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Ionizing Radiation-Induced Mutant Frequencies Increase Transiently in Male Germ Cells of Older Mice

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Abstract

Spontaneous mutant frequency in the male germline increases with age, thereby increasing the risk of siring offspring with genetic disorders. In the present study we investigated the effect of age on ionizing radiation-induced male germline mutagenesis. *lacI* transgenic mice were treated with ionizing radiation at 4-, 15- and 26-month-old, and mutant frequencies were determined for pachytene spermatocytes and round spermatids at 15 days or 49 days after ionizing radiation treatment. Cells collected 15 days after treatment were derivatives of irradiated differentiating spermatogenic cells while cells collected 49 days later were derivatives of spermatogonial stem cells. The results showed that 1) spontaneous mutant frequency increased in spermatogenic cells recovered from nonirradiated old mice (26-months-old), particularly in the round spermatids; 2) mutant frequencies were significantly increased in round spermatids obtained from middle-aged mice (15-months-old) and old age mice (26-month-old) at 15 and 49 days after irradiation compared to the sham-treated old mice; and 3) pachytene spermatocytes obtained from 15- or 26-month-old mice displayed a significantly increased mutant frequency at 15 days post irradiation. This study indicates that age modulates the mutagenic response to ionizing radiation in the male germline.

Keywords

ionizing radiation; age; mutagenesis; spermatogenic cells; *lacI* mouse

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5. Conflict of interest statement None.

1. Introduction

U.S. birth rates have increased 40% for men in the age group of 35- to 49-years-old since 1980 due to the increased life expectancies, changes in family-planning practices, and advances in assisted reproductive techniques [1]. Advanced paternal age (about 40 years or older at the time of conception) is associated with an increased incidence of a wide range of genetic and epigenetic diseases in offspring [2]. For example, *de novo* mutations (C to G in fibroblast growth factor receptor 2 (FGFR2) gene) in 57 Apert cases were all of paternal origin; and the age of the fathers was significantly increased at the time of birth with a mean of 33.0 years of age compared with 30.7 year of age in an age-matched control group [3]. A man's risk to father a child with Apert syndrome when more than 50-years-old is 9.5-fold higher than that of a man 25-to 29-years-old [4]. In addition to several diseases with clear Mendelian inheritance, advanced paternal age is also linked to an increased risk for diseases with a genetic component such as childhood cancers [5–7], diabetes mellitus type I [8], multiple sclerosis [9], autism [10] and congenital malformations [11] and others. The association between paternal age and increased risk for diseases in offspring may be at least partially ascribed to mutagenesis in male gametes [12–16].

Transgenic mouse models carrying mutation reporter transgenes have provided a much needed tool to assess directly *in vivo* mutagenesis in male germ cells [17–23]. A low spontaneous mutant frequency was consistently detected in male germ cells compared with age-matched somatic tissues [18–22]. However, with regard to germline mutant frequency at old ages, the results have varied. Some studies have detected a significantly increased mutant frequency in male germ cells or testis obtained from old mice [21]. However, other studies did not detect a significant change in mutant frequency at old ages [18,19]. In studies where there were no changes with old age, the mutant frequency of the entire testis was often assessed and would necessarily include somatic cells that have a greater mutant frequency when analyzed as relatively pure populations [21]. In at least one case with no change in germline mutant frequency at old age, mixed germ cell enrichment protocols were used [19]. The mutant frequency for the mixed germ cells was greater than that detected for enriched populations of defined spermatogenic cells suggesting that again the preparations may have reflected somatic cell contamination. Studies assessing DNA repair activities (e.g. base excision repair [24] and nucleotide excision repair [25]) in the male germline with old age detected decreased repair activity. The decreased repair activity is consistent with increased mutagenesis and suggests that spermatogenic cells may be compromised in their ability to respond to induced DNA damage in older animals. However, such information on induced germline mutagenesis with advancing male age is not available.

