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Source: Radiation Research, 186(4) : 360-366

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RR14385.1

RADIATION RESEARCH **186**, 360–366 (2016)
0033-7587/16 $15.00

DOI: 10.1667/RR14385.1

Irradiation at Different Fetal Stages Results in Different Translocation Frequencies in Adult Mouse Thyroid Cells

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Hamasaki, K., Landes, R. D., Noda, A., Nakamura, N. and Kodama, Y. Irradiation at Different Fetal Stages Results in Different Translocation Frequencies in Adult Mouse Thyroid Cells. *Radiat. Res.* 186, 360–366 (2016).

While it is generally believed that fetuses are at high risk of developing cancers, including leukemia, after low doses of radiation, it has been reported that atomic bomb survivors exposed *in utero* did not show a dose response for translocations in blood T lymphocytes when they were examined at approximately 40 years of age. Subsequent mouse studies confirmed that animals irradiated during the fetal stage did not show evidence of radiation effects in lymphocytes and bone marrow cells when they were examined after reaching adulthood. However, in a study of rat mammary epithelial cells, radiation effects were clearly observed after fetal irradiation. These results indicate that the fate of chromosome aberrations induced in a fetus could vary among different tissues. Here we report on translocation frequencies in mouse thyroid cells, which were irradiated at different stages of fetal development. Cytogenetic examination was conducted using fluorescence *in situ* hybridization (FISH) painting of chromosomes 1 and 3. Adult mice, 2 Gy X-ray irradiated at 15.5-day-old fetuses (E15.5), showed a higher translocation frequency (30/1,155 or 25.3×10^{-3}) than nonirradiated adult controls (0/1,007 or 0.1×10^{-3}), and was near that experienced by irradiated mothers and non-pregnant adult females (43/1,244 or 33.7×10^{-3}). These results are consistent with those seen in rat mammary cells. However, when fetuses were irradiated at an earlier stage of development (E6.5) before thyroid organogenesis, the resulting observed translocation frequency was much lower (3/502 or 5.8×10^{-3}) than that in E15.5 mice. These results suggest that after fetal irradiation, tissue stem cells record radiation effects primarily when the exposure occurs in cells that have been integrated into tissue. Embryonic stem cells that have been damaged prior to integration into the niche may undergo negative selection due to apoptosis, mitotic death or stem cell-niche cell interactions. The implications of these results in interpreting cancer risks after fetal irradiation are also discussed. © 2016 by Radiation Research Society

INTRODUCTION

Embryos or fetuses are considered to be highly sensitive to radiation and can undergo developmental anomalies or death, and the threshold dose for malformation to occur in human fetuses is estimated to be around 0.1 Gy (*1*). Studies of radiation risks leading to cancer after fetal irradiation originated in the Oxford Survey of Childhood Cancers (OSCC), which was initiated in the 1950s. Specifically, the OSCC study was a case-control study, and the results indicated that women whose children later died from leukemia or cancer before the age of ten exhibited a slightly higher frequency of having received diagnostic X-rays (e.g., ~15%) during pregnancy compared to mothers whose children had no such diseases (e.g., ~10%) (*2, 3*). The X-ray doses to fetuses were later estimated to be approximately 10 mGy (*4–7*). Therefore, the relative risk of developing childhood leukemia or cancer was estimated as 1.5 at 10 mGy. In other words, the relative risk at 1 Gy could increase to as high as ~50 if the increased risks were caused by X-ray exposures and a linear dose response is assumed (*4, 7*). The estimated relative risk is more than one order of magnitude higher than that observed for solid cancers seen in atomic bomb (A-bomb) survivors who were exposed as adults (*8*), although such a large relative risk is not unprecedented. Specifically, the relative risk for leukemia, which appeared within a few years after A-bomb exposure among those who were at the age of approximatley 10 years, was estimated to be over 50 at 1 Gy (*9*). However, as for solid cancers, the magnitude of the increased relative risk seems substantially smaller than that of leukemia (*10*, *11*).

After the OSCC study, a number of similar case-control studies were conducted at various locations around the world, and the results were consistent while the observed relative risks were small (e.g., 1.13–1.70) (*5, 7*). Therefore,

Editor's note. The online version of this article (DOI: 10.1667/RR14385.1) contains supplementary information that is available to all authorized users.

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it appeared to be generally accepted that fetuses exposed to diagnostic X-rays would have higher risks of developing childhood leukemia and solid cancer. However, while epidemiological case-control studies can indicate the existence of an association between fetal radiation exposure and subsequent risks for childhood leukemia and solid cancer, they do not prove a causal association. In fact, subsequent animal studies have consistently demonstrated no indication of such extremely high cancer risks after fetal irradiation (*1, 12, 13*). The challenge to investigate this discrepancy is ongoing.

