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Mutagenic Effects of Ionizing Radiation on Immature Rat Oocytes

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Estimates of genetic risks from radiation delivered to humans are derived largely from mouse studies. In males, the target is spermatogonia and a large amount of information is available. In contrast, in females, immature oocytes are the target, but extrapolations from mice to humans are not very definitive because immature mouse oocytes are highly sensitive to radiation and die by apoptosis, which is not the case in humans. Since mouse offspring derived from surviving immature oocytes have to date not shown any signs of mutation induction, two alternative hypotheses are proposed: 1. Apoptotic death effectively eliminates damaged oocytes in mice and therefore human immature oocytes may be highly mutable; and 2. Immature oocytes are inherently resistant to mutation induction and apoptotic death is not relevant to mutagenesis. To test these hypotheses, rat immature oocytes, which are not as sensitive as those in mice to radiation-induced apoptosis were exposed to 2.5 Gy of gamma rays and the offspring were examined using a two-dimensional DNA analysis method. Screening of a total of 2.26 million DNA fragments, we identified 32 and 18 mutations in the control and exposed groups, respectively. Of these, in the two groups, 29 and 14 mutations were microsatellite mutations, two and one were base changes, and one and three were deletions. Among the four deletions most relevant to radiation exposure, only one was possibly derived from the irradiated dam (but not determined) and three were paternal in origin. Although the number of mutations was small, the results appear to support the second hypothesis and indicate that immature oocytes are generally less sensitive than mature oocytes to mutation induction. © 2014

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INTRODUCTION

A large amount of information on genetic (i.e., trans-generational) risks from exposure to ionizing radiation has

been derived from data from specific-locus tests in mice [the data from the 7 loci used at the Oak Ridge National Laboratory is the most extensive; for reviews see refs. (1, 2)]. In males, spermatogonia cells are considered as the target cells, and dose-response data for mutations after acute or chronic exposure conditions as well as after exposure to different qualities of radiation have been obtained. In females, the target cells are immature oocytes, but in mice these cells are hypersensitive to radiation-induced apoptosis [e.g., 10–20 day-old female mice lose 90% of their immature oocytes as a result of an exposure to 0.18 Gy of gamma rays (3)], while offspring derived from irradiated immature oocytes are essentially mutation free. In contrast, mature or maturing oocytes are not sensitive to radiation-induced apoptosis but their mutation induction rates are twice as high as those in spermatogonia cells after a dose of 4 Gy. In humans, such high sensitivity to radiation-induced apoptosis is not observed in immature oocytes. In fact, an exposure of 1 or 1.5 Gy of X rays delivered to the ovary was once a standard medical procedure for treatment of infertility (4). These differences among species thus make it difficult to estimate genetic risks from radiation delivered to human immature oocytes.

The BEIR III report addresses and fully discusses this issue (5). Briefly, two alternative scenarios that may apply to human immature oocytes are presented. First, human immature oocytes might be sensitive to radiation-induced mutagenesis if it is assumed that the high sensitivity to radiation-induced apoptosis observed in mice actually functions to eliminate damaged gametes and to eliminate mutations. Alternatively, a second scenario suggests that immature mammalian oocytes are inherently insensitive to mutation induction, and human immature oocytes are no exception. This assumes that the high sensitivity to radiation-induced apoptosis observed in immature mouse oocytes is irrelevant to mutagenesis. This is a reasonable assumption because immature oocytes in mammals remain quiescent for a long period of time after birth, and hence they may be equipped to withstand various types of DNA damage that may occur during their resting period. Another report, which suggests a lack of association between apoptosis and mutagenesis, comes from a study that showed

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that dominant lethal mutations were less frequent in immature oocytes in both guinea pigs and golden hamsters, although the frequency of apoptotic death was less frequent in immature oocytes than in maturing oocytes in guinea pigs, whereas the converse was true in golden hamsters (6).

An ongoing problem in the field of radiation genetics has been to decide which of the two scenarios described above is more likely to apply to humans (the BEIR III report indicated that the latter hypothesis is more likely). Although immature rat oocytes are known to be more resistant to radiation-induced apoptosis than those of mice, conducting specific-loci tests in rats was not thought to be feasible since it requires a large number of offspring that require significantly more space to house than is needed for mice. In recent years, various new molecular technologies have been developed, one of which is the Restriction Landmark Genome Scanning method (i.e., two-dimensional gel electrophoresis of radio-labeled DNA fragments or 2DE of DNA), making it possible to examine over 1,000 genomic sites for each individual. We previously reported on radiation effects on spermatogonia in mice using the 2DE method to analyze DNA (7, 8), and in this current study, we present data on immature oocytes in rats.

