Fetal Irradiation of Rats Induces Persistent Translocations in Mammary Epithelial Cells Similar to the Level after Adult Irradiation, but not in Hematolymphoid Cells

Mimako Nakano,[a] Mayumi Nishimura,[b] Kanya Hamasaki,[a] Shuji Mishima,[a] Mitsuaki Yoshida,[c] Akifumi Nakata,[c] Yoshiya Shimada,[b] Asao Noda,[a] Nori Nakamura[a] and Yoshiaki Kodama[a,1]

[a] *Department of Genetics, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Miami-ku, Hiroshima 732-0815, Japan;* [b] *Radiobiology for Children's Health Research Program, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba, 263-8555, Japan; and* [c] *Department of Radiation Biology, Institute of Radiation Emergency Medicine, Hirosaki University, Hirosaki 036-8564, Japan*

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In both humans and mice, fetal exposure to radiation fails to induce a persistent increase in the frequency of chromosome aberrations in blood lymphocytes. Such a low-level response to radiation exposure is counterintuitive in view of the generally accepted belief that a fetus is sensitive to radiation. To determine if this is a general phenomenon, both mammary epithelial cells and spleen cells were studied in rats. Fetuses of 17.5 days postcoitus were irradiated with 2 Gy of gamma rays, and mammary tissues were removed 6–45 weeks later. Subsequently, short-term cultures were established to detect translocations using the two-color FISH method. The results showed that translocation frequencies were not only elevated in rats irradiated as fetuses, but were also almost as high as those in rats that were irradiated as adults (12 weeks old, pregnant mothers or young virgins) and examined 6–45 weeks later. There was no evidence of higher sensitivity in fetal cells with respect to the induction of translocations. In contrast, translocation frequencies in spleen cells were not elevated in adult rats irradiated as fetuses but were increased after irradiation of adults as previously seen in mouse spleen cells and human T lymphocytes. In the case of irradiation of adult rats, the induced translocation frequencies were similar between spleen cells and mammary epithelial cells. If we take translocation frequency as a surrogate marker of potential carcinogenic effect of radiation, the current results suggest that fetal irradiation can induce persistent potential carcinogenic damage in mammary stem/progenitor cells but this does not contribute to the increased risk of cancer since it has been reported that irradiation of fetal rats of the SD strain does not increase the risk of mammary cancers. Possible reasons for this discrepancy are discussed. © 2014 by Radiation Research Society

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[1] Address for correspondence: Department of Genetics, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima 732-0815, Japan; e-mail: ykodama@rerf.or.jp.

INTRODUCTION

It is generally believed that the fetus is sensitive to radiation exposure. This belief is derived from two main sources. The first is the classic observation in the early 20th century by Bergonié and Tribondeau that the degree of radiation sensitivity is inversely related to the degree of differentiation of a cell; e.g., bone marrow or male germ cells which consist of actively dividing stem and progenitor cells can be sterilized with relatively small doses of radiation while terminally differentiated cells such as muscle and nerve cells require much larger doses (*1*). A fetus must contain a proportionally larger number of immature, actively dividing cells than an adult, and may well be sensitive to the killing effects of radiation. This in turn leads to the supposition that a fetus is also sensitive to some of the adverse effects of radiation such as cancer induction. The second source is derived from epidemiologic case-control studies that indicated diagnostic low-dose exposures to the pelvic region of pregnant women (therefore to the fetus as well) on the order of 10 mGy increase the risk of childhood leukemia and cancer by 30–50% above background in the irradiated offspring (*2*, *3*). This result corresponds to a huge relative risk of 30–50 per Gy if a linear dose response is assumed.