Epidemiological studies of A-bomb survivors have not provided definitive evidence to support the extremely high risks of childhood cancers (including leukemia) after exposures *in utero* (*14, 15–17*). To explain the apparent disparate results, various possibilities have been considered, which include selective loss of radiosensitive individuals from the survivor cohort due to exposure to relatively large doses of radiation (*18*). However, it is now understood that the cohort of A-bomb survivors exposed *in utero* is not large enough to negate or support the OSCC data (*14*); therefore, the results are not incompatible with the OSCC results (*4*).

In another published study, translocation frequencies were examined in blood lymphocytes from about 300 A-bomb survivors who had been exposed *in utero*. The translocation frequencies were examined when the survivors were approximately 40 years old. Contrary to expectations, the translocation frequencies did not increase with an increase in radiation dose (*19*). Because the mothers showed a clear pattern of dose-related increases in translocation frequencies, the possibility of dosimetry errors to the fetuses was formally excluded. Subsequent mouse studies confirmed the survivor data; i.e., when mice were irradiated as 15.5-day fetuses (E15.5) and examined when they reached adulthood (20 weeks old), translocation frequencies were quite low, as was observed in A-bomb survivors exposed *in utero* (*20*). It was thought that this lack of radiation effect might be unique to hematolymphoid cells, and subsequent experiments focused on mammary gland epithelial cells in rats. In that study, it was reported that the irradiated rat fetuses did show radiation effect in breast tissues when examined as adults, and the translocation frequency was nearly the same as that in the mothers as well as females irradiated as adults (*21*). These studies may well indicate that the persistence of radiation-induced chromosome aberrations after fetal irradiation depends on the tissues being examined. Additionally, with respect to the settlement of tissue stem cells into the niche resulting in a functional tissue organ, the behavior of hematopoietic stem cells (HSCs) during fetal development is believed to differ from that of nonhematopoietic cells; thus, radiation effect differences among tissues may also be related to irradiation timing relative to the tissue developmental stage.

Because of the known susceptibility of the thyroid to radiation-induced carcinogenesis (*22*) and well-established culture methods for primary mouse thyroid cells (*23*), we chose thyroid epithelial cells as the second nonlymphoid tissue to examine the radiation effects in those exposed as fetuses, that is, to determine if the frequency was low as in lymphoid cells or high as in mammary epithelial cells. We also considered the timing of *in utero* irradiation relative to organogenesis in both thyroid epithelial cells and spleen lymphocytes.

MATERIALS AND METHODS

Mice and Irradiation

Pathogen-free B6C3F1 mice (8 weeks old, both male and female) were purchased from the Experimental Animal Co. (Hiroshima, Japan). Approximately two weeks after their arrival, mice were mated, and pregnant females at day 6.5 (E6.5 fetuses) or 15.5 (E15.5 fetuses) after mating were whole-body X-ray irradiated with 2 Gy. This study used a Shimadzu HF-320 machine (Kyoto, Japan) operating at 220 kVp, 8 mA, with a 0.5 mm aluminum and 0.3 mm copper filter at a dose rate of 1.0 Gy/min, and a Faxitron® CP-160 machine (Tucson, AZ) operating at 160 kVp, 6.3 mA with a 0.5 mm aluminum and 0.21 mm copper filter at a dose rate of 0.77 Gy/min. Nonpregnant adult female mice (9–15 weeks old) were also irradiated to evaluate translocation frequencies after adult exposures. We examined 96 mice after fetal irradiations (74 mice at E15.5 and 22 mice at E6.5), 17 mothers, 61 irradiated adult females (nonpregnant) and 36 nonirradiated adult females (control group) (Table 1). All mice were kept at an animal facility in the Radiation Effects Research Foundation (RERF; Hiroshima, Japan) and housed in sterile cages placed in a microisolator and fed a sterile regular diet *ad libitum*. The study plan was approved by the Experimental Animal Care Committee at RERF.

