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Translocations in Spleen Cells from Adult Mice Irradiated as Fetuses are Infrequent, but Often Clonal in Nature

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We previously reported that mouse fetuses or neonates exposed to 2 Gy of X rays showed an unexpectedly low incidence of chromosome damage in lymphocytes, bone marrow, and spleen cells when the mice were subsequently examined at 20 weeks of age. However, cells bearing translocations were occasionally observed that, on the basis of 2-color whole chromosome painting appeared to be clonal descendants. Unfortunately, this approach typically did not permit unequivocal confirmation of their clonality. To overcome this problem, multi-color FISH (mFISH) was employed, which assigns all 21 individual chromosome types of the mouse a unique color. After mFISH analyses of the same cell samples studied previously, it was confirmed that spleen cells of 20-week-old mice irradiated either as 15.5-day fetuses or as 3- to 4-day-old neonates showed translocation frequencies close to zero. Translocations previously suspected as being clonal in nature were confirmed as such by mFISH, which also revealed the presence of an additional clone not previously detected or suspected. Since no evidence of clonality was observed in the irradiated mother, we concluded that in both fetuses and neonates, there exists a small fraction of stem cells that are distinct from the bulk of the stem cell compartment in terms of their ability to acquire and transmit radiation-induced chromosome damage through clonal expansion. © 2012 by Radiation Research Society

INTRODUCTION

It has long been thought that fetuses are sensitive to radiation in terms of increased risks for childhood leukemia and solid cancers (1–5). Therefore, it was surprising to find that fetuses exposed to atomic-bomb radiation did not show a dose-related increase of translocation frequencies in blood lymphocytes when they were examined at around 40 years of age (6). This observation was in sharp contrast to the

clear dose response results from survivors who were exposed to radiation at various ages (7). Because cytogenetic damage is one of the best characterized biomarkers of radiation exposures and represents one of the most important classes of DNA damage that may contribute to the development of malignancy, further investigations were conducted in mice. The mouse results not only confirmed the lack of a dose response after irradiation as fetuses, but also disclosed that neonates (3–4 days old) had the same characteristics (8). In this study, spleen cells were examined with a 2-color FISH method by painting chromosomes 1 and 3 when the animals reached 20 weeks of age. Further, irradiation of mice at various ages (but with all cytogenetic examinations made at a fixed age of 20 weeks) showed that the translocation frequency was low when mice were irradiated at 1–2 weeks after birth, but thereafter it became progressively higher with increasing age at the time of irradiation and reached a plateau level when they were irradiated at 6 weeks of age or older. (It is noted that human neonates may not show the same characteristics because humans appear to be born relatively more mature than mouse neonates.) Genetic makeup with regard to the knockout conditions of the p53 gene (either heterozygous or homozygous) did not affect these results. Interestingly, although the frequencies were generally low, those translocations that were occasionally detected within the same individuals often looked identical (i.e., they appeared to be clonal descendants). This seems to be a unique characteristic associated with fetal irradiation, if it is confirmed; this is because clonal translocations are usually associated with high aberration frequencies in A-bomb survivors (9) or those who were exposed to radiation during the Chernobyl nuclear plant accident (10).

The aims of the present study are twofold. First, to apply the mFISH method to the same spleen cell samples that were examined previously to understand how often translocations observed in the same animals are clonally related. Second, to confirm that the low translocation yield is not caused by a technical bias due to the usage of a partial genome painting method (FISH painting of chromosomes 1 and 3 only). Although the probability would be low, if the lymphocyte pool consisted of a small number of hematopoietic stem cells (e.g., 10–20), detecting translocations

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involving only a few painted chromosomes could have overlooked translocations that occurred between two unpainted chromosomes.

