RESEARCH ARTICLE

Inflammation Enhanced X-irradiation-Induced Colonic Tumorigenesis in the Min mouse

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Abstract

Inflammation is potential risk factor of various human malignancies. Inflammatory bowel syndromes such as ulcerative colitis are well known as risk factors for colon cancer. Here, we examined enhancing effects of dextran sulfate sodium (DSS)-associated inflammation on X-irradiation induced colonic tumorigenesis in Min and wild-type (WT) mice. Animals were X-irradiated at 1.5 Gy at 5 weeks of age (at 0 experimental week) and 2% DSS in drinking water was administered at 5 or 11 experimental weeks. Mice were sacrificed at 16 weeks and incidence and multiplicity of colonic tumors were assessed. Incidence of colonic tumors in Min mouse was increased from 33.3% to 100% ($p<0.05$) with X-irradiation alone, whereas no tumors were developed in WT mice. In DSS-treated Min mice, X-irradiation increased the number of colonic tumors. Total number of colonic tumors was increased 1.57 times to 30.7±3.83 tumors/mouse with X-irradiation+DSS at 5 weeks comapared to 19.6±2.9 in corresponding DSS alone group ($p<0.05$). When the duration of inflammation was compared, longer period of DSS effect promoted more colonic tumorigenesis. Collectively, we conclude that X-irradiation and DSS-induced inflammation act synergistically for colonic tumorigenesis.

Keywords: Min mouse - X-irradiation - DSS - colon

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Introduction

Inflammation has been widely known as strong risk and promoting factor of carcinogenesis (Balkwill and Mantovani, 2001) in various types of human cancers (Ohshima et al., 2003). Among them, ulcerative colitis is in high risk condition in colonic carcinogensis (Munkholm, 2003). In the animal counterpart, dextran sulfate sodium (DSS) (Okayasu et al., 1990) showed powerful tumor promoting effect in murine colonic carcinogenesis models initiated with azoxymethane (AOM) (Tanaka et al., 2003), 1,2-dimethylhydrazine (DMH) (Kohno et al., 2005), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Tanaka et al., 2005).

Familial adenomatous polyposis (FAP) is an inherited human disease characterized by numerous colorectal tumorigenesis (Kinzler and Vogelstein, 1996). FAP is caused by mutation in the adenomatous polyposis coli (APC) tumor suppressor gene (Powell et al., 1993). Min (multiple intestinal neoplasia) mouse is a murine model of human FAP (Moser et al., 1990), which has nonsense mutation at codon 850 in Apc gene (Su et al., 1992). The mouse develops multiple intestinal adenomas with inactivation of wild type allele. Min mouse have been revealed to be highly susceptible to carcinogenic agents. Subcarcinogenic low-dose *N*-ethyl-*N*-nitrosourea (ENU) increased the tumor incidence in the intestine and mammary gland in Min mice (Shoemaker et al., 1995). Other colonic carcinogens including PhIP (Steffensen et al., 1997) and AOM (Paulsen et al., 2003) also increased intestinal tumors. Besides chemical carcinogens, Min mice have been revealed to be susceptible to ionizing radiation (Luongo and Dove, 1996) in the age-dependent manner (Okamoto and Yonekawa, 2005). Inflammatory stimuli by DSS strongly induced colonic neoplasia in Min mice (Tanaka et al., 2006).

In this study, we investigated whether DSS-induced inflammation enhanced colorectal tumorigenesis initiated with low-dose X-irradiation in Min mice.

Materials and Methods

Animals and genotyping

Male C57BL/6J-ApcMin/J (Min) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Female wild type (WT) C57BL6/J were obtained from Clea Japan (Tokyo, Japan). They were housed in plastic cages with hardwood chips in an air-conditioned room