Preparation of Primary Cultures of Thyroid Epithelial Cells

When the animals irradiated at day E6.5 or E15.5 reached 8 to12 weeks old, mice were euthanized with carbon dioxide and the thyroid lobes were removed (timeline shown in Fig. 1). Primary cultures were established with the method described in Jeker *et al.* (*23*) with slight modifications. Briefly, minced lobes from several animals of the same group (exposed or control) were mixed and digested with type 1A collagenase (125 U/ml, Sigma-Aldrich®, St. Louis, MO) and Dispase I (500 PU/ml; Sanko Junyaku, Japan) dissolved in 1 ml of Eagle's minimal essential medium (EMEM) (Wako Pure Chemical Industries, Osaka, Japan) for 15 min in a 37°C incubator while the tubes were gently rotated. Moderately dispersed lobes were then plated into 24-well culture dishes (one lobe equivalent was plated into 2 wells). Culture media consisted of Ham's F12 media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (HyClone™ Laboratories, Logan, UT), 5 μg/ml of human transferrin (Sigma-Aldrich), 10 μg/ml of bovine insulin (Sigma-Aldrich), 4 ng/ml of hydrocortisone (Wako), 10 ng/ml of somatostatin (Sigma-Aldrich), 2 ng/ml of Gly-His-Lys acetate salt (Sigma-Aldrich) and 0.2 mIU/ml of thyrotropic hormone from bovine pituitaries (Sigma-Aldrich). In addition, 50 μg/ml of gentamicin antibiotic (Sigma-Aldrich) was added. After culturing for one day, dishes were washed with phosphate buffered saline (PBS) and the media was replaced with fresh media. Thyroid epithelial cells were cultured for about 65 h at 37°C in a 5% CO_2 incubator before they were harvested for chromosome tests.

Spleen T Lymphocytes

Spleens were also removed from some animals, and spleen cells were cultured as described previously (*20*). Briefly, spleen cells were suspended in RPMI 1640 media supplemented with 20% FBS and phytohemagglutinin (PHA) (0.18 mg/ml; Remel Europe Ltd./Thermo Scientific, Basingstoke, UK). Cells were then cultured for 26 h.

TABLE 1
Summary Results of Mice 2 Gy X-Ray Irradiated as Fetuses in Thyroid Study

	Age at 2 Gy irradiation			
	Fetuses (E6.5)	Fetuses (E15.5)	Adults[a] (9–15 week)	No irradiation (controls)
Number of cultures	6	11	18	6
Number of mice	22	74	78	36
Number of cells	502	1,155	1,244	1,007
Number of stable events	3[b]	30	43	0
Stable frequency (95%CI)	5.8 (1.5, 14.7)	25.3 (17.3, 35.5)	33.7 (24.5, 44.9)	0.1 (0, 2.3)
Number of unstable events	0	4	33	0
Unstable frequency (95% CI)	0.3 (0, 4.5)	3.4 (1.1, 7.9)	25.9 (18.0, 35.8)	0.1 (0, 2.3)

Note. Stable and unstable aberration frequencies estimated by the Bayesian model are expressed per 1,000 cells.
[a] These include mothers of the irradiated fetuses.
[b] These include one clonal event, which was found in six cells.

Cell Harvest and Slide Preparation

Colcemid (100 ng/ml; Serva Electrophoresis, Heidelberg, Germany) was added during the last 18 h of the thyroid cell cultures and the last 2 h of the spleen cell cultures. Cells were then harvested after trypsin treatment, treated with a hypotonic solution of potassium chloride, and fixed with 3:1 methanol/acetic acid. Chromosome slides were made using a standard air-drying method (*20*).

Fluorescence In Situ Hybridization (FISH)

FISH painting of metaphase chromosomes was performed as previously described (*20*). Briefly, metaphase chromosomes were denatured and hybridized for 18 h with whole DNA probes (Applied Spectral Imaging Inc., Carlsbad, CA) for mouse chromosomes 1 and 3 labeled with FITC and Rhodamine, respectively. Slides were then washed and counterstained with DAPI in an anti-fade/glycerol solution and stored at −20°C until use. In most cases, 100–200 metaphase cells were scored with FISH per culture, which consisted of thyroid cells from about six mice. Aberrant chromosomes bearing color changes were detected in the arms of FISH-painted chromosomes (i.e., translocations, insertions and dicentrics). Chromosome aberrations were counted, as we have described previously (*20*); translocations and dicentrics were counted as single events while insertions were counted as two translocations. When cells bearing apparently identical translocations in terms of breakpoint locations in FISH-labeled and nonlabeled chromosomes were observed, we considered these to be clonal cells and the aberrations were counted as a single event.

Immunostaining of Cultured Thyroid Cells

To confirm that the cells in culture were primarily derived from thyroid epithelial cells, thyroglobulin protein levels were examined with immunostaining. Briefly, cells cultured in slide chambers (8 chambers per slide) were fixed and incubated with polyclonal rabbit anti-human thyroglobulin antibody (Dako Inc., Carpinteria, CA), and subsequently with Alexa Fluor® 488-labeled goat anti-rabbit IgG (H+L) (Molecular Probes®, Grand Island, NY) as the second antibody. More than 90% of cultured cells showed the same levels of thyroglobulin expression as observed in FRTL-5 cells (a thyroid cell line derived from Fisher rats) used for positive control even on day 10 after the initiation of the cultures (unpublished results). Therefore, the cells harvested after 65 h of culture and used for chromosome analyses were considered to be primarily thyroid epithelial cells.