However, despite these observations, it was previously observed that atomic bomb (A-bomb) survivors exposed to radiation as a fetus did not show elevated frequencies of translocations in blood lymphocytes when examined at about 40 years of age, although they had clear mothers

evidence of dose response (4). These findings were confirmed in subsequent studies using mice, which showed that not only irradiated fetuses, but also neonates, were refractory to preserving or expressing a record of radiation damage. The low yield of translocations was seen not only in blood T cells, but also spleen cells and bone marrow cells (5). A cautionary note should be sounded here as newborn mice are relatively immature when compared to humans, and consequently experimental results from mouse neonates/infants may not necessarily apply to humans. Because those studies were restricted to hematolymphoid cells in both human and mouse studies, the question arises of whether the findings were common to different types of cells or restricted to limited cell types. In view of this, testing this model on epithelial cells appeared to be important. Among candidate animals and tissue models, mammary tissues were chosen because breast cancer shows one of the highest relative risks among malignancies in A-bomb survivors after radiation exposures (6). Because mammary tumorigenesis in mice has been indicated to involve mammary tumor virus (7), which is not relevant in humans, rats are frequently utilized for such investigations. In addition, culture methods of rat mammary epithelial cells are well established (8), and rats would provide data as the third species after humans and mice in regard to investigation of the refractory nature of hematolymphoid cells in recording radiation damage when irradiated as a fetus.

MATERIALS AND METHODS

All animals were treated in accordance with the Safety and Health Regulations compiled by the Animal Care and Use Committee at the National Institute of Radiological Sciences (NIRS). Sprague Dawley strain male and female rats (10 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in a rat facility at the NIRS. Virgin or pregnant female rats [17.5 days postcoitus (dpc)] were exposed at the NIRS to 2 Gy of ^{137}Cs gamma rays (0.5 Gy/min) when they were 12 weeks old. Adult animals and the offspring were maintained for 6, 9 or 45 weeks after irradiation and a total of 35 female rats were examined. Specifically, 23 mice were irradiated as fetuses, seven were irradiated as adults (i.e., five as mothers and two as virgins), and five were nonirradiated controls. Animals were then sacrificed under anesthesia with ether and mammary glands were removed. Removed tissues were minced with surgical scissors and treated overnight with collagenase (0.2% type II/III: [(Worthington) dissolved in DMEM/F-12 (50:50) medium in the presence of 5% fetal bovine serum (FBS) and 50 µg/ml Gentamicin]. Incubation times of approximately 14 h in a humidified CO_2 incubator at 37°C were utilized. After digestion, cells were passed through stainless mesh (pore size: 77 µm) to remove undigested tissues or clumps, and were suspended in a culture medium, which consisted of DMEM/F-12 supplemented with 10% FBS and other factors (insulin, apotransferrin, prolactin, mouse EGF, progesterone, hydrocortisone and ascorbic acid) as specified by Darcy *et al*. (8). Cells were inoculated into 10 cm culture dishes at a density of 1 to 4×10^6 cells with 10 ml of medium. Within 2–9 days (usually within 2–5 days) of incubation, the cultures were treated overnight with colcemid (0.02 µg/ml) and then removed with trypsin. Chromosome slides were prepared following standard hypotonic treatment and fixation with Carnoy's solution (5).

For FISH analysis, whole-chromosome probes for chromosome 2 (FITC) and chromosome 4 (Rhodamine) were used (Applied Spectral Imaging, Carlsbad, CA). These were the probes that were commercially available for the two largest chromosomes. For each sample, 800 cells were examined bearing at least a pair of chromosomes 2 and 4. The FISH technique measures the frequency of translocations with one breakpoint in a painted chromosome (5).

Spleen cells were also examined in some animals. Culture methods have been described previously (5) and were used with minor modifications. Briefly, spleen cells were suspended in RPMI 1640 medium supplemented with 20% FBS, phytohemagglutinin (PHA; 0.18 mg/ml, Murex Diagnostics, Inc.), 2-mercaptoethanol (50 µM), Concanavalin A (5 µg/ml), and lipopolysaccharide (LPS; 10 µg/ml). They were cultured for 48 h. The cultures were treated with colcemid (0.02 µg/ml) for the final 24 h before preparation of slides. Because both PHA and LPS were used, T and possibly B cells were expected to respond to the stimuli and were subjected to FISH analysis. A total of 800 cells were scored for each sample.

When apparent clonal aberrations were detected, cells were analyzed again after treatment of the slides for Q-banding analysis (9). This was to identify the unpainted chromosome involved in the translocation by referring to the rat karyotype (10). When the results failed to identify the unpainted chromosome, FISH examinations were conducted anew with probes specific to the suspected (unpainted) chromosome involved in the translocation. For calculation of translocation frequencies, clonal aberrations were counted as single events.

