**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 compared 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

**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 with a 12-hour light-dark cycle. Mice were divided into two groups: one group was given 2% DSS in drinking water for 14 days starting at 5 weeks of age, and the other group was given DSS for 11 weeks starting at 11 weeks of age. The X-irradiation group was given 1.5 Gy X-irradiation at 5 weeks of age.

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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 consisted 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 oIMR0033 (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 body-whole 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 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.

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.

Statistical analysis

The significance of difference in the incidence of colonic tumors was calculated using Fisher’s exact test. Tumor multiplicities were analyzed with Mann-Whitney U test. Differences were considered as statistically significant if p<0.05.
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Table 1. Incidence of Colon Tumors

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Min</th>
<th>WT vs. Min</th>
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<tbody>
<tr>
<td></td>
<td>Effective No. (male/female)</td>
<td>No. of mice with colon tumors (Incidence)</td>
<td>No. of mice with colon tumors (Incidence)</td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>12 (6/6)</td>
<td>0 (0%)</td>
<td>12 (10/2)</td>
</tr>
<tr>
<td>B</td>
<td>14 (7/7)</td>
<td>3 (21.4%)</td>
<td>9 (4/5)</td>
</tr>
<tr>
<td>C</td>
<td>17 (8/9)</td>
<td>0 (0%)</td>
<td>11 (4/7)</td>
</tr>
<tr>
<td>D</td>
<td>9 (3/6)</td>
<td>3 (33.3%)*</td>
<td>8 (3/5)</td>
</tr>
<tr>
<td>E</td>
<td>15 (9/6)</td>
<td>0 (0%)</td>
<td>7 (3/4)</td>
</tr>
<tr>
<td>F</td>
<td>10 (5/5)</td>
<td>2 (20.0%)</td>
<td>6 (5/1)</td>
</tr>
</tbody>
</table>

*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

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 survivors (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

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 alone without DSS was assessed to confirm the effect of X-irradiation.
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 endometrial 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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