Statistical Analyses

In the thyroid study, there were four treatment groups: nonirradiated controls; mice irradiated as adults, at E15.5 or E6.5. From each of the four treatment groups (Table 1), cultures of thyroid cells were obtained. Each culture contained V cells pooled from between 1 to 9 mice; the median number of animals represented in each culture was 6. In each culture, two types of aberrations were counted: stable type (translocations, insertions) and unstable type (dicentrics). For each aberration type, the number of aberrations and the number of cells per culture were counted. The observed aberrations were modeled with Poisson distributions. The single parameter in each treatment group-specific Poisson distribution was $V\lambda$, where λ represents the treatment group aberration frequency (expressed per 1,000 cells). Using a Bayesian approach to the data analysis, the 8 λs (4 treatment groups × 2 types of aberrations) were modeled with a gamma distribution having hyperparameters α and β. Finally, independent uniform $(0, 10^6)$ priors were assigned to α and β. For purposes of comparison, ratios of aberration frequencies and their 95% credible intervals (CIs) are presented. (Credible interval is a Bayesian term analogous to a confidence interval.) We analyzed counts of aberrations in spleen T lymphocytes with a model similar to that used in the thyroid study. Differences were: 1. Cells from a single mouse rather than pooled cells from several mice provided the V cells; and 2. Only stable aberrations were counted, thus, there were 4 λs instead of 8 λs, as in the thyroid study. In both the thyroid and spleen studies, CIs for ratios not containing the null value, 1, were statistically significant at the 0.05 significance level. WinBUGS version 1.4 was used to perform these analyses, and sensitivity analyses were performed to evaluate the impact of the priors on the final results. A more thorough description of the statistical model and WinBUGS code used in this analyses is found in the Supplementary Information (http://dx.doi.org/10.1667/RR14385.1.S1).

RESULTS

Because thyroid lobes from several mice (approximately 6) were pooled for primary cultures due to the difficulty of establishing primary cultures from single mice, the results including summary statistics and estimated aberration

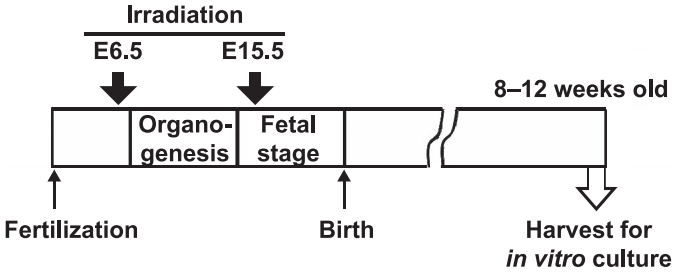

FIG. 1. Timeline of the embryonic stages at irradiation and at tissues harvesting for chromosome analysis.

frequencies (per 1,000 cells) are shown with the group of animals (i.e., irradiated or nonirradiated and age at exposure; see Table 1). Specifically, the estimated frequencies of stable-type aberrations (primarily translocations and some insertions involving chromosome 1 or 3) were estimated to be very near 0 per 1,000 cells in nonirradiated adult female mice (i.e., controls).[2] In the females irradiated as adults (including the mothers), the stable-type aberration frequency of 33.7 per 1,000 cells was 249 times greater [95% CI (13.8, $>10^8$)] than controls; and the frequency of E15.5-irradiated mice (examined at 8 to 12 weeks of age) was 25.3 per 1,000, which was 188 times greater [25.3, 95% CI (10.3, $>10^8$)] than controls. The stable aberration frequency after 2 Gy irradiation in adult females was 1.3 times higher than in adult mice irradiated as E15.5 fetuses, but the difference was not statistically significant [CI 95% (0.8, 2.1)]. Therefore, adult mouse thyroid cells that were 2 Gy X-ray irradiated at E15.5 tended to retain radiation-induced stable-type aberrations at levels similar to their mothers.

Noting that timing of irradiation may be a reason for differences seen in radiation effects among tissues, we also explored timing of irradiation in thyroid development. Specifically, prior to thyroid organogenesis, E6.5 fetuses were 2 Gy irradiated using the same methods as for the E15.5 (post-organogenesis) mice. The E6.5 group's stable-type aberration frequency was 5.8 per 1,000, which was significantly higher than that in the control group's [by 43 times, 95% CI (1.8, $>10^7$)], but was significantly lower by 0.23 times, 95% CI (0.06, 0.63) the frequency in E15.5 group. Consequently, it can be concluded that, in contrast to thyroid cells irradiated at a post-organogenesis stage, precursor cells for thyroid glands irradiated at a pre-organogenesis period were less capable of recording radiation effects.