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with 12 h light-12 h dark cycle and were given basal diet (OA-2, Clea Japan) and water *ad libitum*. One male Min mouse and 5 female wild type C57BL6/J were mated and offsprings were subjected to genotyping. DNA samples were extracted from their tails using a QIAamp tissue kit (QIAGEN, Tokyo, Japan). The 10 µl PCR reaction mixture consited of TITANIUM Taq DNA polymerase (Clontech, Mountain View, CA, USA), 1x buffer provided, 1x dNTP, 1µM of PCR primers (named oIMR0033, oIMR0034, and oIMR0758) and 1µl of genomic DNA. oIMR0033 (5'- GCC ATC CCT TCA CGT TAG -3') and oIMR0034 (5'- TTC CAC TTT GGC ATA AGG C -3') are forward and reverse wild type primers, respectively, to amplify common region. oIMR0758 is forward Min specific primer (5'- TTC TGA GAA AGA CAG AAG TTA -3') in which final adenosin residue corresponds to the mutation. PCR was performed using a Veriti thermal cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) as follows: 1 cycle of 95°C for 1 min; 35 cycles of 95°C for 30 sec, 65°C for 30 sec, 68°C for 30 sec; 1 cycle of 68°C for 3 min. The reaction mixture was electrophoresed in 0.8% SeaKem GTG agarose gel (Cambrex, East Rutherford, NJ, USA). Animals were judged as Min, if 340 bp band was appeared; both of wild type and Min genotypes possessed 600 bp band encompassing the mutation residue.

Experimental design

The experimental design is shown in Figure 1. Min mice and littermate WT mice were randomly divided into 6 groups (groups A–D). Single whole-body irradiation was given to five week-old animals at 1.5 Gy using an X-ray irradiator (MBR-1520R-3, Hitachi Power Solutions Co., Ltd., Tokyo, Japan) with a 0.5 mm Al+0.2 mm Cu filter in Groups C, D, and F. Dose of X-irradiation was determined to be lower than the previous report (Okamoto and Yonekawa, 2005) to compare enhancing effect of dextran sulfate sodium (DSS). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA), dissolved in water at a concentration of 2% (w/v), and administered at 5 experimental week in Groups E and F or at 11 week in Groups B and D for 1 week. The animals were sacrificed at 16 week. Total colon and cecum were fixed in 10% neutral buffered formalin or methacarn and stained with 0.2% methylene blue. Colon segments (S) were divided from S1 to S4 and cecum as S5 (Figure 2). The number of colonic tumors was counted in each segment. The experimental design was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute, and the animals were cared for in accordance with institutional guidelines as well as the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

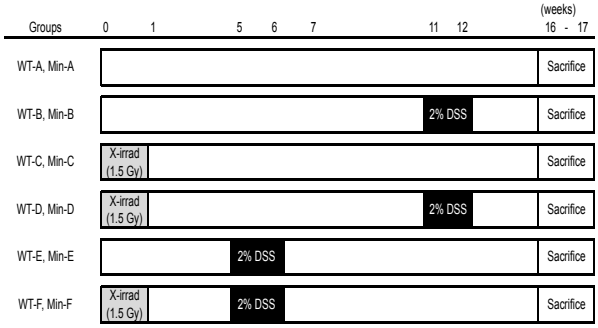

Figure 1. Experimental Protocol

Statistical analysis

The significance of difference in the incidence of colonic tumors was calculated using Fischer's exact test. Tumor multiplicities were analyzed with Mann-Whitney *U* test. Differences were considered as statistically significant if $p<0.05$.

Results

Incidence of colon tumors in WT and Min mice

Effective numbers of animals are described in Table 1. In the wild type mice, incidence of Group WT-D (33.3%) is significantly higher than that of Group WT-C (0%), suggesting enhancing results of DSS on X-irradiated tumorigenesis ($p<0.05$).

When Groups Min-A and Min-C were compared in Min mice, incidence of Group C (100%) was higher than that of Group A (33.3%), proving the aggravating effect of X-irradiation especially in Min mice ($p<0.002$).

When the two genotypes were compared in each group, Min mice showed increased incidence with statistical significance in Groups B-F (Table 1).

Multiplicity of colon tumors in Min mice

X-irradiation at 1.5 Gy alone [the number of tumors=0.91±0.21 (Ave±SE)/mouse in Group C] increased the number of colon tumors in S2 compared with the corresponding region in Group A (0.25±0.13/mouse, $p<0.05$). Total number was also augmented (2.55±0.41 and 1.33±0.45/mouse in Groups C and A, respectively,

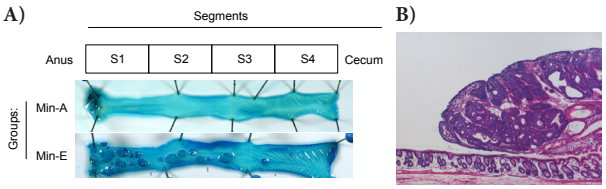

Figure 2. Macroscopic View and Histology of Colon Tumor. A) Macroscopic view of colonic mucosa. Total colon is divided to S1-S4. Cecum is S5 (not shown). Methylene blue staining. **B)** Representative histology of colonic tumor. Hematoxylin and eosin staining. Original magnification, 50x

