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Sex-dependent Differences in Intestinal Tumorigenesis Induced in Apc1638N/+ Mice by Exposure to γ Rays

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Summary

A study was carried out in an adenomatous polyposis coli (APC) mutant mouse model to assess the effect of 1 and 5 Gy of radiation on intestinal tumorigenesis, and to compare susceptibility of females vs males. We report sex-dependent differences in susceptibility to radiation induced intestinal tumorigenesis and provide evidence that exposure to radiation doses as low as 1 Gy can induce a significant increase in intestinal tumor multiplicity and enhance tumor progression *in vivo*.

Purpose—The purpose of the present study was to assess the effect of 1 and 5 Gy radiation doses and to investigate the interplay of gender and radiation with regard to intestinal tumorigenesis in an adenomatous polyposis coli (APC) mutant mouse model.

Methods and Materials—Apc1638N/+ female and male mice were exposed whole body to either 1 Gy or 5 Gy of γ rays and euthanized when most of the treated mice became moribund. Small and large intestines were processed to determine tumor burden, distribution, and grade. Expression of proliferation marker Ki-67 and estrogen receptor (ER)- α were also assessed by immunohistochemistry.

Results—We observed that, with both 1 Gy and 5 Gy of γ rays, females displayed reduced susceptibility to radiation-induced intestinal tumorigenesis compared with males. As for radiation effect on small intestinal tumor progression, although no substantial differences were found in the relative frequency and degree of dysplasia of adenomas in irradiated animals compared with controls, invasive carcinomas were found in 1-Gy- and 5-Gy-irradiated animals. Radiation exposure was also shown to induce an increase in protein levels of proliferation marker Ki-67 and sex-hormone receptor ER- α in both non tumor mucosa and intestinal tumors from irradiated male mice.

Conclusions—We observed important sex-dependent differences in susceptibility to radiation-induced intestinal tumorigenesis in Apc1638N/+ mutants. Furthermore, our data provide evidence

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that exposure to radiation doses as low as 1 Gy can induce a significant increase in intestinal tumor multiplicity as well as enhance tumor progression *in vivo*.

Introduction

In the last 4 decades, significant progress has been achieved in the treatment and cure of cancer, either by radiation therapy alone or in combination with surgery and/or chemotherapy. Subsequently, concern for the risk of radiation-induced second neoplasms among long-term survivors has been growing, especially in the case of childhood cancer patients (1, 2). From epidemiological studies such as the Childhood Cancer Survivor Study (CCSS), it has been shown that among subsequent malignant neoplasms occurring 5 years or more after childhood cancer, risk was increased also for small intestine and colorectal cancer (CRC), typically occurring in older adults (3). To the present date, mechanisms underlying development of radiation-induced intestinal neoplasms are unknown.

Important data on carcinogenic risk of exposure to ionizing radiation (IR) are obtained from the Life Span Study (LSS) on the Hiroshima and Nagasaki atomic bomb (A-bomb) survivors. In the LSS cohort, between 1958 and 1998, significant radiation-associated increase in cancer risk has been observed for most sites, including the colon (4). Notably, for colon cancer, a sex difference was observed for the excess relative risk (ERR) per Gy (at age 70 years after exposure at age 30 years). The ERR gender ratio (ie, $ERR_{CRCFemale}/ERR_{CRCMale}$) for colon cancer was reported to be significantly lower than for persons with any other solid cancer in the LSS cohort (4).

Mutations in the adenomatous polyposis coli (APC) gene, and its subsequent inactivation, are present in 80% of lesions from both familial adenomatous polyposis (FAP) patients and individuals affected by sporadic colon cancer. In the present study, we used the Apc1638N/+ mouse model, which, in the C57BL/6J background, develops <10 intestinal adenomas during their lifetime and can live beyond 1 year (5). Furthermore, in this model some of the small intestinal lesions spontaneously progress to carcinomas. Previously published *in vivo* studies carried out in the Apc1638N/+ model and in other Apc mutant models have focused only on the effect of radiation doses ≥ 2 Gy on intestinal tumorigenesis (6, 7) (for additional primary papers on this topic, see also SR4 and SR5 in S4 Supplementary References), whereas no data are available on effect of low doses (< 1 Gy), which are the most relevant in radiation protection. Moreover, *in vivo* investigation of gender-dependent differences for radiation-induced intestinal tumorigenesis is limited to only a few studies (8), despite epidemiological evidence and animal data supporting a role of gender-dependent factors in colorectal tumorigenesis in the general population (9-12).