Translocation frequencies were also examined in adult spleen cells of mice irradiated during the fetal stage (Table 2) or as adults. Compared to their mothers' frequency of 48.3 per 1,000, the frequency of 3.4 per 1,000 in adults that were irradiated *in utero* at E15.5 was 0.071 times [95% CI (0.047, 0.103)] the mothers' frequency. Similar results were obtained from adult mice irradiated *in utero* at E6.5; namely, E6.5 group's frequency of 1.1 per 1,000 was 0.023 times [95% CI (0.009, 0.048)] the mothers' frequency. Further, the frequency from E6.5 irradiated mice was 0.32 times [95% CI (0.120, 0.711)] that from E15.5 irradiated mice. For adult mice having never been irradiated (i.e., controls), the observed translocation frequency was 0.0 [95% CI (0.0, 0.9) per 1,000 cells]. Of note, clonal translocations were found after irradiation in both the E15.5 fetuses as well as the E6.5 fetuses, although the frequency of clonal events was definitely lower than that after adult irradiations (Table 2)[2].

Although we did not statistically compare translocation frequencies between thyroids and spleens within adult mice exposed to irradiation *in utero*, the thyroid appears to be more susceptible to induction of stable-type aberrations by irradiation; within each of the E6.5 and E15.5 irradiated mouse groups, the thyroid stable aberration frequency was, respectively, over 5 and 7 times greater than the corresponding spleen's frequency (comparing estimated frequencies within treatment groups between Tables 1 and 2).

Unstable-type aberrations (dicentric chromosomes) were also detected in the thyroid cell cultures (Table 1). In contrast to the results for stable-type aberrations, considerable difference was observed in the frequencies between mice exposed as E15.5 fetuses (3.4×10^{-3}) and adults, including the mothers (25.9×10^{-3}). Because cells bearing unstable-type aberrations are subject to negative selection after cell divisions, these results likely reflect the fact that adult mice irradiated as fetuses had more thyroid cell divisions than adult mice irradiated as adults.

DISCUSSION

During the development of most organs in the mouse, tissue stem cells settle into the appropriate niche and begin to function at E7–E14 (the organogenesis stage), but the precise timing for migration, settling and subsequent differentiation may differ among tissues (*24*). The thyroid gland is already observed in E8.5 fetuses and the gland undergoes a split into multiple buds by E15, but with no follicle formation yet (*25*). In contrast, the hematolymphoid system is exceptional because the majority of HSCs do not locate into their bone marrow niches prior to birth, but only at 3–4 weeks after birth (*26*).

Given these considerations, past and present results can be interpreted among other possibilities, as follows. When fetal stem cells were irradiated before migrating into their final niches, most of the aberrant cells could have been lost and were consequently unable to participate in organogenesis. Examples are lymphoid and bone marrow cells irradiated at the fetal stage in mice (*20*), blood lymphocytes in A-bomb survivors exposed *in utero* (*19*) and mouse thyroid cells irradiated at E6.5 (in the current study). In contrast, when fetal stem cells were irradiated after locating into their niches, aberrant cells were able to persist as they did in the mothers, and subsequently contribute to organogenesis as was seen in rat mammary cells (*21*) and mouse thyroid cells (in the current study using irradiation at E15.5). Also, Jacquet *et al*. reported that cells from mouse embryos irradiated at day E8 and examined 7 h later showed high frequency of chromatid aberration but very low frequency of exchange-type chromatid aberration (*27*), which is an indication of deficient DNA rejoining. This model is shown schematically in Fig. 2.

[2] The observed frequency was 0; however, the Bayesian model estimated the frequency to be slightly above 0. This type of result also occurred for unstable aberration frequencies in controls and E6.5 mice (see Table 1), and for control aberration frequencies in the spleen (see Table 2).

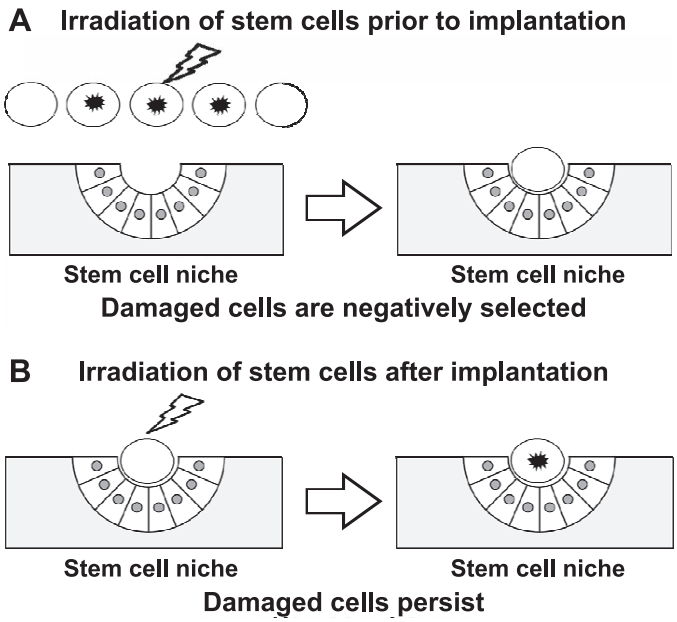

FIG. 2. Schematic model of the observed differences in translocation frequencies in different tissues of adult mice after fetal irradiation. Panel A: When stem cells are irradiated before implantation into the niche, damaged cells are negatively selected and can only poorly contribute to organogenesis. Panel B: In contrast, such a negative selection does not occur when stem cells are irradiated after implantation into the niche.

