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X-rays induce distinct patterns of somatic mutation in fetal versus adult hematopoietic cells

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Abstract

There are a variety of mechanisms and pathways whereby cells safeguard their genomes in the face of environmental insults that damage DNA. Whether each of these pathways is equally robust at specific developmental stages in mammals and whether they are also modulated in a tissue-specific manner, however, are unclear. Here we report that ionizing radiation (IR) produces different types of somatic mutations in fetal cells compared with adult cells of the same lineage. While 1 Gy of X-ray significantly induced intragenic point mutations in T cells of adult mice, no point mutational effect was observed when applied to fetuses. Fetal exposure to IR, on the other hand, led to a significant elevation of mitotic recombination in T cells, which was not observed in adults. Base excision repair (BER) activity was significantly lower in fetal hematopoietic cells than in adult cells, due to a low level of DNA polymerase β , the rate-limiting enzyme in BER. In fetal hematopoietic cells, this low BER activity, together with a high rate of proliferation, causes X-ray-induced DNA lesions, such as base damage, single strand breaks and double strand breaks to be repaired by homologous recombination, which we observe as mitotic recombination. Higher BER activity and a relatively lower rate of cell proliferation likely contribute to the significant induction of DNA point mutations in adults. Thus, the mutational response to IR is at least partly determined by the availability of specific repair pathways and other developmentally-regulated phenotypes such as mitotic index.

Prenatal irradiation with low doses of X-rays imposes a serious risk toward developing childhood leukemia [1]. Both human and animal studies have demonstrated that exposure to IR during the late fetal period causes the greatest induction of cancers [1]. Furthermore, the types of cancer induced by IR exposure *in utero* are different from those seen in adults. Sasaki and Kasuga [2] reported that B6C3F1 mice exposed to X-rays on day 17 post-conception developed solid tumors, but not leukemia. In contrast, irradiation of young adults (15 weeks of age) was associated with development of myeloid leukemia. These differences in the type of cancer induction by IR at different developmental stages suggest the presence of tissue- and/or developmental stage-specific response factors. The mechanisms underlying developmentally modulated IR-induced carcinogenesis are poorly understood. Since there is a clear linkage between DNA repair, mutagenesis and cancer, we propose that cellular responses to IR-induced DNA damage may differ between developmental stages. In the present

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study, we asked whether mutational events that predominate at the autosomal *Aprt* (adenine phosphoribosyltransferase) and X-linked *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) loci in T-cells of mice exposed to IR as adults are the same or different from those in T-cells of mice that had been exposed *in utero*. We showed that IR exposure at different developmental stages generated distinct mutational spectra in T-cells.

Materials and methods

Animals and irradiation

Pregnant C57BL/6 mice that had been mated to C3H males (thus, carrying B6C3F1 fetuses) were subjected to whole body irradiation on days 16–18 of gestation with a single dose of 0.5, 1, or 2 Gy of X-rays. Offspring of irradiated animals were raised under controlled conditions of light and temperature. Two-month old adult mice were also subjected to a single 1 Gy dose of whole body X-irradiation. The *Aprt* genotype of the B6C3F1 mice was determined by allele-specific PCR [3].

Isolation of 2,6-diaminopurine-resistant (DAP^r) and 6-thioguanine-resistant (6-TG^r) T lymphocytes and characterization of mutant clones.

At specific times following X-irradiation (20, 40, or 60 days), groups of the B6C3F1 *Aprt*^{+/-} mice were sacrificed and DAP^r and 6-TG^r splenic T cell colonies were selected, as previously described [3-5].

Molecular and cytogenetic characterizations of T cell clones are described elsewhere [3;4].

Nuclear protein extracts isolated from fetal and adult tissues

Fetal spleens were collected on day 16 of gestation and macerated in ice-cold RPMI-1640 medium. Spleens and thymuses from adult mice (two months old) were also crushed in ice-cold RPMI-1640 medium. Spleen or thymus cell suspensions were passed through cell strainers (70 µm) and harvested by centrifugation. Adult bone marrow cells were obtained by flushing the medullary cavities of femurs with ice-cold RPMI-1640 medium using a 25-gauge needle. Nuclear protein extracts were prepared as described [6].