In the present study, we aimed to assess the effect of 1-Gy and 5-Gy radiation doses and to investigate the interplay of sex and radiation with regard to intestinal tumorigenesis in Apc1638N/+ mice. To this purpose, young female and male mice were exposed whole body (WB) to 1 Gy or 5 Gy of γ rays, and intestinal tumor number, distribution, and grade in these mice were assessed, along with molecular parameters relevant to proliferation and sex-hormone receptors expression in the intestinal epithelium.

Methods and Materials

Mouse husbandry and genotyping

Male and female C57BL/6J-Apc1638N/+ mice were generated and genotyped as previously described (6).

Radiation exposures

Female and male mice (6-8 weeks old) were exposed whole body to either 5 Gy or 1 Gy of γ rays. Radiation exposures were performed as previously described (6).

Analysis of intestinal tumors and histology

Control and irradiated mice were euthanized by CO₂ asphyxiation at ~180 (for 5 Gy) or ~260 (for 1 Gy) days of age, when most of the treated mice became moribund. At least 10 animals were used for each experimental group. Intestines were harvested and processed as described in S1 Supplementary Methods for tumor counts and histology.

Immunohistochemistry

Immunohistochemistry was performed by standard procedures (13) on all tumors from at least 3 animals per group. Incubation with primary antibodies for Ki-67 (1:750, NCL-ki67P, Novocastra, Buffalo Grove, IL, USA) or ER- α (1:600, 04-227, Millipore, Darmstadt, Germany) was performed at room temperature for 1 hour.

Statistical analysis

When comparing 2 groups, statistical significance was determined by 2-tailed, unpaired Student's *t* test. *P* values <.05 were considered significant, and fold change was calculated with a 95% confidence interval. Proliferative index was calculated for all experimental sections as described in S1 Supplementary Methods.

Results

Female mice show reduced susceptibility to intestinal tumorigenesis after 5 Gy of γ rays compared with males

Most of the 5-Gy-treated mice became moribund ~ 140 days after irradiation, regardless of sex, whereas the average lifespan of our control cohort was ~ 12 months. At the time of euthanasia, we observed that control male mice displayed significantly more intestinal lesions compared with females (5.4 vs 2.8; *P*<.05) (Table 1). We observed a significant difference in total intestinal tumor burden also between irradiated males compared with irradiated females (56.7 vs 8.3; *P*<.001) (Table 1). Overall, these data translated into a ~ 11-fold increase in intestinal tumor multiplicity (*P*<.001) for male mice vs. a ~3-fold increase (*P*<.001) for female mice compared with age- and sex-matched controls (Table 1).

Furthermore, when different regions of the intestinal tract (duodenum, jejunum, ileum, colon) were considered individually, we found that average number of adenomas in duodenum was significantly increased in both irradiated female (*P*<.05%) and male mice compared with age- and sex-matched controls, whereas tumor multiplicity in jejunum, ileum, and colon was significantly increased in irradiated males only (Fig. 1).

Intestinal tumorigenesis in mice exposed to 1 Gy vs. 5 Gy of γ rays

Mice exposed to 1 Gy became moribund ~210 days after exposure, therefore had a lifespan averaging 1.6 times that of 5-Gy-irradiated animals (moribund ~ 140 days after irradiation). With both doses, intestinal tumor load corresponded to onset of the moribund phenotype, and death was due to intestinal obstruction and/or intussusception by tumors, as determined at the time of necropsy. No death was reported to be due to metastatic disease or non-GI-tumors. We found that the 1-Gy dose induced in males a lower increase in intestinal tumor multiplicity compared with 5 Gy (3.8-fold vs 10.4-fold compared with control) (Table 1). This effect was even more pronounced in females that displayed, compared with controls, no

significant increase after 1 Gy vs a statistically significant 2.9-fold increase measured in 5-Gy-irradiated female mice (Table 1).