An important question remains concerning the mechanism that might be involved in the elimination of aberrant stem cells from organogenesis when they are irradiated prior to settling into their niches in developing organs. Two alternative models may be proposed to explain the observations. In one model, stable-type aberrations (as well as unstable-type aberrations) are actually induced after misrepair of radiation-induced DNA double-strand breaks (DSBs), but they are subsequently negatively selected against. For example, translocation-bearing cells may carry persistent subtle abnormality and be outcompeted by nonaberrant cells, as was reported by Bondar and Medzhitov between irradiated and nonirradiated HSCs (*28*). *A priori*, replicative disadvantage of the aberrant cells as well as defective interactions between stem cells and the niche can be the cause. In the second model, DNA breaks are induced, but efficient DNA DSB repair, which may lead to formation of exchange-type aberrations, does not take place effectively in the immature precursors or stem cells. In this scenario, persistent DNA breaks can cause either mitotic death after cell division or apoptotic death without division. This possibility is supported by the following reported findings. In one study, early mouse embryonic cells (E6.5 and E7.5) but not extra-embryonic cells underwent ATM- and p53-dependent apoptosis after low-dose irradiation (<0.5 Gy) (*29*). And in another study, mouse HSCs from fetal liver (E14.5) expressed relatively low levels of the ATM gene product, one of the key players involved in the repair of DNA DSBs, compared to HSCs from the bone marrow of postnatal mice (*30*).

The seemingly dissimilar findings between the epidemiological data (high radiosensitivity for cancer induction) and animal data (low yield of chromosome aberrations) requires an explanation. One possible explanation is that a cytogenetic end point was not appropriate to indicate susceptibility to developing leukemia after irradiation because the underlying mechanisms leading to aberrations may be different from those that lead to somatic mutations relevant to carcinogenesis. Alternatively, fetal lymphoid stem or progenitor cells that had acquired translocations spontaneously, specific to childhood leukemia (i.e., preleukemic cells) (*31*), are under altered physiological conditions compared to their normal counterparts and are not susceptible to negative selection after radiation-induced DNA breaks. A third possibility is that a small fraction of HSCs is already active in an early stage of embryogenesis. We have previously reported on the presence of clonal translocations observed in adult mice that were irradiated as fetuses on E15.5 (*32*). In the current study, we confirmed that irradiation of E15.5 fetuses gives rise to clonal translocations, but this was also the case after irradiation of E6.5 fetuses (Table 2).[2] A fourth possibility is that the apparently increased risks seen in the OSCC study and other related studies may not have been caused by low-dose X ray exposure, but by other unknown confounding factors.

TABLE 2
Summary Results of Mice Mice 2 Gy X-Ray Irradiated as Fetuses in Spleen Lymphocyte Study

	Age at 2 Gy irradiation			
	Fetuses (E6.5)	Fetuses (E15.5)	Adults[a] (9–15 week)	No irradiation (controls)
Number of mice[b]	7	11	4	3
Number of cells	5,550	8,763	3,097	2,400
Number of stable events	6[c]	30[d]	151[e]	0
Stable frequency (CI 95%)	1.1 (0.4, 2.2)	3.4 (2.3, 4.8)	48.3 (41.0, 56.5)	0.0 (0.0, 0.9)

Note. Frequencies of stable-type aberrations (translocations and insertions) were estimated by the Bayesian model and are expressed per 1,000 cells.

[a] Mothers of irradiated fetuses.

[b] All mice used for the spleen study were part of the group used for thyroid cell cultures shown in Table 1.

[c] These include three clonal events, each of which was found in 14–20 cells out of 800.

[d] These include eight clonal events, each of which was found in 3–5 cells except for one which was found in 18 cells out of 800.

[e] These include 17 clonal events, each of which was found in 3–18 cells out of 800.

