 100% in the Balb/c mice and 50% in the C57BL/6N mice and a statistically significant difference ($P = 0.0163$) was found between these two strains of mice. However, this malignancy was not found in the C3H/HeN and DBA/2N mice. As shown in Figure 4B, the multiplicity of colonic neoplasms (/mouse) was 11.4 ± 5.9 in Balb/c mice, 0.7 ± 1.5 in C3H/HeN mice, 2.5 ± 2.1 in C57BL/6N mice and 0.2 ± 0.4 in DBA/2N mice. The value for the Balb/c mice was significantly higher ($P < 0.001$) than that of other strains of mice. The order of the multiplicity of adenoma was Balb/c mice (3.7 ± 3.3) > C57BL/6N mice (1.5 ± 1.3) > C3H/HeN mice (0.7 ± 1.5) > DBA/2N mice (0.2 ± 0.4). The value for multiplicity of adenoma in the Balb/c mice was statistically greater than in the C3H/HeN

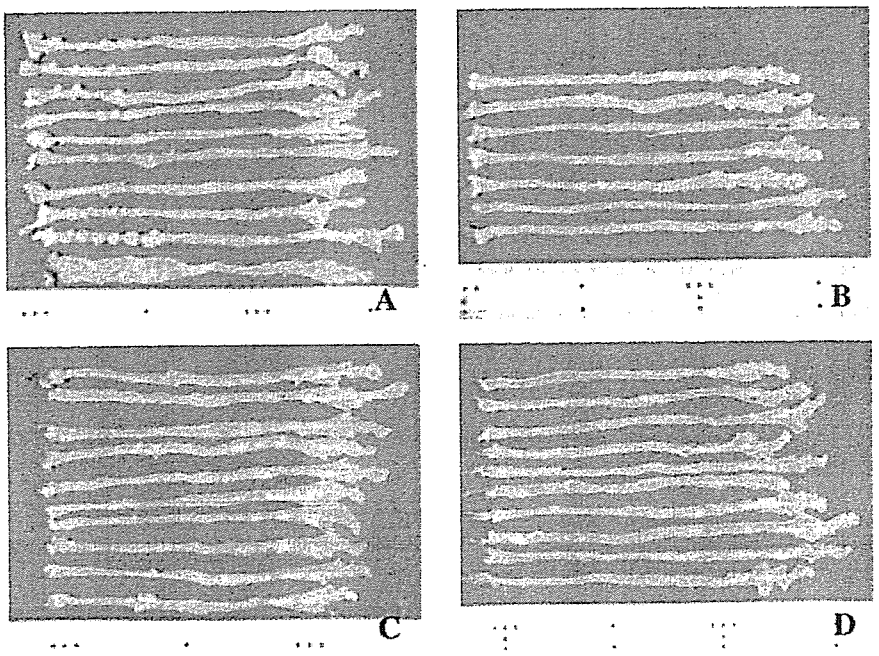

Fig. 2. Macroscopic view of the large bowel treated with AOM and 1% DSS. (A) Numerous colon tumors (2–21 tumors per mouse) develop in all Balb/c mice. (B) One or four colonic tumors are seen in two out of seven C3H/HeN mice. (C) One to five colonic tumors are found in 8 out of 10 C57BL/6N mice. (D) One colonic tumor is present in 2 out of 10 DBA/2N mice.

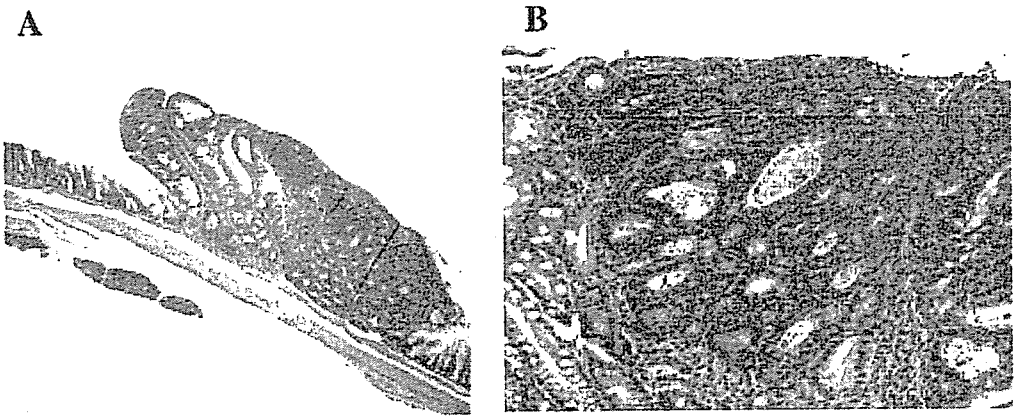

Fig. 3. Histopathology of colonic neoplasms in male Balb/c mice treated with AOM and 1% DSS. (A) Tubular adenoma and (B) moderately-differentiated adenocarcinoma. Hematoxylin and eosin stain. Original magnification, A, 2× and B, 20×.

mice ($P < 0.05$) and DBA/2N mice ($P < 0.01$). The multiplicity of adenocarcinoma in the Balb/c mice (7.7 ± 4.3) was the greatest among the four strains and it was significantly larger than that in the C3H/HeN mice (1.0 ± 1.2, $P < 0.001$).

The scores of inflammation and nitrotyrosine

As shown in Figure 5, the inflammation scores of each strain of mice initiated with AOM and followed by DSS exposure were 1.2 ± 1.1 in Balb/c, 2.3 ± 1.3 in C3H/HeN, 0.4 ± 0.7 in C57BL/6N and 0.6 ± 0.7 in DBA/2N, respectively. The score of C3H/HeN was significantly greater than that for C57BL/6N ($P<0.01$) and DBA/2N ($P<0.01$). As for the mice that received 1% DSS alone, the inflammation score of the C3H/HeN mice (1.4 ± 0.5) was the highest among the strains (1.0 ± 1.2 in Balb/c mice and 0.2 ± 0.4 in DBA/2N mice). C57BL/6N mice given 1% DSS alone had quite a low score of inflammation. The mice treated with AOM alone and the untreated mice demonstrated extremely weak inflammation in the colon.

Nitrotyrosine immunoreactivity was mainly observed in the neoplastic cells, cryptal cells, blood endothelial cells and mononuclear cells, which infiltrated the colonic mucosa (Figure 6). The stainability was relatively weak for infiltrative mononuclear cells in comparison with the cryptal cells and endothelial cells (Figure 6). As shown in Figure 7, the nitrotyrosine immunohistochemistry findings for the Balb/c mice (3.6 ± 0.5) treated with AOM and DSS were significantly higher than those for C3H/HeN (1.7 ± 0.8, $P<0.001$) and DBA/2N mice (1.6 ± 0.5, $P<0.001$). The score of nitrotyrosine-positivity in C57BL/6N mice (3.4 ± 0.5) was

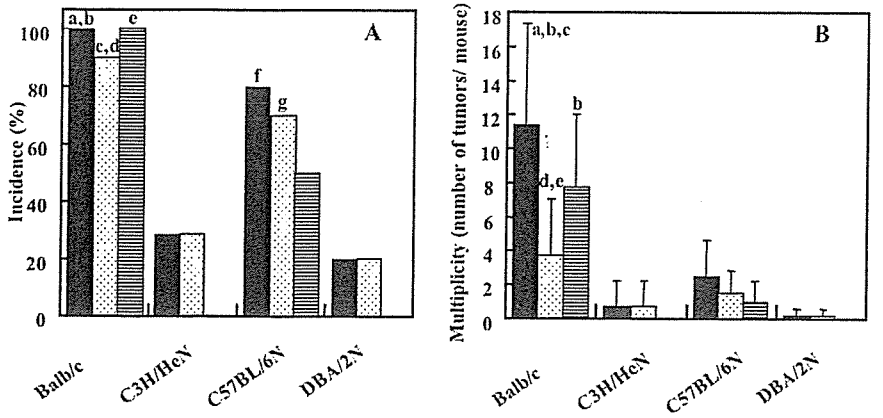

Fig. 4. Incidence and multiplicity of colonic tumors. (A) Incidence of colonic tumors. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN (P=0.0034); b, significantly different from DBA/2N (P = 0.0004); c, significantly different from C57BL/6N (P=0.0175); d, significantly different from DBA/2N (P=0.0027); e, significantly different from C57BL/6N (P=0.0163); f, significantly different from DBA/2N (P=0.0115); and g, significantly different from DBA/2N (P=0.0349). (B) Multiplicity of colonic tumors. Values are the mean ± SD. Black columns represent total; white column filled with dots represent adenoma and striped columns represent adenocarcinoma. a, Significantly different from C3H/HeN ($P<0.001$); b, significantly different from C57BL/6N ($P<0.001$); c, significantly different from DBA/2N ($P<0.001$); d, significantly different from C3H/HeN ($P<0.05$); and e, significantly different from DBA/2N ($P<0.01$).