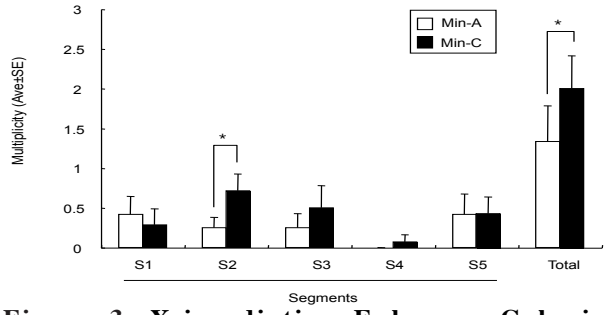

Figure 3. X-irradiation Enhances Colonic Tumorigenesis in Min mice without DSS. Group Min-A (Open bar) and Min-C (Closed bar). *p<0.05

Table 1. Incidence of Colon Tumors

		WT		Min		
		Effective No. (male/female)	No. of mice with colon tumors (Incidence)	Effective No. (male/female)	No. of mice with colon tumors (Incidence)	WT vs. Min
Groups	A	12 (6/6)	0 (0%)	12 (10/2)	4 (33.3%)	p=0.09
	B	14 (7/7)	3 (21.4%)	9 (4/5)	9 (100%)	p<0.002
	C	17 (8/9)	0 (0%)	11 (4/7)	11 (100%)**	p<0.0001
	D	9 (3/6)	3 (33.3%)*	8 (3/5)	8 (100%)	p<0.001
	E	15 (9/6)	0 (0%)	7 (3/4)	7 (100%)	p<0.0001
	F	10 (5/5)	2 (20.0%)	6 (5/1)	6 (100%)	p<0.001

*p<0.05 vs. WT-C, **p<0.002 vs. Min-A

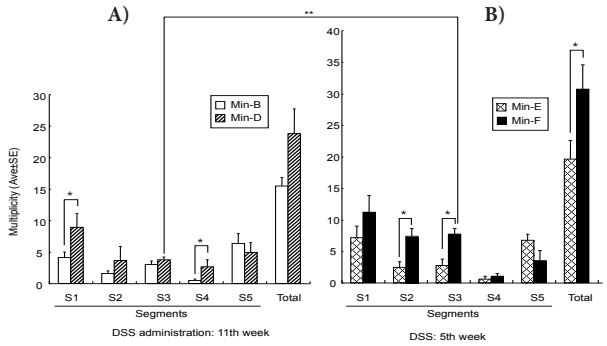

Figure 4. X-irradiation Promoted Colonic Tumorigenesis in DSS Treated Min mice. Group Min-B (Open bar), Min-D (Hatched bar), Min-E (Cross-hatched bar), and Min-F (Closed bar). *p<0.05 and **p<0.01

p<0.05) (Figure 3).

When 2% DSS was administered at 11 experimental week (Groups B and D), the number of colonic tumors were 8.88±2.20 (p<0.05) and 2.63±1.15 (p<0.05) in S1 and S4 in Group D, compared to 4.11±0.87 and 0.44±0.24, respectively, in Group B. It suggested enhancing effect of X-irradiation in Group D (Figure 4A).

If DSS was given at 5 week (Groups E and F), the numbers were 7.33±1.26 and 7.67±0.96 in S2 and S3, respectively, in Group F, compared with 2.43±0.87 and 2.71±1.04 in Group E, also indicating stimulating effect of X-irradiation in Group F (Figure 4B). Total numbers of colonic tumors was 30.67±3.83 and 19.57±2.9 in Groups F and E, respecively; the former was significantly higher than the latter (p<0.01).

When the two X-irradiation+DSS groups (Groups D and F in Figure 4 crossing left and right panels) were compared, earlier administration of DSS (7.67±0.96, Group F) was more effective in increment of colonic tumors in S3 compared to Group D (3.75±0.45) (p<0.05).

Multiplicity of colon tumors in WT mice

The number of total colonic tumors were 0.00±0.00, 0.50±0.27, 0.00±0.00, 0.78±0.46, 0.00±0.00, and 0.20±0.13/mouse in Groups A, B, C, D, E, and F, respecively, showing no significant differences among these groups.