While the etiology of childhood leukemia has yet to be clarified, it is well established that risks are affected by various factors including ethnic group, socioeconomic status, birth order and maternal age, among others (*33, 34*). Among the potential confounding factors, birth weight is of greatest interest. While large fetal size has traditionally been viewed as a strong indicator of healthy development, the combination of large fetal size and small pelvis size of the mother may have compelled obstetricians to obtain pelvimetric X-ray photographs as they decided on approaches to Caesarean section. Later on, heavy birth weight was associated with an increased risk of developing childhood leukemia (*35, 36*) and probably solid cancers as well (*37*), which can be attributed to higher levels of maternal growth factors such as IGF.

In at least one reported study, birth weight was taken into account in estimating radiation risks after fetal X-ray exposure and the conclusion remained the same (*38*). Nevertheless, the issue is complicated and knowledge of the birth weights from the original OSCC data would be of significant help to better understand the biological mechanisms involved in increased cancer risks after low-dose irradiation of the fetus. This data collection process is currently ongoing (*39*).

SUPPLEMENTARY INFORMATION

Description of the Bayesian analyses for this study.

ACKNOWLEDGMENTS

The authors are grateful to Ms. K. Muramoto, T. Matsumoto and Mr. S. Mishima for excellent technical assistance, to Dr. K. Hamatani and Mr. K. Koyama for valuable advice for thyroid cultures, to Mr. N. Kajitani for help with animal care, to Drs. R. Ullrich and L. Kapp for careful reading of the manuscript and to Ms. M. Utaka for manuscript preparation. Dr. R. Wakeford and one anonymous reviewer are also acknowledged for their insightful comments. The Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan is a public interest foundation funded by the Japanese Ministry of Health, Labor and Welfare (MHLW) and the U.S. Department of Energy (DOE). This research was also funded in part through DOE award no. DE-HS0000031 to the National Academy of Sciences. This publication was supported by RERF Research Protocol RP6-11. The views of the authors do not necessarily reflect those of the two governments. This work was supported in part by JSPS KAKENHI grant no. 24710067: Grant-in-Aid for Young Scientists (B).

Received: January 18, 2016; accepted: June 17, 2016; published online: September 14, 2016

REFERENCES

1. Streffer C, Shore R, Konermann G, Meadows A, Uma Devi P, Preston Withers J, et. al. Biological effects after prenatal irradiation (embryo and fetus). A report of the International Commission on Radiological Protection. 2003; 33:5–206.
2. Stewart A, Kneale GW. Radiation dose effects in relation to obstetric x-rays and childhood cancers. Lancet 1970; 1:1185–8.
3. Bithell JF, Stewart AM. Pre-natal irradiation and childhood malignancy: A review of British data from the Oxford survey. Br J Cancer 1975; 31:271–88.
4. Wakeford R, Little MP. Risk coefficients for childhood cancer after intrauterine irradiation: a review. Int J Radiat Biol 2003; 79:293–309.
5. Doll R, Wakeford R. Risk of childhood cancer from fetal irradiation. Br J Radiol 1997; 70:130–9.
6. Boice Jr JD, Miller RW. Childhood and adult cancer after intrauterine exposure to ionizing radiation. Teratology 1999; 59:227–33.
7. Wakeford R. Childhood leukaemia following medical diagnostic exposure to ionizing radiation in utero or after birth. Radiat Prot Dosimetry 2008; 132:166–74.
8. Ozasa K, Shimizu Y, Suyama A, Kasagi F, Soda M, Grant EJ, et al. Studies of the mortality of atomic bomb survivors, Report 14, 1950–2003: an overview of cancer and noncancer diseases. Radiat Res 2012; 177:229–43.
9. Richardson D, Sugiyama H, Nishi N, Sakata R, Shimizu Y, Grant EJ, et al. Ionizing radiation and leukemia mortality among Japanese Atomic Bomb Survivors, 1950–2000. Radiat Res 2009; 172:368–82.
10. Shimizu Y, Kato H, Schull WJ. Studies of the mortality of A-bomb survivors. 9. Mortality, 1950–1985: Part 2. Cancer mortality based on the recently revised doses (DS86) Radiat Res 1990; 121:120–41.
11. Preston DL, Kato H, Kopecky K, Fujita S. Studies of the mortality of A-bomb survivors. 8. Cancer mortality, 1950–1982. Radiat Res 1987; 111:151–78.
12. Upton AC, Odell TT, Sniffen EP. Influence of age at time of irradiation on induction of leukemia and ovarian tumors in RF mice. Proc Soc Exper Biol Med 1960; 104:769–72.
13. Sasaki S. Influence of the age of mice at exposure to radiation on life-shortening and carcinogenesis. J Radiat Res supplement 1991; 2:73–85.
14. Delongchamp RR, Mabuchi K, Yoshimoto Y, Preston DL. Cancer mortality among atomic bomb survivors exposed in utero or as young children, October 1950–May 1992. Radiat Res 1997; 147:385–95.
15. Jablon S, Kato H. Childhood cancer in relation to prenatal exposure to atomic-bomb radiation. Lancet 1970; 2:1000–3.
16. Yoshimoto Y, Kato H, Schull WJ. Risk of cancer among children exposed in utero to A-bomb radiation, 1950–84. Lancet 1988; 2:665–9.
17. Yoshimoto Y, Delongchamp R, Mabuchi K. In utero exposed atomic bomb survivors: cancer risk update. Lancet 1994; 344:345–6.
18. Stewart AM, Kneale GW. Prenatal radiation exposure and childhood cancer. Lancet 1970; 2:1190.
19. Ohtaki K, Kodama Y, Nakano M, Itoh M, Awa AA, Cologne J, et al. Human fetuses do not register chromosome damage inflicted by radiation exposure in lymphoid precursor cells except for a small but significant effect at low doses. Radiat Res 2004; 161:373–9.
20. Nakano M, Kodama Y, Ohtaki K, Nakashima E, Niwa O, Toyoshima M, et al. Chromosome aberrations do not persist in the lymphocytes or bone marrow cells of mice irradiated in utero or soon after birth. Radiat Res 2007; 167:693–702.
21. Nakano M, Nishimura M, Hamasaki K, Mishima S, Yoshida M, Nakata A, et al. Fetal irradiation of rats induces persistent translocations in mammary epithelial cells similar to the level after adult irradiation, but not in hematolymphoid cells. Radiat Res 2014; 181:172–6.
22. Preston DL, Cullings H, Suyama A, Funamoto S, Nishi N, Soda M, et al. Solid cancer incidence in atomic bomb survivors exposed in utero or as young children. J Natl Cancer Inst 2008; 100:428–36.
23. Jeker LT, Hejazi M, Lynne Burek C, Rose NR, Caturegli P. Mouse