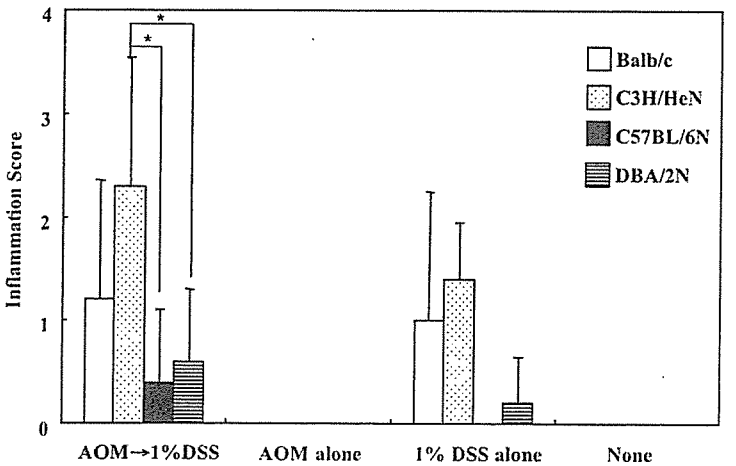

Fig. 5. Inflammation score in the colon for four strains of mice. Values are the mean ± SD. white column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *$P<0.01$.

statistically higher than those in C3H/HeN (P < 0.001) and DBA/2N ($P<0.001$) mice. In mice that received 1% DSS alone, the scores in Balb/c (2.8 ± 0.8) and C57BL/6N (2.4 ± 1.1) mice were higher than those in C3H/HeN (1.6 ± 0.5) and DBA/2N mice (1.4 ± 0.5); however, no significant differences were observed among the strains. As for the mice given AOM alone, the scores of nitrotyrosine in the Balb/c mice and C57BL/6N mice were 0.5 ± 0.6 and 0.2 ± 0.4, respectively. C3H/HeN mice and DBA/2N mice treated with AOM alone showed either no or faint stainability of nitrotyrosine. The degree of nitrotyrosine stainability in untreated mice was almost null.

Discussion

The present investigation demonstrated the different susceptibilities of the four strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice to colon tumorigenesis induced by the combination treatments with AOM and DSS. Apparently, Balb/c mice were extremely sensitive to AOM/DSS-induced colon carcinogenesis in the present experimental condition. The sensitivity of Balb/c mice observed in the present study was almost similar to those found in ICR mice (8,32,35). Colonic adenocarcinoma also developed in C57BL/6N, but the incidence was lower than in Balb/c. In contrast, the susceptibility of C3H/HeN and DBA/2N to the administration of AOM and DSS was quite low and only a few colonic adenomas developed in both the strains of mice.

Regarding the sensitivity of the mice to AOM initiation, the Balb/CJ mice were reported to have a remarkable susceptibility to the formation of distal colon tumors after treatment with AOM (26), whereas C3H, C57BL/6J, and DBA/2 mice were found to have a low incidence of colonic tumors by AOM initiation (25,26,29). Strain differences in the susceptibility to DSS have also been demonstrated: Balb/c, C3H/HeJ and C57BL/6J are relatively susceptible to DSS, whereas DBA/2J mice are virtually resistant based on the frequency of ulceration or the histological score of inflammation in the colon

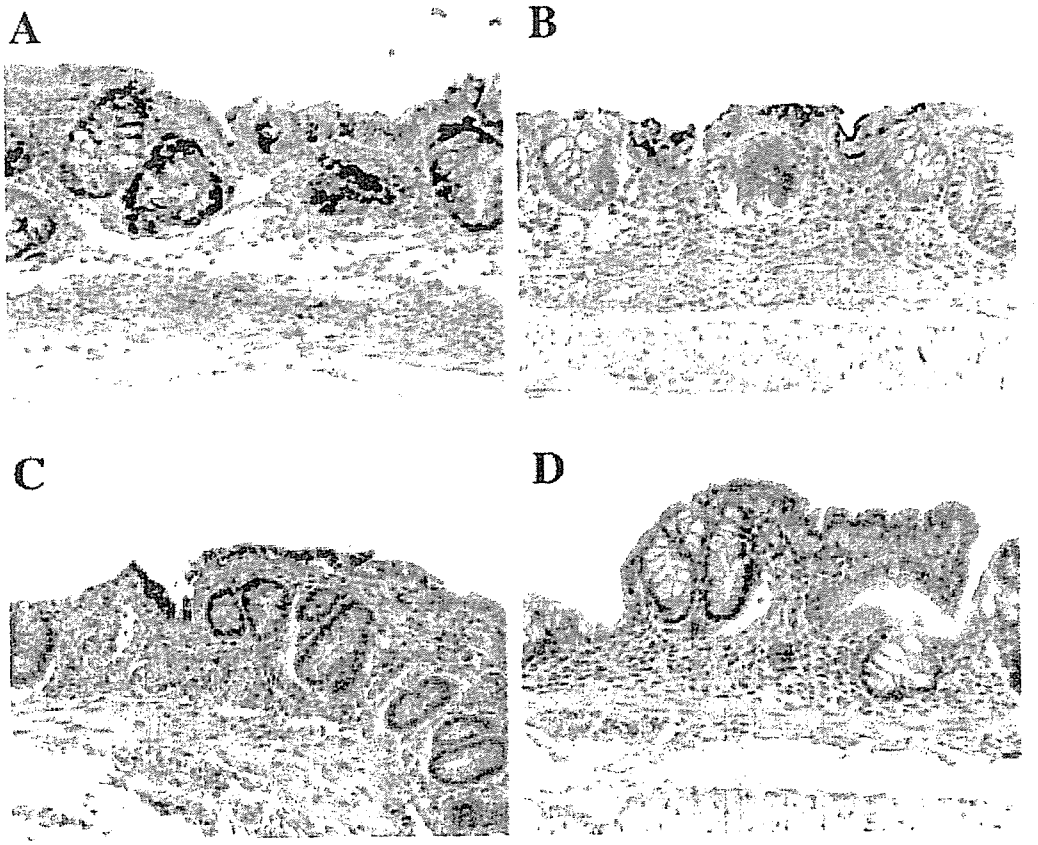

Fig. 6. Nitrotyrosine immunohistochemistry of the colon from four strains of mice given 1% DSS. (A) Balb/c; (B) C3H/HeN; (C) C57BL/6N; and (D) DBA/2N.Original magnification, (A–D), 20×.

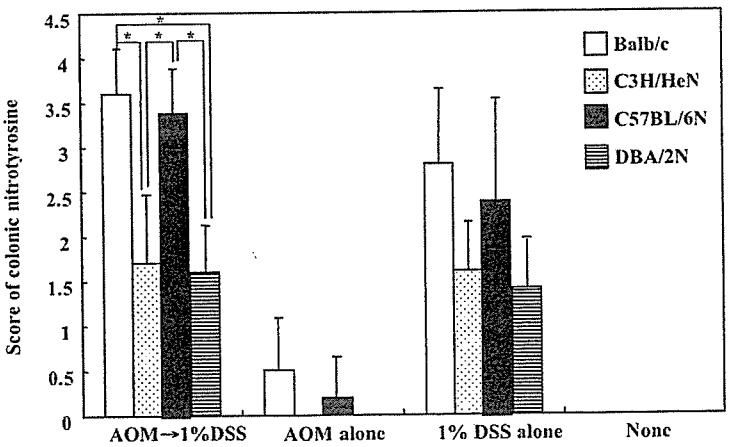

Fig. 7. Score for nitrotyrosine immunohistochemistry. Values are the mean ± SD. White column, Balb/c; white column with dots, C3H/HeN; black columns, C57BL/6N; striped columns, DBA/2N. *$P < 0.001$.