From microscopic examination on the colons of irradiated Apc1638N/+ male mice, lesions were often detected in all regions of the large intestine, ie, proximal, middle, distal (Supplementary Figure e1). Furthermore, irradiated male mice displayed in the distal colon the highest relative increase after both 5 Gy (Table 1) and 1 Gy of whole body γ -ray-exposure (Fig. 2). Male and female control mice, including moribund controls, as well as irradiated females in our cohort, did not develop adenomas in the distal colon (Table 1).

Radiation exposure enhances tumor progression in small intestine

To assess the effect of radiation exposure on tumor progression, 47 small intestinal tumor samples isolated from ten 5-Gy-irradiated and 41 from ten 1-Gy-irradiated male mice were subjected to histopathological examination. Similarly, a total of 32 tumor samples from control sex- and age-matched animals were employed for comparison. Some of the intestinal fragments from irradiated animals contained multiple lesions (3 to 5 lesions for 5-Gy-irradiated, 2 to 3 lesions for 1-Gy-irradiated). No significant difference was observed in the proportion and the degree of dysplasia for small intestinal adenomas in irradiated animals compared with age-matched controls (Table 2). However, although *in situ* carcinomas were more frequent in control animals, invasive carcinomas were found only in the 1-Gy- and 5-Gy-irradiated animals and not in control age-matched male mice (Table 2). No significant differences were observed in regard to tumor grade in irradiated female mice compared with age- and sex-matched controls.

We also carried out experiments in which 1-Gy- 5-Gy-irradiated and control animals were euthanized at 3 months post-irradiation to assess whether major changes occurred in regard to latency time of intestinal carcinomas with the 2 different radiation doses. After sacrifice, the number of intestinal lesions was scored, and pathological evaluation was performed on all lesions. Although for 1-Gy-irradiated mice the frequency and grade of intestinal lesions at 3 months post exposure were not significantly different from control animals, 5-Gy-irradiated male mice displayed a significantly higher tumor burden (~ 10 -fold greater than control and 1-Gy-irradiated male mice, $P<.05$). No carcinomas were found in 1-Gy- or 5-Gy-irradiated animals 3 months after exposure. In addition, regardless of the dose, most lesions were classified as tubular adenomas with low-grade dysplasia.

Effect of radiation exposure on proliferation and hormonal markers in intestinal non tumor epithelium and tumors

To understand whether the difference in the number of intestinal adenomas in irradiated vs control mutants, and in female vs male mice reflected differences in proliferation index in the intestinal mucosa, we performed immunohistochemistry for the proliferation marker Ki-67 (Figs. 3 and 4). A summary of proliferative indexes is reported in Table e1 (S2 Supplementary Tables). In non tumor small intestine of control male animals, localization of Ki-67 positive cells was mostly restricted at the crypt base and walls (Fig. 3a), whereas a more circumferential and more intense staining pattern was detected in non tumor small intestine of irradiated male animals (Fig. 3e). Similarly, in non tumor colonic epithelium from control mice, Ki-67-positive cells were localized at the bottom of the crypts (Fig. 4a), whereas in tissue from irradiated male animals the Ki-67 positive region extended to the crypt walls (Fig. 4b). Carcinomas from small intestine of irradiated animals also exhibited higher expression levels for Ki-67 compared with carcinomas from control mice (Figs. 3c, 3g). The reported observations were radiation dose independent in male mice, whereas in female mice no striking differences were observed between control and irradiated animals (Supplementary Fig. e2).

Based on the reported sex differences, we speculated that radiation exposure might have induced changes in regard to sex-specific factors involved in intestinal homeostasis. Because it had been previously shown that expression of ER- α in non tumor intestinal epithelium was restricted to crypt enterocytes residing at the crypt base to approximately three-fourths of their length (reference SR2 in S4 Supplementary References), with a distribution pattern similar to that of Ki-67 positive cells, we asked whether IR would induce any change also in ER- α levels in both tumors and non tumor tissue. When we looked at ER- α expression in small intestine from male mice, we observed an increase in both staining distribution and intensity in non tumor tissues (Fig. 3b, f) and a *de novo* expression of ER- α in carcinomas (Fig. 3d, h) for irradiated mice compared with controls (Table e2 in S2 Supplementary Tables). ER- α expression was observed also in colonic adenomas and, in particular, strong nuclear ER- α expression was detected in colonic adenomas from 1-Gy-irradiated male mice (Fig. 4f). In tissues from female mice, baseline (control) expression of ER- α was higher than in age-matched control males, and no increase was detected in female mice following exposure to γ rays (Supplementary Figure e3).