thyroid primary culture. Biochem Biophys Res Commun 1999; 257:511–5.

24. Niwa O. Roles of stem cells in tissue turnover and radiation carcinogenesis. Radiat Res 2010; 174:833–9.

25. Kaufman M, Nikitin AY, Sundberg JP. Histologic basis of mouse endocrine system development, a comparative analysis. Boca Raton: CRC Press; 2010.

26. Bowie MB, McKnight KD, Kent DG, McCaffrey L, Hoodless PA, Eaves CJ. Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. J Clin Invest 2006; 116:2808–16.

27. Jacquet P, van Buul P, van Duijn-Goedhart A, Reynaud K, Buset J, Neefs M, et al. Radiation sensitivity of the gastrula-stage embryo: Chromosome aberrations and mutations induction in lacZ transgenic mice: The roles of DNA double-strand break repair systems. Mutat Res 2015; 792:26–34.

28. Bondar T, Medzhitov R. p53-mediated hematopoietic stem and progenitor cell competition. Cell Stem Cell 2010; 6:309–22.

29. Heyer BS, MacAuley A, Behrendtsen O, Werb Z. Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. Genes Dev 2000; 14:2072–84.

30. Bowie MB, Kent DG, Dykstra B, McKnight KD, McCaffrey L, Hoodlesfs PA, et al. Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. Proc Natl Acad Sci U S A 2007; 104:5878–82.

31. Mori H, Colman SM, Xiao Z, Ford AM, Healy LE, Donaldson C, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A 2002; 99:8242–7.

32. Nakano M, Kodama Y, Ohtaki K, Nakamura N. Translocations in spleen cells from adult mice irradiated as fetuses are infrequent, but often clonal in nature. Radiat Res 2012; 178:600–3.

33. Sources, effects and risks of ionizing radiation. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) 1988 Report. New York: United Nations; 1988.

34. Doll R. The epidemiology of childhood leukemia. J R Statist Soc A 1989; 152:341–51.

35. Caughey RW, Michels KB. Birth weight and childhood leukemia: a meta-analysis and review of the current evidence. Int J Cancer 2009; 124:2658–70.

36. Tower RL, Spector LG. The epidemiology of childhood leukemia with a focus on birth weight and diet. Crit Rev Clin Lab Sci 2007; 44:203–42.

37. O'Neill KA, Murphy MF, Bunch KJ, Puumala SE, Carozza SE, Chow EJ, et al. Infant birthweight and risk of childhood cancer: international population-based case control studies of 40,000 cases. Int J Epidemiol 2015; 44:153–68.

38. Monson RR, MacMahon B. Prenatal X-ray exposure and cancer in children. In: Boice JD, Fraumeni JF. Radiation carcinogenesis: epidemiology and biological significance. New York: Raven Press; 1984. p. 97–105.

39. Wakeford R, Bithell JF. Childhood cancer- the role of birthweight and antenatal radiography. Int J Epidemiol 2015; 1–3.