In the present study, a *lacI* transgenic mouse model was used to study the relationship between paternal age and the mutagenic effects of ionizing radiation (IR) in spermatogenic cells. A cell type-specific pattern has been reported previously for IR-induced mutagenesis in the germline of young rodents. Spermatids were identified as the most susceptible cell type using indirect mutation assays such as the dominant lethal mutation and minisatellite locus assays [26,27]. In contrast, when mutant frequencies were measured directly in differentiating derivatives of spermatogenic cells exposed to ionizing radiation, it was demonstrated that all tested cell types were susceptible to induced mutagenesis, but that the male germline has the capability to restore a low mutant frequency as cells progress through spermatogenesis [23]. The present study was designed to determine if paternal age impacts the ability of the male germline to maintain a low mutant frequency after exposure to an exogenous genotoxin.

2. Materials and Methods

2.1. Animals

Six mating pairs of *lacI* homozygous transgenic mice in the C57BL/6 genetic background were obtained from Taconic (Hudson, NY), and used to generate the male animals needed in these studies. The number of animals per treatment group and their ages are listed in Table 1. All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. All procedures were approved by the Institutional Animal Care and Use Committee prior to implementation. The specific-pathogen-free animals were maintained *ad libitum* on standard food and water and a 12:12 light:dark light cycle.

2.2. Treatment with ionizing radiation

Male *lacI* mice at different ages as shown in Table 1 were whole-body irradiated with 1.2 Gy of gamma-radiation. Mice were placed in an acrylic holding rack for exposure to IR in a GammaCell 40 (Atomic Energy of Canada Limited, Ontario, Canada). The dose rate of the ^{137}Cs source was 1.2 Gy per minute. The mock-treated control mice were placed into the exposure chamber, but the chamber was not lowered to the radiation source for exposure. The control mice were mock-exposed for the same period of time as the IR-treated mice. The mice were anesthetized with isoflurane and sacrificed by cervical dislocation at 15 days or 49 days after irradiation. The testes were then removed for mutant frequency analyses. To minimize the number of animals used in the study, one mock-treated group was sacrificed 30 days after mock treatment rather than using two groups of mice for sacrifice at each 15 and 49 days post treatment. This is justified because the spontaneous mutant frequency in spermatogenic cells does not significantly change within this time frame as observed in previous studies [28,29].

2.3. Preparation of defined spermatogenic cell types

Mice were anesthetized at the appropriate ages (Table 1) with isoflurane (Abbott Laboratories, Chicago, IL) followed by cervical dislocation. Testes from each mouse within the same group were pooled together for spermatogenic cell preparations. Pachytene spermatocytes and round spermatids were prepared using a Sta Put gradient system as described previously [30] and identified morphologically by phase contrast microscopy. The purity of pachytene spermatocytes was greater than 90%, and greater than 94% for round spermatids. Cells were centrifuged at $1000 \times g$ for 10 minutes, snap frozen in liquid nitrogen, and then stored at -80°C until use.

2.4. Genomic DNA isolation and mutagenesis assay

High molecular weight genomic DNA was prepared using the RecoverEase™ DNA isolation kit according to the manufacturer's recommendations (Agilent Technologies, Santa Clara, CA) as described previously [23]. Lambda phage shuttle vectors harboring the bacterial *lacI* gene were recovered from high molecular weight genomic DNA samples using Agilent's Transpack *in vitro* packaging extracts. Packaged phage were mixed with *E. coli* SCS-8 cells and added to top agarose containing 5-bromo-4-chloro-3-indoyl-beta-galactopyranoside (X-gal) and plated on NZY agar assay trays. After incubation overnight at 37°C , plaque-forming units (pfus) were counted. Blue mutant plaques were visually identified, cored and re-plated at low density under the same incubation conditions for confirmation. Mutant frequency was determined by dividing the number of confirmed mutant plaques by the total number of pfus obtained.

The required numbers of animals and pfus depend on the spontaneous mutant frequency, the inter-animal variability, and the desired power and detectable effects [31–34]. The general

recommendations for the Big Blue™ mutagenesis assays were based on the data from somatic tissues [34,35], which was followed in some studies on male germ cells [18,19]. In this study, the packaging efficiency for the Big Blue™ assay was determined by the minimum plaque counts of 9,000 in a 25 × 25 cm plate. A minimum of five *in vitro* packaging reactions were performed on each pooled DNA sample. Approximately one million pfus for each sample were obtained except for the lower pfus in pachytene spermatocytes in 26-month-old mice at 15 days post IR due to the limiting amount of DNA (Table 1). With this number of pfus, we had the ability to detect a 2.5-fold to 4-fold difference between pachytene spermatocytes and round spermatids with a power greater than 0.80 based on the recommendations [33,36].