(27,28). In the current study, the sensitivities of the four strains to DSS were somewhat dissimilar to those of previous studies (27,28). The inflammation score of colonic mucosa revealed a severe and moderate inflammation to be present in the C3H/HeN and Balb/c mice treated with both AOM and DSS, respectively, while C57BL/6N and DBA/2N mice had only a relatively weak inflammation. In the case of the receptivity of C57BL mice to lipopolysaccharide (LPS), C57BL/10ScCr mice were resistant to LPS, whereas C57BL/10ScSn mice responded to LPS (37). Similarly, C3H/HeJ and C3H/HeN are LPS-responder and LPS-non-responder mice, respectively (38,39). As a result, the discrepancy in the response of DSS in mice might be due to differences in the substrains. In the current study, the highest incidence of colonic tumors was

found in Balb/c. C57BL/6N had the second highest incidence among the strains tested. On the other hand, C3H/HeN and DBA/2N had only a few benign colonic tumors (adenomas). The shortening of colon length in the mice that received DSS is one of the biological markers of severity of colonic inflammation (8–10,32,35). When comparing the colon length in mice treated with AOM and DSS with that in untreated mice, the order of the shortening rate of the colon length of mice was Balb/c (7%) > C57BL/6N (4%) > DBA/2N (3%) > C3H/HeN (−6%). These results suggest that the different susceptibilities of the inbred mouse strains to AOM/DSS-induced colon carcinogenesis might correlate with different sensitivities to AOM or DSS, with only slight contradictions among the substrains.

AOM is widely used as a colonic carcinogen to investigate the pathogenesis and modification of colon carcinogenesis in rodents (11–13). AOM requires metabolic activation to exert its carcinogenic action. Cytochrome P450 (CYP) is known to play a prominent role in the modulation of the xenobiotic metabolism, including chemical carcinogens. CYP 2E1 is one of the important factors for converting AOM to methylazoxymethanol, which can produce DNA adduct formation and also produce the initiation event (40,41). Although we did not investigate the activity of CYP 2E1, it may be possible that the expression and/or content of CYP 2E1 differ among the strains examined. This may be indicated by the findings that the relative liver weight of Balb/c, which had the highest susceptibility of AOM/DSS-induced colon carcinogenesis, was higher than that of other strains of mice in the current study (data not shown).

The influence of nitrosation stress caused by DSS is also an important factor for AOM/DSS-induced mouse colon carcinogenesis, since a powerful tumor-promoting activity of DSS has been observed in this model (8,32,35,42). We found a close association between the score of nitrotyrosine and the occurrence of tumors in the current study. Nitrotyrosine-immunohistochemical scores of each strain of mice in the 'AOM → DSS' and 'DSS alone' groups were much greater than those of the 'AOM alone' and 'untreated' groups. The scores of the 'AOM → DSS' group were relatively higher than those of the 'DSS alone' group in all strains of mice and the order was Balb/c > C57BL/6N > C3H/HeN > DBA/2N in these two groups. Such inflammation could influence tumorigenesis, although the inflammation score did not completely correspond with the frequency of colonic tumors in the current study. Indeed, the score of inflammation in the mice receiving both AOM and DSS was higher than that of the mice administered DSS alone. An investigation of additional factors is needed to precisely elucidate the strain differences in the susceptibility to colon carcinogenesis. Recently Greten *et al.* (43) reported interesting findings, namely that a specific inactivation of the IκB kinase (IKK)/NF-κB pathway can attenuate the formation of inflammation-associated colon tumors in *villin-Cre/Ikkβ*$^{F/\Delta}$ mice. They also suggested that IKKβ might be involved in inflammation-related carcinogenesis.

In conclusion, we herein demonstrated the differences in the genetic susceptibility to AOM/DSS-induced colon tumorigenesis among four inbred strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) of mice and found the Balb/c mice to be the most sensitive. Our findings suggest that the genetic background thus plays an important role in the cancer risk in colitis-related colon tumorigenesis. In addition, strain differences in the susceptibility of colon carcinogenesis induced by AOM and DSS might be influenced by the response to nitrosation stress due to inflammation as determined by the genetic background.

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Predominant T helper type 2-inflammatory responses promote murine colon cancers

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Colon cancer is one of the most serious complications of inflammatory bowel diseases, especially ulcerative colitis (UC). Previous studies have shown that characteristic immunological event during inflammation in UC is the expression of T helper-type 2 (Th2) cell-derived cytokines. In this study, we investigated the influence of a predominant Th2-type cytokine response in colitis on carcinogen-induced colon tumors. Wild type (WT), interferon gamma (IFN-γ) gene deficient (−/−) [Th2 dominant] or interleukin (IL)-4$^{-/-}$ [Th1-dominant] mice of BALB/c background were used in this study. To compare tumor formation, mice were given the carcinogen azoxymethane (AOM) and intrarectal administration of trinitrobenzene sulfonic acid (TNBS), to induce colitis. Thirty-three weeks after initial treatment, the total colon was examined. When IFN-γ$^{-/-}$ mice were treated with AOM and TNBS, significantly higher number of tumors were seen (8.4 ± 1.7) than in WT (3.3 ± 2.9) or IL-4$^{-/-}$ (3.1 ± 3.4) mice, which received identical treatments. A separate set of experiment, using less doses of AOM and TNBS also showed the higher frequency of tumor formation in IFN-γ$^{-/-}$ mice than in IL-4$^{-/-}$ mice. Histologically, the tumors were well- or moderately-differentiated adenocarcinomas. No invasion into the submucosal or serosal layers of the intestine was seen. In immunohistological staining, some tumors in IFN-γ$^{-/-}$ mice showed distinct nuclear expression of β-catenin, in contrast to the strong membrane staining seen in tumors of IL-4$^{-/-}$ mice. In conclusion, colonic inflammation associated with Th2-donimant cytokine responses enhanced the formation of malignant neoplasms.
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Key words: colitis; cancer; interferon-γ; interleukin-4; carcinogenesis

Colorectal cancer is one of the most serious complications in inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD).[1] Of note, patients with longstanding, extensive UC have a high cancer risk, ~16.5% at 30 years, after initial diagnosis.[2,3] It has long been noted that cancer arises from regions of chronic inflammation, and the inflammatory cells and cytokines of the immune system found in tumors are more likely to contribute to tumor growth and progression.[4] In animal models, colitis induced by dextran sulfate sodium (DSS) are associated with dysplasia and cancer.[5–7] Recent studies on liver cancer[8] and colon cancer[9] models suggested that transcription factor NF-κB, which is a key player of inflammatory responses, does not affect initiation, but acts in tumor promotion. Thus, inflammation may significantly affect the process of cancer in UC. In fact, cancers in UC have several distinct features from colorectal cancers in non-IBD patients.[10] First, tumors in UC are often multiple, which is to be expected from precancerous dysplastic changes found in UC mucosa. Second, cancer in UC is often flat and infiltrating. Third, there is a higher incidence of high-grade, mucinous carcinomas than seen in non-IBD cancer. At a molecular level, p53 gene mutations or p53 protein overexpression, which is a late event in the development of sporadic colorectal carcinoma, have been commonly reported as early events in the dysplasia–carcinoma sequences in UC-associated carcinomas.[11–14] These results provide evidence that UC-associated cancer may develop along a pathway that is different from that of sporadic colorectal cancer.