To determine the histologic origin of cells in the mammary cultures, a portion of the cell population was isolated in a few cell cultures at the time of slide preparation and the cells subjected to immunohistochemical staining. Anti-human cytokeratin antibody (αCK18 Ab, Abcam, Tokyo, Japan) was used as the primary antibody to stain epithelial cells (11). Anti-vimentin antibody (Dako Japan, Inc., Tokyo, Japan) and anti-smooth muscle Actin antibody (αSMA Ab, Abcam, Tokyo) were used to stain mesenchymal or myoepithelial cells. For the secondary antibody, Histofine® Simple Stain™ Rat MAX PO (MULTI) Ab (Nichirei Biosciences Inc., Tokyo, Japan) was used with a DAB substrate kit (Nichirei, Biosciences Inc., Tokyo). It has been confirmed, using formalin-fixed paraffin-embedded sections of rat mammary tissues, that αCK18 Ab stains luminal epithelial cells, αSMA Ab stains myoepithelial cells and anti-vimentin Ab stains stromal fibroblasts but not cells of the ducts (Mishima, unpublished results). Under the current study conditions, about 70% of the cells used for FISH examinations were αCK18 Ab positive, often round shaped and were therefore considered as epithelial cells. In contrast, cells that were positively stained with anti-vimentin Ab were scattered and fibroblast-like, and αSMA Ab positive cells were mostly large and elongated. These cells were probably mesenchymal cells or fibroblasts (results not shown).

RESULTS

The frequency of translocations in primary cultures of mammary cells from rats exposed as a fetus varied from 2.7–5.5%. The mean individual frequency was 3.74% (SD 0.78%) at the 6th week after irradiation (n = 10). The corresponding means were 3.79% (SD 1.35%) at the 9th week (n = 5) and 3.36% (SD 1.03%) at the 45th week (n = 8) (Fig. 1, closed circles and see Supplementary materials; http://dx.doi.org/10.1667/RR13446.1.S1). The differences between these means were not statistically significant (Student's *t* test, results not shown). Thus, the frequency did not seem to change with increasing time intervals between the exposures and the cytogenetic tests. These
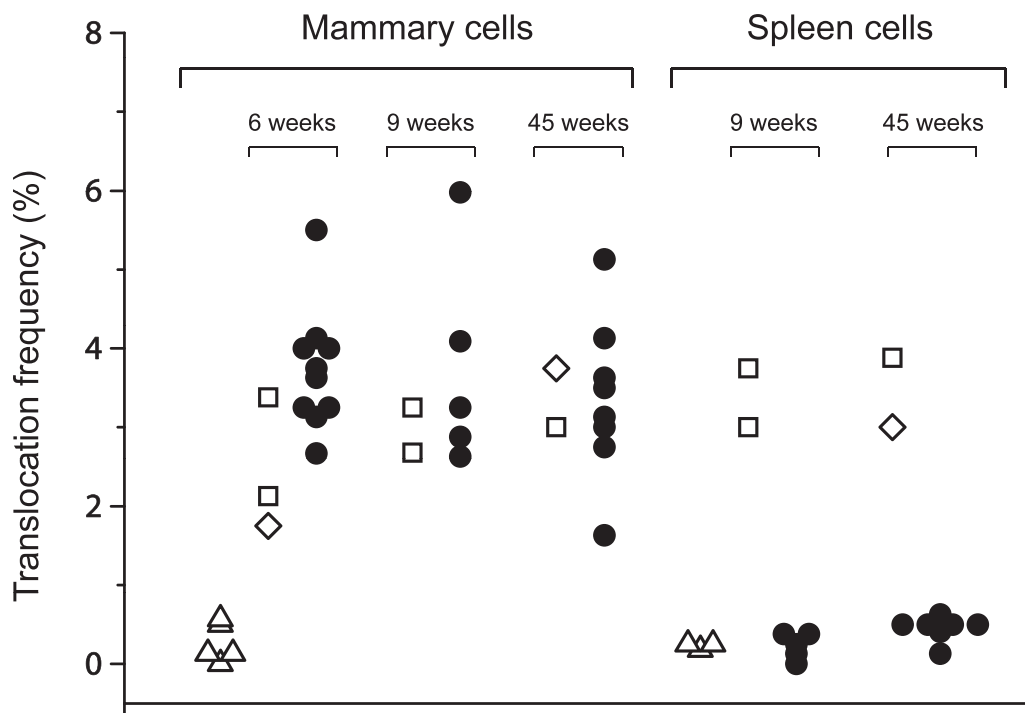
FIG. 1. Observed translocation frequencies involving painted chromosomes 2 or 4 in mammary cell cultures or spleen cells in rats gamma irradiated with 2 Gy: Fetus (●); mother, 12 weeks old (□); virgin, 12 weeks old (◇); and nonirradiated control rats (△). The time intervals between irradiation and FISH examinations are shown at the top of the figure. Each point was derived from scoring 800 cells.