In vitro analysis of base excision repair (U:G mismatch repair assay)

An *in vitro* base excision repair (BER) assay that measures the short patch BER efficiency of cell extracts was used [7]. Briefly, a 30-mer oligonucleotide (5'-GAGCCGGCACTGGUGCCCAGCTGATATCGC-3') containing a uracil at position 14 was annealed to the oligonucleotide (5'-GCGATATCAGCTGGGCGCCAGTGCCGGCTC-3'). The corresponding "normal" duplex containing a CG base pair was used as control. The DNA duplex was incubated in a reaction mixture (25 µl) with an equivalent amount (6 µg) of nuclear extract from mouse fetal or adult cells at 37°C for 30 min. The reaction mixture contained 40 mM phosphocreatine di(tris) salt, 5 mM MgCl₂, 1 mM dithiothreitol, 2 mM ATP, 20 µM each of dATP, dGTP, dTTP and dCTP, 1 µCi [α -³²P]dCTP, 2.5 µg creatine phosphokinase, 50 mM NaCl, and 0.5 µg 30 bp duplex oligonucleotide (UG or CG substrate). Reactions were stopped by proteinase K treatment (1 µl of 10 mg/ml proteinase K per reaction) at 37°C for 10 min. Samples were loaded onto a 15% polyacrylamide gel and electrophoresed at 150 V for 60 min. The BER activity is determined by the incorporation of the [α -³²P]dCTP, in replacing the uracil in the 30 bp DNA duplex. The radiolabeled 30 bp fragment was visualized and quantified with a Typhoon PhosphoImager using ImageQuant software (Molecular Dynamics).

Immunoblotting

Equivalent amounts of nuclear extracts from fetal and adult cells were separated with SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked at room temperature for 30 min in blocking buffer (3% non-fat dry milk in PBS containing 0.1% Tween 20 (T-PBS)) and incubated at 4°C overnight with primary antibody diluted in blocking buffer. After five washes with T-PBS, membranes were incubated at room temperature for 1 hr with secondary antibody diluted in blocking buffer. Immunoblots were visualized by enhanced chemiluminescence. Primary antibodies used in this study were: mouse anti-DNA polymerase β (Lab Vision corporation, Fremont, CA), and rabbit anti- β -actin (Abcam, Cambridge, MA).

Results

Prenatal X-irradiation increases the *Aprt* but not the *Hprt* mutant frequency in T cells

We and others have observed that a single dose of 1 Gy X-irradiation given to mice at two months of age significantly increases the mutant frequency (MF) at *Hprt* in T cells, but does not significantly increase the MF at the *Aprt* locus in the same cell type [8;9]. Specifically, 1 Gy X-ray increased the *Hprt* MF by 3.3-fold as compared to unirradiated controls (11×10^{-6} v.s. 3.3×10^{-6} , in irradiated mice and controls, respectively) [8]. PCR amplification of *Hprt* indicated that intragenic deletions in *Hprt* were significantly induced by X-irradiation (Nguyen, Liang and Tischfield, unpublished data). Molecular analyses of *Aprt* mutants also demonstrated that intragenic alterations in *Aprt* (base substitutions and deletions) but not large chromosome alterations were induced by 1 Gy X-ray [8]. However, the putative induction in intragenic alterations (3.2×10^{-6}) at *Aprt* was not enough to produce a significant increase in overall *Aprt* MF due to the high spontaneous *Aprt* MF (24×10^{-6}). Thus, X-irradiation induces intragenic alterations at both loci.

We hypothesized that fetal cells respond to X-rays differently than adult cells and, as a consequence, harbor a different spectrum of somatic mutations after exposure. To test this hypothesis, we exposed fetuses to X-rays and characterized the mutations using *Hprt* and *Aprt* as reporters. Pregnant C57BL/6 mice carrying B6C3F1 fetuses were irradiated with a single dose of 1 Gy X-rays on days 16 – 18 of gestation and the B6C3F1 *Aprt*^{+/-} mice were sacrificed at 20, 40 or 60 days following irradiation. As shown in Figure 1A, the spontaneous *Aprt* and *Hprt* MFs in T cells of control mice were unchanged with maturation. The *Aprt* MF in T cells from mice irradiated *in utero* was about two-fold greater than that of controls at day 40 post exposure, and remained at a high level at day 60. This time-dependent increase in the *Aprt* MF after exposure may be attributed to a combination of the time required for the phenotypic expression of 2,6-diaminopurine resistance and the time required for the migration of mutants from hematopoietic tissues to the spleen and peripheral lymphatic tissues. In contrast to the post-exposure time-dependent increase in the *Aprt* MF, the *Hprt* MF in T cells of mice irradiated *in utero* was unchanged compared with control values, at all time points tested (Fig. 1A).

To determine whether the T cell MF displays a dose-dependent response at the *Aprt* and *Hprt* loci of mice treated *in utero*, pregnant mice carrying B6C3F1 fetuses were irradiated with a single 0.5 Gy, 1.0 Gy, or 2.0 Gy dose of X-rays on days 16 – 18 of gestation. The mice were sacrificed 60 days later and T-cells were isolated and placed into culture in appropriate selection media. As shown in Figure 1B, the mean *Aprt* MF of all treated groups is significantly higher than that of the control ($P=0.004$, one way ANOVA). However, the dose-response curve does not show linearity with increasing dose. The curve peaks at 1 Gy (44×10^{-6}) and drops at 2 Gy (32×10^{-6}). Interestingly, in T-cells from irradiated mice, the MF at *Hprt* remained flat

with dose and was not elevated over that of the unirradiated mice ($P=0.62$, one way ANOVA, Fig. 1B).