Although pathogenesis of IBD is unknown, fluctuating but constant inflammatory responses at the local site is the major pathological finding. Past studies have shown that local immunological events during chronic inflammation in UC and CD are different. The presence of activated Th1 cells in the intestine is the characteristic of CD, with high expression of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α).[15,16] On the other hand, elevated expression of T helper-type 2 (Th2) cell-derived cytokines is often seen in UC.[17,18] By use of various murine models, we and others have shown that such distinct cytokine responses actually involved in the unique pathological changes in CD and UC. For example, we noted distinct pathological differences in the hapten-induced colitis in Th1 *versus* Th2-dominant mice.[19,20] In Th2 dominant mice, fibrosis and diffuse atrophic changes in epithelial cells were seen, while acute ulcers were the major lesions of colitis in Th1 dominant mice. Other groups have reported that administration of the sensitizing agent, oxazolone, induced colitis with diffuse epithelial damage. In this model, a Th2 type cytokine, interleukin (IL)-13 was the major effector cytokine.[21,22] Further, transfer of Th2-dominant T cells to T cell-deficient recipient mice resulted in ileal villus atrophy and goblet cell metaplasia,[23] while transfer of Th1 dominant T cells induced erosive gastritis with enhanced surface epithelial cell apoptosis.[24] These results suggest that a predominance of either Th1- or Th2-type cytokines in inflammatory responses has a major influence on the pathology and tissue remodeling in the chronic inflammation, and eventually affects controlling epithelial cell differentiation, as well as their turnover. Thus, differential upregulation of inflammatory cytokines in UC may directly contribute to malignant progression. However, data on the participation of a predominance of Th1 or Th2 cytokines in mucosal immunity in colonic carcinogenesis is limited.

The possible factors which lead to dysplasia and malignant transformation in UC need to be more thoroughly investigated. In this study, we used models of Th1- or Th2-dominant colitis model together with azoxymethane (AOM)-induced carcinogenesis and sequential and morphological analysis, paying particular attention to the tissue tropism of carcinogenesis. Our findings show that a Th2 cytokine dominant colitis increases the frequency and changes in pathological features of colonic neoplasms.

Abbreviations: AOM, azoxymethane; DSS, dextran sulfate sodium; GI, gastrointestinal; H&E, hematoxylin and eosin; IFN, interferon; IL, interleukin; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

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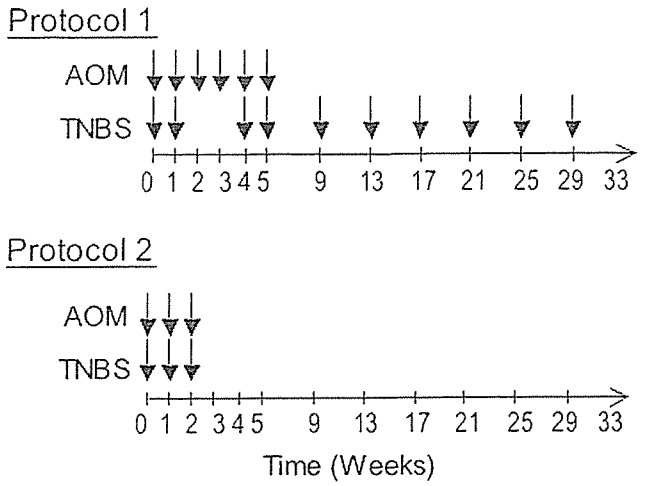

FIGURE 1 – Experimental design used in this study.

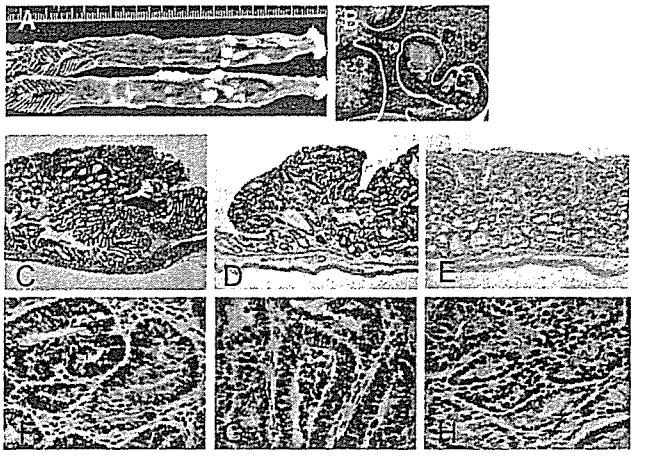

FIGURE 2 – Pathological features of colonic tumors. (*a*) Macroscopic appearance of typical tumors. Colons from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS are shown. (*b*) Zoom stereomicroscopic appearance of tumors in panel A after staining with methylene blue. Polypoid cancer seen in WT (*c*) and IL-$4^{-/-}$ (*d*) mice treated with AOM plus TNBS, stained by H&E. (*e*) Flat-elevated cancer seen in IFN-$\gamma^{-/-}$ mice (H&E staining). Immunostaining for β-catenin of well-differentiated adenocarcinomas from WT (*f*) and IL-$4^{-/-}$ (*g*) mice showing a cell membrane pattern and moderately differentiated adenocarcinoma from IFN-$\gamma^{-/-}$ mice (*f*) showing a nuclear pattern.

Material and methods

Mice

Wild type (WT), IFN-γ gene knockout (IFN-$\gamma^{-/-}$), IL-4 gene disrupted (IL-$4^{-/-}$) mice, all on the BALB/c background were originally obtained from Jackson Laboratory (Bar Harbor, ME). The colony was maintained under pathogen-free conditions in the Immunocompromized Mouse Facility of the Research Institute, International Medical Center of Japan (IMCJ). All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and according to the approval of the local ethics committee in the IMCJ.

Induction of colitis and colon tumors

Treatment of mice was initiated when mice were 8 weeks of age. Trinitrobenzene sulfonic acid (TNBS) colitis was induced, as described previously.[19,20] A 2% solution of TNBS (Research Organics, Cleveland, OH) in PBS:ethanol (1:1 by volume) was administered intrarectally to mice, anesthetized with ketamine (Sankyo Co. Ltd., Tokyo, Japan) and xylazine (Sigma, St. Louis, MO). A dose of TNBS of 36 μg/g of body weight was given. The colon carcinogen AOM was purchased from Sigma, and a dose of 10 μg/g of body weight in saline was injected intraperitoneally. TNBS and AOM were given, as indicated (Fig. 1). In some experimental groups, treatment with either TNBS or AOM was performed. In protocol 1, six doses of AOM or 4 doses of TNBS were given during the first 5 weeks, and then TNBS was given every 4 weeks on 6 occasions, to mimic recurrence of inflammation. In protocol 2, three doses of TNBS and AOM were given together in the first 2 weeks, and colonic tissues were examined after 33 weeks. To examine the spontaneous development of tumors, some cytokine knockout mice were kept untreated, until they were 40 weeks of age.

Macroscopic and histological examination

Colonic tissues were opened longitudinally, fixed in 10% formalin overnight at 4°C, washed in PBS, stained with 2% methylene blue for contrast, and then the numbers of macroscopically visible tumors were assessed. Some specimens were examined with a zoom stereomicroscope. Tumors were removed along with the surrounding normal colonic tissues and embedded in paraffin blocks; then, 4-μm sections were prepared and stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, 3-μm-thick paraffin sections were deparaffinated in xylene, rehydrated and heated at 95°C in 10 mM citrated buffer (pH 6.0) for 10 min. After treatment with 3% H_2O_2, followed by 0.25% goat serum in PBS for blocking, sections were incubated with monoclonal antibodies to p53 (DO-1, 1:200) or β-catenin (1:800, both from BD Transduction Laboratories, CA) overnight at 4°C. Mouse IgG was used as a negative control. Bound antibody was detected using Vectastain ABD-kit (Vector Laboratories, Burlingame, CA) according to vendor's protocol, and deaminobenzidine was used as substrate for peroxidase.

Statistics

The results were compared by the Mann–Whitney test or χ^2 test, using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for the Macintosh computer.

Results

Effects of inflammation and cytokine deficiency on the formation of colon tumors

In our study, polypoid or sessile elevated lesions were macroscopically visible in the middle to distal colon, and these were enumerated (Figs. 2*a* and 2*b*). The results of protocol 1 are summarized in Table I. When WT mice were treated with only AOM in protocol 1, 3.3 tumors/mouse were seen after 33 weeks. In IFN-$\gamma^{-/-}$ mice, the incidence of tumors induced with AOM was not significantly different from that of WT mice. IL-$4^{-/-}$ mice, treated with AOM only, did not develop tumors, except for 1 mouse with a single tumor, although aberrant crypt foci (ACF) were frequently seen by following zoom stereomicroscopy. Induction of TNBS colitis, in addition to AOM treatment, did not change the numbers of tumors in WT mice. In contrast, induction of colitis induced enhanced formation of tumors in both IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice. Of note, the number of tumors in IFN-$\gamma^{-/-}$ mouse reached to 8 tumors/mouse, which was significantly higher when compared with those in IL-$4^{-/-}$ or WT mice. The incidence of tumor bearing mice was 100% in the IFN-$\gamma^{-/-}$ group. There were no significant differences in the size of the tumors, which developed in each experimental group. In protocol 1, most of the deaths occurred mostly in the first 8 weeks. The exceptions were that some IL-$4^{-/-}$ mice treated with TNBS died in later period, coinciding with our previous report that IL-$4^{-/-}$ mice are more susceptible to TNBS colitis.[19] Further, we did not see any tumors in the mice which died before 33 weeks, including IL-$4^{-/-}$ mice.