MATERIALS AND METHODS

Animals and Cells

Details have been described previously (8). Briefly, pregnant B6C3F₁ mice at 15.5-day p.c. or 3- to 4-day-old neonates were exposed to 2 Gy of X rays. When the offspring or neonates reached approximately 20 weeks of age, their spleen cells were isolated and cultured for metaphase preparations. Spleen cell samples from 8 animals were examined in the present study. These included samples from 4 and 3 mice irradiated as fetuses and neonates, respectively, and from one mother. Samples that showed low or high translocation frequencies in the previous study with FISH painting of chromosomes 1 and 3 were selected from the fetal or neonatal irradiation group. The mouse ID numbers shown in the present study correspond to those used in the previous study (8). Fixed cells were used (some of which were previously examined) which had been kept at -20°C in Carnoy's fixative to prepare new metaphases. Metaphase slides were also used that had been examined previously, and that had been found to contain possibly identical translocations (see below).

Multi-Color FISH (mFISH)

Metaphase chromosomes were hybridized with a set of chromosome probes (mFISH probes) according to the manufacturer's instructions (Cambio, Cambridge, UK). Spectrum gold, spectrum red, spectrum far red, spectrum green, and Cy5.5 were used as the fluorochromes. The resulting mFISH figures were analyzed with a Genus/Chromofluor System (Applied Imaging International, Newcastle upon Tyne, UK). Five images were sequentially acquired through fluorochrome-specific optical filters, and pseudo-colors were assigned to each image based on gray scale intensity values using custom-designed software. One hundred metaphases were analyzed for each animal with the exception of the N2-6 individual, for which 73 metaphases were scored.

Examination of Metaphases Previously Suspected to Contain Clonal Translocations

In the previous study, only chromosomes 1 and 3 were painted. Thus, to identify clonal translocations, it was necessary to conduct additional FISH painting on the same metaphases to identify the unpainted cooperating chromosomes that had been involved in the translocation events. However, because there were no clues regarding the interacting chromosomes, this was a difficult task. Therefore, in the present study, mFISH data was first analyzed from newly prepared metaphases from spleen cells from the same mice to obtain clues about the interacting chromosomes. Subsequently, probes for the most likely candidate chromosomes were selected and used on the metaphases that were previously screened and known to contain possible clonal translocations. Every translocation detected in the previous study was photographed with color film (ISO 800, Fuji, Tokyo, Japan), and the microscope coordinates were recorded. Chromosome slides were then put into a 100% ethanol solution for 30 min to remove the cover slips, washed twice in a 100% ethanol solution for 2 min to remove the mounting medium, and air dried. Slides were then soaked in 70% formamide/2×SSC at 72°C for 2 min to denature the DNA. They were then cooled quickly with successive rinses; twice with 2×SSC at 4°C, and dehydrated in an ethanol series (70, 85 and 100%), and air-dried. Metaphase slides suspected to contain clonal translocations were then rehybridized with a new set of chromosome probes. Translocations were judged as identical (i.e., clonal) if the two chromosomes involved

in each translocation were the same, and the estimated chromosome break points were reasonably similar.

Calculation of the Genome-Equivalent Translocation Frequency

The previously used 2-color FISH method detected translocations involving chromosome 1 or chromosome 3, and the genome-equivalent frequency of translocations (F_G) was estimated with the equation of Lucas *et al.* (11). That is:

$$F_G = \frac{F_{rg}}{\{2.05 \times [f_g(1 - f_g) + f_r(1 - f_r) - f_r f_g]\}}$$

where F_{rg} represents the observed translocation frequency, and f_g and f_r are the fractions of the genome painted with green (chromosome 1) and red (chromosome 3), respectively. Since chromosomes 1 and 3 comprise 7% and 6% of the genome, respectively (12), four times the observed frequency is equal to the genome-equivalent frequency, or $F_G = F_{rg}/0.24$.

RESULTS

Translocation Frequencies Measured with mFISH

Table 1 summarizes the translocation frequencies obtained with the mFISH method along with the frequencies previously obtained with the 2-color FISH method. The results are expressed as genome-equivalent translocation frequencies so that direct comparisons can be made. It is evident that the paired results are in reasonable agreement; specifically, the frequency in the mother was 25% with the mFISH method and 20% with the 2-color FISH method. Also, the mean translocation frequencies of mice irradiated as fetuses or neonates were nearly the same with either method (about 4%), which was clearly lower than that of the mother. (The frequency of 4% itself does not specifically represent a particular event or parameter, however, because this is a mean of arbitrarily selected samples of animals as mentioned in the Materials and Methods.)