Discussion

In the present study, we analyzed promoting effect of DSS-induced inflammation on X-irradiated colorectal carcinogenesis in WT and Min mice. Firstly, X-irradiation alone without DSS was assessed to confirm the effect of X-irradiation. In the Min mouse, incidence of colonic tumors was increased to 100% compared to non-irradiated group (33.3%). The number of colonic tumors in S2 was also increased with X-irradiation compared with non-irradiated Min mice. On the other hand, WT mice were not influenced with X-irradiation. It suggested that *Apc* locus might be more sensitive to loose normal APC function in Min mice although chromosome abberation might have occurred independent of their sequence (Rydberg, 1996). Okamoto and Yonekawa (Okamoto and Yonekawa, 2005) reported that 10 days old Min mice were more sensitive than other ages. In this study, since the mice were X-irradiated around 5 weeks old (35 days old), enhancing effect may have become unclear.

In the Min mice, DSS alone has known to enhance colonic tumorigenesis (Tanaka et al., 2006). X-irradiation was further added to assess if it may have enhanced colonic tumorigenesis. When Group F was compared with E, tumor multiplicity was increased in S2 and S3. Then, Group D vs. B, the number of tumors was increased upon X-irradiation in S1 and S4. Although localization of colonic tumors were different, X-irradiation was proved to exacerbate DSS-associated colonic tumorigenesis in Min mice. When the timing of DSS treatment was compared whether duration of inflammation could affect promoting effect, longer duration of inflammation had more enhancing effect (Group F) compared with shorter period (Group D). Similar phenomenon was observed in carcinogen induced murine colonic carcinogenesis model (Tanaka et al., 2003) and DSS alone induced model (Tanaka et al., 2006).

Considering WT mice, tumor incidence was increased in Groups D and F compared with non-treated or X-irradiation alone Groups, although enhancing effect was not as clear as that of Min mice. It was suggested that DSS treatment could bring latent genetic damage to apparent colonic tumors.

In the serial research in Life Span Study cohort of atomic bomb surviviors (Ozasa et al., 2012), the additive radiation risk of solid cancers continues to increase throughout life with a linear dose-response relationship. The estimated lowest dose range with a significant excessive relative risk (ERR) for all solid cancer was 0 to 0.20 Gy indicating no threshold. The risk of cancer mortality increased significantly for most major sites, including colon, whereas rectum did not. In the current study, multiplicities of colonic tumors (S2-S4 regions) were significantly increased with X-irradiation in Min-C (S2), Min-D (S4), and Min-F (S2 and S3) groups. In the

rectum (S1 region), although tumor multiplicity was not significantly different in DSS non-treated groups between Min-C and Min-A regardless of X-irradiation, Min-D receiving X-irradiation showed higher tumor multiplicity in the rectum compared with Min-B group. It suggested DSS-induced inflammation might have influenced rectal tumorigenesis as well as in colon.

Exposure to ionizing radiation is associated with an increased risk of cancer. The majority of radiation exposure and risk associated with gastrointestinal malignancy comes from CT scans, especially of the abdomen and pelvis; the colon carries the highest lifetime attributable risk of radiation associated malignancy (Chang and Hou, 2011). Besides patients, cancer risk of colon and rectum cancers in male diagnostic radiation workers in Korea also showed a significantly increasing trend according to the increase of the average annual radiation dose (HR: 2.37) (Choi et al., 2013).

Oncology patients treated for childhood cancer tended to develop secondary colorectal carcinomas. This risk was reported to be proportional to dose and volume of radiation; tumors were more likely localized in an irradiated segment of the colon (Nottage et al., 2012). Radiotherapy is a powerful tool for the treatment of gynecological malignancies including cervical (Tamai et al., 1999) and endemetrial cancers (Brown et al., 2010) and prostate cancer (Bolla et al., 2013). Patients treated with radiotherapy likely have significantly increased risk of subsequent primary malignancies including bladder, vagina, colon, and soft-tissue (Brown et al., 2010).

Low-level ionizing radiation (mean colon dose=0.18 Gy) has been revealed to influence the development of soft tissue sarcomas in atomic bomb survivors (Samartzis et al., 2013). However, no sarcomas were found in the current study, suggesting the DSS-induced inflammation has rarely affect stromal cells at least in this experimental condition.

In summary, our results suggest that colonic inflammation enhanced occult X-irradiated tumorigenesis not only in Min mice but also WT animals. It should be noted that those with colorectal inflammation including inflammatory bowel disease might exacerbate risk of colorectal cancer development in people with previous X-ray exposure. Patients should be carefully followed up especially with bowel inflammation.

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