TABLE I – NUMBER OF TUMORS IN CYTOKINE DEFICIENT MICE (PROTOCOL 1)

Mice	Treatment	No. of mice survived/total	Incidence of tumor	No. of tumors/mouse	Size of tumors (mm^3)	Total number of tumors
WT	AOM	7/12 (58)[1]	5/7 (71)	3.3 ± 2.9	2.2 ± 1.3	23
WT	AOM + TNBS	10/17 (59)	6/10 (60)	2.0 ± 2.5	2.9 ± 1.3	20
WT	TNBS	5/5 (100)	0/5 (0)	0 ± 0		0
IFN-$\gamma^{-/-}$	AOM	7/16 (43)	2/7 (28)	1.4 ± 2.3	2.7 ± 1.9	10
IFN-$\gamma^{-/-}$	AOM + TNBS	7/11 (64)	7/7 (100)[2]	8.3 ± 1.8[3]	2.8 ± 1.8	58
IFN-$\gamma^{-/-}$	TNBS	8/10 (80)	0/8 (0)	0 ± 0		0
IFN-$\gamma^{-/-}$	Untreated	8/8 (100)	0/8 (0)	0 ± 0		0
IL-$4^{-/-}$	AOM	15/17 (88)	1/15 (7)	0.1 ± 0.3[4]	1.6	1
IL-$4^{-/-}$	AOM+TNBS	7/11 (64)	5/7 (71)[5]	3.1 ± 3.4	1.7 ± 1.2	22
IL-$4^{-/-}$	TNBS	2/7 (29)	0/2 (0)	0 ± 0		0
IL-$4^{-/-}$	Untreated	4/4 (100)	0/4 (0)	0 ± 0		0

[1]Values in parentheses indicate percentages.–[2]Difference from IFN-$\gamma^{-/-}$ or IL-$4^{-/-}$ mice treated AOM was statistically significant ($p<0.02$).–[3]Differences from all other experimental groups were statistically significant ($p < 0.02$).–[4]Differences from WT mice with AOM or AOM+TNBS were statistically significant ($p<0.02$).–[5]Difference from IL-$4^{-/-}$ mice treated AOM was statistically significant ($p < 0.02$).

TABLE II – NUMBERS OF TUMORS IN CYTOKINE DEFICIENT MICE (PROTOCOL 2)

Mice	Treatment	No. of mice survived/total	Incidence of tumor formation	No. of tumors/mouse	Total number of tumors
IFN-$\gamma^{-/-}$	AOM + TNBS	19/24 (79)[1]	10/19[2] (53)	0.8 ± 0.9[2]	15
IL-$4^{-/-}$	AOM + TNBS	12/24 (50)	0/12 (0)	0	0

[1]Values in parentheses indicate percentages.–[2]Differences from IL-$4^{-/-}$ mice were statistically significant ($p < 0.02$).

It indicated that mortality was not associated with cancer, but more with susceptibility to TNBS colitis or toxicity of AOM in each mouse strain.

Next, to conform the difference between IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice treated with both AOM and TNBS, we have chosen protocol 2 (Fig. 1), reducing the dose of AOM and TNBS. A change in the dose of AOM improved the survival of IFN-$\gamma^{-/-}$ mice; however, this dose change did not affect the survival of IL-$4^{-/-}$ mice, probably because of their higher sensitivity to TNBS colitis than IFN-$\gamma^{-/-}$ mice (Table II). In this protocol, death occurred within the first 3 weeks, and no tumor-caused death was observed in both groups. Although the numbers of tumor/mouse decreased in this protocol, we saw similar differences between the IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice groups (Table II). No visible tumors were seen in IL-$4^{-/-}$ mice. In contrast, 10 of 19 IFN-$\gamma^{-/-}$ mice formed more than 1 tumor in the colon.

Microscopic findings

Tumors formed in protocol 1 were subjected to microscopic examination. The majority of these were polypoid lesions (Figs. 2*c* and 2*d*), and some of them were flat, elevated lesions (Fig. 2*e*). Tumors histologically examined were all diagnosed as well-differentiated or moderately differentiated adenocarcinoma. There was also colonic dysplasia involving 1–2 crypts, in mouse groups that had treatment with AOM. In each groups of mice, there were no differences in the severity of cell infiltration into tumors. In IFN-$\gamma^{-/-}$ mice with TNBS colitis, deformity of crypts was the most evident, as previously reported[20]; however, there were no clear relations to tumor location. In WT mice, the ratio of moderately differentiated adenocarcinoma from mice treated with both AOM and TNBS tended to be higher than those treated only with AOM, in which well-differentiated adenocarcinoma was the major type (Fig. 3). The majority of tumors from IFN-$\gamma^{-/-}$ mice were moderately differentiated adenocarcinomas in both AOM or AOM plus TNBS treated groups (Fig. 3). In protocol 2, fourteen out of 15 tumors from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS were also diagnosed as moderately differentiated adenocarcinomas. One tumor was a well-differentiated adenocarcinoma.

Immunohistochemistry

Immunostaining for p53 and β-catenin was performed. Staining with anti-p53 monoclonal antibody was generally weak, and obviously enhanced nuclear staining was seen in only 2 tumors in IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS, 2 from IL-$4^{-/-}$ mice

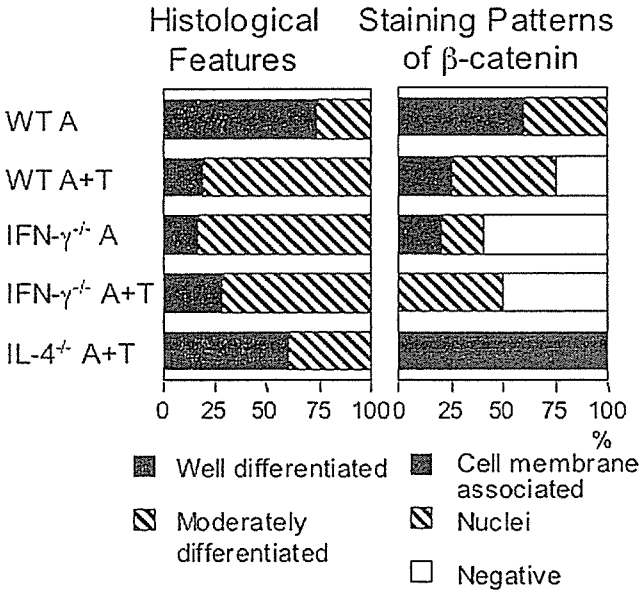

FIGURE 3 – Percentage of histological diagnosis and β-catenin staining pattern of colon tumors. Tumors (6–10) from each mouse group in protocol 1 were evaluated for their histological grading and expression of β-catenin. A, treated with AOM; T, treated with TNBS.

treated with AOM plus TNBS and 2 tumors from WT mice treated with AOM (data not shown). In contrast, a differential staining pattern with β-catenin was seen. Well-differentiated adenocarcinomas in WT and IL-$4^{-/-}$ mice showed typical staining of cell membrane, which was much stronger than that in normal epithelial cells (Figs. 2*f* and 2*g*). In contrast, nuclear staining was frequently observed in tumors from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS (Fig. 2*h*). All tumors from IL-$4^{-/-}$ mice showed a cell membrane pattern, including the one from mice treated with only AOM. There was no tumor with a membrane-staining pattern from IFN-$\gamma^{-/-}$ mice treated with AOM plus TNBS (Fig. 3). Thus, nuclear and membrane localization of β-catenin was the characteristics of moderately differentiated adenocarcinomas in IFN-$\gamma^{-/-}$ mice and well-differentiated adenocarcinoma in IL-$4^{-/-}$ mice, respectively (Fig. 3).