Clonal Translocations

In the previous study that used the 2-color FISH method, only one clonal translocation event could be confirmed, although several candidate clones were recognized (8). Thus, in the present study, attempts were made to detect additional translocations because mFISH is the method best suited for this purpose (i.e., the two chromosomes involved in a translocation are generally identifiable). The results are included in Table 1. For example, mFISH analysis detected 5 translocations in spleen cells of the F2-1 mouse, and 4 of the 5 were identical, t(6;6), whereas, no translocations were detected previously in this spleen cell sample. Likewise, in the F2-7 mouse, the mFISH method detected 9 translocations of which 5 were judged as clonal. Specifically, one cell had t(1;12), and 4 cells had t(3;4), and both of them were detected in the previous study, i.e., 5 cells had t(1;12) and 2 cells had t(3;4). In the N2-7 mouse, cells bearing t(1;4) or t(1;13) were found in both the previous and present slides repeatedly and were considered as clonally derived. It

TABLE 1
Results of mFISH and 2-Color FISH Analyses of Spleen T-Lymphocytes of Mice Irradiated as Fetuses or Neonates

Mouse ID ^b	mFISH	2-color FISH ^a
	Translocation frequency clone information ^c	Translocation frequency ^d clone information
Mother		
M2-1	25/100 (25%) No clones detected	38/800 (20%) No clones detected
Irradiated as fetuses ^e		
F2-1	5/100 (5%) 4 × t(6q+;6q-)	0/800 (0%)
F2-2	0/100 (0%)	0/800 (0%)
F2-7	9/100 (9.0%) 1 × t(1q-;12q+) 4 × t(3q-;4q+)	16/800 (8.4%) 5 × t(1q-;12q+) 2 × t(3q-;4q+)
F2-8	1/100 (1%)	18/800 (9.5%) 18 × t(1q-;19q+)
(Mean %)	(3.8%)	(4.5%)
Irradiated as neonates		
N2-2	1/100 (1%)	2/800 (1%) No clones detected
N2-6	4/73 (5%) No clones detected	3/800 (1.6%) No clones detected
N2-7	5/100 (5%) 1 × t(1q-;4q+) 2 × t(1q+;13q-)	14/800 (7.4%) 2 × t(1q-;4q+) 8 × t(1q+;13q-)
(Mean %)	(3.7%)	(3.4%)

^a The 2-color FISH data shown here are new data except for the clonal translocation t(1q-;19q+) found in F2-8 individual which was reported previously (8).

^b The animal IDs correspond to those in the previous report (8).

^c For example, 4 × t(6q+;6q-) stands for 4 cells had the same t(6q+;6q-).

^d Percentages in the parentheses indicate genome-equivalent frequencies of translocations so that direct comparisons can be made between the mFISH and 2-color FISH data. The frequencies in this column are reproduced from the previous report (8).

^e Offspring F2-1, F2-2, F2-7 and F2-8 were born to the mother M2-1.

should be noted that although many translocations that were previously suspected to be clonal could be identified as clones in the present mFISH analysis, there are still metaphases in the previously examined slides in which it was difficult to determine the unpainted interacting chromosomes because re-hybridization with the new FISH probes did not work sufficiently well [i.e., t(1;12?) in 3 cells and t(3;4?) in 5 cells of the F2-7 mouse].

As a considerable fraction of the translocations were confirmed as being clonally related, the translocation frequencies of the clone bearing animals was consequently reduced substantially because clonal translocations should be counted as single events. For example, when adjusted for clonality, the translocation frequency of the F2-1 mouse with the mFISH method (i.e., the observed translocation frequency $F_G = 5\%$) becomes 2% (two events among 97 cells). In contrast, in the mother mouse, the translocation frequency was much higher and no clonal translocations were detected among 100 cells scored with the mFISH method.