Discussion

Exposure to radiation has been shown to enhance the frequency and onset of colon cancer in exposed individuals (3, 14). Inheritance of mutations in the Apc gene predisposes carriers to spontaneous intestinal tumorigenesis (15) and the human phenotype is recapitulated to some extent in Apc mutant mouse models. The Apc1638N/+ mouse model is well suited for studies on risk of radiation-induced intestinal tumorigenesis due to both its low tumor background and high signal-to-noise ratio (6).

Multiple independent *in vivo* studies have demonstrated that exposure to high radiation doses increases intestinal tumor burden in susceptible Apc mutant models (6, 7) (SR4 in S4 Supplementary References). Here we provide evidence that exposure to doses as low as 1 Gy of low-linear energy transfer (LET) radiation produced a significant increase in intestinal tumor multiplicity in small intestine and colon, as well as in the frequency and proportion of carcinomas and invasive carcinomas in the small bowel. From our study it has also emerged that female mutants displayed significant resistance to γ radiation-induced intestinal tumorigenesis compared with males. In a previously published study, Nakayama et al reported that ApcMin/+ male mutants exposed to 5 Gy of X-rays displayed higher multiplicity of invasive carcinomas compared with females (8). However, the investigators did not observe any difference between male and female mice in regard to total intestinal tumor multiplicity after radiation exposure. This may be due to different criteria used for tumor scoring in their study compared with ours.

We also report that only irradiated Apc1638N/+ males, but not females, developed lesions in the distal colon. *In vivo* studies have indicated that tumor regionality reflects the mechanism of tumor initiation, and that different genetic pathways dominate the carcinogenic process in the proximal and distal portion of the small intestine (SR3 in S4 Supplementary References.). Therefore, our model can be a valuable system for future studies aimed at elucidating genetic or epigenetic mechanisms responsible for sex-dependent differences in radiation-induced colorectal cancer risk.

Although the precise role of estrogen in colorectal carcinogenesis remains unclear, it is believed that the balance between ER- α and ER- β may be of importance for cell growth and tumor formation in the gut, as it is in other organs (16, 17). Selective loss of ER- β protein has been reported in malignant colon as compared with non tumor mucosa from the same individuals, in parallel with increase in ER- α :ER- β ratio (18). While the role of ER- β in mediating the anti tumor properties of estrogen in the colon has been widely investigated

(19), our understanding of ER- α involvement in colorectal tumorigenesis is far more limited. In the course of our study, we observed a striking increase in ER- α in non tumor mucosa and adenomas/carcinomas in the small intestine of irradiated male mice only, whereas ER- β levels were unchanged in all treatment and sex groups (data not shown). Further investigations are awaited to elucidate molecular mechanisms responsible for the sex-dependent radiation effects that we describe here.

Persistent or late molecular changes in non tumor tissues, such as the increase in Er- α protein levels discussed above, can be very informative of the effect of radiation exposure, and very important in explaining carcinogenesis resulting from it. We observed that in irradiated male mice, proliferation was significantly increased in the epithelium of both small intestine and colon. It is intriguing that such a field effect was observed as late as 7 months after radiation exposure. This push toward enhanced tissue renewal may have contributed to the increase in intestinal tumorigenesis observed for irradiated mice compared to controls. Notably, also intestinal carcinomas from irradiated mice showed higher relative numbers of Ki-67 positive cells compared with the same type of lesions from control animals. This suggests that radiation exposure may be capable, by inducing lesions with higher proliferative potential, of leading to higher risk of invasive carcinomas, as described here.

Conclusion

In conclusion, we have shown here that male and female Apc1638N/+ mutants displayed different susceptibility to radiation-induced intestinal tumorigenesis. Our data also provide evidence that *in vivo* exposure to radiation doses as low as 1 Gy can produce a significant increase in intestinal tumor multiplicity and in the risk of invasive carcinomas in susceptible rodent models. Further studies are awaited to identify molecular mechanisms involved in the pathogenesis of intestinal and other radiation-induced neoplasms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Total intestinal tumor distribution and burden in Apc1638N/+ male and female mice 140 days after exposure to 5 Gy of γ rays. Average number of tumors is reported for duodenum, jejunum, ileum, colon and whole intestinal tract. Standard deviations and statistical significance are also indicated (* $P<.001$).