MATERIALS AND METHODS

All animal experiments were carried out at Asahikawa Medical College under approval of the in-house Animal Care Committee. Female Sprague-Dawley (SD) rats (7 weeks old) were purchased from CLEA Japan (Sapporo, Japan). After an acclimation period of 1 week, an i.p. injection of Somnopentyl (Pitman-Moore, Ontario, Canada) was administered at a concentration of 25–28 mg (as sodium pentobarbital) per kilogram of body weight to anesthetize the rats, after which the animals were placed in a lead block to shield their heads and upper trunks and exposed to 2.5 Gy of ^{137}Cs gamma rays (0.83 Gy/min). Shielding the head from radiation was done to avoid perturbation of hormone production by the radiation exposure, and shielding the upper trunk was done to avoid development of radiogenic thymic lymphomas. To collect offspring derived from immature oocytes at the time of the radiation exposure, irradiated females were mated with nonirradiated males [Brown Norway (BN) strain] starting at 80 days after irradiation. The irradiated females had deliveries three to four times and up to 56 offspring per dam. F1 rats were humanely sacrificed at 3 weeks of age, and the spleen, kidney and liver were collected and stored at -80°C until use. Fifty-two SD females (dams) and 16 BN males (sires) were used, a total of 1,500 F1 rats were examined, of which 750 were derived from 21 nonirradiated SD females and 750 from 31 irradiated SD females.

Detailed methods for 2DE of ^{32}P -labeled genomic DNA fragments have been previously described in refs. (7, 8). Briefly, DNA isolated from the spleen was digested with *NotI* and *EcoRV* restriction enzymes. Subsequently, 5' overhangs were filled with ^{32}P -labeled dGTP and dCTP, and the DNA was subjected to first dimensional gel electrophoresis to separate the fragments by size. Gels containing 1–4 kb DNA fragments and 4–10 kb fragments were subjected to digestion with a third enzyme *HinfI* *in situ* and were placed on top of polyacrylamide gels to perpendicularly separate the DNA in the second dimension. Autoradiograms were prepared after exposure of X-ray films, and ^{32}P -labeled DNA fragments were visualized as spots. The autoradiograms were scanned and the images were screened for the presence of spots with an intensity reduced by 50% (the loss of one of the two alleles in an autosome) or the loss of an entire spot (the loss

of alleles that are either from the X chromosome or specific to either parent). The intensity of each spot was quantified with the value generated by the density \times area.

Cloning and sequencing of DNA fragments that had undergone mutations was done as previously described (8). Multiple bacterial clones were isolated and sequenced for each mutation, and a consensus sequence was mapped on the rat reference sequence, Baylor 3.4/m4, to obtain chromosomal locations and upstream and downstream sequence information.

Microsatellite sequences were PCR amplified with fluorescence-labeled primers flanking the repeats, and the amplicon size of each microsatellite mutation was estimated by capillary electrophoresis with size markers to determine the number of repeat units (9).

RESULTS AND DISCUSSION

Menstrual Cycle

If the menstrual cycle is prolonged after a radiation exposure, collecting offspring derived from fertilization ≥ 80 days after irradiation does not guarantee that they were derived from irradiated immature oocytes. To ensure that the cycles were not disturbed, five female rats irradiated with 4 Gy were examined for regularity of cycles with a vaginal smear technique for up to one year after exposure. No evidence of prolongation or perturbation of the cycle was observed (results not shown).

Selection of Spot

An example of an autoradiogram of an F1 hybrid from an irradiated SD strain (dam) and a nonirradiated BN strain (sire) is shown in Fig. 1A and B. Spots for screening purposes were selected based on conditions requiring good reproducibility and the absence of nearby spots that might overlap the spot of interest. After examination of scanned autoradiograms from four families (50 offspring and four pairs of parents), 162 spots unique to the SD strain, 179 spots unique to the BN strain and 1,387 spots common to both strains were selected (i.e., 1,549 spots for the SD genome and 1,566 spots for the BN genome).