In the F2-8 mouse, all of the 18 translocations detected previously were t(1;19), but none of them was detected in the present mFISH study. This is probably attributable to a chance observation of analyzing 100 cells with mFISH compared with 800 cells with 2-color FISH (the expected number of translocations is 2.3 with the estimated prediction interval of $-0.645.6$ to 5.6 in 100 cells if the observed frequency 18/800 is regarded as the true frequency) (13).

DISCUSSION

Previously, a 2-color FISH method was used to examine 19 adult mice that were exposed to 2 Gy of X rays as fetuses or neonates. The majority of the animals (9 out of 12 mice irradiated as fetuses, and 6 out of 7 mice irradiated as neonates) had only 0–4 translocations which involved the painted chromosomes 1 or 3 among 800 cells examined, or a genome-equivalent frequency (F_G) of less than 2%. This result is in sharp contrast to that of the mother (F_G of 20% and 25%) (8). In contrast, a minor fraction of the animals (3 out of 12 mice irradiated as fetuses, and 1 out of 7 irradiated as neonates) had 10 or more translocations among 800 cells (F_G of $\geq 5\%$), and the translocations were often identical as confirmed with the present mFISH study (Table 1). Thus, the frequency of cells with a translocation (without correcting for the clonal aberrations) appears to show either close to zero or larger than 5% in F_G , and the latter often contains clonal translocations. The results indicate that when the stem cell pool of 15.5-day fetuses started to recover from radiation damage after an exposure to 2 Gy, a small number of stem cells could record radiation damage as persisting translocations and subsequently clonally expanded. The results are in line with the observations that 10–11-day-old fetuses already carry a large number of hematopoietic stem cells that may contribute to at least 10% of adult hematopoiesis (14) whereas fetal stem pool still does not seem to be a simple homogeneous cell population.

This observation is in sharp contrast to the findings among the A-bomb survivors exposed after birth, namely, clonal translocations were found preferentially among those survivors with high frequencies of translocations (i.e., those who received large doses of radiation) (9). This is reasonable because in adults, a large number of hematopoietic stem cells were already present at the time of radiation exposure, and exposure to larger doses affected a larger fraction of the stem cell populations chromosomally. It also facilitated clonal expansions of the surviving (and affected) stem cells through the recovery processes taking place after the extensive cell death caused by the exposure.

The present results shed light on a previous finding that one A-bomb survivor exposed *in utero* was found to bear clonal aberrations; i.e., 6 translocations and 3 inversions were identical out of 48 aberrant cells when 144 cells were examined with the G-banding method (15). The mother's estimated uterine dose was 1.5 Gy with the DS02 dosimetry

system. These A-bomb results stood out and appeared unusual, but are now supported by the present study.

The reasons why irradiated fetuses cannot record radiation damage may be related to the specific nature of hematopoietic tissues. Our recent studies indicate that both rat mammary epithelial cells (Nakano *et al.*, unpublished data) and mouse thyroid cells (Hamasaki and Kodama *et al.*, unpublished data) irradiated as fetuses do record radiation damage when examined as adults. It is well known that hematopoietic stem cells move during fetal life and most enter the bone marrow niche several weeks after birth in mice (16). Additionally, their gene expression levels change before and after settlement in the bone marrow niche. For example, the expression level of the *ATM* gene is reportedly considerably lower before entering the bone marrow niche (17). This observation indicates that hematopoietic stem cells might be incapable of triggering the ATM-dependent repair of so-called dirty double-strand breaks of DNA induced by irradiation. This hypothesis might be testable by creating animals with forced expression of the *ATM* gene (and/or additional related genes) in fetuses.

In summary, ordinary cytogenetic tests of blood T cells are not applicable to retrospective biodosimetry for those who were exposed to radiation as fetuses and possibly as neonates. However, because it seems likely that other tissues with the exception of hematology cells may record radiation damage after fetal irradiation, a possibility remains to use cells that are available with the least invasive method, such as skin cells.

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