Fig. 2. Tumor distribution in the colon of male and female mice 210 days after exposure to 1 Gy of γ rays. Average total numbers of tumors are reported for proximal, middle and distal colon. Standard deviations and statistical significance are also indicated (* $P < .001$).

Fig. 3.

Expression and localization of Ki-67 and Er- α proteins in small intestine from 1-Gy- γ -irradiated and control male mice at ~210 days after exposure. Ki-67 was detected in crypts in non tumor small intestine from control and irradiated animals (a and e) with comparable intensity. However, in irradiated animals, Ki-67 expression (e) in intestinal crypts was characterized by higher distribution and more circumferential pattern compared with controls (a). Ki-67 intensity and distribution was higher in small intestinal carcinomas from irradiated mice (c) compared with controls (g). In control mice, Er- α expression was low and cytoplasmic in non tumor small intestine (b) but was absent in small intestinal carcinomas (d). In irradiated mice, higher cytoplasmic expression for Er- α was detected in crypts from non tumor epithelium (f), and even stronger expression was observed in small intestinal carcinomas (h). Magnification $\times 20$ (a, b, e, f) and $\times 10$ (c, d, g, h). These observations are also summarized in Table e2 in the Supplementary Data.

Fig. 4. Expression and localization of Ki-67 and Er- α proteins in large intestine from 1-Gy- γ -irradiated and control male mice at ~210 days after exposure. Ki-67 was increased in non tumor mucosa of irradiated (b) compared with control mice (a) and was also highly expressed in colonic adenomas from irradiated mice (c). Er- α expression was absent in non tumor colonic epithelium from both control and irradiated mice (b, e), whereas, in adenomas from irradiated mice, both nuclear and cytoplasmic expression was detected (f). Magnification $\times 20$ (a, b, d, e) and $\times 40$ (c, f).

Table 1
Total intestinal tumor multiplicity and frequency of distal colonic adenomas in γ -irradiated male and female Apc1638N/+ mice

Radiation dose (Gy)	Intestinal tumor no. (Mean $\pm$ SD)		LI tumor no. (Mean $\pm$ SD)		Intestinal tumor fold-increase*		Distal LI tumor frequency		Intestinal polyp size (mm) (mean $\pm$ SD)	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
0	5.4 $\pm$ 2.4	2.8 $\pm$ 2.0	0.1 $\pm$ 0.3	0.2 $\pm$ 0.4	-	-	N/A	N/A	1.8 $\pm$ 0.8	1.8 $\pm$ 0.9
1	19.8 $\pm$ 9.0	5.0 $\pm$ 2.3	1.6 $\pm$ 1.7	0.2 $\pm$ 0.5	3.8	1.8	0.8	0	2.3 $\pm$ 1.4	1.8 $\pm$ 1.0
5	57 $\pm$ 15	8.3 $\pm$ 4.0	4.7 $\pm$ 2.0	1.0 $\pm$ 1.3	10.4 [†]	2.9 [*]	0.9	0	1.6 $\pm$ 0.9	1.7 $\pm$ 0.9

Abbreviations: LI = large intestine; N/A = not applicable; SD = standard deviation.

* Fold increase was calculated using the corresponding control values as reference.

[†] P-values <.05 were considered significant.

Table 2
Proportion of adenomas and carcinomas in control and irradiated Apc1638N/+ male mice

Group/treatment	Relative proportion* (%) $\pm$ SE (%)			
	Low-grade adenomas	High-grade adenomas	Carcinomas	Invasive carcinomas
Age-matched controls (n=32)	70 $\pm$ 8	10 $\pm$ 5	20 $\pm$ 7	0
1 Gy γ -rays (n=41)	80 $\pm$ 6	0	3 $\pm$ 3	17 $\pm$ 6
5 Gy γ -rays (n=47)	78 $\pm$ 6	0	15 $\pm$ 5	8 $\pm$ 6

Abbreviations: n = total number of small intestinal tumor analyzed per each experimental group; SE = standard error.

Tissues from 15 control mice and 10 of each treatment group were used for this analysis. Size of individual tumors ranged from 0.5-5 mm in diameter.

* Proportion of adenomas and carcinomas was calculated using the total number of small intestinal lesions screened for each experimental group.