2.5. Statistical analysis

The numbers of mutations were described by a Poisson regression model with parameter estimates obtained by the method of maximum likelihood [37]. The statistical model included the effects of age, time after irradiation, cell type, all two-factor interactions, and the three-factor interactions. Statistical tests of differences used the likelihood ratio test. Comparisons among ages for mice not irradiated (mock) were regarded as *a priori* statistical tests; other comparisons among groups were Bonferroni adjusted. Computations were made using SAS PROC GENMOD (Version 9.2, SAS Institute, Cary, NC).

3. Results

3.1. Ionizing radiation (IR) dose determination

A pilot study was conducted to identify a radiation dose that did not impair spermatogenesis. Four month old male mice were subjected to whole body exposure to 1.2 Gy (7 mice) or 2.4 Gy (8 mice) of IR. A dose of 1.2 Gy of IR was chosen based on normal histology within the seminiferous tubules (H&E staining) at 15 days and 49 days after irradiation. In contrast, 2.4 Gy of IR was associated with disruption of spermatogenesis at 15 days and 49 days after irradiation. The histological changes included seminiferous tubules devoid of cells in the adluminal compartment or with detached spermatogenic cells.

3.2. Spontaneous mutant frequencies increased with age in spermatogenic cell

Pachytene spermatocytes displayed an increase in spontaneous mutant frequency with age, although no statistically significant difference was found (Table 1). In contrast, round spermatids from 26-month-old control mice showed a significantly elevated spontaneous mutant frequency ($1.26 \pm 0.35 \times 10^{-5}$) relative to those from 4-month-old ($0.41 \pm 0.18 \times 10^{-5}$, $p=0.032$) and 15-month-old mice ($0.32 \pm 0.18 \times 10^{-5}$, $p=0.032$) (Table 1). There were no significant differences in spontaneous mutant frequencies between pachytene spermatocytes and round spermatids from mice of the same age.

3.3. Mutagenic response to ionizing radiation (IR) was greater and transient in spermatogenic cells from middle-aged and old mice

A single dose of IR treatment (1.2 Gy) did not produce an elevated mutant frequency in spermatogenic cells obtained from 4-month-old mice at 15 or 49 days post irradiation (Table 1). However for 15-month-old mice, significant increases in mutant frequencies were observed in pachytene spermatocytes ($2.54 \pm 0.50 \times 10^{-5}$) and round spermatids ($2.50 \pm 0.50 \times 10^{-5}$) 15 days after IR treatment compared to the corresponding cell types in sham treated control mice ($0.63 \pm 0.28 \times 10^{-5}$ and $0.32 \pm 0.18 \times 10^{-5}$, respectively) ($p<0.05$). At 49 days post IR, only round spermatids ($1.46 \pm 0.35 \times 10^{-5}$) were significantly elevated compared to the corresponding controls ($0.32 \pm 0.18 \times 10^{-5}$, $p<0.05$) (Table 1). For 26-month-old mice, pachytene spermatocytes displayed a significantly greater mutant

frequency 15 days post IR ($2.62 \pm 0.68 \times 10^{-5}$) compared to the corresponding control mice ($0.77 \pm 0.26 \times 10^{-5}$, $p < 0.05$), whereas round spermatids ($1.20 \pm 0.32 \times 10^{-5}$) were similar to the control group ($1.26 \pm 0.35 \times 10^{-5}$) at each timepoint after IR.

Comparisons between different age groups showed that mutant frequencies in pachytene spermatocytes in 15-month-old or 26-month-old mice 15 days post IR were significantly increased compared to the same IR treatment sub-group in 4-month-old mice (Table 1). Round spermatids in 26-month-old 49 days after IR displayed significantly higher mutant frequency than that in the 4-month-old same IR treatment sub-group. No significant differences in mutant frequency between pachytene spermatocytes and round spermatids were found at the same IR-treatment sub-groups regardless of age (Table 1).