Thus, irradiation of adult T-cells with 1 Gy of X-rays significantly elevated the MF at the *Hprt* locus, but not at *Aprt*. In fetal cells, however, the same dose of X-rays had the opposite effect, increasing mutations at the *Aprt* locus, but not at *Hprt*.

Mitotic recombination (MR) is the major mechanism for the X-ray-induced *Aprt* mutants in the fetus

To determine the types of mutational events that contributed to the increased MF at *Aprt* in T cells of mice exposed to 1 Gy X-rays *in utero*, we characterized the *Aprt* mutant T cells (DAP⁺ T cells). Based on molecular and cytogenetic analyses, the frequency with which variants arose as a consequence of MR was calculated to be 2.6 fold higher than that of unirradiated controls (37×10^{-6} vs. 14×10^{-6}) (Fig. 2). The frequency of variants derived from interstitial deletion/gene conversion was also increased although these mutational events were still rare (Fig. 2). DNA sequencing, however, indicated that the frequency of variants caused by intragenic point mutations, including small scale deletions, insertions, and base substitutions, was not increased (Fig. 2). These data are consistent with our observation that the frequency of *Hprt* mutants, which are primarily caused by intragenic point mutations, is not increased when mice are exposed *in utero*. In contrast, the frequency of intragenic point mutations was significantly increased when mice were irradiated with 1 Gy X-rays at two months of age (Fig. 2). Thus, these results clearly demonstrate that X-irradiation produces different types of mutations in fetal cells than in adult cells of the same lineage.

Base excision repair activity is lower in mouse fetal hematopoietic cells than in adult hematopoietic cells.

The base excision repair (BER) pathway repairs altered bases and DNA single-strand breaks induced by IR [10]. We hypothesized that the absence of X-ray induced point mutations at the fetal stage was caused by higher BER activity. To test this proposition, we measured BER efficiency at different developmental stages in nuclear extracts prepared from tissues that harbor hematopoietic stem and precursor cells. We isolated nuclear extracts from bone marrow, spleen and thymus cells of adult mice as well as fetal spleen cells, since the major site of hematopoiesis switches from the fetal liver to the spleen in late fetal development [11]. Surprisingly, fetal spleen cells displayed the lowest BER activity, about 5-fold lower than cells from adult bone marrow, spleen or thymus (Fig. 3A). Since DNA polymerase β (Pol β) has been reported to be the rate-limiting enzyme in the BER pathway [12], responsible for both repair synthesis and excision of the deoxyribose phosphate moiety generated during the repair process, we measured the Pol β protein level by Western blot. As shown in Figure 3B, the protein level of Pol β in fetal spleen cells is much lower than that in adult cells. Thus, the lack of X-ray-induced point mutations at the fetal stage is not a consequence of elevated BER.

It has been reported that cells with BER deficiency exhibit increased homologous recombination (HR) activity that is reflected by an increased frequency of sister chromatid exchange and increased Rad51 focus formation [13;14]. Our finding that X-rays induced MR, but not point mutation, in fetal hematopoietic tissues is consistent with these reports that BER deficiency can be accompanied by an increase in HR.

Discussion

We have shown that adult mice exposed to 1 Gy of X-rays display a significant elevation of intragenic alterations (including base substitutions and deletions/insertions) at *Aprt* in T cells (Fig. 2). In contrast, when the same dose was administered to fetuses, MR, but not intragenic

alteration, was significantly induced (Fig. 2). Thus, the same DNA damaging agent induced different kinds of somatic mutations following X-ray exposure of adults or fetuses. This developmental stage-specific induction of either MR or intragenic point mutations may reflect preferential use of different DNA repair pathways at distinct developmental stages.

Ionizing radiation causes various types of DNA damage, including single-strand DNA breaks (SSBs), double-strand DNA breaks (DSBs), and base damage (BD) [15]. BD and SSB are primarily repaired by the BER pathway [10]. However, BER can lead to base substitutions since Pol β , a key enzyme in BER, lacks proofreading activity [16]. DSBs are repaired by two distinct types of repair pathways, homologous recombination repair (HR) and non-homologous end-joining (NHEJ). NHEJ can be mutagenic, resulting in deletions and insertions at the DSB site. Although repair by HR is presumed to be precise, it has the potential to result in loss of heterozygosity when recombination occurs between homologous chromosomes with a heterozygous locus distal to the crossover.