frequencies were nearly the same or slightly higher than those in the irradiated mothers (2.91%, SD 0.54%, n = 5) (Fig. 1, open squares). Two virgin females irradiated as young adults (16 weeks old) showed translocation frequencies of 1.8 and 3.8% when examined at the 6th and 45th weeks, respectively, after irradiation (Fig. 1, open diamonds). These do not seem to be higher than the means detected in the mother rats that raised the offspring. The results are notable in that there does not appear to be evidence for higher radiation sensitivity in fetal cells.

In sharp contrast to the observations in mammary epithelial cells, spleen cells from rats irradiated as fetuses and examined at 9th and 45th weeks after irradiation did not show an increased frequency of translocations, which is in line with previous observations in mice (5). Translocation frequencies in spleen cells from two mother rats were 3.0 and 3.8% at the 9th week, and 3.9% at the 45th week in another mother rat after irradiation. The translocation frequencies in spleen cells of irradiated adults rats were similar to those found in mammary epithelial cells regardless of the time interval since exposure (9 vs. 45 weeks) (Fig. 1).

Identical translocations were occasionally observed in 2 or more cells (clonal translocations) among the 800 cells scored; these cells arise as a result of proliferation of stem/progenitor cells *in vivo* and hence were counted as single events. In the mammary cell cultures, 12 rats among the 23 rats irradiated at the fetal stage showed 16 different clonal

translocations (individual cases are shown in the Supplementary materials; http://dx.doi.org/10.1667/RR13446.1. S1). Specifically, the number of clonal cells varied from 2–8 out of 800 cells scored (0.3–1%). Among five mothers and one virgin female, one mother was found to show 2 translocation clones (2 and 3 cells among 800 cells, respectively, 0.3–0.4%). In contrast, in the spleen cells, only one rat irradiated as a fetus had a clonal translocation (7 cells out of 800 cells, 0.9%).

DISCUSSION

The current study demonstrated that radiation exposure induces translocations in fetal stem/progenitor cells that may later develop into mammary glands. It further demonstrated that the frequency of translocations after irradiation at the fetal stage is not higher than that resulting from adult exposures and the frequency does not change during 6–45 weeks after irradiation. In contrast, radiation-induced cytogenetic damage to hematolymphoid cells did not persist after fetal irradiation but it did persist after adult exposures in humans (4), mice (5) and rats (current study). Therefore, in both hematolymphoid cells and mammary epithelial cells, no evidence was found of higher radiosensitivity in fetal cells leading to the induction of chromosome aberrations. One may ask which tissue response is common after fetal irradiation, full recording (mammary epithelial cells) or no recording (hematolymphoid cells) of radiation-

induced damage. It appears that the former is more likely for the following reasons:

- Our recent study indicated that irradiation of fetal mice could increase the frequency of translocations in thyroid cells when the animals were examined as adults (Hamasaki *et al.*, unpublished results). The results are similar to the observations made in the current study in rat mammary epithelial cells;
- Hematopoietic stem cells (HSCs) remain in fetal liver at birth and start to migrate into final niches in bone marrow during a few weeks after birth in the mouse (*12*), which closely corresponds to the period when radiation-induced translocations start to persist (*5*);
- Finally, fetal hematopoietic stem cells express only a very low level of ATM gene (*13*), which indicates that initial recognition processes of DNA damage response may be defective exclusively in fetal HSCs.

In our experiments, clonal translocations were observed, and the results can be used to estimate the number of surviving mammary stem cells in a fetus after irradiation with 2 Gy, assuming that clonal expansion occurs by chance and does not accompany a growth advantage. In addition, it was previously shown that the number of surviving stem cells is inversely related to the clone size (*14*). Thus, a clone size of 0.3–1% as observed in the current study indicates that the total number of surviving stem cells is 1/0.003–1/0.01 or 300–100.