4. Discussion

Spontaneous mutant frequencies in defined spermatogenic cells were found significantly elevated only in old mice (28-month-old) compared to young (4-months-old) and middle-aged (15-months-old) mice in our previous study [21], suggesting that male germ cells at middle age are still able to maintain a background level of mutant frequency. DNA repair mechanisms play a pivotal role in mutagenesis. Long-patch base excision repair activity is significantly decreased in cells from middle-aged mice, short-patch base excision repair is reduced in cells from old mice, and nucleotide excision repair activity is significantly decreased in spermatogenic cells from middle-age mice [24,25]. These findings suggested that DNA repair mechanisms in male germ cells may be less able to counter IR-induced DNA damage at middle and old age as manifested by increased mutant frequency. Indeed, in the present study when middle-aged and old mice were challenged by IR, mutant frequencies were significantly increased in a cell type dependent manner compared to young mice (4-month-old).

The decreased ability to ameliorate induced DNA damage in spermatogenic cells at middle and old ages may have a significant impact on paternal age-related genetic diseases. DNA damage is an unavoidable aspect of life; there are many sources of DNA damage. For example, the principal sources of background exposure to IR are cosmic radiation and radionuclides that occurs naturally, whereas internal background exposure arises from decay of radionuclides, principally potassium-40, deposited naturally within tissues. These sources of radiation collectively deliver whole body dose rates to humans in the United States that range from 1–2 mSv (milli-sievert, equal to 0.1–0.2 cGy) per year [38]. Thus, IR is constantly damaging germline DNA. Spermatogonial stem cells in older males have been subjected to more DNA damage than their counterparts in younger males. The combination of decreased DNA repair and greater DNA damage could easily result in greater mutant frequencies in spermatogenic stem cells and the resulting differentiated progeny. As a result, the risk of producing offspring with certain genetic diseases would increase.

The effects of ionizing radiation on the mouse testes were carefully studied previously by Oakberg [39,40]. Spermatogonial cell death was examined in hybrid males ($101 \times C3H$) after γ -ray or X-ray exposure. Cell death peaked within 72 h of treatment [40]. 0.2 Gy of γ -ray depleted approximately 22% of type A spermatogonia 15 h after irradiation. 1 Gy of X-ray resulted in loss of more than 70% of type A spermatogonia by 72 h after irradiation, whereas nearly complete depletion of type B spermatogonia was observed 36 h after irradiation [40]. More differentiated cells were then lost by maturation depletion. In other words meiotic cells that were present when the animals were irradiated continued differentiating but were not replenished because spermatogonia had been killed. Eventually spermatogenesis was restored from surviving stem cells [39]. A later study [41] showed that the number spermatogonia displaying cell death indicators in C57BL/6J mice was positively

proportional to the dose of γ -ray at 12 h after irradiations (0.5 Gy, 2.5 Gy, 5 Gy), and returned to baseline levels at 24 h post irradiations. In the Hasegawa et al study, a dose of 2.5 Gy of γ -ray irradiation did not kill all the spermatogonia. The apparent discrepancy between the Oakberg and Hasegawa studies [40,41] regarding the spermatogonial sensitivity to radiation, can be explained, at least in part, by variation in sensitivity to ionizing radiation among different mouse strains, i.e. a hybrid of 101 X C3H vs. C57BL/6J. In the present study, 1.2 Gy of γ -ray was chosen based on normal testicular histology at 15 days and 49 days post irradiation. We did not examine the testes at earlier time points. It was expected that spermatogenesis would be completely restored by 49 days after irradiation based on the 35 day cycle of spermatogenesis in mouse [39] and the results from a similar dose of irradiation in Oakberg's previous study [40]. However, our finding of normal histology in the testis 15 days after irradiation is discordant with Oakberg's previous study [40]. If type B spermatogonia were completely depleted 36 h after irradiation as observed in Oakberg study [40], we would expect to observe maturation depletion of pachytene spermatocytes at 15 days after irradiation. This was not observed in the present study. Our results suggested that 1.2 Gy of γ -ray irradiation was not high enough to kill all the spermatogonia in mouse model used, notably a different strain than that used by Oakberg. Thus the surviving spermatogonia in our study were sufficient to maintain normal spermatogenesis. This interpretation is supported by the results of the Hasegawa et al, in which spermatogonia survived even after 2.5 Gy of γ -ray irradiation [41]. Notably, Hasegawa et al used the same strain of mice, C57BL/6J that we used in the present study. Abnormal histology was observed at 15 days and 49 days after 2.4 Gy of γ -ray irradiation in the present study, indicating normal spermatogenesis is at least transiently compromised at this dose of irradiation.