Discussion

In the present study comparing WT, IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice, several new findings in colitis-related colon cancer were revealed. First, in the absence of IFN-γ, induction of TNBS colitis and AOM strongly enhanced tumor formation in the colon. Importantly, colitis also affected the histological degree of differentiation, and frequently induced nuclear translocation of β-catenin.

Although the precise mechanism of enhancement of tumor formation is not yet clear, one possibility is that a defect of tumor immunosurveillance occurs in IFN-$\gamma^{-/-}$ mice. Previous studies showed that IFN-$\gamma^{-/-}$ mice develop chemical carcinogen methylcholanthrene-induced sarcoma,[25] spontaneous lymphomas and lung adenocarcinomas[26] more frequently than do WT mice. In our studies, there were no significant differences in the frequency of tumor formation between WT and IFN-$\gamma^{-/-}$ mice, when inflammation was not induced. In contrast, in the presence of colitis, the number of tumors in IFN-$\gamma^{-/-}$ mice was markedly increased, while tumor frequency in WT mice was not increased. This result in WT mice is distinct from studies reporting the development of AOM-induced tumors within a short term in WT mice when DSS colitis was induced,[27,28] although there has been no report testing IFN-$\gamma^{-/-}$ mice. In DSS colitis, diffuse loss of epithelium is the primary pathological finding, which might increase the chance of carcinogenesis and promotion during vigorous epithelial cell regeneration. On the other hand, since TNBS colitis is characterized by a focal ulcer and a strong hapten (trinitrophenyl residue)-specific immune response of T and B cells, IFN-γ produced as a part of adoptive immunity in the inflammation seems to be important for protection from the colonic tumor. In other words, IFN-γ production may not be required for tumor surveillance in non-inflamed, steady state colons of BALB/c mice. A significant incidence of lung carcinoma, but no report of colon carcinoma during the life span of more than 600 days in BALB/c IFN-$\gamma^{-/-}$ mice, also supports this notion.[26] The importance of IFN-γ in tumor surveillance produced by T cells as a part of adoptive immunity was also shown in the previous study that $RAG2^{-/-}$ x $STAT1^{-/-}$ double knockout mice showed increased susceptibly to methylcholanthrene-induced carcinogenesis, but did not display a significantly greater tumor incidence, when compared with mice that lacked either RAG2 or STAT1 genes.[25] Thus, it is feasible that the colonic mucosa of UC, which fails to induce prevailing upregulation of IFN-γ but produces Th2-type cytokines, can frequently develop colonic carcinoma. On the other hand, our results also showed that addition of TNBS colitis to AOM increased the incidence of tumor in IL-$4^{-/-}$ mice, although the number was much less than in IFN-$\gamma^{-/-}$ mice. This result suggested that aberrant Th1-dominant inflammatory responses might also increase the tumor risk, although it was not comparable to that in Th2 dominant condition.

Since there is an interaction between Th1 and Th2 cytokines that they suppress the effect of each other, the presence of an excess amount of Th2 cytokines in IFN-$\gamma^{-/-}$ would have a significant effect. We have previously analyzed the cytokine production by colonic $CD4^+$ cells in WT, IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice in naïve colon, as well as in acute (day 1) and chronic (day 10) phase of TNBS colitis. Colonic $CD4^+$ T cells from IFN-$\gamma^{-/-}$ mice produced very large amounts of IL-4 and IL-5. In contrast, secretion of IFN-γ by colonic $CD4^+$ T cells from IL-$4^{-/-}$ mice was higher than in colonic $CD4^+$ T cells from WT mice.[19,20] In protocol 1 of current study, the number of tumors in IL-$4^{-/-}$ mice was much lower than those of WT or IFN-$\gamma^{-/-}$ mice. In protocol 2, none of the IL-$4^{-/-}$ mice developed tumors, in contrast to the 53% incidence in IFN-$\gamma^{-/-}$ mice. These results suggest that IL-4 may have a direct effect on promoting tumor formation. In fact, many malignant tumors express the IL-4 receptor, which is able to bind to both IL-4 and IL-13 and also the high affinity decoy receptor of IL-13, IL-13 receptor α2.[29] However, the function of IL-4 and IL-13 in tumor cells, especially in colonic cancer is not yet clear, and the output effect via these receptors still remains to be investigated. Early studies showed that IL-4 and IL-13 had antitumor activity in mice by growth inhibition[30] through IL-4 receptor.[31] However, subversion of host antitumor defenses has also been demonstrated for IL-13. Recent studies using tumor cell lines demonstrated that STAT6, IL-4 and IL-13 were capable of inhibiting tumor rejection.[32–34] Thus, Th2 type cytokines appear to antagonize tumor immunosurveillance.

In our study, IL-$4^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice showed distinct expression patterns for β-catenin, cell membrane and nuclear staining in IL-$4^{-/-}$ and IFN-$\gamma^{-/-}$ mice, respectively, while WT mice had tumors of both patterns. This again suggests that Th2-cytokine predominance directly influences the mutation of DNA in epithelial cells. Indeed, exogenous IL-4 and IL-13 decreased the levels of membrane staining of β-catenin in keratinocytes, without changes of total protein levels of β-catenin, while IFN-γ enhanced membrane staining.[35] In our model, gene mutation induced by AOM might facilitate nuclear translocation of β-catenin, which had localized in cytoplasm, but not in cell membrane by the action of IL-4 and IL-13, secreted as inflammatory responses of IFN-$\gamma^{-/-}$ mice. Thus, we propose that enhancement of tumor formation in IFN-$\gamma^{-/-}$ mice is due both to a lack of immunosurveillance by IFN-γ and promotion of carcinogenesis by excess Th2 type cytokines. In this context, epithelial cells in the process of tissue repair in the inflamed colon are susceptible to the absence of IFN-γ and the presence of excess amount of IL-4 and IL-13.

In summary, our results show that a predominance of Th2 type cytokines in the inflamed colon, which mimics mucosal immunity in UC, promotes aberrant β-catenin expression and tumor formation. This model will be useful to further clarify the mechanism of colitic-cancer and for our search for targets or new agents for prevention of colon cancer.

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Parp-1 deficiency does not enhance liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline in mice

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Abstract

The susceptibility of poly(ADP-ribose) polymerase-1 (Parp-1) knockout mice to 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-induced liver carcinogenesis was analyzed. Twelve-week-old male *Parp-1*$^{+/+}$, *Parp-1*$^{+/-}$ and *Parp-1*$^{-/-}$ mice of the C57BL/6 congenic strain were fed a diet containing IQ at a concentration of 300 ppm or a control diet for 60 weeks. Hepatocellular carcinomas were observed only in 1/19, 2/18 and 1/17 of the *Parp-1*$^{-/-}$, *Parp-1*$^{+/-}$ and *Parp-1*$^{+/+}$ mice, respectively. *Parp-1* deficiency did not affect the susceptibility of mice to carcinogenicity of IQ, which produces bulky DNA adducts that are repaired mainly through the nucleotide excision repair pathway. This result is in sharp contrast to the increased susceptibility of *Parp-1*$^{-/-}$ mice to carcinogenesis induced by alkylating agents that produce DNA damage repaired mainly through base excision repair and DNA strand break repair pathways.

Keywords: PARP; IQ; Knockout mice; Liver; Carcinogenesis

1. Introduction

Cellular DNA damage is constantly generated by physical and chemical stimuli from the environment, such as ultraviolet (UV) radiation, exhaust fumes, as well as many chemicals in foods, and various systems of DNA repair have evolved which prevent mutations and thus tumor induction.