Taken together these results raise an interesting question: if cytogenetic damage is a surrogate marker of the carcinogenic effect of radiation, will the translocation frequency be directly relevant to the carcinogenic risks after irradiation? With regard to hematolymphoid cells, the answer is yes, e.g., Upton *et al.* irradiated RF mice of various ages with 3 Gy and followed them to observe the development of acute myeloid leukemia (AML) (*15*). The results showed that fetuses are the *least* sensitive to the induction of AML after radiation exposure and the sensitivity steadily increases as the age of the animals increases at the time of irradiation up to 100 days. The results are in close agreement with our previous study on the induction of persistent translocations in the hematolymphoid cells of mice varying from fetal to young adult stages (*5*). Specifically, the frequency was nearly zero for mice irradiated as fetuses or neonates (cytogenetic examinations were made when the animals reached 20 weeks of age), while the frequency steadily increased with an increase in the mouse age at the time of irradiation. The close correlation between the induction of AML and translocations in hematolymphoid cells is reasonable if it can be assumed that both end points are involved with the fate of damaged hematopoietic stem cells.

With regard to induction of tumors other than AML, however, the answers are much less clear, specifically, it appears that fetal radiosensitivity differs depending on the tissues (e.g., *16*, *17*) and there also appears to be no general trend suggesting that fetuses are more sensitive than neonates or young adults. It should be cautioned, however, that currently available information is so limited that a general conclusion cannot be derived on this issue; e.g., the radiation effect may be affected not only by the fetal stage of development at the time of irradiation but also by the radiation dose employed since the dose-response curve can be humped at low doses (*17*). As for the current study on mammary glands, cytogenetic damage to fetal mammary stem/progenitor cells is not correlated with risk of tumorigenesis because fetal irradiation of SD rats is reported to not increase the risk of mammary tumors (*18*, *19*) even though translocations were induced as effectively as in adults. Several possible explanations, not mutually exclusive, may reconcile this discrepancy.

- The number of surviving target cells at risk after 2 Gy exposure is too small to preserve the carcinogenic effect of radiation, e.g., the results of clonal translocations indicated that after 2 Gy exposure the number would be only 100–300 surviving stem cells. These cells cannot record radiation damage quantitatively if we assume that irradiation adds oncogenic mutations to the cells, because mutation induction rates at a specific gene are on the order of 10^{-5}–10^{-4} per Gy (*20*, *21*).
- Exposure to 2 Gy can partly destroy the oocyte pool in a fetus (*22*), leading to subsequent low estrogen levels or earlier menopause and resulting in reduced promotion of precancerous cells.
- Stem cells and their descendants in mammary glands might escape cytogenetic tests. This may happen when *in vitro* tissue culture conditions are inadequate and for example, stimulate stroma cells to grow rather than epithelial cells. However, this explanation appears to have a low probability because about 70% of the cells examined were αCK18Ab positive and are hence regarded as epithelial cells.
- The possibility, as described by Degregori (*23*), that certain mutations required for a carcinogenic process may actually impair rather than stimulate growth of hematopoietic stem cells. In any event, further studies are required to understand the refractory nature of a fetus for the development of mammary cancers after radiation exposure.

In humans, mammary glands are among the most sensitive tissues in terms of developing malignancies after postnatal exposure to radiation. Specifically, after radiation exposure in infancy, increased risk levels were observed in studies of patients treated with radiation for hemangioma (*24*) or enlarged thymus (*25*, *26*) and in studies of individuals exposed to A-bomb radiation (*6*, *27*). After fetal exposure, however, even the total cancer risk is not well established in the A-bomb survivors because the epidemiologic cohorts of A-bomb survivors are fairly small and the number of subjects with known doses is about 2,500

(28, 29). Therefore, the risk of breast cancer after fetal exposure to radiation is still unclear.

In summary, the current results suggest that ordinary cytogenetic tests using blood lymphocytes will not work for biological dose evaluations of irradiated fetuses. However, it is anticipated that cells from solid tissues (e.g., skin fibroblasts) could be an alternative source for biodosimetry.

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