These results of our study demonstrated that age impacts the short term mutagenic effects of IR exposure on spermatogenic cells resulting in an increased mutant frequency in derivatives of irradiated differentiating spermatogenic cells. Disasters, such as the nuclear accidents at Chernobyl, and Fukushima and natural sources of IR induce DNA damage in living organisms. At Fukushima, older workers were selected as volunteers that stayed to contain the disaster. Young workers were not selected as volunteers because the long-term effects of IR exposure had a greater chance of being realized in them. However, short term effects on male reproductive biology should also be considered.

In summary, the data from the present study indicate that mutant frequencies in spermatogenic cells from middle and old age mice are elevated immediately after exposure to IR, but that the male germline largely recovers from this effect. A restoration of the spontaneous mutant frequency was achieved in subsequent waves of spermatogenesis suggesting that spermatogonial stem cells are either resistant to the mutagenic effects of IR or there are mechanisms to prevent spermatogenic cells with high mutant frequencies from progressing through spermatogenesis in subsequent waves of differentiation.

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Highlights

- Spontaneous mutant frequency is elevated in round spermatids from old mice
- Age and IR together increase round spermatid mutant frequency
- Age and IR together transiently increase pachytene spermatocyte mutant frequency

Table 1

Experimental design and mutant frequencies in different spermatogenic cell types from different aged mice

Age	No. of animals	Ionizing radiations	Cell type	No. of mutants	Pfu	Mutant frequency (Mean $\pm$ SE $\times 10^{-5}$)
4 mo	5	mock	P	3	997,160	0.30 $\pm$ 0.17
			RS	5	1,231,690	0.41 $\pm$ 0.18
	10	15 days	P	9	1,066,300	0.84 $\pm$ 0.28
			RS	13	1,151,250	1.13 $\pm$ 0.31
	10	49 days	P	12	1,222,700	0.98 $\pm$ 0.28
			RS	8	1,315,720	0.61 $\pm$ 0.21
15 mo	6	mock	P	5	798,000	0.63 $\pm$ 0.28
			RS	3	945,900	0.32 $\pm$ 0.18
	8	15 days	P	26	1,022,330	2.54 $\pm$ 0.50 ^{a,b,c,d}
			RS	25	1,001,700	2.50 $\pm$ 0.50 ^{b,d}
	8	49 days	P	18	1,155,150	1.56 $\pm$ 0.37
			RS	17	1,165,100	1.46 $\pm$ 0.35 ^{b,d}
26 mo	10	mock	P	9	1,163,259	0.77 $\pm$ 0.26
			RS	13	1,034,913	1.26 $\pm$ 0.35 ^{a,c}
	10	15 days	P	15	572,915	2.62 $\pm$ 0.68 ^{a,b,c,d}
			RS	14	1,171,114	1.20 $\pm$ 0.32
	10	49 days	P	20	1,396,470	1.43 $\pm$ 0.32
			RS	19	1,163,569	1.63 $\pm$ 0.37 ^{a,c}

P, pachytene spermatocytes; RS, round spermatids; mo, month old; Pfu, plaque forming unit.

^a significantly different from 4 months old (mo), same cell type and mock group, p=0.032;

^b significantly different from 15 months old (mo), same cell type and mock group, p=0.032;

^c significantly different from 4 months old (mo), same cell type and ionizing radiation group, p<0.05 (Bonferroni adjusted);

p significantly different from mock group, same cell type and age, $p < 0.05$ (Bonferroni adjusted).