Detection of Mutations

Two autoradiograms (first dimension sizes of 1–4 kb and 4–10 kb) were obtained for each rat and 1,500 offspring (750 from the control and 750 from irradiated groups) were screened along with the parents. In total, 32 and 18 mutations were detected in the control and exposed groups, respectively, among about 1.13×10^6 spots screened in each group (Table 1). Theoretically, the total number of spots screened should be $1,549 \times 750 = 1.16 \times 10^6$ spots for the SD strain and $1,566 \times 750 = 1.17 \times 10^6$ for the BN strain. However, autoradiograms were not always ideal and portions of a gel were occasionally unsuitable or unusable, resulting in a 3–4% loss of spot information. These mutations were all confirmed using DNA from the liver and kidney, indicating that they are germ cell mutations in origin (if the mutation occurred after fertilization, the zygote

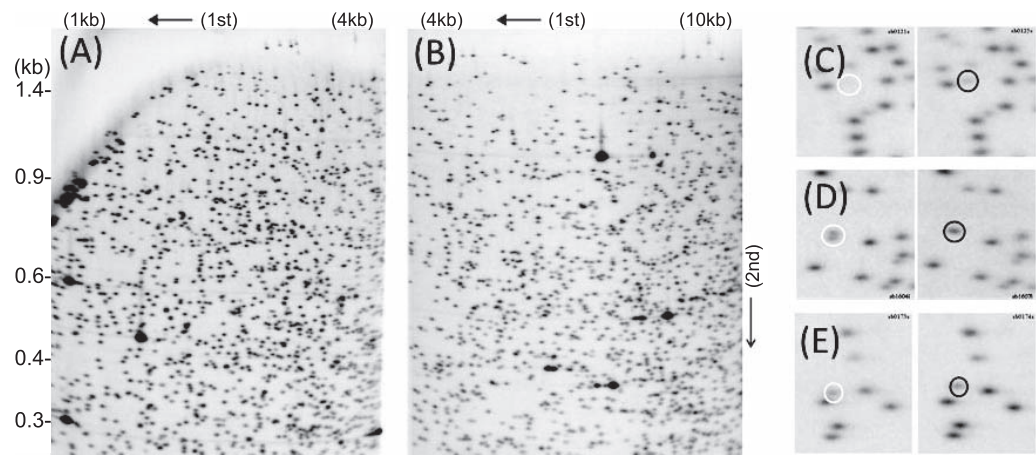


FIG. 1. Two-dimensional autoradiograms of ³²P-labeled rat DNA fragments. Panel A: First dimension size of 1–4 kb. Panel B: First dimension size of 4–10 kb. Panels C–E: Examples of mutations, right panels represent normal patterns and left panels include mutations. Mutant spots are indicated with white circles and corresponding normal spots are indicated with black circles. Panel C: One spot was lost; panel D: one spot divided into two spots; and panel E: one spot shifted downward.

will have a mosaic structure and consequently may show discordant results depending on the tissues examined).

Characterization of the Mutations

The majority of mutations detected had lost spot intensity by nearly one half, and were accompanied by a new one-copy spot near the original position (29 out of 32 in the control and 14 out of 18 in the exposed groups) (examples are shown in Fig. 1C–E). Our past experience strongly suggested that these were likely to be mutations at microsatellites (repeats of 2–8 core sequences), and indeed they were found to be microsatellite mutations after cloning and sequencing the normal and the affected spots. The contribution of the paternal and maternal origins seemed roughly equal: in the control group, 11 were paternal, 11 were maternal and 7 were undetermined; in the exposed group, 7 were paternal, 3 were maternal and 4 were undetermined (Table 2). Thus, the results do not indicate any radiation-induced effect and are consistent with previously published results showing that microsatellite mutations were not induced in germ cells (9, 10). Lastly, it should be noted that several highly mutable spots were detected, the most mutable being the BN-specific spot RS158 that was mutated 15 times. The second most mutable was the SD-specific spot RS163 that was mutated 10 times, and the third most mutable was the spot RL1388, common

to both the SD and BN strains, which was mutated 4 times. Sequence data showed that RS158 and RS163 are BN- and SD-specific alleles of the same microsatellite locus that contain 25 and 20 repeats of GCGGGGC sequences, respectively (Table 2). It is possible that long GC-rich repeat sequences may tend to undergo slippage after replication stress as observed in the *FMRI* locus of fragile X syndrome (11).

Other Mutations

In the irradiated group, there was one base-change mutation (the parental origin could not be determined) and three deletions (two were paternal and one could not be determined). In the control group, there were two base-change mutations (both were maternal) and one deletion mutation (paternal). Therefore, there was only one deletion mutation that was possibly derived from irradiated immature oocytes.