Poly(ADP-ribose) polymerase-1 (Parp-1) has multiple functions in DNA repair/recombination [1], maintenance of genomic stability [2,3], and induction of cell death accompanying nicotinamide adenine dinucleotide (NAD) depletion [4,5]. Parp-1 is also involved in transcriptional regulation and control of

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differentiation [6–8] and thus may play important roles in preventing carcinogenesis [9]. Among the five major DNA repair pathways [10], accumulating evidence suggests that Parp-1 contributes mainly to base excision repair (BER) [2,11] as well as single- and double-strand break repair [12,13]. In *Parp-1* knockout mice disrupted in *Parp-1* exon 1 [14], increased sensitivity regarding *N*-nitrosobis(2-hydroxypropyl)amine (BHP)-induction of liver hemangiomas and hemangiosarcomas [15] and azoxymethane-induced colon and liver tumors [16] has been demonstrated. Although spontaneous tumors were not observed at 7 and 9 months of age in mice having an ICR:129/Sv mixed genetic background [15,16], the incidences of hepatocellular carcinomas were increased at 18–24 months of age with a 129/Sv: 129/Ev:C57BL/6: albino mixed genetic background [17], and in the ICR:129/Sv case [18]

In the present study, to clarify the impact of *Parp-1* deficiency on carcinogenesis induced by a different type of chemical, the susceptibility of *Parp-1* knockout mice to the carcinogenic activity of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a mutagenic and carcinogenic heterocyclic amine [19] produced during cooking of meat and fish [20], was analyzed. After enzymatic activation, IQ produces bulky adducts in DNA which have been suggested to be repaired mainly through nucleotide excision repair (NER) [21], in contrast to the case of alkylating agents, especially methylating agents, in which the induced lesions are repaired mostly through BER [22] and DNA strand break repair. Comparison of the results of tumorigenesis induced by IQ and alkylating agents may provide useful information to elucidate the impact of *Parp-1* deficiency on DNA repair in relation to carcinogenesis.

2. Materials and methods

2.1. Animals and chemicals

Parp-1 knockout mice were generated by disrupting the *Parp-1* exon 1 through insertion of a neomycin resistance gene cassette [14] and then serial backcross mating was carried out to establish a C57BL/6 congenic strain [23]. Eleven-week-old male wild type ($Parp\text{-}1^{+/+}$), hetero ($Parp\text{-}1^{+/-}$) and null ($Parp\text{-}1^{-/-}$) littermate male mice were produced by in vitro fertilization and housed, five to a cage on wood-chip bedding, in an air-conditioned animal room targeted to 23 ± 2 °C and 50% humidity. Food and water were available *ad libitum* throughout the experiment. Body weights were measured weekly. Use of the animals in carcinogenesis experiments was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Graduate School of Medical Sciences, and was approved by the Institutional Animal Care and Use Committee. The animals were also treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of other participating institutions. IQ was dissolved in ethanol and added at 300 ppm to powdered CE2 diet (CLEA JAPAN, Tokyo, Japan), which was then pelleted at 100 °C for a few seconds and dried at 70 °C for 2 h. The presence of approximately 100% of the initial dose of IQ in the IQ-containing diet one year after its preparation was confirmed by HPLC after extraction with methanol, as described elsewhere [19].

2.2. Treatment and analysis

After a one-week initial observation period with the control diet, starting from the 12 weeks of age, the IQ-containing diet was given to mice of groups 1–3 (20 mice each for $Parp\text{-}1^{+/+}$, $Parp\text{-}1^{+/-}$ and $Parp\text{-}1^{-/-}$). Groups 4–6 (6, 10, 8 mice each for $Parp\text{-}1^{+/+}$, $Parp\text{-}1^{+/-}$ and $Parp\text{-}1^{-/-}$) received the control diet. All surviving mice were killed under ether anesthesia at the end of experimental week 60. Most of the animals killed upon becoming moribund were also analyzed. After a careful and gross examination, liver, kidneys and spleen were removed and weighed. Macroscopic lesions, and liver, kidneys, spleen, lungs, stomach and brain were fixed in buffered 10% formalin, trimmed and routinely embedded in paraffin. Sections cut at 3 μm thickness were stained with hematoxylin and eosin for histological examination.

2.3. Statistical analysis

Kruskal–Wallis and Bonferroni/Dunn analysis was used for statistical analysis of the quantitative data and χ^2 analysis was performed for the incidence data.

3. Results

Initial mean body weights of the *Parp-1*$^{-/-}$ mice (24.7±1.8 g) were significantly lower than those of their *Parp-1*$^{+/+}$ (27.1±2.4 g) and *Parp-1*$^{+/-}$ (27.3±2.2 g) counterparts, at $P<0.001$. Body weight gain and relative kidney weights were apparently suppressed by IQ in all genotypes (Table 1). Food consumption of the mice per body weight on the IQ-containing diet was lower than with the control diet, but no variation was observed among the different genotypes (data not shown). All of the control diet and 85, 80, and 85% of *Parp-1*$^{+/+}$, *Parp-1*$^{+/-}$, and *Parp-1*$^{-/-}$ mice, respectively, given IQ survived until the termination of the experiment. Final body weights and relative kidney and spleen weights in the *Parp-1*$^{-/-}$ mice receiving IQ were significantly lower than those of *Parp-1*$^{+/+}$ mice ($P<0.01$, 0.001 and 0.01, respectively). Final body weights and relative kidney weights were also lower in *Parp-1*$^{-/-}$ than *Parp-1*$^{+/+}$ controls, although the difference was not significant. Relative liver weights in *Parp-1*$^{+/+}$ mice given IQ were significantly higher than those in *Parp-1*$^{+/+}$ mice given the control diet ($P<0.01$).

Liver nodules were observed macroscopically in two *Parp-1*$^{+/-}$ and one *Parp-1*$^{-/-}$ mice treated with IQ that became moribund before the termination of the experiment. Data for incidences and multiplicities of pre- and neoplastic liver lesions are summarized in Table 2. Hepatocellular adenomas were observed in 3 *Parp-1*$^{+/+}$ mice given IQ and one each of the *Parp-1*$^{+/-}$ and *Parp-1*$^{-/-}$ mice given IQ, as well as one *Parp-1*$^{+/+}$ mouse maintained on the control diet, and hepatocellular carcinomas (HCC) were seen in one *Parp-1*$^{+/+}$, 2 *Parp-1*$^{+/-}$ and one *Parp-1*$^{-/-}$ mice given IQ, indicating that 300 ppm was not a saturating concentration for tumor induction in the liver. No significant differences were observed in the incidences of these lesions among the *Parp-1* genotypes. Most hepatocellular lesions in *Parp-1*$^{+/+}$ mice were found as single adenomas or adenocarcinomas, but some *Parp-1*$^{+/-}$ and *Parp-1*$^{-/-}$ mice demonstrated more than one tumor. In the lungs, alveolar cell hyperplasias and adenomas were occasionally noted, regardless of the treatment and genotype. Squamous cell hyperplasias of the forestomach ranging from 0.2 to 2.0 mm in diameter were observed more frequently in IQ-treated than in control mice, with no apparent link with the genotype (Table 3).

The relative spleen weights were also significantly higher in *Parp-1*$^{+/+}$ mice given IQ ($P<0.01$) than in their *Parp-1*$^{+/-}$ and *Parp-1*$^{-/-}$ counterparts. This was associated with a higher incidence of splenomegaly in *Parp-1*$^{+/+}$ (5/17 (29%)) than in *Parp-1*$^{-/-}$ mice (0/19 (0%)), although the difference was not statistically significant (Table 3). Three *Parp-1*$^{+/+}$ mice (18%) and one *Parp-1*$^{-/-}$ mouse (6%) developed malignant lymphomas in the spleen, but the inter-group variation was not significant. In these cases, small foci of atypical lymphocytes were also observed in other organs, such as the liver and kidney.

Table 1
Final body and relative organ weights

Group	Genotype	IQ	No. of mice	Body weight (g)	Relative organ weights (%)		
					Liver	Kidneys	Spleen
1	+/+	+	17	29.2±4.1[a]	6.38±1.59[b]	1.23±0.16[a]	0.65±0.59
2	+/−	+	16	29.4±2.4[a]	5.53±0.68	1.13±0.12[a]	0.32±0.12[d]
3	−/−	+	17	26.5±1.4[a,d,e]	5.93±0.55	1.06±0.08[a,c]	0.28±0.07[d]
4	+/+	−	6	34.8±2.2	4.85±0.59	1.50±0.25	0.31±0.05
5	+/−	−	10	33.4±3.2	5.15±0.85	1.37±0.18	0.43±0.29
6	−/−	−	8	31.8±2.2	5.68±0.43	1.30±0.13	0.39±0.25

[a] Significantly different from control diet group of the same genotype at $P<0.001$.
[b] Significantly different from control diet group of the same genotype at $P<0.01$.
[c] Significantly different from +/+ group of the same treatment at $P<0.001$.
[d] Significantly different from +/+ group of the same treatment at $P<0.01$.
[e] Significantly different from +/− group of the same treatment at $P<0.01$.