In the absence of BER, HR plays an important role in the repair of BD and SSB in mammalian cells, since stalled and collapsed DNA replication forks at unrepaired apurinic sites and SSB sites serve as a signal for recombination repair [17;18]. It was reported recently that lack of Pol β -dependent BER activity leads to SSB accumulation in G1 phase [13]. Cells entering into S phase with unrepaired SSBs exhibit a rapid phosphorylation of histone H2AX, indicating that the unrepaired SSBs have been converted to DSBs. This is followed by the relocalization of Rad51 protein that is essential for HR [13]. The lower level of BER activity in mouse fetal hematopoietic cells compared to their adult counterparts (Fig. 3A), due to diminished Pol β protein (Fig. 3B), suggests that BDs and SSBs may not be repaired efficiently by BER in fetal hematopoietic cells. Such lesions could produce stalled or collapsed replication forks, and then be repaired by HR if they persist into S phase.

The preferential induction of MR as a consequence of prenatal exposure to X-rays could also be attributable to a higher frequency of target cells in S/G2 phase. During fetal development, hematopoietic cells, including hematopoietic stem cells (HSCs), multiply dramatically to keep pace with growth. Indeed, fetal HSCs proliferate more rapidly than those in adult bone marrow [19]. On the other hand, most mature T lymphocytes are in G0 phase *in vivo* until they are activated by specific mitogens or antigens [20]. Thus, it is reasonable to assume that the fraction of fetal hematopoietic cells in S-phase is greater than that of adult cells. Since the choice of repair pathways for DSBs, HR and/or NHEJ, is dependent on the phase of cell cycle when DSBs occur, with HR predominant during the S and G2 phases and NHEJ in G0/G1 phase [21], more actively proliferating cells, such as those in the fetus, are more likely to use HR to repair DSBs.

Therefore, a low level of BER coupled with high proliferative activity in fetal hematopoietic cells results in HR as the predominant pathway for the repair of X-ray-induced DNA damage, including BDs, SSBs, and DSBs. In contrast, BER and NHEJ may be the major repair pathways for X-ray-induced lesions in less actively dividing adult cells. The fact that HR, NHEJ and BER produce different mutagenic effects explains the characteristic induction of MR or intragenic point mutations at specific developmental stages. Our finding that cells of the same lineage at different developmental stages use distinctive DNA repair mechanisms, some of which are error prone, may partially explain why different developmental stages are more susceptible to specific cancers. The genetic risk(s) of prenatal exposure to IR should be reevaluated in light of this new concept.

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Abbreviations:

Ap^rt, adenine phosphoribosyltransferase; BER, base excision repair; DSB, double strand break; Hp^rt, hypoxanthine-guanine phosphoribosyltransferase; IR, ionizing radiation; MF, mutant frequency; MR, mitotic recombination.

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Figure 1. Prenatal X-irradiation increases the *Aprt* but not the *Hprt* mutant frequency in T cells
 A. Time course of mutagenic effects of X-rays on T-cells of B6C3F1 mice. Pregnant C57BL/6 mice carrying B6C3F1 fetuses were irradiated with a single dose of 1 Gy X-rays on days 16 – 18 of gestation. T cells were isolated from irradiated offspring and assayed for mutations at *Hprt* and *Aprt* at 20, 40 and 60 days after exposure. Each point represents the mean mutant frequency (N = 5 – 22 mice/group). The error bar represents the standard error of the mean.
 B. Effect of X-ray dose on the *Aprt* and *Hprt* MFs in T-cells from B6C3F1 mice irradiated *in utero*. B6C3F1 fetuses were irradiated with a single dose of 0.5, 1, or 2 Gy of X-rays on days 16 – 18 of gestation. T cells were isolated 60 days after prenatal exposure. Each point represents the mean mutant frequency (N = 5 – 22/group). The error bar shows standard error of the mean.

Figure 2. Mitotic recombination, but not point mutation, is the major cause of the increased *Aprt* MF in T cells from mice irradiated *in utero*

A minimum of 120 DAP^r colonies per treatment group were characterized as described in the Methods and Materials. The value of each bar represents the *Aprt* MF caused by a specific mutation type and is calculated by multiplying the *Aprt* MF by the fraction of each given mutation type.

Figure 3. Fetal hematopoietic cells have a lower level of DNA polymerase β -dependent BER activity than adult hematopoietic cells

A. In vitro BER activity in extracts of cells from fetal and adult mice. BER reactions contained the 30 bp DNA duplex with a UG mismatch, [α - 32 P]dCTP, and nuclear extracts. Replacement by BER of an unpaired U with a paired C [α - 32 P]dCTP produces a radioactive 30 bp band. The corresponding “normal” duplex containing the CG base pair served as a control. The radiolabeled 30 bp fragment was visualized and quantified with a Typhoon PhosphoImager using ImageQuant software. **B.** Western blot analysis of DNA polymerase β in extracts of fetal and adult cells.