Uncertainty

A total of over 1 million spots were screened in both the control and irradiated groups, however only one deletion mutation was detected in the irradiated group (but the parental origin could not be determined). The paucity of deletion mutations does not necessarily mean that immature

TABLE 1
Summary of Mutations Derived from Immature Rat Oocytes Irradiated with 2.5 Gy of Gamma Rays

Group	Total number of spots screened (×10 ⁵)	Number of mutations			
		Total	Microsatellite mutations	Base change mutations	Deletion mutations
0 Gy	11.3	32	29	2 (maternal)	1 (paternal)
2.5 Gy	11.3	18	14	1 (undetermined)	2 (paternal) 1 (undetermined)

TABLE 2
Parental Origins and Sequence Changes in
Microsatellite Mutations

Dose	Spot ID	Parental origin*	Sequence change
0 Gy	RL1388	—	(CGA) 8 → 7
	RL1388	—	(CGA) 8 → 7
	RL1388	—	(GGA) 8 → 7
	RL1388	—	
	RL184	P	(GC)4(AC)17 → (GC)6(AC)17
	RL650	—	(CA)29 → 25
	RS488	M	(TAG)22 → 21
	RS007	—	(TAG)22 → 21
	RS007	—	Not cloned
	RS1028	—	(GT)26 → 25
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 19
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 22
	RS163	M	(GCGGGGC)25 → 23
	RS163	M	(GCGGGGC)25 → 24
	RS163	M	(GCGGGGC)25 → 24
	RS163	M	(GCGGGGC)25 → 26
	RS163	M	(GCGGGGC)25 → 23
	RS163	M	(GCGGGGC)25 → 26
	RS163	M	(GCGGGGC)25 → 23
	RS163	M	(GCGGGGC)25 → 22
	RS379	P	(TAG)25 → 24
	RS379	P	(TAG)25 → 24
	RS488	M	(TAG)22 → 21
	RS488	M	(TAG)22 → 21
2.5 Gy	RL174	—	(GT)23 → 20
	RL357	—	(ATTT)11 → 10
	RL953	M	(CA)27 → 12
	RS214	—	(AGGGGC) 29 → 30
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 22
	RS158	P	(GCGGGGC)20 → 21
	RS158	P	(GCGGGGC)20 → 22
	RS163	M	(GCGGGGC)25 → 23
	RS163	M	(GCGGGGC)25 → 27
	RS1028	—	(GT)31 → 29

* P = paternal; M = maternal; dash (—) = undetermined parental origin.

oocytes are refractory to recording radiation damage because it may well be that the number of spots screened was not large enough. In this context, it may be useful to compare these results with those previously obtained after irradiation of mouse spermatogonia [for a summary of these results, see Table 3 in ref. (8)] with the same 2DE method. Briefly, about 1,800 spots were screened per offspring, and 5 deletion mutations, most likely caused by radiation exposure, were observed among 500 offspring derived from spermatogonia irradiated with 4 Gy. In contrast, only one deletion mutation was observed in the offspring of the

control group consisting of a similar number of mice. Although the number of mutations is rather small and hence the confidence intervals are wide, taking the results at face value it appears that one deletion mutation may occur among 100 offspring after males were irradiated with 4 Gy. From this, it is possible to calculate the expected number of deletion mutations in the current study under the assumptions of equal radiation sensitivity between mouse spermatogonia and rat immature oocytes. Compared with the mouse data, the current rat data is derived from a 40% lower radiation dose (2.5 vs. 4 Gy), and utilized 15% fewer spots screened per individual (1,500 vs. 1,800). Thus, the expected number of deletion mutations is calculated as: $750 \times (1/100) \text{ deletion mutation} \times (2.5 \text{ Gy}/4 \text{ Gy}) \times (1,500 \text{ spots}/1,800 \text{ spots}) = 3.9$. This number does not seem large enough to have a high probability of being detected, which leads us to conclude that compared with mouse spermatogonia, rat immature oocytes do not seem to be more sensitive to the induction of deletion mutations.

CONCLUSION

We propose that death of immature mouse oocytes from apoptosis after radiation exposure is likely to be irrelevant in the elimination of germ cells that have acquired mutational DNA damage. This proposal is also supported by a report indicating that the target of radiogenic apoptosis in mouse immature oocytes is likely to be plasma membrane (12). Currently, screening of deletion mutations using microarrays (array-based CGH studies) is underway, and the preliminary data from these studies also support our proposal (unpublished results).

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