Table 2
Incidence and multiplicity of liver lesions

Group	Genotype	IQ	No. of mice	Clear cell foci		Hepatocellular carcinoma		Hepatocellular adenoma	
				Incidence (%)	Multiplicity (No./tumor bearing mice)	Incidence (%)	Multiplicity (No./tumor bearing mice)	Incidence (%)	Multiplicity (No./tumor bearing mice)
1	+/+	+	17	1 (6)	2	3 ###	1	1 (6)	1
2	+/−	+	18	1 (6)	1	1 (6)	3	2 ###	2.5 ± 2.1
3	−/−	+	19	0		1 (5)	5	1 (5)	1
4	+/+	−	6	0		1 ###	1	0	
5	+/−	−	10	0		0		0	
6	−/−	−	8	0		0		0	

No brain tumors were noted in our present experiment, unlike the case with *Parp-1*$^{-/-}$ *p53*$^{-/-}$ mice, which have been reported to show a high incidence of brain tumors at week 24 [24].

4. Discussion

The present study demonstrated no increase with *Parp-1* deficiency in the incidences of tumors induced by IQ in the liver, lung and forestomach, which are the main targets for IQ-induced carcinogenesis in mice with the CDF1 genetic background [19]. Although Ohgaki et al. [19] and we used the same dose of IQ (300 ppm in the diet), we observed lower incidences of tumors in the target organs with the present C57BL/6 genetic background. However, the difference could be at least partly explained by the shorter experimental period we applied (60 versus 96 weeks) or a lower susceptibility of C57BL/6 mice to IQ carcinogenicity than with CDF1 mice.

IQ is metabolically activated, mainly in the liver, to form a mutagenic metabolite, *N*-hydroxy-IQ [25], which can be further activated by *O*-acetyl transferase to *N*-acetoxy-IQ [26]. Both *N*-hydroxy-IQ and *N*-acetoxy-IQ covalently bind to DNA, producing *N*-(deoxyguanosin-8-yl)-IQ adducts [27]. Another direct acting mutagen, *N*-nitro-IQ, is also reported to be generated by extrahepatic peroxidases such as prostaglandin H synthase [28]. IQ adducts produced by *N*-hydroxy-IQ, *N*-acetoxy-IQ and *N*-nitro-IQ are suggested to be repaired through nucleotide excision repair (NER) [21], in which Parp-1 is not involved [3]. Therefore, the absence of any difference in susceptibility to IQ-induced carcinogenesis between *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice is explainable and provides a sharp contrast to the much elevated susceptibility of *Parp-1*$^{-/-}$ mice on an ICR/129 Sv mixed genetic background to azoxymethane-induced colon and liver tumorigenesis [16], as well as *N*-nitrosobis(2-hydroxypropyl)amine (BHP)-induction of liver hemangiomas and hemangiosarcomas [15]. In BHP-treated mice, we observed a higher frequency of mutations, such as deletions, insertions/rearrangement in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice [29], providing direct evidence of decreased

Table 3
Incidences of the lesions and abnormality in the lungs, forestomach and spleen (%)

Group	Genotype	IQ	No. of mice	Lungs		Forestomach		Spleen	
				Alveolar cell hyperplasia	Adenoma	Hyperplasia	Papilloma	Splenomegaly	Malignant lymphoma
1	+/+	+	17	2 (12)	0	7 (41)	0	5 (29)	3 (18)
2	+/−	+	18	0	2 (11)	4 (22)	1 (6)	1 (6)	0
3	−/−	+	19	0	0	8 (42)	0	0	1 (6)
4	+/+	−	6	0	0	1 (17)	0	0	0
5	+/−	−	10	0	1 (10)	0	0	3 (30)	2 (20)
6	−/−	−	8	0	0	1 (13)	0	2 (25)	2 (25)

efficiency either in BER or DNA strand break repair. The evidence that Parp-2 is also activated by DNA damages and functions in base excision repair [30] implies the possibility that Parp-2 may complement the activity of Parp-1 in carcinogenesis by IQ. Although it has been suggested that main function of Parp-1 in suppressing the carcinogenesis induced by alkylating agents could not be complemented by Parp-2 or other members of Parp family [15,16], we could not completely exclude this possibility. This point might be further elucidated by studying carcinogenesis induced by IQ in animals with combined Parp-1 and other Parp family member deficiencies.

In line with our results, administration of 4-nitroquinoline 1-oxide (4NQO), which mimics UV induced-DNA damage mainly repaired by NER [31], did not result in a higher incidence of tumors in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice on an ICR:129/Sv mixed genetic background (Gunji et al., unpublished). Therefore, although the genetic background sometimes affects the outcome of carcinogenesis experiments, in both C57BL/6 and ICR:129/Sv cases, *Parp-1* deficiency did not elevate susceptibility to carcinogenesis induced by carcinogens that generate bulky DNA adducts. Taking all available data together, among the multiple functions of Parp-1, its role in BER and DNA strand break repair is suggested to be most important in the prevention of carcinogenesis. In contrast, *XPA*$^{-/-}$ mice, which lack an important enzyme, XPA, for NER, were found to demonstrate elevated susceptibility to the carcinogenic activity of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) [32], another mutagenic and carcinogenic heterocyclic amine, as well as 4NQO-induced oral tumorigenicity [33].

IQ is also reported to induce sister chromatid exchange [34], presumably through non-enzymatic reduction of *N*-nitro-IQ in the presence of NADH and Cu (II) and generation of H_2O_2, which results in oxidative DNA damage [35] in vitro. Free radical generation in the presence of IQ and NADH by recombinant human cytochrome b5 reductase has also been reported [36]. Analysis of mutation frequencies and patterns using Big Blue® rats indicated IQ-DNA adducts rather than IQ-induced oxidative DNA damage to be the major DNA lesions induced by IQ in vivo [37]. The notion that IQ-induced DNA adducts but not IQ-induced oxidative DNA damages are primarily responsible for the IQ-induced carcinogenesis is supported by the present findings. If IQ produces a significant amount of DNA strand breakage or oxidative DNA damage, which is mostly repaired through BER pathway [38], *Parp-1* deficiency would be expected to result in greater tumorigenesis in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice. The results also provide further evidence that susceptibility to tumorigenesis induced by carcinogens is primarily determined by the efficiency of DNA-repair capacity.

We here noted that, relative liver weights were increased in *Parp-1*$^{+/+}$ mice by IQ treatment ($P < 0.01$), whereas no increase was observed in their *Parp-1*$^{-/-}$ counterparts. Relative spleen weights were also increased in *Parp-1*$^{+/+}$ mice receiving the carcinogen ($P < 0.01$). A defect of S-phase entry in *Parp-1*$^{-/-}$ splenocytes on the C57BL/6 congenic background [39] might be connected with the smaller spleen in *Parp-1*$^{-/-}$ than other genotypes. However, the average relative weight data excluding animals harboring either liver nodules or splenic malignant lymphomas did not show significant differences among the genotypes. Thus, the differences in relative weights might have simply been due to the presence of tumors.

It would be of interest to examine the effect of *Parp-1*-deficiency on IQ-induced carcinogenesis at advanced age after a lower-dose and longer-term treatment with IQ because accumulating studies have indicated that Parp-1 is involved in cell death induction through NAD depletion [4,5]. A role in genome stability could be envisaged through regulation of centrosome function and also cell differentiation.

In conclusion, *Parp-1* deficiency in the present study did not alter the susceptibility to carcinogenesis induced by IQ, which produces bulky DNA adducts that may repaired through NER. This result is in sharp contrast to the elevated susceptibility of *Parp-1*$^{-/-}$ mice to carcinogenesis induced by alkylating agents that produce DNA damage repaired mainly through BER and DNA strand break repair pathways.

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